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ORIGINAL ARTICLE

TWO CONTRADICTORY ROLES OF HYALURONAN IN AN EXPERIMENTAL RAT ACUTE PANCREATITIS MODEL

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Abstract Background & Aims: Hyaluronan plays a role not only as a structural component but also in the regulation of inflammatory processes. However, the role of hyaluronan in acute pancreatitis is not clear. In this study, we have explored the role of hyaluronan in a rat acute pancreatitis model using a hyaluronan synthesis inhibitor, 4-methylumbelliferone (4-MU). **Methods**: Six-week-old male Sprague-Dawley rats were fed a standard diet or the same diet containing 5% 4-MU beginning two weeks before induction of cerulean- and lipopolysaccharide-induced acute pancreatitis. The severity of acute pancreatitis and pro-inflammatory cytokine levels were evaluated with or without pretreatment with 4-MU. **Results**: Before development of acute pancreatitis, 4-MU pretreatment reduced serum hyaluronan levels. After development of acute pancreatitis, 4-MU pretreatment increased serum amylase level and blood urea nitrogen/creatinine ratio. However, 4-MU pretreatment suppressed tumor necrosis factor-α (TNF-α) production in acute pancreatitis. **Conclusions**: Systemic inhibition of hyaluronan exacerbated acute pancreatitis in spite of suppression of TNF-α production. These data suggest that hyaluronan has two completely different effects, i.e., a barrier function and as a regulator of inflammation in acute pancreatitis. The results emphasize that further analysis that takes into account the molecular size of hyaluronan is needed since it may explain the contradictory findings.

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Key words: hyaluronan; 4-methylumbelliferone; acute pancreatitis; pro-inflammatory cytokine.

Introduction

Acute pancreatitis is a common disease of the pancreas in clinical practice with rapid disease progression. Inflammation of the pancreas originates from the intracellular activation of digestive enzymes in pancreatic acinar cells[1]. Further excessive inflammatory cytokines result in systemic inflammatory response syndrome and multiple organ failure. Mild acute pancreatitis, which is seen in approximately 80% of pancreatitis patients, is usually associated with a low mortality rate. In contrast, patients with severe acute pancreatitis, who account for about 10-20% of acute pancreatitis patients, are associated with a 25-50% mortality rate despite improvements in critical care[2, 3]. Therefore, novel avenues of effective treatment need to be identified. The pathogenesis of acute pancreatitis has been extensively studied; however, the specific mechanisms underlying acute pancreatitis have still not been completely elucidated[4].

Hyaluronan is an essential component of the extracellular matrix in every tissue of the body, and it provides a favorable microenvironment for cell function such as proliferation and motility[5, 6]. Recently, hyaluronan has been recognized, beyond merely a structural component, as a dynamic substance that can participate in the regulation of inflammatory processes[7]. Low-molecular-weight hyaluronan, in particular, promotes local inflammatory responses by driving the release of pro-inflammatory cytokines[8, 9]. Thus, understanding the role of hyaluronan in the pathophysiology of inflammatory disease could allow us to develop novel therapeutics for

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critical inflammatory diseases including acute pancreatitis. However, the role of hyaluronan in acute pancreatitis has not been investigated.

4-methylumbelliferone (4-MU), a coumarin derivative, inhibits hyaluronan production by depleting cellular uridine diphosphate glucuronic acid and down-regulating hyaluronan synthase (HAS) 2 and HAS3 expression[10, 11]. As HAS2 and HAS3 produce high-molecular-weight hyaluronan and low-molecular-weight hyaluronan, respectively[12], it is speculated that 4-MU inhibits hyaluronan synthesis regardless of molecular weight. In the past, 4-MU had been used to treat functional and obstructive spasms of the biliary tract in clinical practice. Recent animal studies have shown that 4-MU treatment has other potential therapeutic properties for inflammation and autoimmunity in the lungs, kidneys, joints, and central nervous system[13].

In the present study, we evaluated the role of hyaluronan in acute pancreatitis with an experimental rat model by using 4-MU as a hyaluronan synthesis inhibitor.

