ANTICANCER RESEARCH *38*: 5799-5804 (2018)
doi:10.21873/anticanres.12919

4-Methylumbelliferone Decreases the Hyaluronan-rich Extracellular Matrix and Increases the Effectiveness of 5-Fluorouracil

ERI YOSHIDA[1], DAISUKE KUDO[1], HAYATO NAGASE[1], AKIKO SUTO[1], HIROSHI SHIMODA[2], SHINICHIRO SUTO[3], IKUKO KAKIZAKI[3], MASAHIKO ENDO[3] and KENICHI HAKAMADA[1]

Departments of [1]Gastroenterological Surgery, [2]Anatomical Science, [3]Glycotechnology, Center for Advanced Medical Research, Hirosaki University, Graduate School of Medicine, Hirosaki, Japan

Abstract. *Background/Aim: Pancreatic cancer responds poorly to most chemotherapeutic agents. Several studies have reported that hyaluronan (HA)-rich extracellular matrix (ECM) is a biological barrier against chemotherapeutic agents. 4-methylumbelliforone (MU) led to inhibition of HA synthesis and its preservation in ECM, which may enhance 5-fluorouracil (5-FU) cytotoxicity. Thus, new therapy with MU and 5-FU may be developed for pancreatic cancer. Materials and Methods: A 5-fluorouracil (5-FU) concentration and 4-methylumbelliferone (MU) dosage was analyzed by high-performance liquid chromatography (HPLC). Change in antitumor efficacy of 5-FU in combination with MU was also examined in vivo and in vitro. Results: Combined 5-FU and MU treatment inhibited cell proliferation better than 5-FU alone; 0.01 mM 5-FU alone decreased cell proliferation by 37.7 %, while 0.01 mM 5-FU with 0.5 mM MU decreased cell proliferation by 57.4%. MU enhanced the intracellular concentration of 5-FU by 47.3% compared to control. Mice tumors treated with 5-FU and MU decreased in size and animal survival was prolonged. Moreover, MU decreased cohesiveness of the intercellular space. Conclusion: Combination therapy of 5-FU with MU was effective. A novel therapy can be designed for pancreatic cancer by using ECM modulation.*

Pancreatic cancer responds poorly to most chemotherapeutic agents (1). The desmoplastic reaction is a prominent pathological characteristic of pancreatic cancer. It is accompanied by increased deposition of many extracellular matrix (ECM) components. Changes in stromal cell proliferation and the deposition of ECM result in dramatic changes in overall tissue heterogeneity and elasticity. These changes have been suggested to contribute to chemoresistance. Hyaluronan (HA) is a major component of the stroma of many common tumors, and correlates with their growth and spreading. HA-rich environments enhance tumor invasion and metastasis and are associated with worse clinical course in severe kinds of human adenocarcinomas (2). Several studies have reported the importance of the extracellular HA matrix as a biological barrier. Delivery of macromolecules to cancer cells is inhibited by ECM, because HA supplies high intratumoral fluidic pressure preventing diffusion and penetration of anticancer agents into the tumor tissue (3).

We have reported that 4-methylumbelliferone (MU) inhibits HA synthesis without any influence on other glycosaminoglycan production in cultured human skin fibroblasts (4). MU has been widely utilized as a specific inhibitor of HA synthesis and proposed as a promising anticancer agent (5-7). Some studies have shown that MU enhanced the efficacy of several anticancer agents (8, 9), but the exact mechanism of this effect was not revealed. In addition, effect of ECM modulation has not been reported. In this study, the control of ECM concentration of 5-fluorouracil (5-FU) by MU *in vitro* was analyzed. Effect of MU on the anticancer activity of 5-FU was also analyzed *in vivo*. HA surrounding cancer cells was depleted by MU. If it enhances the cytotoxic activity of 5-FU, novel combination therapy of 5-FU with MU can be developed for pancreatic cancer.

