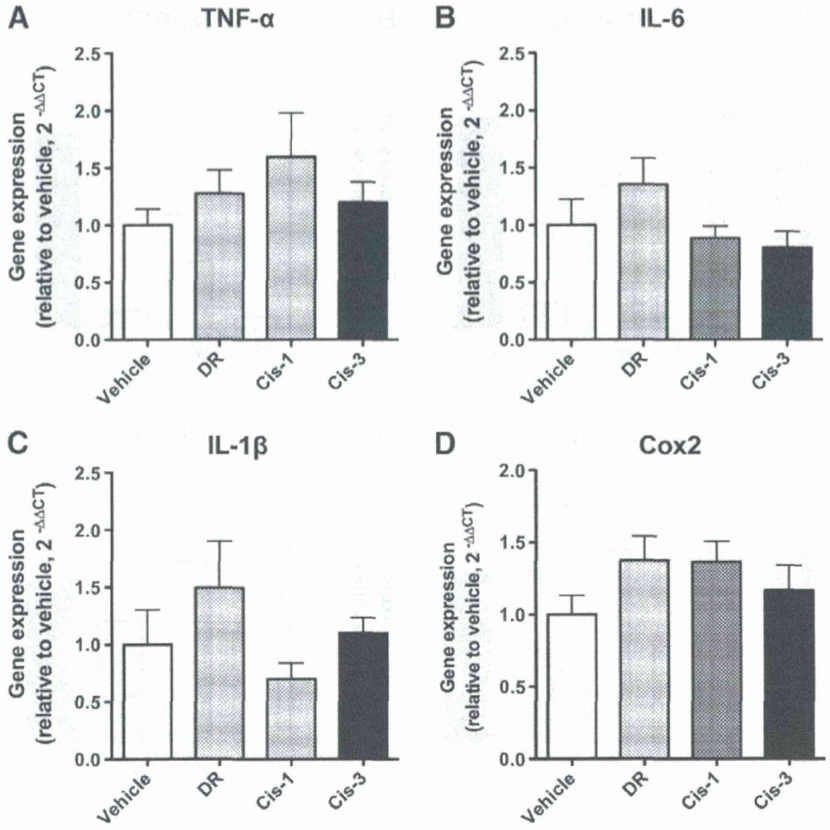


Fig. 4. Effects of DR and cisplatin on the gene expression of TNF-α (A), IL-6 (B), IL-1β (C) and Cox2 (D) in the quadriceps muscle of mice. Each value represents the mean ± S.E.M. from 4 independent mice.

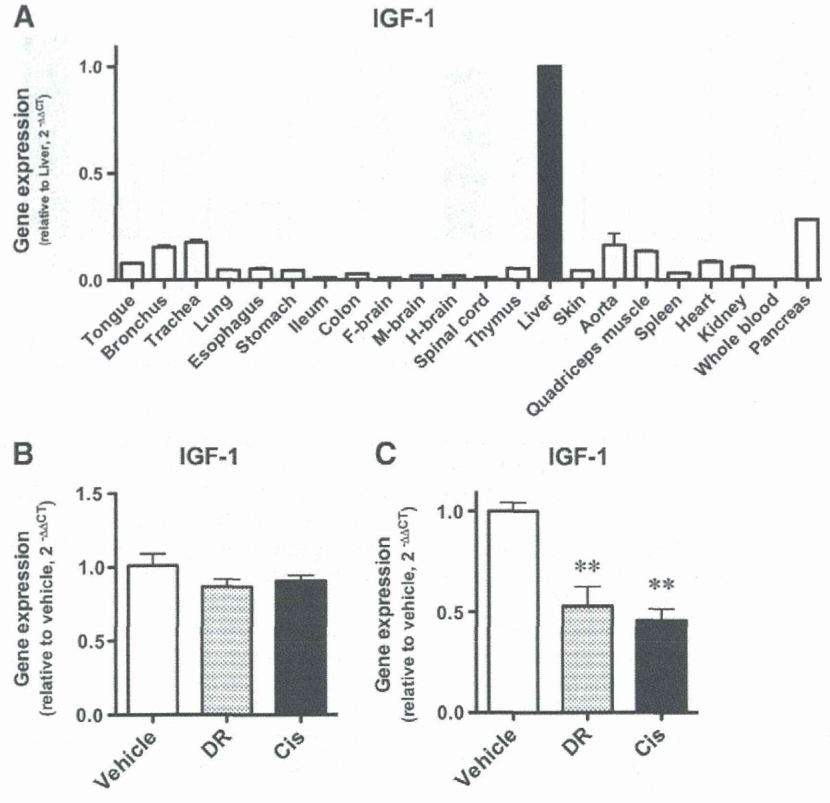


Fig. 5. Effects of DR and cisplatin on the gene expression of IGF-1 in quadriceps muscle of mice. Tissue gene distribution of IGF-1 in the various tissues of mice (A). Gene expression is shown as $2^{-\text{deltadeltaCT}}$ values, and indicates the fold-amount relative to that in the liver. Effects of DR and cisplatin on the gene expression of IGF-1 in the liver (B) and quadriceps muscle (C) of mice. Each value represents the mean ± S.E.M. from 4 to 8 independent mice. **$p < 0.01$ vs. vehicle control (saline).

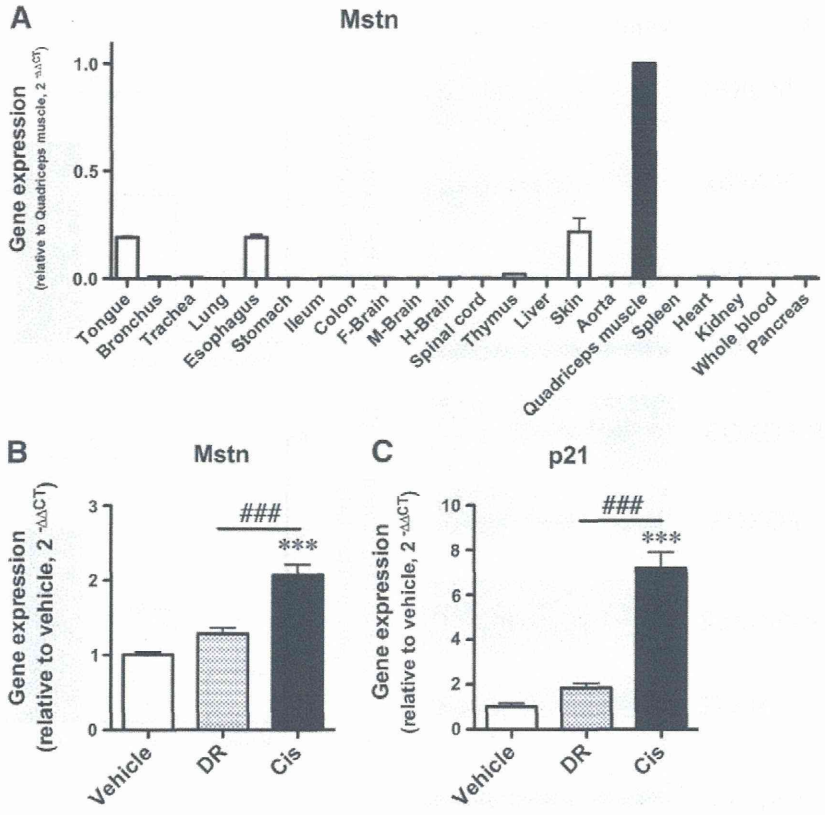


Fig. 6. Effects of DR and cisplatin on the gene expression of Mstn in quadriceps muscle of mice. Tissue gene distribution of myostatin (Mstn) in various tissues of mice (A). Gene expression is shown as $2^{-\text{deltadeltaCT}}$ values and indicates the fold-amount relative to that in the quadriceps muscle. Effects of DR and cisplatin on the gene expression of Mstn (B) and p21 (C) in quadriceps muscle of mice. Each value represents the mean ± S.E.M. from 4 to 8 independent mice. ***$p < 0.001$ vs. vehicle control (saline). ###$p < 0.001$ vs. DR.

