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ORIGINAL ARTICLE

Hideaki Nakazawa · Shuichi Yoshihara · Daisuke Kudo
Hajime Morohashi · Ikuko Kakizaki · Atsushi Kon
Keiichi Takagaki · Mutsuo Sasaki

4-methylumbelliferone, a hyaluronan synthase suppressor, enhances the anticancer activity of gemcitabine in human pancreatic cancer cells

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Abstract Hyaluronan (HA) is a ubiquitous, major component of the pericellular matrix and is necessary for various physiological processes. It plays a very important role in biological barriers. We previously reported that 4-methylumbelliferone (MU) inhibits HA synthesis and pericellular HA matrix formation in cultured human skin fibroblasts, *Streptococcus equi* FM100, and B16F10 melanoma cells. We hypothesized that MU-mediated inhibition of HA synthesis and pericellular HA matrix formation would increase the efficacy of anticancer drugs. We have already demonstrated in vitro, using a sandwich binding protein assay and a particle exclusion assay, that MU inhibits HA synthesis and formation of the pericellular HA matrix, respectively, in human KP1-NL pancreatic cancer cells. AlamarBlue assay revealed that the anticancer effect of gemcitabine in KP1-NL cells was increased by pretreatment with MU. In vivo simultaneous administration of MU and gemcitabine to tumor-bearing mice with severe combined immunodeficiency disease (SCID) decreased the size of the primary and metastatic tumors more than did gemcitabine alone. These data strongly suggest that a combination of MU and gemcitabine is effective against human pancreatic cancer cells. MU may have potential as a chemosensitizer and may provide us with a new anticancer strategy.

Keywords Hyaluronan · 4-methylumbelliferone · Gemcitabine · Pancreatic cancer · Pericellular matrix · Chemosensitizer

H. Nakazawa · S. Yoshihara (✉) · D. Kudo · H. Morohashi
M. Sasaki
Department of Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8562, Japan
E-mail: yoshi2@cc.hirosaki-u.ac.jp
Tel.: +81-172-395079
Fax: +81-172-395080

I. Kakizaki · A. Kon · K. Takagaki
Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, 036-8562, Japan

Abbreviations HA: Hyaluronan · HAS: Hyaluronan synthase · MU: 4-methylumbelliferone · SCID: Severe combined immunodeficiency disease · FCS: Fetal calf serum · DMSO: Dimethyl sulfoxide · PBS: Phosphate-buffered saline · UGT: UDP-glucuronyltransferase

Introduction

Hyaluronan (HA) is a high-molecular-weight glycosaminoglycan composed of repeating disaccharide units of GlcNAc-β (1 → 4)-GlcUA-β (1 → 3) [24]. It has the ability to trap large amounts of water because of its physiochemical composition, and its solution has viscosity and elasticity. It is synthesized from the precursors UDP-GlcUA and UDP-GlcNAc [23] at the inner leaflet of the plasma membrane by a membrane-associated hyaluronan synthase (HAS), and it is present in many tissues as a major component of the pericellular matrix and the extracellular matrix, non-covalently in association with other components. In normal tissues, it provides an environment that facilitates cellular proliferation and migration and is necessary for various physiological processes such as embryonic development and wound healing [17]. In cancer, however, these properties appear to enhance tumor invasion, growth, angiogenesis, and metastasis [1, 17, 25, 26].

We previously reported that 4-methylumbelliferone (MU) inhibits HA synthesis and pericellular HA matrix formation in cultured human skin fibroblasts [19], *Streptococcus equi* FM100 [11], and B16F10 melanoma cells [14]. Electron microscopic studies have revealed that the structure of the extracellular matrix of cultured human skin fibroblasts is changed by MU [5]. Moreover, we have examined the mechanism by which MU inhibits HA synthesis in *Streptococcus equi* FM100 and have demonstrated that MU dose not affect expression of the HA synthesis gene but instead alters the lipid environment of the membrane, especially the distribution of different cardiolipin species, surrounding HAS [11]. In

addition, using rat 3Y1 fibroblasts stably expressing HAS2, we have also recently demonstrated a novel mechanism of MU-mediated inhibition of HA synthesis involving the glucuronidation of MU by endogenous UDP-glucuronyltransferase, resulting in depletion of UDP-GlcUA [10].