Materials and methods

1. Animal and reagents

Male six-week-old Sprague-Dawley rats were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). They were housed in cages in a temperature- and humidity-controlled room with a 12-hour light/dark cycle and given free access to water and diet. All animals received humane care, and the experiment was performed in accordance with Hirosaki University's Guidelines for Animal Experimentation. 4-MU was purchased from Sigma-Aldrich (St. Louis, MO, USA). The diet containing 4-MU was pelleted by Oriental Yeast Co., Ltd. (Tokyo, Japan).

2. Animal treatments

In order to inhibit hyaluronan production, rats were given a standard diet or the same diet containing 5% 4-MU beginning two weeks before induction of pancreatitis[14]. Rats were deprived of food but were allowed access to water 24 hours before induction of acute pancreatitis. Acute pancreatitis was induced as previously described by administration of intraperitoneal injection of cerulein (100 μg/kg) and lipopolysaccharide (LPS) (30 mg/kg)[15, 16]. Rats were sacrificed before development of acute pancreatitis to assess the effect of hyaluronan inhibition in normal pancreas, or after development of acute pancreatitis to assess the effect of hyaluronan inhibition in acute pancreatitis. Rats that developed acute pancreatitis were divided into three groups. In the first group, rats were given a standard diet and saline injection (control group). In the second group, rats were given a standard diet and acute pancreatitis was induced (AP group). In the third group, rats were given a diet containing 4-MU and acute pancreatitis was induced (AP+4-MU group) (Fig. 1). To evaluate the severity of pancreatitis, rats were sacrificed 2 hours after the LPS injection, and specimens from the pancreas, jejunum, lung, ascites, and blood were rapidly harvested for the study.

3. Serum biochemical analysis

Serum amylase, blood urea nitrogen (BUN), creatinine (Cre), total protein (TP), albumin, total bilirubin (T-bil), aspartate transferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), calcium, and glucose were measured by an automated analyzer (Spotchem EZ SP-4430; Arkrey Inc., Kyoto, Japan). Hyaluronan levels were measured by latex coagulation immunonephelometry, which can detect both high- and low-molecular-weight hyaluronan. Cytokine levels in sera and ascites were measured according to the Bio-Plex cytokine assay system (Bio-Rad Laboratories, Hercules, CA, USA) to

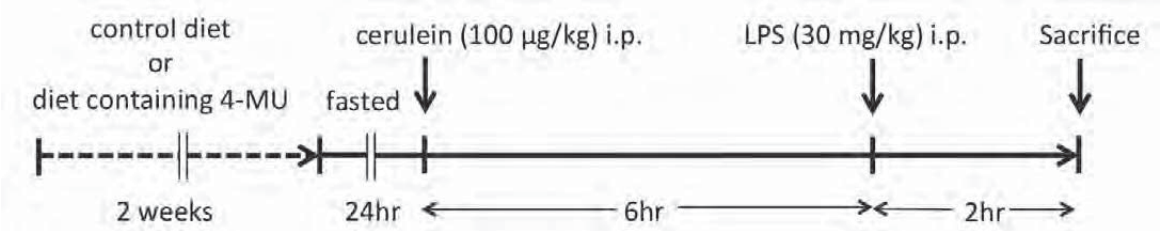

Figure 1 Experimental protocol. Rats were given a standard diet or the same diet containing 5% 4-MU beginning two weeks before induction of pancreatitis. For induction of acute pancreatitis, rats received intraperitoneal injection of cerulein (100 μg/kg body weight) after fasting for 24 hours, and in addition, rats were injected with LPS intraperitoneally (30 mg/kg body weight) 2 hours after cerulein injection. Rats were sacrificed before (after fasting for 24 hours) or after development of acute pancreatitis (2 hours after LPS injection), and samples were collected.

quantify the concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, and monocyte chemotactic protein-1 (MCP-1).

4. Histological analysis

For light microscopy, a part of the pancreas, jejunum, and lung were fixed in 10% formaldehyde neutral buffer solution and embedded in paraffin wax. Hematoxylin and eosin (H&E) staining was performed on 5-μm sections from the paraffin-embedded pancreatic specimens. The severity of acute pancreatitis was graded by a semiquantitative assessment of edema, acinar cell necrosis, and inflammatory cell infiltration as described previously[17].