DR in this study. Taken together, it is possible that the muscle atrophy is responsible for augmentation of Mstn expression induced by cisplatin. It is well known that the administration of cisplatin causes neuropathy (Jaggi and Singh, 2012). Furthermore, denervation or nerve injury is believed to upregulate Mstn signaling (Liu et al., 2007). Against this background, we hypothesized that peripheral neuropathy by cisplatin accompanied by the activation of Mstn signaling pathways could, at least in part, contribute to cisplatin-induced muscle atrophy. Therefore, ActRIIB antagonism can be a new approach for cisplatin-induced muscle atrophy.

A recent study revealed an unexpected connection between TNF-α signaling and MuRF1/MAFbx expression. Specifically, treatment with TNF-α has been reported to upregulate MuRF1 and MAFbx in skeletal muscle (Bonaldo and Sandri, 2013; Minetti et al., 2011). Therefore, we examined the possible changes in the mRNA expression of inflammatory cytokines and cyclooxygenase2 (COX2) in the DR and cisplatin groups. Damrauer et al. (2008) has been reported that cisplatin is able to induced NF-κB activity in mouse muscles, suggesting that a side effect of cisplatin treatment is the regulation of muscle atrophy, which is independent of the commonly implicated ubiquitin proteasome system. However, there were no differences in the mRNA levels of TNF-α, IL-6, IL-1β and Cox2 among the groups in the present study. These findings suggested that absence of proinflammatory cytokine activation NF-κB signaling pathway is most likely not upregulated under condition of the present study. Although it is unclear about this inconsistency in detail, it may be difference of an administration route or dose of cisplatin.

It has been reported that unloading stress results in skeletal muscle atrophy through the induction and activation of Cbl-b (Nakao et al., 2009). Cbl-b induces the ubiquitination and degradation of insulin receptor substrate-1 (IRS-1), the IGF-1 signaling intermediate. In turn, the loss of IRS-1 causes the FOXO3-dependent induction of muscle atrophy MAFbx. It has also been demonstrated that Cbl-b-deficient mice are resistant to unloading-induced atrophy and loss of muscle function (Nakao et al., 2009). On the other hand, recent studies have demonstrated that the expression and activity of TRAF6 are increased in distinct models of muscle atrophy (Kumar et al., 2012). Muscle-specific ablation of TRAF6 inhibits the induction of atrophy in response to starvation, denervation, or cancer cachexia. In the present study, the mRNA levels of Cbl-b and TRAF6 in the quadriceps muscle were increased by DR. However, cisplatin did not further increase the levels of Cbl-b and TRAF6.

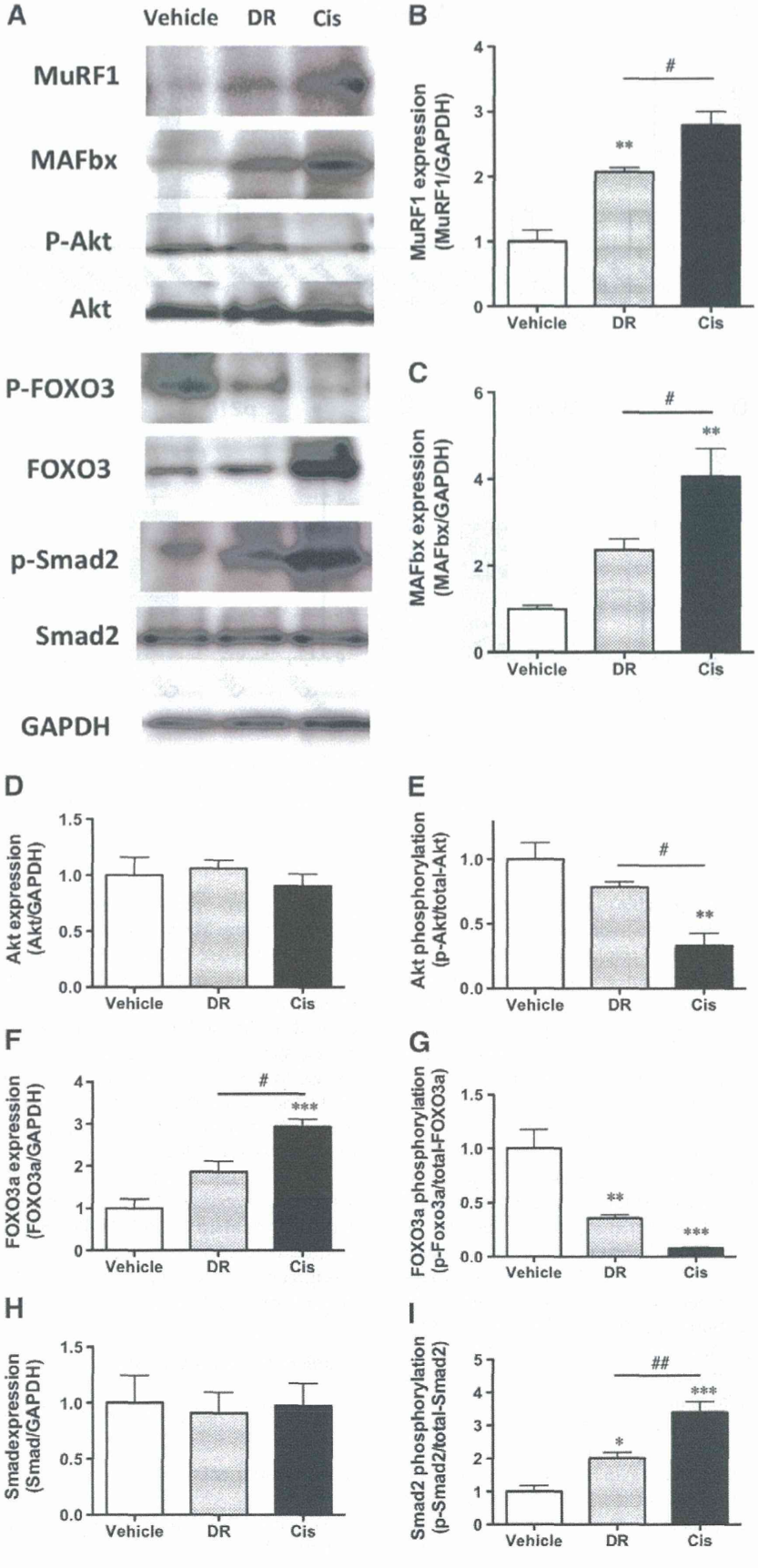
In conclusion, the administration of cisplatin that induced the loss of body weight without TNFα-dependent inflammation strongly activated the Mstn/ActRIIB/MuRF1/MAFbx pathways associated with ubiquitination and the degradation of proteins in muscle, thereby resulting in muscle atrophy. This muscle atrophy may, at least in part, explain cisplatin-induced muscle fatigue.

Conflict of interest statement

The authors have no conflict of interest.