Several studies have reported the importance of the pericellular HA matrix as a biological barrier. The pericellular HA matrix inhibits lymphocytes from approaching cancer cell membranes and protects cancer cells from lymphocyte-mediated cytolysis [16], inhibiting penetration of chemotherapeutic agents [8]. On gene therapy the uptake of DNA complexes to the target cells varies, depending on the amount of cell-surface glycosaminoglycans that inhibit transgene expression [21]. Furthermore, it has been reported that extracellular matrix proteins play important roles in acquired resistance to anticancer drugs [18].

Our aim here was to investigate whether reduction of the pericellular HA matrix by MU in cancer cells would enhance the efficacy of anticancer agents. We chose a human pancreatic cancer cell line as our cancer cells and gemcitabine as our anticancer agent. In vitro, we investigated whether MU inhibited HA synthesis of pancreatic cancer cells and formation of the pericellular HA matrix. We then investigated whether reduction of the pericellular HA matrix influenced the anticancer activity of gemcitabine. In vivo, we investigated whether administration of both MU and gemcitabine influenced primary tumor growth and inhibited liver metastasis as compared with the administration of gemcitabine alone in mice that had been implanted with pancreatic cancer cells and that also had severe combined immunodeficiency disease (SCID).

Materials and methods

Materials

MU was purchased from Wako Pure Chemicals (Osaka, Japan). Gemcitabine was purchased from Nippon Eli Lilly (Kobe, Japan). *Streptomyces* hyaluronidase was purchased from Seikagaku Corporation (Tokyo, Japan). AlamarBlue was purchased from Biosource International (Camarillo, CA, USA). RPMI-1640 was purchased from Nissui Pharmaceutial Corporation (Tokyo, Japan). Lab Animal Diet (MF) was purchased from Oriental Yeast Corporation (Tokyo, Japan). All other reagents were of analytical grade and were obtained from commercial sources.

Tumor cells

The human pancreatic cancer cell line KP1-NL [9] was obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were routinely maintained as monolayer cultures in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, amphotericin B, and sodium bicarbonate solution at 37°C in a mixture of 5% CO_2 and 95% humidified air. In all experiments cells at passage 8–15 were used.

Mice

Male C.B-17/Icr-scid mice were purchased from Clea (Tokyo, Japan). The mice were housed in specific pathogen-free conditions under controlled light–dark cycles (every 12 h), temperature (about 22°C), and humidity (about 47%) with water and food (Lab Animal Diet (MF)) ad libitum, and used when they were 7–12 weeks of age, at the weight of about 20 g.

Cell-growth assay

Cell proliferation was assessed by the AlamarBlue assay [20]. Briefly, Ninety microliters of medium containing 6.25×10^2 cells was seeded into 96-well plates (Asahi Technoglass Corp., Chiba, Japan) and allowed to adhere overnight. MU and/or gemcitabine in physiological saline was added to each well in serial concentrations. Cell numbers were assessed every 24 h. 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, respectively. All assays were performed with five replicates.

Analysis of HA synthesis in cells

Nine milliliters of medium containing 6×10^5 cells were seeded into a 100-mm culture dish and allowed to adhere for 24 h. Cells were treated with 0.1 mM MU dissolved in dimethyl sulfoxide (DMSO). After 48 h of incubation the medium was removed. Cells were washed with 5 ml of phosphate-buffered saline (PBS) twice, harvested, and suspended in 0.5 ml of PBS. We then added 0.5 ml of lysis buffer (1% Nonidet P-40, 140 mM NaCl, 10 mM EDTA (3Na), 20 mM Tris-HCl (pH 7.4), and 1 mM Phenylmethanesulfonyl fluoride) to the cell suspension, vortexed for 1 min, and placed it on ice for 30 min. After centrifugation of the suspension at 10,000*g* at 4°C for 10 min, the supernatant containing HA was transferred into another tube and frozen until analysis. HA was measured by the sandwich binding protein assay kit, in accordance with the manufacturer's protocol (Chugai, Tokyo, Japan) [4].

Visualization of cell surface HA

The pericellular HA matrix was visualized by using a particle exclusion assay [12]. Two milliliters of medium

containing 2.5×10^4 cells was suspended in a 35-mm-diameter tissue culture dish and cultured for 24 h. Then 0.1 mM MU dissolved in DMSO was added to the culture medium. As a control, only DMSO was added. The dishes were incubated for 48 h. Glutaraldehyde-fixed sheep erythrocytes were reconstituted in PBS at a density 5×10^8 cells/ml. The pericellular HA matrix was visualized by an addition of 0.75 ml of the erythrocyte suspension to the medium, followed by viewing by phase-contrast microscopy. In the particle exclusion assay, the pericellular HA matrix appeared as a halo surrounding each cell. To demonstrate that this matrix was comprised of retained HA, cells in the dish were treated with 1 U/ml of HA-specific *Streptomyces* hyaluronidase for 1 h before visualization.

