MUC5AC protects pancreatic cancer cells from TRAIL-induced death pathways

HIROTAKA HOSHI[1,3], TETSUJI SAWADA[2], MOTOYUKI UCHIDA[1], HIROKO IIJIMA[1], KENJIRO KIMURA[2], KOSEI HIRAKAWA[2] and HIDEKI WANIBUCHI[3]

[1]Biomedical Research Laboratories, Kureha Corporation, Shinjuku-ku, Tokyo 169-8503; Departments of [2]Surgical Oncology and [3]Pathology, Osaka City University Graduate School of Medicine, Abeno-ku, Osaka 545-8585, Japan

Received September 3, 2012; Accepted November 12, 2012

DOI: 10.3892/ijo.2013.1760

Abstract. We have previously reported that a specific siRNA transfected MUC5AC could knockdown MUC5AC expression and suppress *in vivo* tumor growth and metastasis, although it had no effects on *in vitro* cell growth, cell survival, proliferation and morphology. In the present study, we investigated which host immune cells induced these effects and how the effects were induced using immunocyte-depleted animal models. The tumor growth of SW1990/si-MUC5AC cells, which show no tumor growth when implanted subcutaneously into a nude mouse, was recovered when neutrophils were removed by anti-Gr-1 mAb administration. This result suggests that MUC5AC may suppress the antitumor effects of neutrophils by allowing tumor cells to escape the host immune system. Subsequently, we investigated the effects of MUC5AC on apoptosis induction mediated by TNF-related apoptosis-inducing ligand (TRAIL), one of the antitumor mechanisms of neutrophils. SW1990/si-MUC5AC cells showed significantly increased active caspase 3 expression after the addition of TRAIL. On the other hand, SW1990/si-mock cells showed no such changes. Our results indicate that MUC5AC inhibits TRAIL-induced apoptosis in human pancreatic cancer and may serve as an important indicator in diagnosis and prognosis.

Introduction

Pancreatic cancer is the 8th and 9th leading cause of cancer-related deaths worldwide in men and women. More than 50% of patients are diagnosed with advanced disease and have metastatic disease at presentation, 10 to 15% will be resectable, and the remainder will be locally advanced unresectable disease as well as an incidence of unrecognized metastases. The majority of patients have a median survival with treatment of less than 6 months. Even for those seemingly fortunate enough to have early-stage local and resectable disease, the 5-year survival is only 20% after resection (1). Therefore, pancreatic cancer is a refractory cancer having poor prognosis.

MUC5AC is a member of the secreted mucin family and is expressed as secretory mucin from goblet cells of the stomach, airways and cervical secretion. These cover the epithelium and provide a lubricating barrier that protects the mucosal surface. On the other hand, it is overexpressed as a membrane-bound type in the ductal region of human pancreatic cancer, while remaining undetectable in the normal pancreas. However, there have been few reports on the function of MUC5AC in pancreatic cancer (2-4).

The dominant view of polymorphonuclear neutrophils (PMNs) is that they are of the most abundant and short-lived in total circulating blood leukocytes and their cytoplasm has highly developed cytomatrices and granules containing microbicidal proteins and digestive enzymes. They provide the first-line of defense against various infections, are potent effectors of inflammation, and release chemotactic factors that lead to the recruitment of non-specific and specific immune effectors (5). Studies on the immunological mechanisms have focused mainly on the function of lymphocytes and monocytes/macrophages as important mediators of the host response. However, PMNs are increasingly recognized as an important effector cell population for the rejection of malignant tumors *in vivo*, including Fas ligand-mediated apoptosis, antibody-dependent cellular cytotoxicity (ADCC), direct cell killing by H_2O_2 and superoxide and calprotectin (6,7).