Acknowledgments

We thank Mr Satoshi Hasegawa, Mr Tetsuro Shoji, Ms Tomomi Yamada, Ms Aya Oguchi and Ms Yuko Ishikawa for their technical assistance. This work was supported by KAKENHI, a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (24790582).



References

Banerjee, A., Guttridge, D.C., 2012. Mechanisms for maintaining muscle. Curr. Opin. Support. Palliat. Care 6, 451–456.

Bender, C.M., Sereika, S.M., Berga, S.L., Vogel, V.G., Brufsky, A.M., Paraska, K.K., Ryan, C.M., 2006. Cognitive impairment associated with adjuvant therapy in breast cancer. Psychooncology 15, 422–430.

Bodine, S.C., Latres, E., Baumhueter, S., Lai, V.K., Nunez, L., Clarke, B.A., Poueymirou, W.T., Panaro, F.J., Na, E., Dharmarajan, K., Pan, Z.Q., Valenzuela, D.M., DeChiara, T.M., Stitt, T.N., Yancopoulos, G.D., Glass, D.J., 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294, 1704–1708.

Bonaldo, P., Sandri, M., 2013. Cellular and molecular mechanisms of muscle atrophy. Dis. Model. Mech. 6, 25–39.

Cella, D., Lai, J.S., Chang, C.H., Peterman, A., Slavin, M., 2002. Fatigue in cancer patients compared with fatigue in the general United States population. Cancer 94, 528–538.

Costelli, P., Muscaritoli, M., Bonetto, A., Penna, F., Reffo, P., Bossola, M., Bonelli, G., Doglietto, G.B., Baccino, F.M., Rossi Fanelli, F., 2008. Muscle myostatin signalling is enhanced in experimental cancer cachexia. Eur. J. Clin. Invest. 38, 531–538.

Damrauer, J.S., Stadler, M.E., Acharyya, S., Baldwin, A.S., Couch, M.E., Guttridge, D.C., 2008. Chemotherapy-induced muscle wasting: association with NF-κB and cancer cachexia. Basic Appl. Myol. 18, 139–148.

Foletta, V.C., White, L.J., Larsen, A.E., Leger, B., Russell, A.P., 2011. The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. Pflugers Arch. 461, 325–335.

Fujimoto, S., 2006. Schedule-dependent antitumor activity and toxicity of combinations of 5-fluorouracil and cisplatin/carboplatin against L1210 leukemia-bearing mice. Biol. Pharm. Bull. 29, 2260–2266.

Glass, D.J., 2010. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. Curr. Top. Microbiol. Immunol. 346, 267–278.

Gomes, M.D., Lecker, S.H., Jagoe, R.T., Navon, A., Goldberg, A.L., 2001. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc. Natl. Acad. Sci. U. S. A. 98, 14440–14445.

Jaggi, A.S., Singh, N., 2012. Mechanisms in cancer-chemotherapeutic drugs-induced peripheral neuropathy. Toxicology 291, 1–9.

Jansen, C.E., Cooper, B.A., Dodd, M.J., Miaskowski, C.A., 2011. A prospective longitudinal study of chemotherapy-induced cognitive changes in breast cancer patients. Support. Care Cancer 19, 1647–1656.

Khasabova, I.A., Khasabov, S., Paz, J., Harding-Rose, C., Simone, D.A., Seybold, V.S., 2012. Cannabinoid type-1 receptor reduces pain and neurotoxicity produced by chemotherapy. J. Neurosci. 32, 7091–7101.

Kumar, A., Bhatnagar, S., Paul, P.K., 2012. TWEAK and TRAF6 regulate skeletal muscle atrophy. Curr. Opin. Clin. Nutr. Metab. Care 15, 233–239.

Lecker, S.H., Solomon, V., Mitch, W.E., Goldberg, A.L., 1999. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J. Nutr. 129, 227S–237S.

Lee, S.J., 2008. Genetic analysis of the role of proteolysis in the activation of latent myostatin. PLoS One 3, e1628.

Liu, M., Zhang, D., Shao, C., Liu, J., Ding, F., Gu, X., 2007. Expression pattern of myostatin in gastrocnemius muscle of rats after sciatic nerve crush injury. Muscle Nerve 35, 649–656.

Lynch, G.S., 2001. Therapies for improving muscle function in neuromuscular disorders. Exerc. Sport Sci. Rev. 29, 141–148.

McPherron, A.C., Lawler, A.M., Lee, S.J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387, 83–90.

Minetti, G.C., Feige, J.N., Rosenstiel, A., Bombard, F., Meier, V., Werner, A., Bassilana, F., Sailer, A.W., Kahle, P., Lambert, C., Glass, D.J., Fornaro, M., 2011. Galphai2 signaling promotes skeletal muscle hypertrophy, myoblast differentiation, and muscle regeneration. Sci. Signal. 4, ra80.

Montazeri, A., 2009. Quality of life data as prognostic indicators of survival in cancer patients: an overview of the literature from 1982 to 2008. Health Qual. Life Outcomes 7, 102.

Nakao, R., Hirasaka, K., Goto, J., Ishidoh, K., Yamada, C., Ohno, A., Okumura, Y., Nonaka, I., Yasutomo, K., Baldwin, K.M., Kominami, E., Higashibata, A., Nagano, K., Tanaka, K., Yasui, N., Mills, E.M., Takeda, S., Nikawa, T., 2009. Ubiquitin ligase Cbl-b is a negative regulator for insulin-like growth factor 1 signaling during muscle atrophy caused by unloading. Mol. Cell. Biol. 29, 4798–4811.

Patrick, D.L., Ferketich, S.L., Frame, P.S., Harris, J.J., Hendricks, C.B., Levin, B., Link, M.P., Lustig, C., McLaughlin, J., Reid, L.D., Turrisi III, A.T., Unutzer, J., Vernon, S.W., 2004. National Institutes of Health State-of-the-Science Conference Statement: symptom management in cancer: pain, depression, and fatigue, July 15–17, 2002. J. Natl. Cancer Inst. Monogr. 9–16.

Paul, P.K., Bhatnagar, S., Mishra, V., Srivastava, S., Darnay, B.G., Choi, Y., Kumar, A., 2012. The E3 ubiquitin ligase TRAF6 intercedes in starvation-induced skeletal muscle atrophy through multiple mechanisms. Mol. Cell. Biol. 32, 1248–1259.

Prue, G., Allen, J., Gracey, J., Rankin, J., Cramp, F., 2010. Fatigue in gynecological cancer patients during and after anticancer treatment. J. Pain Symptom Manage. 39, 197–210.

Reardon, K.A., Davis, J., Kapsa, R.M., Choong, P., Byrne, E., 2001. Myostatin, insulin-like growth factor-1, and leukemia inhibitory factor mRNAs are upregulated in chronic human disuse muscle atrophy. Muscle Nerve 24, 893–899.

Rebbapragada, A., Benchabane, H., Wrana, J.L., Celeste, A.J., Attisano, L., 2003. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. Mol. Cell. Biol. 23, 7230–7242.

Rios, R., Fernandez-Nocelos, S., Carneiro, I., Arce, V.M., Devesa, J., 2004. Differential response to exogenous and endogenous myostatin in myoblasts suggests that myostatin acts as an autocrine factor in vivo. Endocrinology 145, 2795–2803.