Materials and Methods

Materials. MU and hyaluronidase from *Streptomyces hyalurolyticus* were purchased from Wako Pure Chemicals (Osaka, Japan). 5-FU was purchased from Kyowa Hakko Kirin Co. (Tokyo, Japan). The Hitachi (Tokyo, Japan) high performance liquid chromatography (HPLC) system which consisted of L-2300

Correspondence to: Prof. Kenichi Hakamada, Department of Gastroenterological Surgery, Hirosaki University, Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8562, Japan. Tel: +187 172395079, Fax: +187 172395080, e-mail: hakamada@hirosaki-u.ac.jp

Key Words: Pancreatic cancer, 4-methylumbelliferone, hyaluronan, 5-fluorouracil, antitumor effect, extracellular modulation.

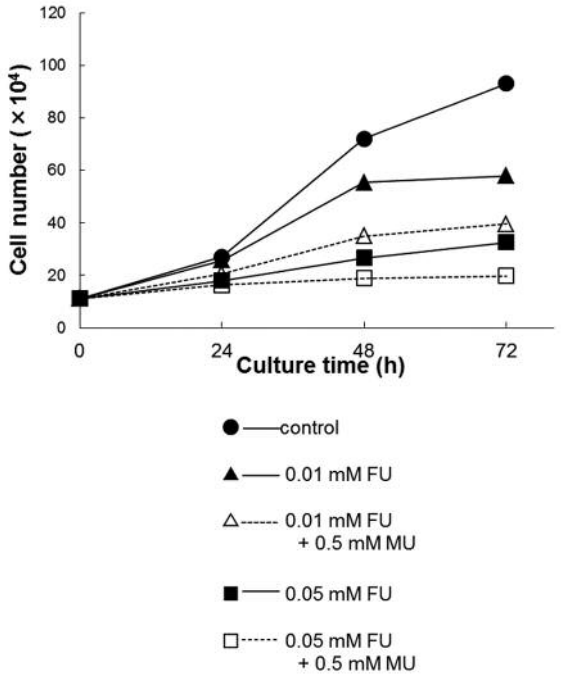

Figure 1. *Effect of MU and 5-FU on the proliferation of MIA PaCa-2 cells. Cells were incubated with MU and/or 5-FU. Cell number was examined as described in Materials and Methods.*

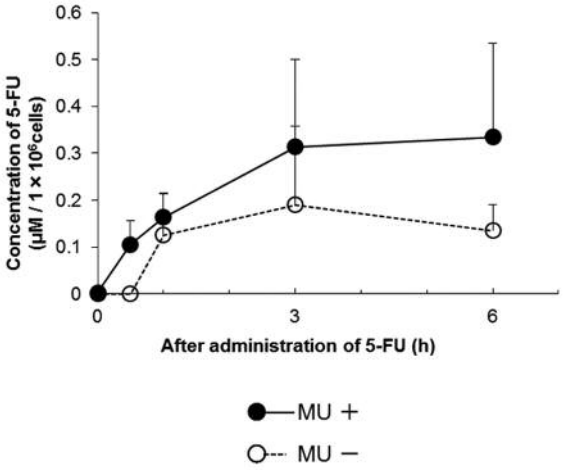

Figure 2. *Effect of MU on the intracellular migration of 5-FU. Concentration of intracellular 5-FU. Concentration was examined as described in Materials and Methods. Each point represents the average of quadrant independent assays.*

liquid chromatography, L-2200 autosampler, L-2420 variable wavelength UV detector and D-2000 Elite integrator was used for analysis of samples with ODS-column (0.46×15.0 cm, Shinwa Chemical, Kyoto, Japan).

Tumor cells. The human pancreatic cancer cell line MIA PaCa-2 was kindly provided by the Department of Pharmacy, Hirosaki University Hospital (Hirosaki, Japan). The cells were routinely maintained as monolayer cultures in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37˚C in a mixture of 5% CO_2 and 95% humidified air.