5. Statistical analysis

Quantitative values are expressed as mean ± standard error of the mean (SEM). Statistical evaluations were performed using two-tailed Student's t-test. Differences were considered to indicate a significant result with *p*-values <0.05.

Results

1. Hyaluronan levels and serum pro-inflammatory cytokine levels before development of acute pancreatitis

Two-week pretreatment with 4-MU reduced body weight in rats (Fig. 2a). The serum hyaluronan level in rats fed a standard diet alone (62.7 ± 4.1 ng/mL) was lower than that in rats fed a diet containing 4-MU (49.7 ± 3.1 ng/mL), although the difference was not statistically significant (P=0.06) (Fig. 2b). In terms of other biochemical data (amylase, TP, albumin, T-bil, AST, ALT, LDH, BUN, Cre, calcium, and glucose), there were no significant differences between rats fed a standard diet alone and rats fed a diet containing 4-MU (data not shown). The serum MCP-1 level was significantly higher in rats fed a diet containing 4-MU (1205.2 ± 108.9 pg/mL) as compared with rats fed a standard diet (675.8 ± 33.2 pg/mL) (P<0.05). The serum IL-1β level was the same in rats fed a standard diet and those fed a diet containing 4-MU. However, TNF-α, IL-6, and IL-10 were not detected in serum in rats in either of the two groups (Fig. 2c).

2. Exacerbation of acute pancreatitis by 4-MU pretreatment

As shown in Fig. 3a, rats that received intraperitoneal injection of cerulein and LPS developed edematous pancreatitis. In terms of local inflammation findings, edema of the pancreas, ascites, and redness of the duodenum were seen. However, any apparent differences were not clear between the AP group and the 4-MU+AP group. In terms of histological findings (Fig. 3b), both the AP group and the 4-MU+AP group showed edema formation