The antitumor molecule TNF-related apoptosis-inducing ligand (TRAIL) has been reported previously (8,9) and is part of the TNF superfamily member, is expressed in a broad range of cells including activated T cells, B cells, natural killer cells, dendritic cells, monocytes and activated neutrophils, and exerts great antitumor activity. It is known that TRAIL is highly expressed on neutrophils selectively inducing apoptosis. TRAIL exerts its activity by interacting with a complex system of 2 death receptors (DRs) (DR4/TRAIL-R1 and DR5/TRAIL-R2) and 3 decoy receptors (DcRs) [DcR1/TRAIL-R3, DcR2/TRAIL-R4

Correspondence to: Dr Tetsuji Sawada, Department of Surgical Oncology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
E-mail: t_sawada@osaka-ekisaikai.jp

Key words: MUC5AC, mucin, pancreatic cancer, short interfering RNA, TNF-related apoptosis-inducing ligand, apoptosis

and osteoprotegerin (OPG)]. Although these receptors are characterized by high sequence homology in their extracellular domains, only DR4/TRAIL-R1 and DR5/TRAIL-R2 contain a functionally active death domain that allows an apoptotic response by TRAIL stimulation (10,11). Some correlations between TRAIL sensitivity and receptor expression have been reported in various tumor cells. TRAIL only acts on cancer cells, it does not act on normal cells. Although there is a theory that this reason is due to a difference in expression levels of death receptors and decoy receptors on these cells, the detailed mechanisms are not known. In our previous study, when MUC5AC-expressing pancreatic cancer cells were knocked down for MUC5AC by specific siRNA, cell survival, proliferation, and morphology *in vitro* were the same as control cells. However, their tumor growth in *in vivo* xenograft studies was significantly lower than that of control cells and most infiltrated leukocytes, particularly neutrophils and B cells, were observed to be accumulated into its tumor challenged site. Moreover, a greater number of antibodies against cancer cells were found in the blood of SW1990/si-MUC5AC cell-challenged mice than that of SW1990/si-mock cell-challenged mice. As a result, it appeared that the tumorigenicity and growth of MUC5AC knockdown cells were significantly lower than that of control cells (12).

In the present study, we assessed the mechanisms of MUC5AC on these immune cells using the neutrophil depletion model by anti-Gr-1 mAb and SCID mice in which B and T cells were depleted. Furthermore, we focused on the anti-tumor effects of neutrophils and hypothesized that MUC5AC was able to inhibit apoptosis via TRAIL signaling pathways. To investigate the function of MUC5AC in pancreatic cancer, we evaluated whether or not MUC5AC knockdown would increase TRAIL responsiveness such as the expression of TRAIL receptor and the effects on apoptosis signaling pathways in pancreatic cancer cells.

Materials and methods

Reagents. Recombinant human TRAIL was purchased from PeproTech GmbH (Paris, France). The tetrapeptide caspase inhibitor z-VAD-fmk was obtained from R&D Systems (Minneapolis, MN). The rat anti-mouse Gr-1 monoclonal antibody used for deplete murine neutrophils was from BD Pharmingen Inc. (San Diego, CA). GeneJuice used for siRNA transfection was purchased from Merck (Darmstadt, Germany).

Animals. Specific-pathogen-free female BALB/c-nu/nu mice (nude mice) and C.B17-scid/scid mice (SCID mice) purchased from Charles River Japan Inc. (Kanagawa, Japan) were acclimatized and then used in experiments at the age of six weeks. The experimental design was approved by the Ethics Committee on Animal Experiments of the Biomedical Research Laboratories of Kureha Corp., and mice were treated in accordance with the guidelines of the committee. All animals were allowed free access to sterilized CE-2 food (Oriental Yeast, Tokyo, Japan) and sterilized tap water. Mice were bred at 25±2˚C, a humidity of 55±7%, laminar flow, and under a 12 h light/12 h dark cycle at 150-300 lux. To maintain a uniform environment, noise was carefully avoided and only experimental staff and keepers were allowed into the animal room.

Cell culture conditions. SW1990 was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation, San Diego, CA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaill, France), 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were grown at 37˚C with 5% CO_2 in a humidified atmosphere and passaged before they reached confluency using 0.25% (w/v) trypsin solution containing 0.04% (w/v) EDTA.