Sakai, H., Sagara, A., Matsumoto, K., Hasegawa, S., Sato, K., Nishizaki, M., Shoji, T., Horie, S., Nakagawa, T., Tokuyama, S., Narita, M., 2013. 5-Fluorouracil induces diarrhea with changes in the expression of inflammatory cytokines and aquaporins in mouse intestines. PLoS One 8, e54788.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S.H., Goldberg, A.L., 2004. FOXO transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117, 399–412.

Seder, C.W., Hartojo, W., Lin, L., Silvers, A.L., Wang, Z., Thomas, D.G., Giordano, T.J., Chen, G., Chang, A.C., Orringer, M.B., Beer, D.G., 2009. Upregulated INHBA expression may promote cell proliferation and is associated with poor survival in lung adenocarcinoma. Neoplasia 11, 388–396.

Stitt, T.N., Drujan, D., Clarke, B.A., Panaro, F., Timofeyva, Y., Kline, W.O., Gonzalez, M., Yancopoulos, G.D., Glass, D.J., 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol. Cell 14, 395–403.

Stromgren, A.S., Goldschmidt, D., Groenvold, M., Petersen, M.A., Jensen, P.T., Pedersen, L., Hoermann, L., Helleberg, C., Sjogren, P., 2002. Self-assessment in cancer patients referred to palliative care: a study of feasibility and symptom epidemiology. Cancer 94, 512–520.

Suzuki, N., Motohashi, N., Uezumi, A., Fukada, S., Yoshimura, T., Itoyama, Y., Aoki, M., Miyagoe-Suzuki, Y., Takeda, S., 2007. NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. J. Clin. Invest. 117, 2468–2476.

Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J., Kambadur, R., 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J. Biol. Chem. 275, 40235–40243.

Wanner, A., Abraham, W.M., Douglas, J.S., Drazen, J.M., Richerson, H.B., Ram, J.S., 1990. NHLBI Workshop Summary. Models of airway hyperresponsiveness. Am. Rev. Respir. Dis. 141, 253–257.

Wildi, S., Kleeff, J., Maruyama, H., Maurer, C.A., Buchler, M.W., Korc, M., 2001. Overexpression of activin A in stage IV colorectal cancer. Gut 49, 409–417.

Wu, H.S., McSweeney, M., 2004. Assessing fatigue in persons with cancer: an instrument development and testing study. Cancer 101, 1685–1695.

Yarasheski, K.E., Bhasin, S., Sinha-Hikim, I., Pak-Loduca, J., Gonzalez-Cadavid, N.F., 2002. Serum myostatin-immunoreactive protein is increased in 60–92 year old women and men with muscle wasting. J. Nutr. Health Aging 6, 343–348.

Zhou, X., Wang, J.L., Lu, J., Song, Y., Kwak, K.S., Jiao, Q., Rosenfeld, R., Chen, Q., Boone, T., Simonet, W.S., Lacey, D.L., Goldberg, A.L., Han, H.Q., 2010. Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. Cell 142, 531–543.

Zinna, E.M., Yarasheski, K.E., 2003. Exercise treatment to counteract protein wasting of chronic diseases. Curr. Opin. Clin. Nutr. Metab. Care 6, 87–93.

Fig. 7. Effects of DR and cisplatin on the levels of expression of MuRF1 and MAFbx, and phosphorylation of Akt, FOXO3 and Smad2 in quadriceps muscle of mice. Typical photographs of bands for MuRF1, MAFbx, phospho-Akt, total-Akt, phospho-FOXO3a, total-FOXO3a, phospho-Smad2, total-Smad2 and GAPDH (A). Values are the means ± S.E.M. of 4 independent experiments (B–G). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. vehicle control. #$p < 0.05$ and ##$p < 0.01$ vs DR.

Pharmacological Research 87 (2014) 71–79

Contents lists available at ScienceDirect

Pharmacological Research

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Neutrophil recruitment is critical for 5-fluorouracil-induced diarrhea and the decrease in aquaporins in the colon

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ARTICLE INFO

Article history:
Received 2 April 2014
Received in revised form 14 May 2014
Accepted 28 May 2014
Available online 25 June 2014

Keywords:
5-Fluorouracil
Diarrhea
Neutrophil recruitment
Neutrophil elastase
Aquaporin
Colon

ABSTRACT

Diarrhea is a common side effect experienced by cancer patients undergoing clinical chemotherapy, such as with 5-fluorouracil (5-FU). However, the precise mechanisms underlying 5-FU-induced diarrhea remain unclear. In the present study, we examined the role of neutrophil in 5-FU-induced diarrhea. Mice were given 5-FU (50 mg/kg, i.p.) daily for 4 days. Sivelestat sodium (100 or 300 mg/kg, i.p., neutorophil elastase inhibitor) or SB225002 (3 or 9 mg/kg, i.p., CXCR2 antagonist) was administered before the administration of 5-FU. Gene expression levels of aquaporin (AQP) 4 and 8, CXCL1, CXCL2, CXCL3, neutrophil elastase (Elane) and myeloperoxidase (MPO) in the colon were examined by real-time RT-PCR. The neutrophil (Ly-6G positive cell) number in the mucosa of colon was measured by flow-cytometric analysis. Administration of 5-FU induced diarrhea and decreased the expression levels of AQP 4 and 8 in the colon. Under the present conditions, the expression levels of CXCL1, CXCL2, CXCL3, the neutrophil markers Elane and MPO, as well as Ly-6G-positive neutrophils, in the colon were significantly increased by 5-FU. Neutrophil recruitment with decreased levels of AQP 4 and 8 were dramatically inhibited by either sivelestat sodium or SB225002. Furthermore, these reagents reduced the 5-FU-induced body weight loss and diarrhea. These findings provide evidence that neutrophil recruitment and neutrophil elastase may decrease the levels of AQP 4 and 8 in the colon of mice treated with 5-FU and contribute to the pathophysiology of 5-FU-induced body weight loss and diarrhea.

Introduction

Diarrhea is a common side effect experienced by cancer patients undergoing clinical chemotherapy [1,2]. 5-fluorouracil (5-FU) is widely used to treat malignant tumors due to its ability to improve the tumor-free status and survival rates [3]. However, serious side effects which include severe diarrhea [1,4,5] induced by 5-FU often necessitate a decrease in the drug dose or even a discontinuation of treatment, which may threaten the success of cancer chemotherapy.

The gastrointestinal (GI) mucositis induced by 5-FU chemotherapy is a consequence of abnormal inflammatory responses that lead to intestinal malabsorption and dysfunctions [6]. The mechanisms that underlie the chemotherapy-mediated induction of mucositis are still poorly understood, but may be associated with the production of proinflammatory cytokines such as TNF-α and IL-1β [7,8]. However, we recently reported that etanercept, a TNF-α inhibitor, significantly reduced the 5-FU-induced increase in gene expression levels of IL-1β, IL-6, IFN-γ, IL-17A and IL-22 in the colon of mouse, and exacerbated 5-FU-induced diarrhea [9].

We recently reported that the genes of aquaporins (AQPs) 4 and 8 were mainly expressed in the murine colon [9]. It is widely thought that AQPs are involved in diseases that are characterized by alterations in water transport. The regulation of transepithelial fluid transport in the GI tract is based on ion transport and

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http://dx.doi.org/10.1016/j.phrs.2014.05.012
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water transport by AQPs [10]. Diarrhea is a common symptom of patients with inflammatory bowel disease (IBD), and a reduction in the expression of AQPs appears to be associated with increased disease activity in patients with ulcerative and Crohn's colitis [11]. Defects in water absorption in the GI tract are important factors in the pathogenesis of diarrhea. The changes in AQP expression in diseases of the digestive system have been useful for understanding the functions of AQPs. Recently, we demonstrated that the expression levels of AQP 4 and 8 in the intestines were significantly decreased by treatment with 5-FU [9].