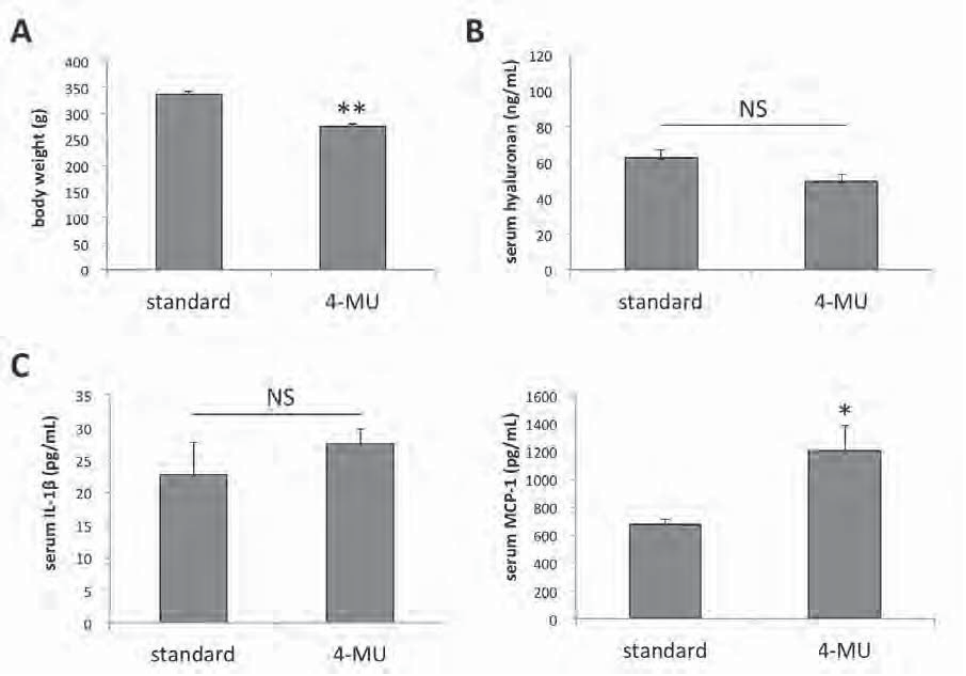

Figure 2 Body weight, serum hyaluronan, and serum cytokines before development of acute pancreatitis in rats fed a standard diet or fed a diet containing 5% 4-MU. Two-week pretreatment with 4-MU reduced the body weight (a) and serum hyaluronan level (b). On the other hand, two-week pretreatment with 4-MU increased the serum MCP-1 level in rats (c) (n=3 in each group). Significant differences were observed in body weight and serum MCP-1 level (a, c) ($^{*}P<0.05$ and $^{**}P<0.01$, respectively, compared with rats fed a standard diet). However, serum hyaluronan level was not significant (P=0.06) (b). Abbreviation: NS, not significant.

and cell infiltration in the pancreas. However, histological scoring for acute pancreatitis did not show a statistical significance between these two groups. No pathological findings in the jejunum and lung were observed in either the control group or the 4-MU+AP group. Cerulein and LPS injection significantly increased the serum amylase level in the AP group (14500 ± 3823 IU/mL) as compared with the control group (2770 ± 120 IU/mL) ($P<0.05$). Moreover, the serum amylase level in the 4-MU+AP group (35275 ± 2700 IU/mL) was significantly higher than that in the AP group ($P<0.01$) (Fig. 4a). BUN/Cre ratio was almost the same in the control group (21.8 ± 1.7) and the AP group (25.7 ± 1.7) after cerulein and LPS injection ($P=0.2$), while it was elevated in the 4-MU+AP group (49.4 ± 8.0) as compared with the control group or the AP group ($P<0.05$ compared with both groups) (Fig. 4b). In terms of other biochemical data (TP, albumin, T-bil, AST, ALT, LDH, calcium, and glucose), no differences were observed among the control group, AP group, and 4-MU+AP group (data not shown). As shown Fig. 4c, cerulein and LPS injection significantly increased the serum hyaluronan level in both the AP group (102.3 ± 9.9 ng/mL) and the 4-MU+AP group (102.8 ± 11.0 ng/mL) as compared with the control group (59.0 ± 1.5 ng/mL) ($P<0.05$ in both groups). However, there was no difference between the AP group and the 4-MU+AP group.

3. Suppression of TNF-α production in acute pancreatitis by 4-MU pretreatment

As shown in Fig. 5, cerulean and LPS injection significantly increased serum levels of pro-inflammatory cytokines in rats. Although pretreatment with 4-MU suppressed the elevation of serum pro-inflammatory cytokine