Therapy for human pancreatic cancer growing in the spleen and liver

Twenty SCID mice were anesthetized with diethyl ether. A left lateral laparotomy was performed and the spleen mobilized. A suspension of cells (1×10^5 cells/50 µl of PBS) harvested from 80% subconfluent cultures was injected directly into the spleen. The tumor-bearing mice were randomized into four groups of five mice each, as follows: (a) no treatment, (control group) oral administration of Arabic gum every day and i.p. injection of physiological saline twice a week; (b) oral administration of 1 g/kg MU every day and i.p. injection of physiological saline twice a week; (c) i.p. injection of 125 mg/kg gemcitabine twice a week and oral administration of Arabic gum every day; (d) oral administration of 1 g/kg MU every day and i.p. injection of 125 mg/kg gemcitabine twice a week. MU was suspended in 1% Arabic gum at a density of 20% and the suspension was administered by gastric sound. The mice were sacrificed 6 weeks after the implantation of the cancer cells into the spleen. Both the spleen containing the primary pancreatic cancer and the liver containing tumor metastases were surgically removed and weighed.

Statistical analyses

Single comparisons were performed by Student's *t*test, and multiple comparisons were performed by the Kruskal–Wallis test and then the Mann–Whitney *U* test. $P < 0.05$ was considered statistically significant.

Results

Effect of MU on KP1-NL cell growth

To determine whether MU had any direct effect on the growth of KP1-NL cells, cells were seeded in 96-well plates (625 cells/well) and incubated in the presence of varying concentrations of MU for 48 h. Live cells were then evaluated by the AlamarBlue assay, as described in Materials and methods. Addition of 0–0.1 mM of MU did not inhibit the proliferation of KP1-NL cells (Fig. 1).

Inhibition of HA synthesis of KP1-NL cells by MU

We had previously shown that MU inhibits HA synthesis in cultured dermal fibroblasts, *Streptococcus equi* FM100, and B16F10 melanoma cells [11, 14, 19]. To determine whether MU also inhibits HA synthesis in KP1-NL cells, we incubated KP1-NL cells with 0.1 mM MU—a noncytostatic concentration—for 48 h. We analyzed the amount of HA by a sandwich binding protein assay, as described in Materials and methods. The amount of HA released into the culture medium decreased when 0.1 mM MU was present (data not shown). The amount of HA present at the cell surface and inside the cells decreased upon treatment with 0.1 mM MU (Fig. 2). The data revealed that treatment with 0.1 mM MU decreased the amount of HA significantly by a mean weight of about 30% (HA content 55.74 ± 1.36 ng/10^6 cells in treated groups versus 76.76 ± 4.0 ng in untreated group).

Next, we used a particle-exclusion assay, as described in Materials and methods, to examine the effect of MU on formation of the pericellular HA matrix. When cells were incubated without MU, a halo was observed (Fig. 3a). In contrast, the halo gradually disappeared upon treatment with 0.1 mM MU and reached a minimum after incubation for 48 h (Fig. 3b).

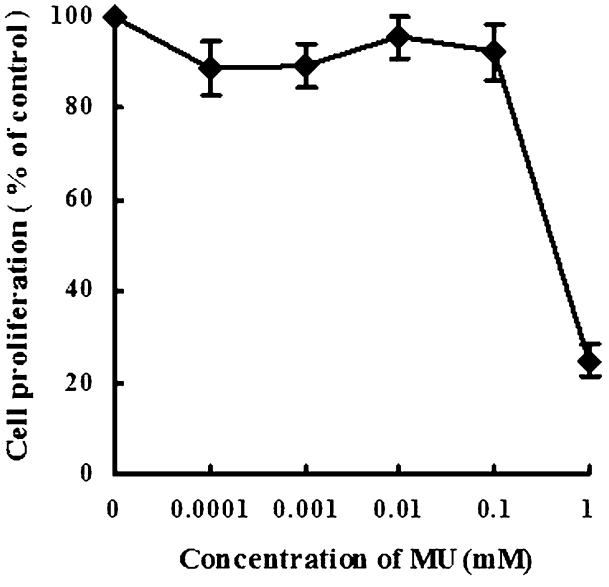


Fig. 1 Effect of MU treatment on the growth of KP1-NL cells. KP1-NL cells were incubated with 0–1 mM MU for 48 h. Live cells were evaluated by AlamarBlue assay, as described in Materials and methods. Results are expressed as percentages of live cells compared with those without MU treatment. *Each point* represents the mean ± SEM of five replications of three separate experiments