Growth-related oncogenes (GROs; GROα; CXCL1, GROβ; CXCL2 and GROγ; CXCL3) are all CXC chemokines, which have neutrophil-activating and neutrophil-chemoattracting properties similar to those of interleukin-8 (IL-8; CXCL8). Neutrophils are innate inflammatory cells in chronic inflammatory diseases and are attracted to the site of inflammation via chemoattractants, such as IL-8 in humans and CXCL1 or CXCL2 in mice [12]. CXCL1, CXCL2 and CXCL3 bind to the chemokine receptor CXCR2, which is predominantly expressed in neutrophils. Activated neutrophils release proteases, which contribute to tissue injury.

Neutrophil elastase is a major secretory product from activated neutrophils and a major contributor to tissue destruction in inflammatory diseases such as acute respiratory distress syndrome (ARDS), lung emphysema, and so on [13,14]. It has been demonstrated that the plasma neutrophil elastase level is associated with the clinical activity of ulcerative colitis. Sivelestat sodium, a specific synthetic inhibitor of neutrophil elastase has been shown to have a protective effect against neutrophil-mediated tissue injury in some animal models, including lung injury, neurologic damage after spinal cord injury, and collagen-induced arthritis [15–18]. In the present study, we speculated that neutrophilic inflammation may cause diarrhea with changes in AQP expression. To investigate the role of neutrophils in 5-FU-induced diarrhea, we examined the effects of sivelestat sodium and a selective CXCR2 antagonist on the development of diarrhea and the downregulation of AQP4 and 8 expression with the administration of 5-FU in the mouse.

Materials and methods

Animals

Male C57BL/6J mice (8–9 weeks of age, 23–27 g) were used. All experiments were approved by the Animal Care Committee at Hoshi University (Tokyo, Japan).

Treatment protocol

Mice were given a single intraperitoneal injection of 5-fluorouracil (5-FU; 50 mg/kg) daily for 4 days, with saline (vehicle) used as a control (Fig. 1A). Twenty-four hour after the final injection of 5-FU (Day 3), animals were killed under deep anesthesia with isoflurane, and the proximal colon, transverse colon, and distal colon were removed, washed with cold saline, and stored in TRI Reagent™ (Sigma–Aldrich) at −80 °C. Sivelestat sodium (100 or 300 mg/kg, Ono Pharmaceutical Co., Ltd.) or SB225002 (3 or 9 mg/kg, CXCR2 antagonist, Cayman Chemical Co., Ltd.) was administered intraperitoneally 30 min before the administration of 5-FU on Days 0–4. Previous study indicated that sivelestat sodium (100 mg/kg/day, i.p.) prevented the development of DSS-induced colitis in mice. Therefore we used doses of 100 and 300 mg/kg/day, i.p. [19]. On the other hand, Manjavachi [20] previously showed that the intraneural injection of CXCL1 in the mouse sciatic nerve elicited long-lasting mechanical hyperalgesia, which was prevented by SB225002 (3 mg/kg, i.p.). Therefore, 3 and 9 mg/kg, i.p. of SB225002 were used in this study.

Diarrhea assessment

A diarrhea score was determined for each mouse. Diarrhea assessment was performed by four blind investigators, and their data were averaged. The severity of diarrhea was scored using the following scale, 0: normal (normal stool), 1: minimal (soft stool), 2: slight (slightly wet and soft stool), 3: moderate (wet and unformed stool with moderate perianal a staining of the coat), 4: severe (watery stool with severe perianal staining of the coat). The incidence of each diarrhea score (0–4) and the average diarrhea score were used to evaluate the severity of diarrhea.

Real-time RT-PCR

Gene expression levels of AQP 4 and 8, CXCL1, CXCL2, CXCL3, Elane and MPO were examined by real-time RT-PCR as described previously [9]. Briefly, total RNA was extracted from various tissues with a one-step guanidinium–phenol–chloroform extraction procedure using TRI Reagent™ (Sigma–Aldrich). cDNAs were prepared from total RNA (1.0 μg) by using QuantiTect Reverse Transcriptase (Qiagen, Germany) after incubation with gDNA wipeout buffer at 42 °C for 3 min to remove contaminating genomic DNA. The reaction mixture (2 μL) was subjected to PCR (50 nM forward and reverse primers, Fast SYBR Green Mastermix; Applied Biosystems) in a final volume of 10 μL. The PCR primer sets used are shown in Table 1. The thermal cycle profile used was (1) denaturing for 30 s at 95 °C, and (2) annealing for 30 s at 60 °C. PCR amplification was performed for 40 cycles. Data are expressed as the expression relative to GAPDH mRNA as a housekeeping gene using the $2^{-\text{deltadeltaCT}}$ method.

Flow cytometry analysis

Colonic mucosal layer, containing epithelial, goblet, immune and various cells, obtained from mouse was incubated in Hanks' balanced salt solution containing 10 mM HEPES (pH7.3), 1 mg/ml collagenase D (Roche Diagnostics, Penzberg, Germany) and 5 μg/ml DNase I (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 20 min with gentle agitation, and filtered. The cells were incubated with a phycoerythrin-labeled anti-mouse Ly-6G antibody (Miltenyi Biotech, Auburn, CA, USA) and a fluorescein isothiocyanate-labeled anti-mouse Epcam antibody (Miltenyi Biotech, Auburn, CA, USA), and subjected to flow cytometry analyses using FACS Verse (BD, Franklin Lakes, NJ, USA).

Immunohistochemical study

Mouse colonic tissue preparation and immunohistochemical procedures were performed as described by Matsumoto et al. [21]. In the frozen section of mouse distal colon, Ly-6B.2 immunoreactivities were detected by indirect staining with rat Ly-6B.2 (1:100; AbD Serotec, Raleigh, NC, USA), respectively. To visualize the each marker labeling, sections were then incubated with fluorescein tetramethylrhodamine isothiocyanate (1:400; Jackson Immunoresearch Laboratories, West Grove, PA, USA). In control experiments, the neutrophil antibody was omitted from the staining procedures to verify the specificity of the staining. No immunolabeling was observed in these controls.

Statistical analysis

The statistical significance of differences was determined by an unpaired Student *t*-test or one-way analysis of variance (ANOVA) with the Bonferroni/Dunn post hoc-test. A value of $p < 0.05$ was considered significant.