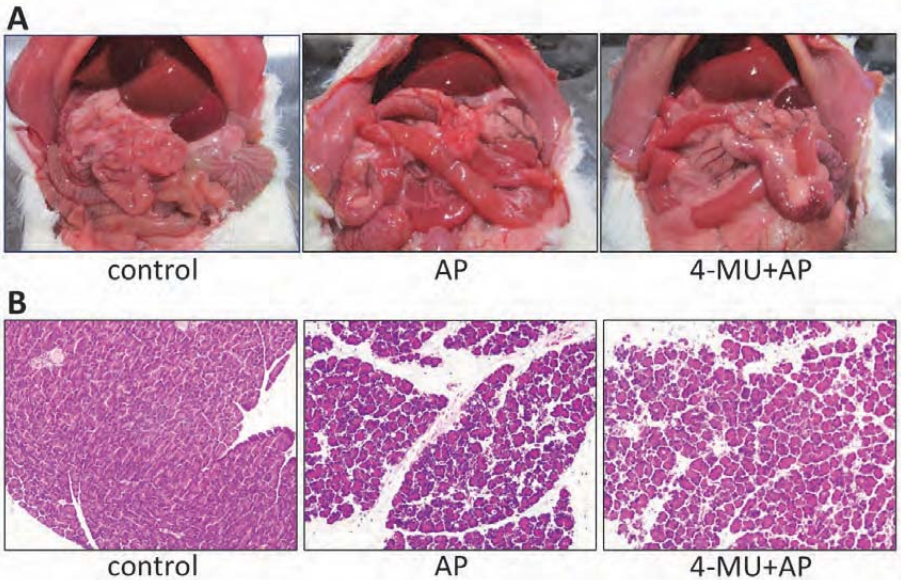

Figure 3 Macroscopic and microscopic findings after development of acute pancreatitis in rats. After cerulein and LPS injection, edema of the pancreas, ascites, and redness of the duodenum were observed macroscopically (a), and edema formation and cell infiltration were identified microscopically. H&E staining (original magnification, ×200) (b). However, any apparent differences were not clear with pretreatment with 4-MU in terms of macroscopic and microscopic findings (n=3 in control; n=4 in AP and 4-MU+AP).

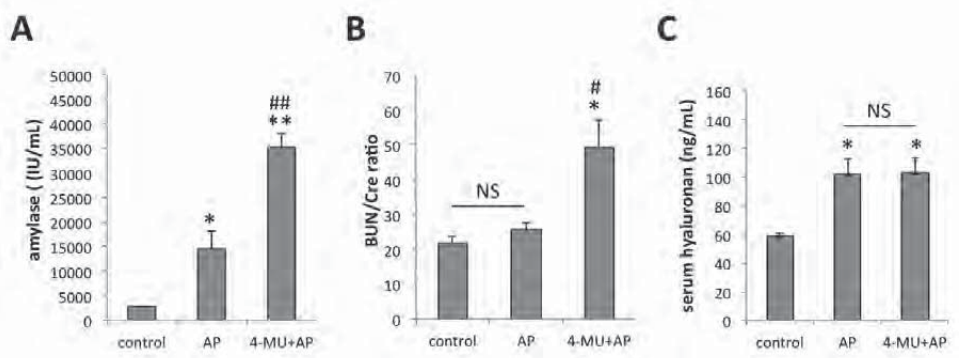

Figure 4 Serum amylase level, BUN/Cre ratio, and serum hyaluronan level after development of acute pancreatitis. (a) Serum amylase level was increased after cerulein and LPS injection. Moreover, pretreatment with 4-MU enhanced the increment of serum amylase level ($^{*}P<0.05$ and $^{**}P<0.01$ compared with control, $^{\#\#}P<0.01$ compared with AP). (b) Pretreatment with 4-MU increased BUN/Cre ratio after cerulean and LPS injection ($^{*}P<0.05$ compared with control, $^{\#}P<0.05$ compared with AP). (c) Serum hyaluronan level was increased after cerulean and LPS injection ($^{*}P<0.05$ compared with control). However, there was no difference between AP and 4-MU+AP (n=3 in control; n=4 in AP and 4-MU+AP). Abbreviation: NS, not significant.

levels, there were no statistically significant differences between the AP group and the 4-MU+AP group (P=0.09 for TNF-α, P=0.07 for IL-1β, P=0.06 for IL-6, P=0.09 for IL-10, P=0.31 for MCP-1). On the other hand, in the case of ascites, pretreatment with 4-MU markedly suppressed the elevation of TNF-α (AP group: 610.0 ± 164.5 pg/mL, 4-MU+AP group: 111.2 ± 15.7 pg/mL, P<0.05). However, the levels of other pro-inflammatory cytokines (IL-1β, IL-6, IL-10, and MCP-1) in ascites did not differ between the AP group and the 4-MU+AP group (Fig. 6). Ascites was not detected in the control group.