Construction of siRNA-MUC5AC and establishing the stable expression cell line. The MUC5AC siRNA target sequence 5'-TTTGAGAGACGAAGGATAC-3' was cloned to generate a stable siRNA expressing construct into the p*Silencer* 3.1-H1 neo vector (Ambion Inc., Austin, TX) as described previously (12). SW1990 cells were transfected with p*Silencer*/si-MUC5AC as a target or p*Silencer*/si-mock as a control using GeneJuice according to the manufacturer's instructions. SW1990 cells were selected by culturing in the presence of geneticin at 600 μg/ml. The efficiency of MUC5AC-knockdown was tested through RT-PCR and FACS analysis (data not shown).

Preparation of neutrophil-depleted mice. Neutrophils were depleted from nude mice using anti-Gr-1 mAb. Anti-Gr-1 mAb was treated at single doses of 100 μg/mice via i.p. injection on days -1, 4, 9, 14, 19, 24, 29, 34 and 39. The depletion of neutrophils was determined before tumor cell implantation by a flow cytometer (13).

Tumorigenicity assay in xenograft models. Cells were implanted subcutaneously (s.c.) at $1x10^7$/mice on the flank of nude mice or SCID mice. Tumor volumes were measured at least once a week. For the determination of tumor volume, two bisecting diameters of each tumor were measured by slide calipers and tumor volumes were calculated using the following formula: tumor volume = length x $(\text{width})^2$ x 0.5236. Eight mice were used for each cell line. Tumor growth curves were plotted as the mean volume ± standard error (SE).

Apoptosis assays. SW1990/si-MUC5AC cells and SW1990/si-mock cells ($2x10^3$ cells/100 μl) were cultured in 96-well plates in medium containing recombinant human TRAIL at fixed concentrations. Cell proliferation was evaluated by the MTT assay as previously described (12). The pan-caspase inhibitor z-VAD-fmk was dosed at 10 μM before 1 h of TRAIL treatment.

Human apoptosis array. The expression profile of apoptosis-related proteins was detected and analyzed using a Proteome Profiler™ (R&D Systems Inc.) according to the manufacturer's instructions. Briefly, protein lysates (500 μg) from SW1990/si-MUC5AC cells and SW1990/si-mock cells were loaded onto an array membrane blocked with blocking reagent. The membrane was incubated overnight at 4˚C, washed thrice with TBST, and then incubated with a detection antibody cocktail for 1 h. After three TBST washes,

spots were visualized by a chemiluminescence assay and the average density of duplicate spots was recorded.

Production of IL-8. SW1990/si-MUC5AC cells and SW1990/si-mock cells (10^4 cells/100 μl) were cultured in 96-well plates for 2 or 4 days. Culture supernatants were collected and measured using a human IL-8 ELISA kit (RayBiotech Inc., Norcross, GA), as directed by the manufacturer.

Statistical analysis. All data are expressed as the means ± SE. Statistical significance was determined by the Student's t-test. P-values <0.05 were considered significant.

Results

Effects of MUC5AC on immunocytes. Previous studies have demonstrated that the tumor growth of MUC5AC-knockdown cells is markedly suppressed when they are subcutaneously implanted into a nude mouse. Furthermore, neutrophils and B cells accumulated in the tumor (12). Thus, in the present study, we investigated the effects of neutrophils and B cells on the tumor growth of MUC5AC-expressing cells and the immune evasion mechanism through MUC5AC.

First, to examine the involvement of B cells on the tumor growth of MUC5AC-expressing cells, we investigated the tumor growth of MUC5AC-expressing and MUC5AC-knockdown cells in SCID mice. SW1990/si-MUC5AC or SW1990/si-mock cells were implanted subcutaneously into SCID mice at $1x10^7$ cells/mice and then tumor volume was assessed. If B cells act directly on the antitumor effects of MUC5AC-knockdown-challenged mice, MUC5AC-knockdown cells should grow comparably to MUC5AC-expressing cells. However, the tumor volume of SW1990/si-mock cells increased over time, while that of SW1990/si-MUC5AC cells barely increased and little tumor growth was observed (Fig. 1A).