When cells were treated with 1.0 U/ml HA-specific *Streptomyces* hyaluronidase, the halo could not be observed (Fig. 3c). These results suggested that MU inhibited HA synthesis in KP1-NL cells, as well as its retention on the cell surface and the formation of the pericellular HA matrix.

Effect of MU on anticancer activity of gemcitabine in cultured cells

To investigate whether the anticancer effect of gemcitabine was increased by MU, KP1-NL cells cultured with 0.1 mM MU for 48 h beforehand were incubated with various concentrations of gemcitabine (0–10 μM) after removal of the MU. Cell numbers were assessed every 24 h for 5 days using the AlamarBlue assay, as described in Materials and methods. Figure 4 showed the cell numbers in 5th day. From 1st day to 4th day, no differences of cell numbers between with MU and without MU were clearly found. Therefore, we described only the cell numbers on 5th day. Gemcitabine dose-dependent decreased cell viability. When >0.1 μM of gemcitabine was added to cells pretreated with MU, cell viability was dramatically reduced by about 50%. Thus, pre-treatment with MU increased the anticancer effect of gemcitabine in KP1-NL cells.

Effect of MU on gemcitabine therapy of human pancreatic cancer cell growing in the spleen and liver

To examine the effect of MU on the anticancer activity of gemcitabine in vivo systems, we implanted 1×10^5 KP1-NL tumor cells directly into the spleens of SCID mice. We then gave MU and/or gemcitabine orally or intraperitoneally to the mice, as described in Materials and methods. All mice were sacrificed on day 42 and the spleen and liver were removed and weighed.

The mean spleen weight was decreased by the administration of MU or gemcitabine alone compared with the control group; furthermore, the mean spleen weight of the gemcitabine plus MU group was significantly ($P<0.05$) lower (60 ± 0.080 mg) than with gemcitabine alone (240 ± 0.047 mg; Fig. 5a). Similarly, the median weight of the liver in the gemcitabine plus MU group was significantly ($P<0.05$) lower (1.1 ± 0.059 g) than in gemcitabine-alone group (1.6 ± 0.095 g) (Fig. 5b). These results suggest that the anticancer effect of gemcitabine was increased in vivo by pretreatment with MU.

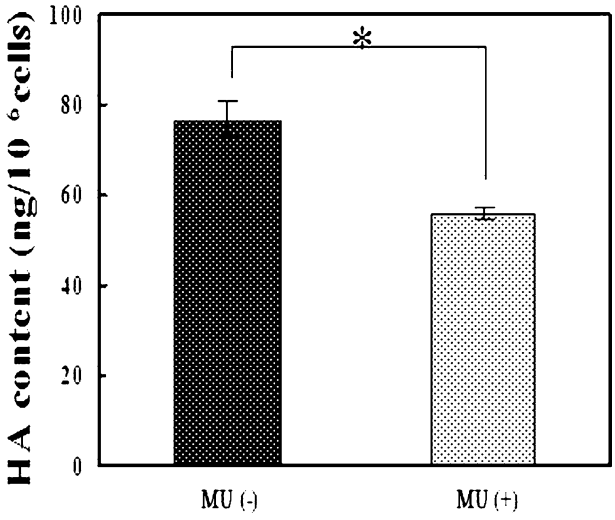


Fig. 2 Effect of MU on the amount of HA in KP1-NL cells. KP1-NL cells were incubated in 0.1 mM MU for 48 h. HA was extracted and quantified as described in Materials and methods. *Each bar* represents the mean ± SEM of five replicattions. $^*P<0.05$

Discussion

We examined the effects of MU on HA synthesis and pericellular HA matrix formation in KP1-NL pancreatic cancer cells, as well as influence of MU-mediated inhibition on the anticancer activity of gemcitabine.