Mice. CB17/Icr-scid mice were used throughout the experiments. All mice were purchased from Japan Clea (Tokyo, Japan). The mice were housed under controlled light–dark cycles, temperature, and humidity with water and food *ad libitum*. Animals were used when they were 6-weeks-old with weights of about 25 g. All animal experiments were performed in accordance wih the Guidelines for Animal Experimentation of Hirosaki University.

Cell proliferation assay. Medium (2 ml) containing 1×10^5 cells was seeded into 6-well plates. Following 12 h incubation, 0.5 mM MU dissolved in DMSO and/or 0.01-0.05 mM 5-FU was added. After incubation with MU and/or 5-FU, each well was washed with PBS twice (5 ml), then detached from the plates by addition of trypsin/EDTA (0.5 ml), and finally suspended in PBS (2 ml). The suspensions were applied onto an automated cell counter (TC20™ , BIO-RAD, Tokyo, Japan), and living cell numbers were counted (10).

Analysis of intracellular 5-FU. Extraction and analysis of cellular 5-FU was performed in accordance with the reports (11). Medium (9 ml) containing 6×10^5 MIA-PaCa2 cells was seeded into a 100 mm culture dish. Following 12 h incubation, MU (0.5 mM) dissolved in DMSO was added. After 48 h of incubation, 5-FU (10 ml; 2mM) dissolved in medium was added to each dish and incubated for 0.5-6 h and then cells were washed with PBS, and suspended in 0.3 ml of PBS. The solution was added to a solution of 5-bromouracil (20 μg/ml, 300 μl) dissolved in isotonic phosphate buffer (pH 7.4) as an internal standard, 1M sodium acetate buffer (pH 4.8, 100 μl), and 20% anhydrous sodium sulfate solution (500 μl). The mixtures were extracted with ethyl acetate. Organic layers were collected and subjected to evaporation. The extracted residues were dissolved in 500 μl of distilled water and washed twice with 1 ml of hexane. Samples (100 μl) were injected into the HPLC column. Reverse phase chromatography on a STR ODS column was carried out. 10 mM sodium acetate buffer (pH 4.0) in water was used as the mobile phase. Flow rate was set to 0.7 ml/min, with column temperature at 25˚C and wavelength of detection, 266 nm.

Analysis of tumor growth and survival time. The suspension of 4×10^6 MIA-PaCa2 cells was injected into the dorsal part of mice subcutaneously. After 2 weeks, MU was orally administered at a dose of 2 g/kg/day, mixed with bait. 5-FU was administered via the intra-peritoneal route at a dose of 50 mg/kg/week. Tumor size of 4 groups, namely, (A) control (n=15), (B) treated with 5-FU (n=14), (C) treated with MU and 5-FU (n=11), was measured using caliper once a week. The PBS was injected into mice in the control group. The tumor volume was calculated as length × width^2 × 0.52 (12). Survival time was set to the endpoint based on UKCCCR guidelines. Statistical comparison was made by log-rank analysis of Kaplan–Meier curves (13).

Transmission electron microscopy. Several small pieces of tissue established by the transplantation of MIA PaCa-2 cells were