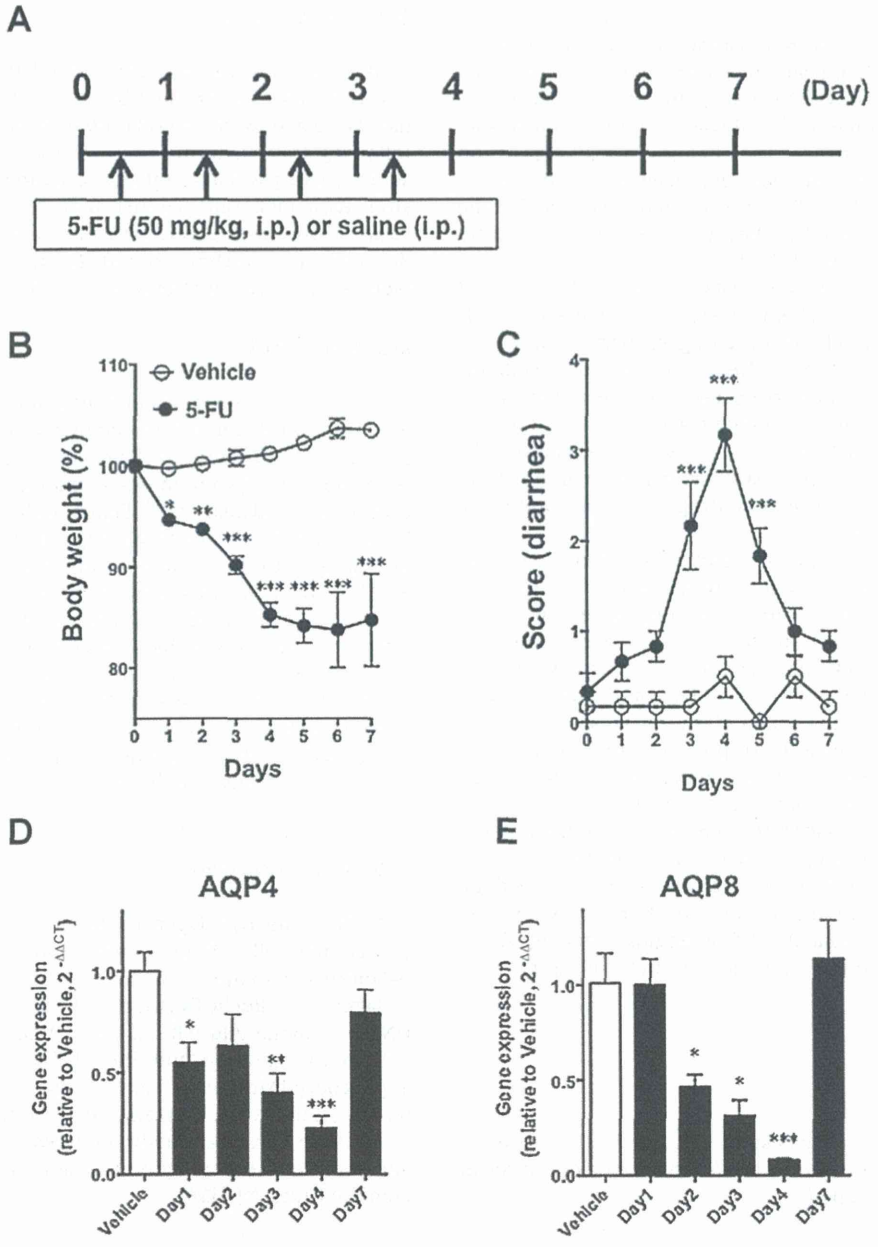

Fig. 1. Schedule for the administration of 5-fluorouracil (5-FU) and the effects of 5-FU on body weight and the diarrhea score. (A) 5-FU (50 mg/kg, i.p.) or vehicle (saline, i.p.) was administered on Days 0–3. (B) Body weight decreased significantly under the repeated administration of 5-FU. (C) Changes in the diarrhea score during and after 5-FU-administration. The diarrhea score significantly increased with 5-FU. Each point represents the mean ± S.E.M. of 6–8 mice. $^{*}p < 0.05$, $p < 0.01$ and $^{***}p < 0.001$ vs. vehicle (saline). Effect of the administration of 5-FU on the expression of AQP 4 and 8 in the distal colon of mouse. 5-FU decreased the expression of the genes for AQP 4 (D) and 8 (E). Each column represents the mean ± S.E.M. of 4–8 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. vehicle (saline).

Results

5-FU-induced body weight loss and diarrhea

The body weight of mice gradually and significantly decreased under the administration of 5-FU (Fig. 1B). The diarrhea scores significantly increased with the administration of 5-FU on Days 3–5 (Fig. 1C). Although the 5-FU-induced increase in the diarrhea score was decreased by the withdrawal of 5-FU, the body weight loss did not recover. The gene expression levels of AQP4 and 8 in the distal colon were gradually and significantly decreased by the administration of 5-FU until Day 4. Furthermore, the downregulation of AQP4 and 8 gene expressions by 5-FU was recovered by the withdrawal of 5-FU at Day 7 (Fig. 1D and E). These findings were associated with diarrhea scores.

5-FU-induced neutrophil recruitment in the colon

To investigate the role of neutrophil-chemoattracting chemokines in 5-FU-induced diarrhea, we examined the changes in the gene expression of CXCL1, CXCL2 and CXCL3 in the colon. The gene expression levels of CXCL1, CXCL2 and CXCL3 were significantly increased by the administration of 5-FU in the proximal, transverse and distal colon. Furthermore, these increases were recovered with the withdrawal of 5-FU (at Day 7) (Fig. 2). To investigate neutrophil recruitment in the colon of 5-FU-treated

Table 1
PCR primers used in the present study.

	Accession number		Primers deoxyribonucleotide sequences	Product size (base pairs)
GAPDH	NM_008084.2	Forward	CCTCGTCCCGTAGACAAAATG	100
		Reverse	TCTCCACTTTGCCACTGCAA	
AQP4	NM_009700.2	Forward	CCTGATGTGGAGCTCAAACGT	130
		Reverse	CGGGCTTCAGGATCAAGTCTT	
AQP8	NM_007474.2	Forward	GTGTGTATGGGTGCTGTCAATGA	110
		Reverse	CAGGCTCCAGAGATGCTACCA	
CXCL1	NM_008176.3	Forward	GCTCCCTTGGTTCAGAAAATTG	97
		Reverse	TCACCAGACAGGTGCCATCA	
CXCL2	NM_009140.2	Forward	CCTGCCAAGGGTTGACTTCA	105
		Reverse	TTTTGACCGCCCTTGAGAGT	
CXCL3	NM_203320.2	Forward	AGGCCCCAGGCTTCAGATAAT	103
		Reverse	AATGCAGGTCCTTCATCATGGT	
Elane	NM_015779.2	Forward	GAGCGCACTCGACAGACCTT	110
		Reverse	ATGGTAGCGGAGCCATTGAG	
MPO	BC053912.1	Forward	GAGCCAGCTACCCGGTTCTC	100
		Reverse	GGTCATTGGGTGGGATCTTG	

mice, we investigated the changes in the gene expression levels of neutrophil markers, neutrophil elastase (Elane) and myeloperoxidase (MPO) in the distal colon. The gene expression of Elane in the distal colon was significantly increased by the administration of 5-FU (Fig. 3A). MPO gene expression was also increased by the administration of 5-FU (Fig. 3B) at Days 3 and 4. Furthermore, Ly-6G-positive neutrophil cells in the colonic mucosa were markedly increased by the administration of 5-FU at Day 4 based on the results of flow cytometry analysis (Fig. 3C–E). With the use of an immunohistochemical technique, we clearly observed that the administration of 5-FU markedly increased Ly-6B.2-positive neutrophil cells in colonic mucosa (Fig. 3F and G).