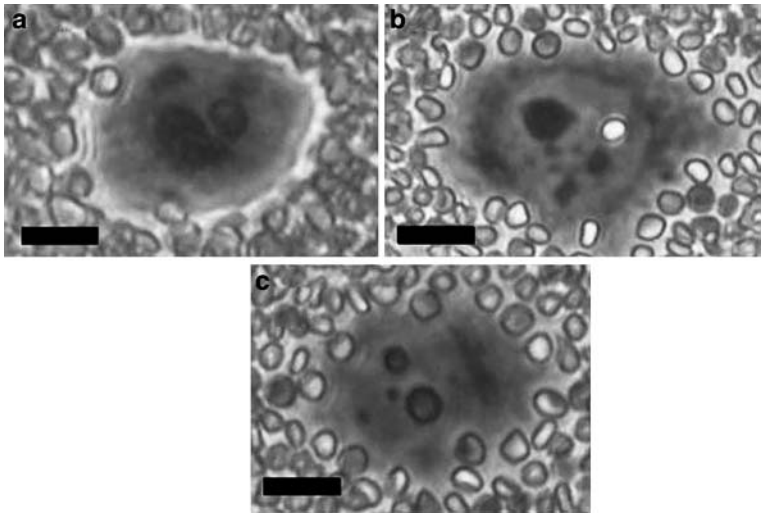


Fig. 3 Effect of MU on the formation of a pericellular HA matrix in KP1-NL cells. The pericellular HA matrix was assessed by particle-exclusion assay, as described in Materials and methods. **a** KP1-NL cells were incubated in the absence of 0.1 mM MU. **b** KP1-NL cells were incubated in the presence of 0.1 mM MU for 48 h. **c** We added 1. 0 U/ml of *Streptomyces* hyaluronidase to the culture dish before the exclusion assay. *Bars* expressed ten micrometers

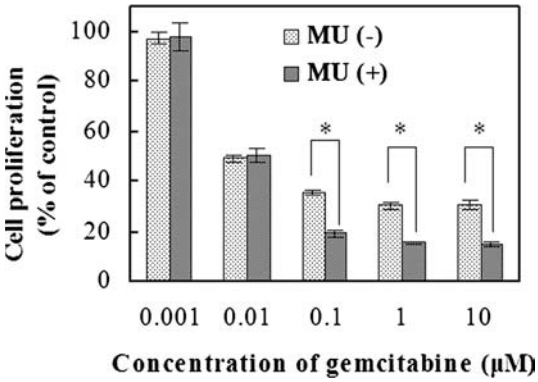


Fig. 4 Mediation of efficacy of gemcitabine by MU. KP1-NL cells were incubated with 0.1 mM MU for 48 h. After removal of the MU, the cells were incubated with varying concentrations of gemiciabine (0–10 M). Cell numbers were assessed every 24 h for 5 days by AlamarBlue assay, as described in Materials and methods. *Each bar* represents the mean ± SEM of five replications experiments of three separate experiments. $*P < 0.05$

Kudo et al. [14] previously demonstrated that MU inhibits HA in B16F10 melanoma cells in a dose-dependent manner. Therefore, we treated cells with the maximum concentration of MU that had no cytotoxicity (0.1 mM), but still maintained maximum inhibition of HA synthesis. The amount of HA in the pericellular matrix and inside the cell gradually decreased (data not shown), finally reaching a minimum after 48 h.

Next, using particle-exclusion assay, we demonstrated that the pericellular HA matrix, which had the appearance of a halo around the cells, gradually thinned and reached a minimum after incubation with 0.1 mM MU for 48 h. When these cells were treated with HA-specific *Streptomyces* hyaluronidase, they no longer had observable pericellular halos.