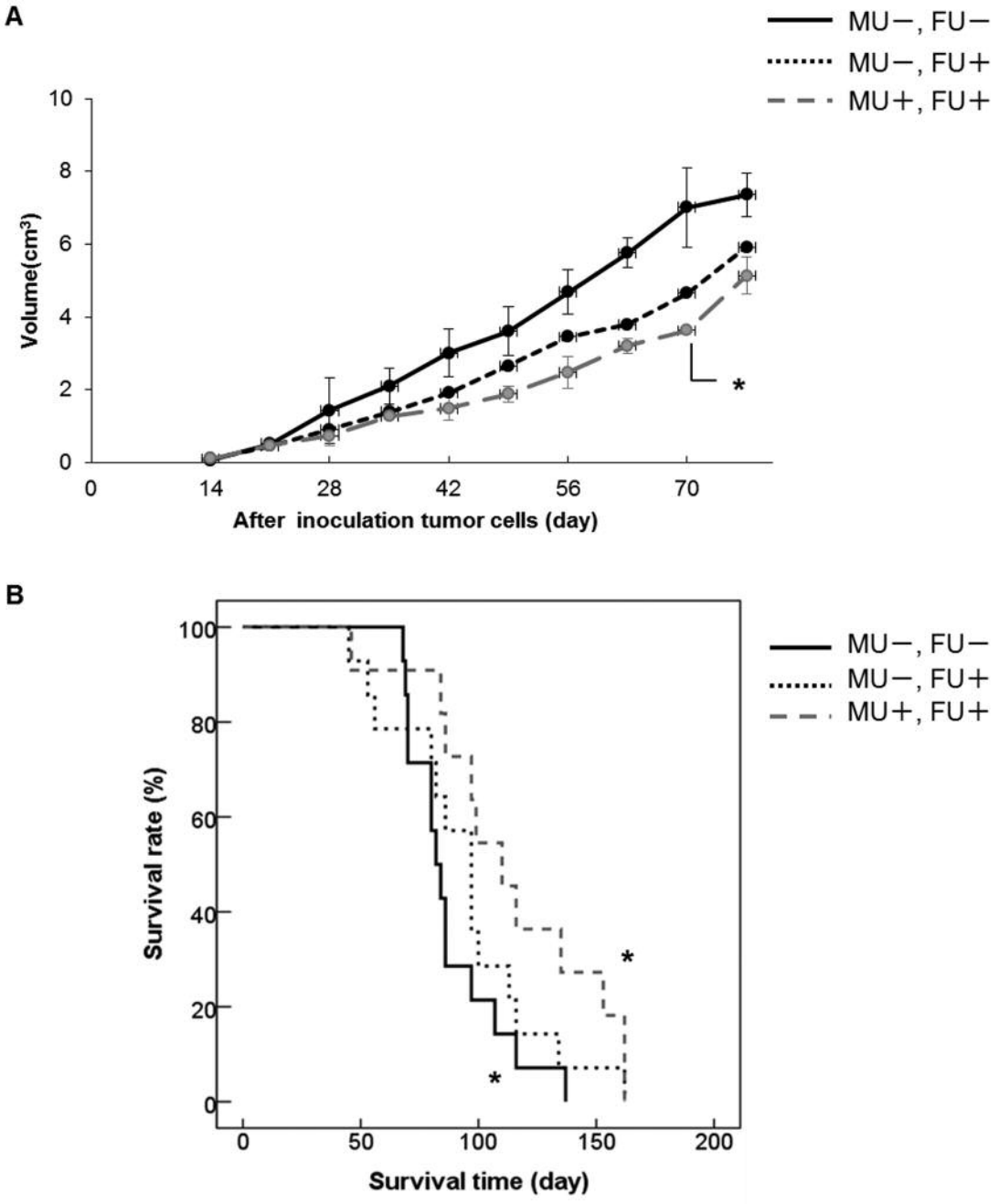

Figure 3. *Antitumor effect of MU and 5-FU. A: Change over time of tumor volume. Tumor size was measured with calipers and its volume was calculated as described in the Materials and Methods section. The data represents the means for 11-14 mice. *p<0.05 (Student's t-test, control versus 5-FU plus MU group). B: Survival time of mice. Survival time was compared by log-rank analysis of Kaplan–Meier curves. *p<0.05 (control versus 5-FU plus MU group).*

immersed in Karnovsky's fixative for over 24 h at 4 ˚C. This was then rinsed in 0.1 M cacodylate buffer (pH 7.2), and postfixed in 0.1% ruthenium red and 2% osmium tetroxide solution overnight at 4˚C. Tissues were dehydrated in a graded ethanol and embedded in Epon 812 (Oken, Tokyo, Japan). Ultrathin sections were prepared, and stained with uranyl acetate and lead citrate. These sections were examined using a JEM-1230EX transmission electron microscope (JEOL, Tokyo, Japan).