The effect of SB225002 and sivelestat sodium on the 5-FU-induced diarrhea

Finally, we investigated the effect of a neutrophil elastase inhibitor, sivelestat sodium, and a CXCR2 antagonist, SB225002, on the 5-FU-induced body weight loss and diarrhea. Sivelestat sodium (300 mg/kg) and SB225002 (9 mg/kg) each attenuated the 5-FU-induced body weight loss. Furthermore, these reagents dramatically inhibited 5-FU-induced diarrhea (Fig. 4). Under the present conditions, the 5-FU-induced downregulation of AQP4 and 8 gene expression was recovered by the administration of sivelestat sodium (300 mg/kg) or SB225002 (9 mg/kg) (Fig. 5). The

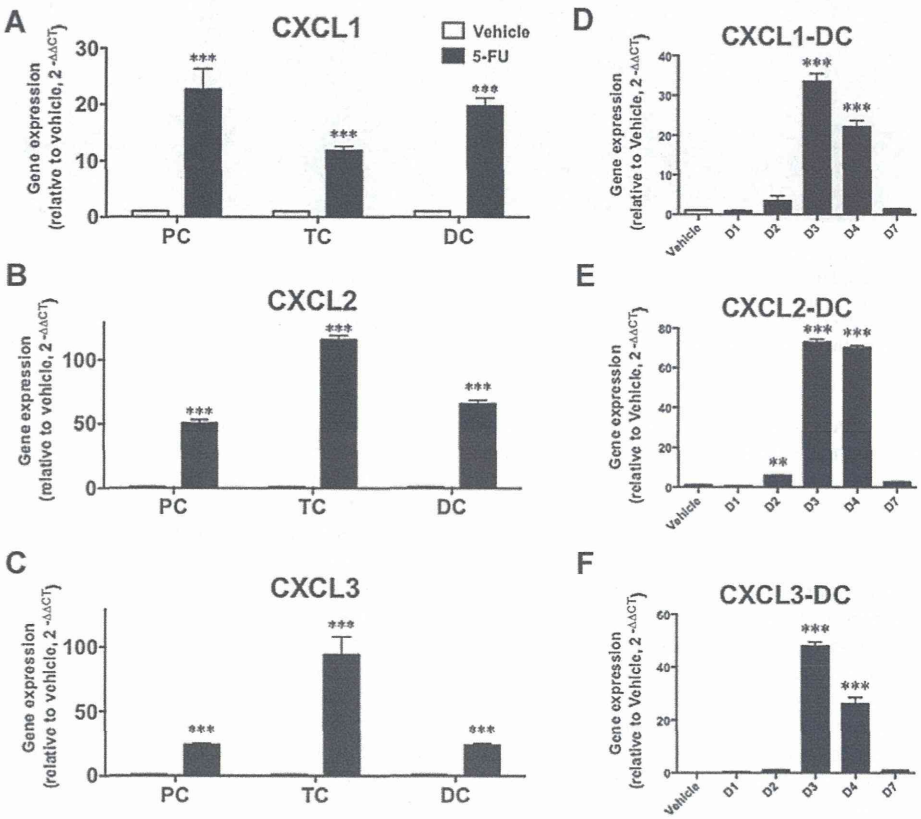

Fig. 2. Changes in gene expression of CXCL1, 2 and 3 in the colon of mouse treated with 5-FU. Treatment with 5-FU increased the gene expression of CXCL1 (A and D), CXCL2 (B and E) and CXCL3 (C and F) in different parts of the colon and on different Days. PC, TC and DC represent the proximal colon, transverse colon and distal colon, respectively. Each column represents the mean ± S.E.M. of 3–6 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. vehicle (saline).

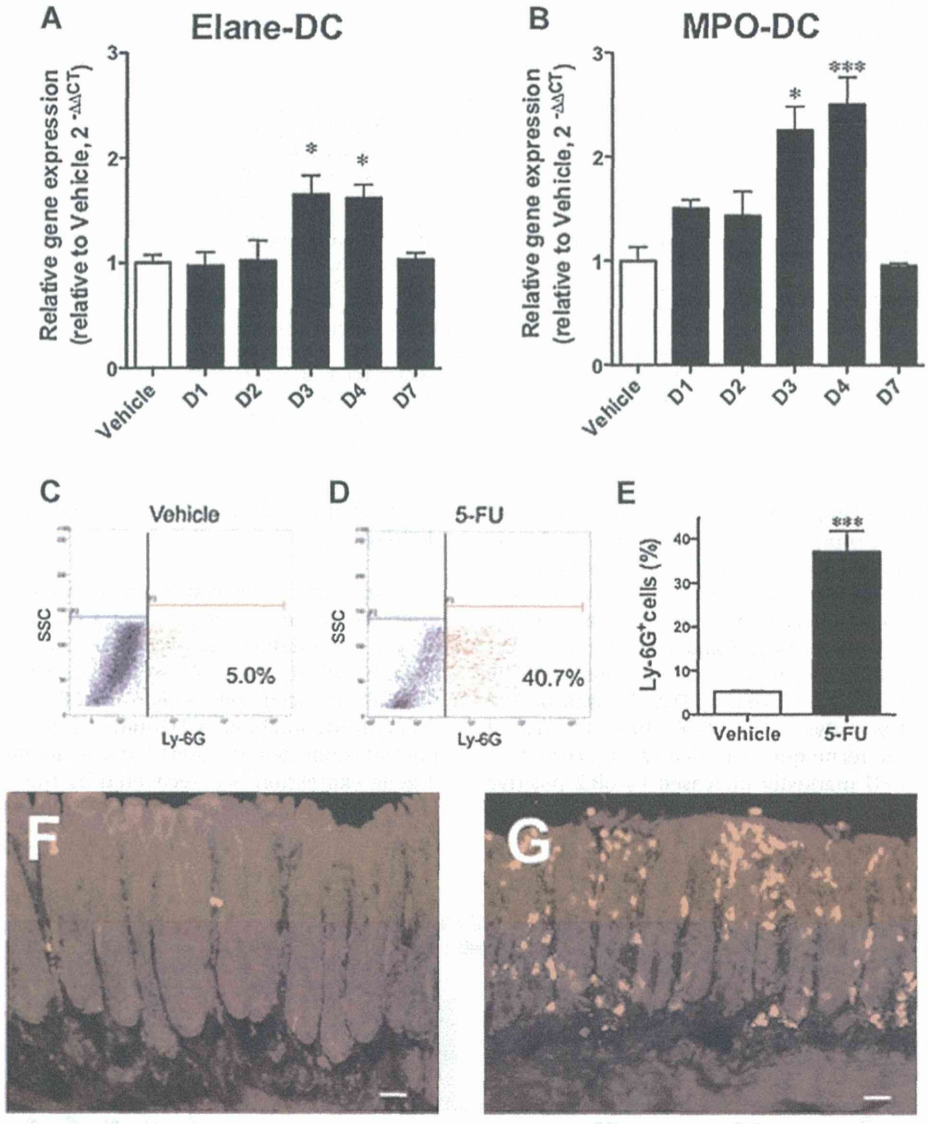

Fig. 3. Changes in the gene expression of neutrophil elastase (Elane) and myeloperoxidase (MPO) in the colon of mouse treated with 5-FU. Treatment with 5-FU increased the gene expression of Elane (A) and MPO (B) in the distal colon (DC) on different Days. Flow-cytometric analysis of the neutrophil number in the colon of mouse treated with 5-FU at Day 4. Ly-6G$^+$ cells (neutrophil marker) were increased by the administration of 5-FU (D), compared to vehicle control (C). These data are summarized in E. Each column represents the mean ± S.E.M. of 3–6 independent experiments. $^{***}p < 0.001$ vs. vehicle (saline). 5-FU increased expression of Ly-6B.2 immunopostive cells in mucosa of mouse distal colon. Immunohistochemical staining of Ly-6B.2 in transverse sections of colonic mucosa obtained from mice treated with vehicle (F) and 5-FU (G).

administration of either sivelestat sodium or SB225002 also reduced the 5-FU-induced increase in the number of Ly-6G-positive cells in mucosa of the colon (Fig. 6).