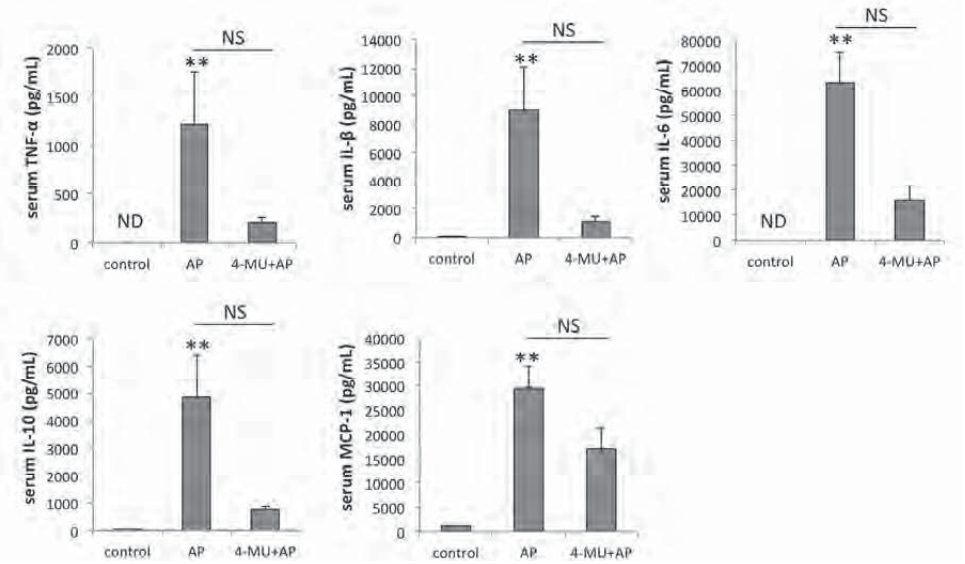

Figure 5 Pro-inflammatory cytokine levels in serum after development of acute pancreatitis. Pro-inflammatory cytokine levels in serum were significantly increased after cerulein and LPS injection (**P<0.01 compared with control). Pretreatment with 4-MU suppressed the elevation of serum pro-inflammatory cytokine levels. However, there was no statistically significant difference between AP and 4-MU+AP (n=3 in control; n=4 in AP and 4-MU+AP). Abbreviations: ND, not detectable; NS, not significant.

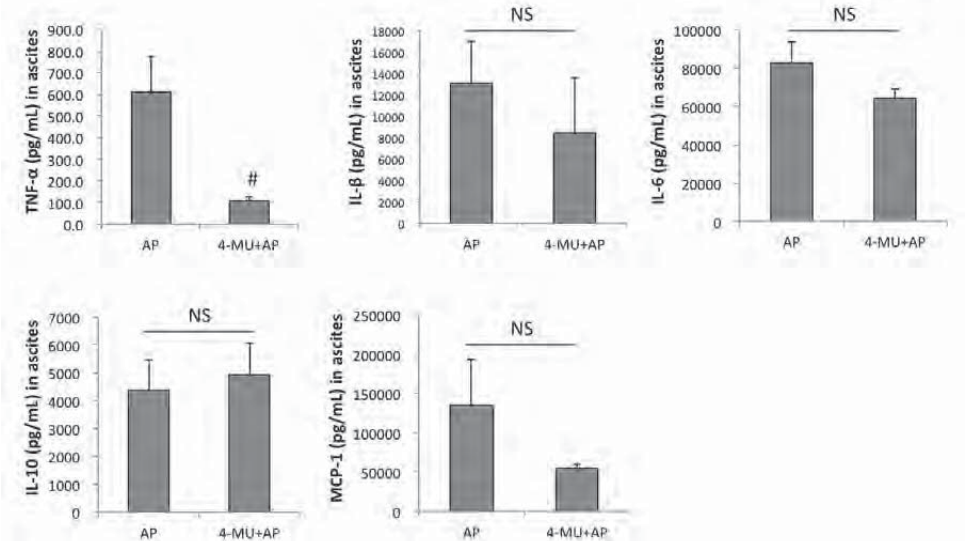

Figure 6 Pro-inflammatory cytokine levels in ascites after development of acute pancreatitis. Pretreatment with 4-MU significantly suppressed the elevation of TNF-α after cerulein and LPS injection (#P<0.05 compared with AP). However, the levels of other pro-inflammatory cytokines (IL-1β, IL-6, IL-10, and MCP-1) in ascites did not differ between AP and 4-MU+AP (n=4 in AP and 4-MU+AP). Abbreviations: ND, not detectable; NS, not significant.