Statistical analysis. Statistical comparisons were made using the Student's *t*-test, log-rank analysis of Kaplan–Meier curves and a value of $p<0.05$ was accepted as statistically significant.

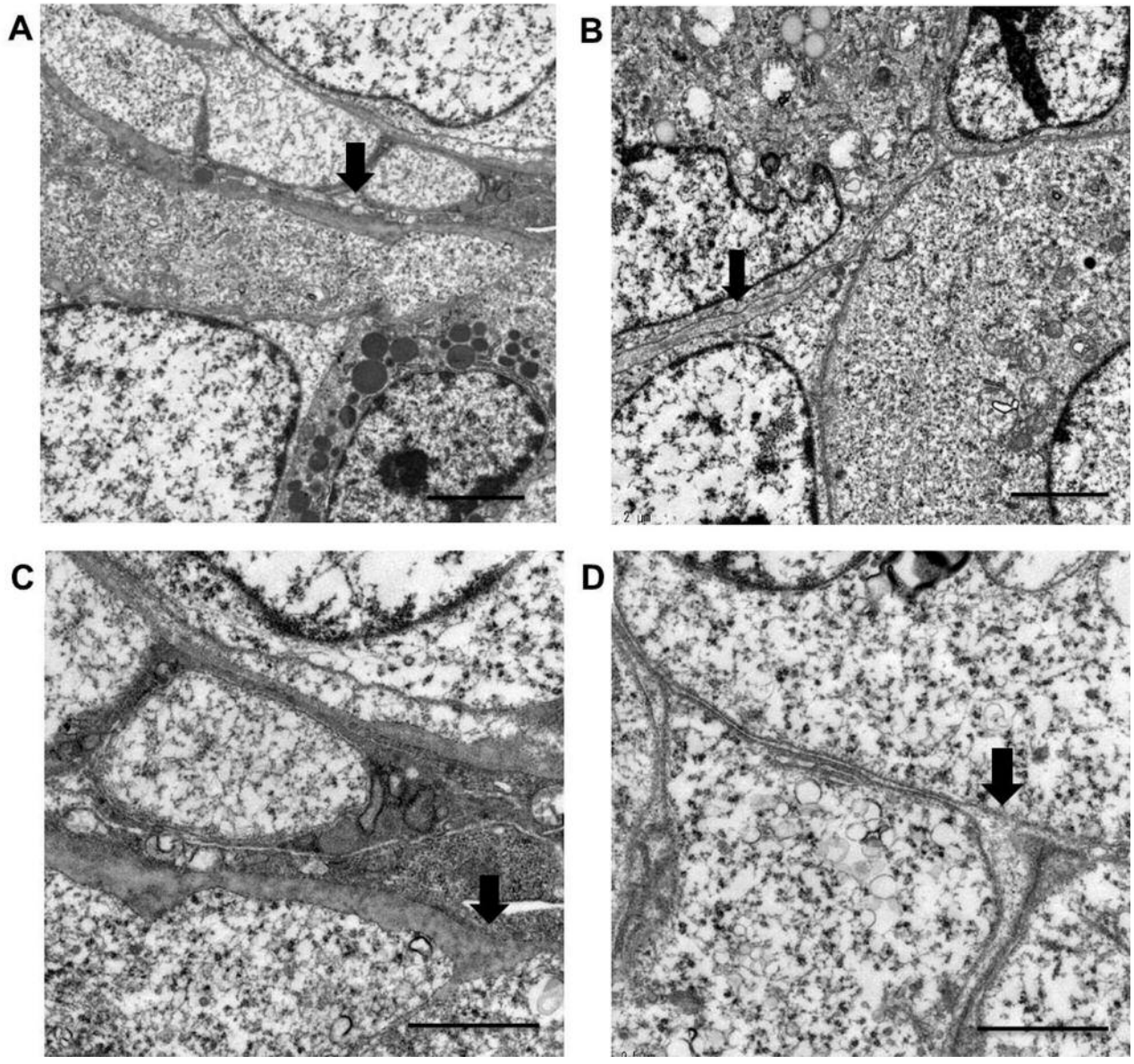

Figure 4. *Electron microscopy of tumors. The stroma of pancreatic cancer tumors. Tumors were observed by electron microscope, A and C: after 5-FU treatment; B and D: after MU and 5-FU treatment. Bar indicates 2.5 μm (A and B) and 1.0 μm (C and D). The arrow shows a stroma.*

Results

MU and 5-FU inhibited proliferation of pancreatic cancer cells. The cell proliferation of MIA-PaCa2 was inhibited by MU and 5-FU. Rate of growth inhibition was higher than 5-FU alone even after 72 h. The combination of 0.01 mM 5-FU with 0.5 mM MU inhibited cell proliferation up to 57.4% (Figure 1).

MU enhanced intracellular concentration of 5-FU in pancreatic cancer cells. HPLC analysis of the concentration of 5-FU showed that MU enhanced intracellular concentration of 5-FU. 6 h after administration of 2 mM 5-FU, cells treated with 0.5 mM MU showed 47.3 % increase in 5-FU concentration compared to the MU-untreated group (Figure 2).

MU and 5-FU decreased tumor volume and prolonged survival time in mice. There were no differences in body weight in all the three groups. The tumor volume was decreased by the administration 5-FU alone compared to the control group; furthermore, tumor volume in the 5-FU plus MU treated group was significantly decreased compared with

the control group and 5-FU treated group as well ($p<0.05$) (Figure 3A). Survival time of mice was prolonged by the administration 5-FU alone compared to the control group. Survival time of the mice treated with 5-FU plus MU was significantly prolonged compared to the control group ($p<0.05$) (Figure 3B).

5-FU plus MU increased the intercellular space in the cancer microenvironment. Electron microscopy was used to detect changes in the microenvironment surrounding inoculated MIAPaCa-2 cells. No morphological difference of the nucleus and cytoplasmic organelle was observed between the 5-FU-treated group and the 5-FU plus MU-treated group (Figure 4A and C). The intercellular space, on the other hand, was increased in the 5-FU plus MU-treated group. Mosaic-like staining pattern for ruthenium red was also observed in this group. In the cytoplasm, mitochondrial swelling was recognized in the 5-FU plus MU group in comparison with the 5-FU-alone group. The cellular shape was also found to be irregular (Figure 4B and D).

Discussion