Discussion

In the present study, we found that 5-FU-induced body weight loss did not recover with the withdrawal of 5-FU, whereas the withdrawal of 5-FU was clearly associated with a discontinuation of diarrhea. Although further studies are required, we hypothesize that the administration of 5-FU could have long-lasting negative effects on energy homeostasis or reduce food intake. On the other hand, the present study provides evidence that treatment with 5-FU may directly induce loose and watery bowel movements.

The results obtained from the present study indicated that CXCR2 antagonist and neutrophil elastase ameliorated 5-FU (50 mg/kg, i.p.)-induced intestinal diarrhea. Consistent with the findings from previous studies [4,5,22], repeated dosing of mice with 5-FU caused severe intestinal mucositis, included diarrhea. In clinical setting of 5-FU sole administration, 5-FU is administered (5–15 mg/kg, i.v.) once per day during five days, and then 5-FU is administered with (5–7.5 mg/kg, i.v.) once per day every other day referenced by package leaflet of 5-FU Injection 250 (Kyowa, Kyowa Hakko Kirin). Although it is difficult to compare the clinical dose and the dose used in this study, it is expected that there is no extreme difference in both doses.

It has been reported that AQP 4 plays a role in the transcellular movement of water across surface colonocytes [23]. Furthermore, the inhibition of AQP 8 expression by small interfering RNA has been shown to significantly decrease water absorption in the rat colon [24]. Changes in the levels of various AQPs in intestinal tissues have been reported in animal models and human diseases. The expression levels of AQP 4 and AQP 8 are significantly decreased immediately after exposure to dextran sodium sulphate (DSS) in a mouse model of colitis. Rapid decreases in AQP 4 and 8 are associated with changes in colonic water transport, which leads to a shift

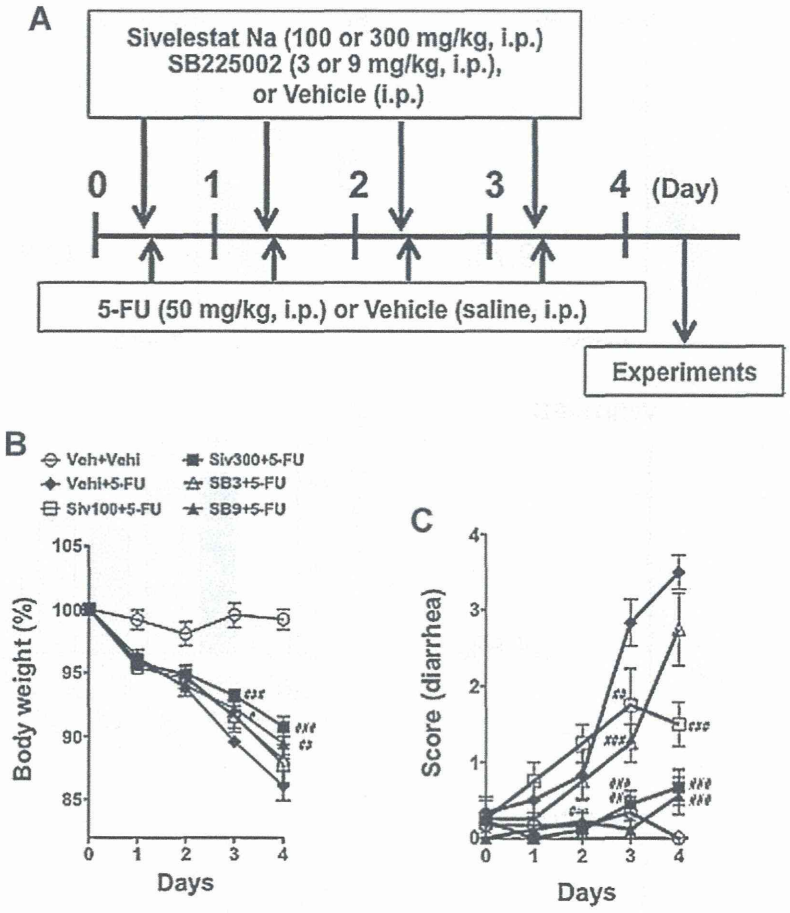

Fig. 4. Effects of the administration of the neutrophil elastase inhibitor sivelestat sodium or the CXCR2 antagonist SB225002 on the 5-FU-induced changes in body weight and the diarrhea score. (A) Thirty minutes before the administration of 5-FU, sivelestat sodium (100 or 300 mg/kg, i.p.; Siv100 or Siv300), SB225002 (3 or 9 mg/kg, i.p.; SB3 or SB9) or vehicle (saline, i.p.) was administered on Days 0–3. The 5-FU-induced changes in body weight loss (B) were significantly attenuated by the administration of sivelestat sodium (300 mg/kg) or SB225002 (9 mg/kg). The development of diarrhea by the administration of 5-FU was inhibited by the administration of sivelestat sodium (300 mg/kg) or SB225002 (9 mg/kg). Each point represents the mean ± S.E.M. of 4 independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. Vehi + 5-FU.

in fluid flux from an absorptive state to a secretory state. Furthermore, colonic fluid transport returns to an absorptive state with increases in AQP 4 and 8 following the cessation of DSS, which suggests that there may be a functional association between fluid transport and levels of AQP 4 and 8 expression in the colon. These results from a mouse model of DSS colitis are consistent with data from clinical investigations of human IBD [25]. On the other hand, patients with active ulcerative colitis, Crohn's colitis or infectious colitis who develop severe diarrhea show similar reductions in the expression of AQP 8 [26]. Taken together, these findings strongly

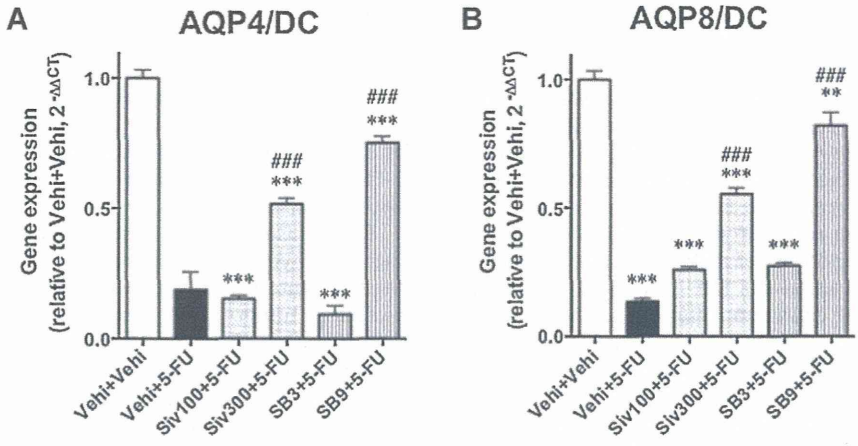

Fig. 5. Effects of sivelestat sodium (100 or 300 mg/kg) and SB225002 (3 or 9 mg/kg) on the 5-FU-induced downregulation of AQP4 and 8 gene expression in the distal colon (DC) of mouse. The 5-FU-induced downregulation of AQP4 (A) and 8 (B) in the distal colon was recovered by the administration of sivelestat sodium or SB225002. Each column represents the mean ± S.E.M. of 4 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Vehi (saline) + Vehi (saline). $^{\#\#\#}p < 0.001$ vs. Vehi (saline) + 5-FU.