Subsequently, to examine the involvement of MUC5AC on the antitumor effects of neutrophils, 100 μg of anti-Gr-1 antibody was administered once every five days to a nude mouse to deplete neutrophils. Neutrophil depletion was confirmed by FACS before tumor implantation (data not shown). SW1990/si-MUC5AC or SW1990/si-mock cells were implanted subcutaneously at $1x10^7$ cells/nude mouse, and then tumor volume was assessed. The tumor growth of SW1990/si-MUC5AC cells was barely observed in the presence of neutrophils (Fig. 1B). However, tumor growth recovered to the same level as SW1990/si-mock cells when neutrophils were depleted with an anti-Gr-1 antibody (Fig. 1C).

MUC5AC suppresses IL-8 production of tumor cells. IL-8 produced by peripheral tissue cells and activated neutrophils is involved in the migration of neutrophils into the tissue (8). Hence, the amounts of *in vitro* IL-8 production were determined in SW1990/si-MUC5AC and SW1990/si-mock cells. IL-8 concentrations in the supernatants of SW1990/si-MUC5AC and SW1990/si-mock cells were 1,529 and 179 ng/ml on day 2 and 4,100 and 1,094 ng/ml on day 4, respectively. Suppressed MUC5AC expression significantly increased IL-8 production (Fig. 2).