HA is a major structural component of the stroma of many tumors. Studies have revealed that HA is involved in tumor metastasis, by playing a role in cell adhesion (14) and migration (15). This suggests that cancer cells are dependent on HA-rich environments to exert malignant potentials. We have previously shown that MU decreased cell surface HA and capsule (16). In this study, we analyzed the antitumor effect of 5-FU in combination with MU *in vitro*. It was found that the intracellular 5-FU concentration increased in the presence of MU. Thus, MU decreases a biological barrier of HA-rich ECM, increasing facilitated diffusion and leading to increased intracellular concentration of 5-FU. The combination of 5-FU with MU was found to be effective in the mice model. The tumor size was decreased, and the survival time of mice was prolonged. On the other hand, MU did not increase the side-effects of 5-FU. These results suggest that MU is useful as a chemosensitizer. We previously showed that the quantity of HA in the tumor was significantly ($p<0.05$) decreased by MU treatment (16, 17). In the current study, intercellular space was found to increase with mosaic-like staining by ruthenium red in the 5-FU plus MU-treated group compared to the 5-FU-alone group. MU enhanced the concentration of 5-FU in cancer cells, and consequently promoted its cytotoxic effect. Mikami *et al*. reported that simultaneous administration of MU and thioacetamide (TAA) increased murine hepatic carcinogenesis (18). This may be because MU decreased a biological barrier of ECM, increasing facilitated diffusion and leading to increased concentration of TAA. When MU is administered, it may be necessary to consider the order of drug administration. A treatment involving remodeling of ECM will become important in the future. Hyaluronidase from bovine testis enhanced activity of adriamycin in a breast cancer model (19). Use of hyaluronidase for the treatment of cancer has been demonstrated in several reports, but has been abandoned due to the fact that it caused an allergic reaction, inflammation or pain in the joints (20, 21). MU influences pericellular environments, but not the nucleus and cytoplasm. This is the reason why MU had low toxicity and few side-effects. These features of MU are a great advantage for its use as a chemosensitizer. In fact, MU has been used previously as a choleretic and antispasmodic agent for patients with motor disorders of duodenal papilla (22). Thus, combination therapy of MU and 5-FU might prove an effective approach to treat pancreatic cancer.