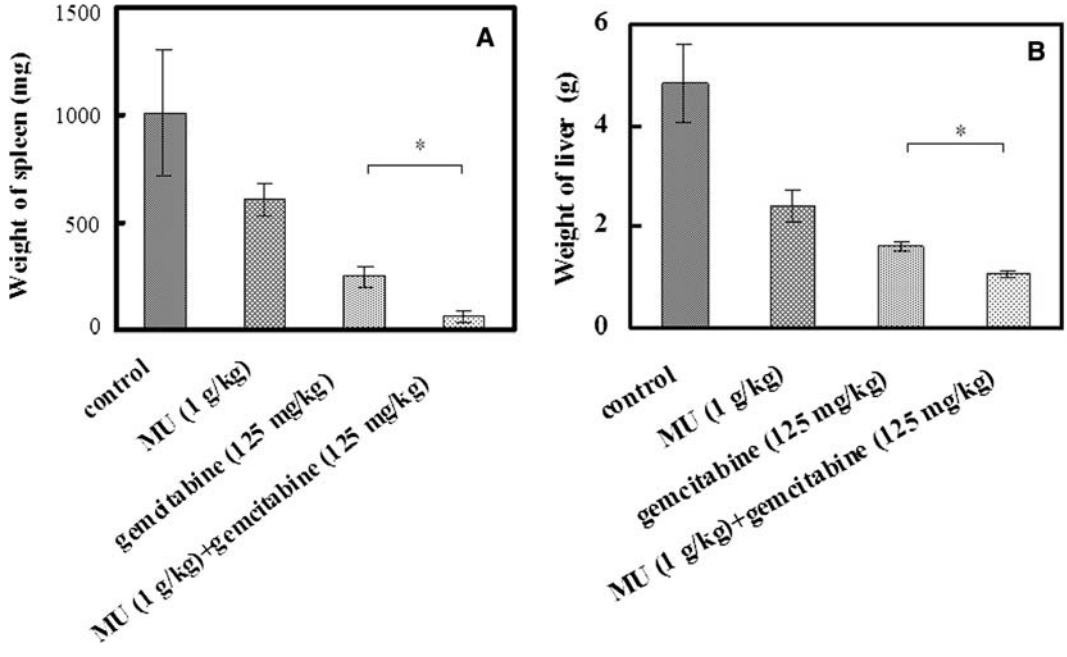
These results indicated that MU inhibited formation of the pericellular HA matrix following inhibition of HA synthesis. These findings are in agreement with those of our previous studies [11, 14, 19]. Therefore, we suggest that MU-mediated inhibition of HA synthesis might not occur in a cell-specific manner.

We examined the effect of MU on the efficacy of gemcitabine in KP1-NL pancreatic cancer cells. In vitro, the anticancer effect of gemcitabine was increased by pretreatment with MU. In vivo, in SCID mice models, the sizes of pancreatic cancer xenografts in the spleen and of metastatic tumors in the liver were decreased significantly by the administration of both MU and gemcitabine compared with gemcitabine alone. Thus, a combination of gemcitabine and MU had higher anticancer activity than gemcitabine alone.

Taken together with the results of several other studies that have shown the importance of the pericellular matrix as a biological barrier [8, 16, 18, 21], our results strongly predict that inhibition of HA synthesis and pericellular matrix formation by MU might promote drug penetration into the cells, resulting in increased drug efficacy.

Our results revealed that hyaluronidase could decrease the formation of the pericellular HA matrix. Preclinical and clinical observations have demonstrated that the administration of hyaluronidase shortly before the administration of chemotherapeutics increases the access of anticancer drugs to the tumor and thus their effectiveness [2, 22]. The hyaluronidase is assumed to facilitate penetration and to decrease interstitial fluid pressure, thus permitting the anticancer agents to reach malignant cells [3]. Hyaluronidase has been utilized as an adjunctive

Fig. 5 Effect of MU in gemcitabine therapy of KP1-NL-cell-bearing SCID mice. KP1-NL cells were injected into the spleens of SCID mice. **a** weight of spleen **b** weight of liver. *Each bar* represents the mean ± SEM of five mice. $*P < 0.05$



chemosensitizer in anticancer chemotherapeutic regimens [2, 13, 15, 22]. However, the hyaluronidase, that was used clinically, is made from the bovine testis and therefore will not be useable in future because of the risk of bovine spongiform encephalopathy. Furthermore, usage of hyaluronidase is limited to local injection, so another drug that can be administered systemically needs to be found. For example, development of new MU inductor, which has the same effect as that of MU without toxic side effects, will be expected.

MU has potential that acts as a novel therapeutic chemosensitizer. In addition, oral MU has already been used clinically in hepatobiliary disease because it has cholagogic activity and also has spasmolytic activity on the sphincter of Oddi [6, 7]. Therefore the clinical application of MU could probably be extended.

In conclusion, we demonstrated that the novel approach of inhibiting HA synthesis by MU increased the efficacy of gemcitabine in KP1-NL pancreatic cancer cells. Our data showed that a combination of MU and gemcitabine was effective against pancreatic cancer cells. Although we did not examine differences in the gemcitabine concentration inside the cells that were treated or not treated with MU, our results suggest that MU may have potential as a chemosensitizer in anticancer chemotherapeutic regimens and therefore offers promise as part of a new anticancer strategy. To our knowledge, this is the first report to show the relationship between the effect of MU on cancer cells and the efficacy of an anticancer drug.

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