Discussion

The present study is the first to investigate the mode in which inhibition of hyaluronan synthesis affects acute pancreatitis with the use of a rat model. We were able to demonstrate that systemic inhibition of hyaluronan synthesis exacerbated acute pancreatitis with elevation of the serum amylase level. On the other hand, it was clearly shown that inhibition of hyaluronan synthesis by 4-MU suppressed the production of TNF-α in

acute pancreatitis. In other words, pancreatic inflammation was enhanced in spite of the suppression of pro-inflammatory cytokines. While at first it seems to be a paradoxical event, this fact might be due to complicated biological functions dependent on differences in the molecular size of hyaluronan[18].

Recent studies showed that changes in the molecular size of hyaluronan have functional consequences; namely, that the differential molecular size has a different biological impact on microenvironment homeostasis and injury responses[7, 18]. Specifically, high-molecular-weight hyaluronan (over 1000 kDa), predominantly present in normal tissues, creates stable extracellular matrices contributing to barrier function and has anti-inflammatory properties[19-21]. Conversely, low-molecular-weight hyaluronan (below 500 kDa), which is fragmented by tissue damage, drives local inflammation, inducing pro-inflammatory cytokines via Toll-like receptors or CD44 signaling[7, 22]. With respect to hyaluronan synthesis, high-molecular-weight hyaluronan is synthesized by HAS2, while low-molecular-weight hyaluronan is synthesized by HAS1 and HAS3[12]. As 4-MU inhibits hyaluronan synthesis through HAS2 and HAS3 inhibition[10, 11], it is considered that 4-MU inhibits both high- and low-molecular-weight hyaluronan. In the present study, 2-week pretreatment with 4-MU induced MCP-1 production, which is speculated to have inhibited high-molecular-weight hyaluronan synthesis. Therefore, to understand the role of hyaluronan in the pathophysiologic condition, it is important to take into account not only the amount but also the molecular size of hyaluronan in tissue injury. Although our results showed a paradoxical effect, there is a possibility that the molecular size of hyaluronan in the pancreas was changed in our experiments by using 4-MU. Actually, the inhibition of hyaluronan synthesis has shown both protective and oppressive effects in several animal inflammatory disease models[23-26]. Unfortunately, however, we could not analyze differences in the molecular size of hyaluronan in the present study due to limitations at our experimental facility at the moment.

4-MU is an approved oral agent in Europe and Asia for the treatment of biliary spasm. Owing to the safety of 4-MU in clinical practice, hence, many studies aimed at clinical applications using 4-MU for various diseases have been reported[12, 27, 28]. In the present study, pretreatment with 4-MU suppressed TNF-α production, which is considered a beneficial effect with clinical implications. A previous study showed that the increment of serum pro-inflammatory cytokines such as IL-6 and TNF-α was associated with multiple organ dysfunction syndrome and a high mortality rate in severe acute pancreatitis[29]. Thus, pro-inflammatory cytokines may be novel targets for the treatment of acute pancreatitis[30]. With respect to the molecular mechanism of cytokine suppression, a recent study reported that 4-MU had an anti-inflammatory effect not only through hyaluronan inhibition but also through antioxidant activity independent of hyaluronan inhibition[31]. Thus, there is a possibility that 4-MU suppressed TNF-α production directly independent of hyaluronan inhibition in our study, as well. Regarding the barrier function, pretreatment with 4-MU increased the elevation of serum amylase levels and BUN/Cre ratio after development of acute pancreatitis, which means the aggravation of pancreatic inflammation and the increment of vascular permeability, respectively. These adverse events might be due to disruption of the barrier function with hyaluronan synthesis inhibition by 4-MU. Therefore, for the purpose of clinical application of 4-MU, it is important to pay attention to an unfavorable effect like barrier dysfunction with 4-MU[26]. In any case, however, 4-MU has been used safely in clinical practice for a long period

of time. Thus, 4-MU will be a promising agent for clinical application for various inflammatory diseases and cancer.

In summary, systemic inhibition of hyaluronan synthesis aggravates pancreatic inflammation, as opposed to suppression of the production of pro-inflammatory cytokines. These results indicate that hyaluronan affects both the barrier function and the immune system in acute pancreatitis. For therapeutic interventions targeting hyaluronan in acute pancreatitis, it is important to consider the multiple effects of hyaluronan.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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