Conflicts of Interest

The Authors who took part in this study declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this article.

Acknowledgements

The Authors thank Prof. Hiroshi Kijima and Dr. Satoko Morohashi, Department of Pathology and Bioscience, Hirosaki University, Graduate School of Medicine for their excellent technical help.

References

1 Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Pere-Verge D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C and Ducreux M: FOLFIRINOX *versus* gemcitabine for metastatic pancreatic cancer. N Engl J Med *364*: 1817-1825, 2011.

2 Sironen RK, Tammi M, Tammi R, Auvinen PK, Anttila M and Kosma VM: Hyaluronan in human malignancies. Exp Cell Res *317*: 383-391, 2011.

3 Buckway B, Wang Y, Ray A and Ghandehari H: Overcoming the stromal barrier for targeted delivery of HPMA copolymers to pancreatic tumors. Int J Pharm *456*: 202-211, 2013.

4 Kakizaki I, Kojima K, Takagaki K, Endo M, Kannagi R, Ito M, Maruo Y, Sato H, Yasuda T, Mita S, Kimata K and Itano N: A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. J Biol Chem *279*: 33281-33289, 2004.

5 Kudo D, Kon A, Yoshihara S, Kakizaki I, Sasaki M, Endo M and Takagaki K: Effect of a hyaluronan synthase suppressor, 4-methylumbelliferone, on B16F-10 melanoma cell adhesion and locomotion. Biochem Biophys Res Commun *321*: 783-787, 2004.

6 Yoshihara S, Kon A, Kudo D, Nakazawa H, Kakizaki I, Sasaki M, Endo M, and Takagaki K: A hyaluronan synthase suppressor, 4-methylumbelliferone, inhibits liver metastasis of melanoma cells. FEBS Lett *579*: 2722-2726, 2005.

7 Piccioni F, Malvicini M, Garcia MG, Rodriguez A, Atorrasagasti C, Kippes N, Piedra Buena IT, Rizzo MM, Bayo J, Aquino J, Viola M, Passi A, Alaniz L and Mazzolini G: Antitumor effects of hyaluronic acid inhibitor 4-methylumbelliferone in an orthotopic hepatocellular carcinoma model in mice. Glycobiology *22*: 400-410, 2012.

8 Lompardia SL, Papademetrio DL, Mascaro M, Alvarez EM and Hajos SE: Human leukemic cell lines synthesize hyaluronan to avoid senescence and resist chemotherapy. Glycobiology *23*: 1463-1476, 2013.

9 Nakazawa H, Yoshihara S, Kudo D, Morohashi H, Kakizaki I, Kon A, Takagaki K and Sasaki M: 4-methylumbelliferone, a hyaluronan synthase suppressor, enhances the anticancer activity of gemcitabine in human pancreatic cancer cells. Cancer Chemother Pharmacol *57*: 165-170, 2006.

10 Nojiri S and Joh T: Albumin suppresses human hepatocellular carcinoma proliferation and the cell cycle. Int J Mol Sci *15*: 5163-5174, 2014.

11 Watanabe J, Hayashi Y, Iwamoto K and Ozeki S: Salivary excretion of 5-fluorouracil. I. Fluctuation of the saliva/plasma concentration ratio and salivary clearance in beagle dogs following bolus intravenous administration. Chem Pharm Bull *33*: 1187-1194, 1985.

12 Andarini S, Kikuchi T, Nukiwa M, Pradono P, Suzuki T, Ohkouchi S, Inoue A, Maemondo M, Ishii N, Saijo Y, Sugamura K and Nukiwa T: Adenovirus vector-mediated *in vivo* gene transfer of OX40 ligand to tumor cells enhances antitumor immunity of tumor-bearing hosts. Cancer Res *64*: 3281-3287, 2004.

13 Workman P, Balmain A, Hickman JA, McNally NJ, Rohas AM, Mitchison NA, Pierrepoint CG, Raymond R, Rowlatt C, Stephens TC *et al*: UKCCCR guidelines for the welfare of animals in experimental neoplasia. Lab Anim *22*: 195-201, 1988.

14 Simpson MA, Reiland J, Burger SR, Furcht LT, Spicer AP, Oegema TR Jr. and McCarthy JB: Hyaluronan synthase elevation in metastatic prostate carcinoma cells correlates with hyaluronan surface retention, a prerequisite for rapid adhesion to bone marrow endothelial cells. J Biol Chem *276*: 17949-17957, 2001.

15 Itano N, Atsumi F, Sawai T, Yamada Y, Miyaishi O, Senga T, Hamaguchi M and Kimata K: Abnormal accumulation of hyaluronan matrix diminishes contact inhibition of cell growth and promotes cell migration. Proc Natl Acad Sci USA *99*: 3609-3614, 2002.

16 Yoshida E, Kudo D, Nagase H, Shimoda H, Suto S, Negishi M, Kakizaki I, Endo M and Hakamada K: Antitumor effects of the hyaluronan inhibitor 4-methylumbelliferone on pancreatic cancer. Oncol Lett *12*: 2337-2344, 2016.

17 Kudo D, Suto A and Hakamada K: The development of a novel therapeutic strategy to target hyaluronan in the extracellular matrix of pancreatic ductal adenocarcinoma. Int J Mol Sci *18*: 600, 2017.

18 Mikami K, Endo T, Sawada N, Igarashi G, Kimura M, Sakuraba H, Fukuda S:Inhibition of systemic hyaluronan synthesis exacerbates munine hepatic carcinogenesis. In Vivo *32*: 273-278, 2018.

19 Beckenlehner K, Bannke S, Spruss T, Bernhardt G, Schonenberg H and Schiess W: Hyaluronidase enhances the activity of adriamycin in breast cancer models *in vitro* and *in vivo*. J Cancer Res Clin Oncol *118*: 591-596, 1992.

20 Baumgartner G, Gomar-Hoss C, Sakr L, Ulsperger E and Wogritsch C: The impact of extracellular matrix on the chemoresistance of solid tumors – experimental and clinical results of hyaluronidase as additive to cytostatic chemotherapy. Cancer Lett *131*: 85-99, 1998.

21 Whatcott CJ, Han H, Posner RG, Hostetter G and Von Hoff DD: Targeting the tumor microenvironment in cancer: why hyaluronidase deserves a second look. Cancer Discov *1*: 291-296, 2011.

22 Garrett ER, Venitz J, Eberst K and Cerda JJ: Pharmacokinetics and bioavailabilities of hymecromone in human volunteers. Biopharm Drug Dispos *14*: 13-39, 1993.

Received August 14, 2018
Revised September 3, 2018
Accepted September 4, 2018