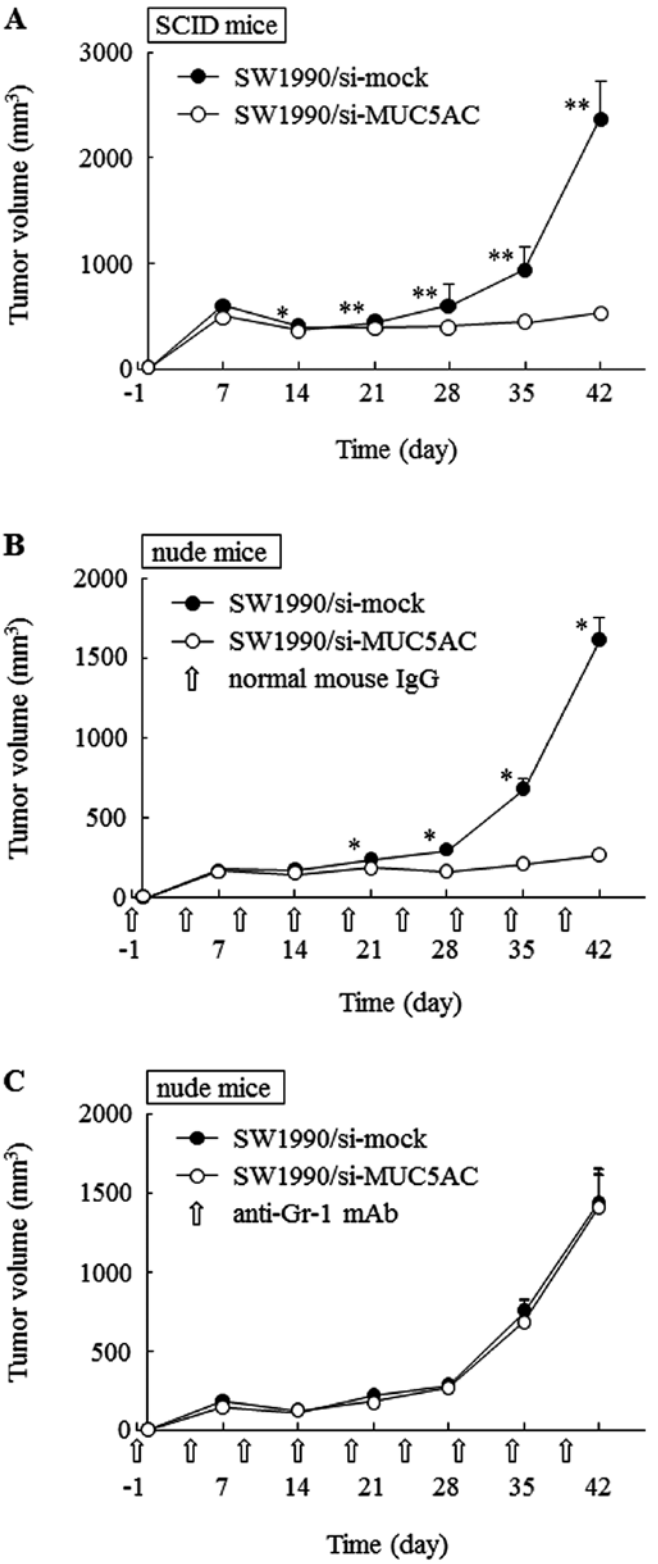

Figure 1. MUC5AC suppresses the antitumor processes of neutrophils. (A) Tumor growth curves of SW1990/si-mock cells (filled circles) and SW1990/si-MUC5AC cells (open circle). Cells were s.c. implanted with $1x10^7$ cells into SCID mice on day 0. (B) Tumor growth curves of SW1990/si-mock cells (filled circles) and SW1990/si-MUC5AC cells (open circle) in normal mouse IgG-treated nude mice. (C) Tumor growth curves of SW1990/si-mock cells (filled circles) and SW1990/si-MUC5AC cells (open circle) in anti-Gr-1 mAb-treated mice. Cells were s.c. implanted with $1x10^7$ cells into nude mice on day 0. Mice were monitored for tumor formation until 42 days and tumor sizes were measured on indicated days. These experiments were performed at least twice and representative data are shown. Points, tumor volume of 8 mice for each group; bars, SE. *P<0.05 and **P<0.001.

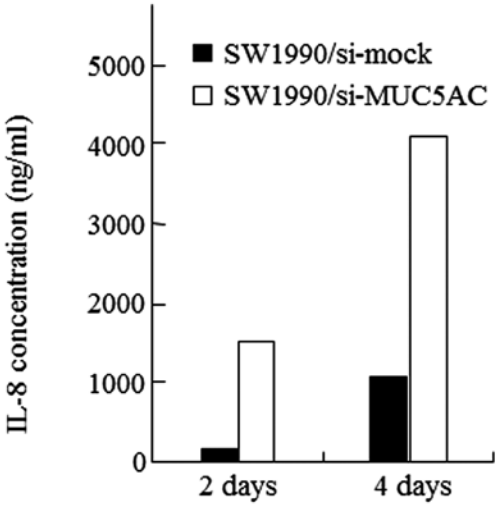

Figure 2. Knockdown of MUC5AC stimulates IL-8 production in pancreatic cancer cells. Cells (10^4 cells/100 μl) were cultured in 96-well plates for 2 or 4 days. Culture supernatants were collected and measured using a human IL-8 ELISA kit. Results were averaged from 3 separate experiments. One representative of at least three similar experiments is shown.

MUC5AC inhibits TRAIL-induced apoptosis. It is known that activated neutrophils produced TRAIL, which in turn, induced apoptosis of cancer cells via its death receptors. Therefore, we evaluated cell viability after TRAIL treatment by the MTT assay. The correlation between the dose of TRAIL and cell growth inhibition is illustrated in Fig. 3A. Our previous reports show that no difference in cell proliferation was observed between SW1990/si-MUC5AC cells and SW1990/si-mock cells under normal conditions *in vitro* (12). After 12 h of TRAIL treatment, cell death was induced in SW1990/si-MUC5AC cells in a dose-dependent manner. In this condition, the IC_{50} value of TRAIL was 5.8 ng/ml. In contrast, treatment of SW1990/si-mock cells at a concentration of TRAIL up to 100 ng/ml resulted in <10% cell death. Concerning cell morphologic changes, SW1990/si-MUC5AC cells were rounded and floating in the medium, while SW1990/si-mock cells still formed a typical epithelioid monolayer at a TRAIL concentration of 100 ng/ml for 4 h (Fig. 3B). Consequently, to examine whether caspases were involved in TRAIL-induced apoptosis in SW1990/si-MUC5AC cells, either the pan-caspase inhibitor z-VAD-fmk (10 μM) or a vehicle control was added to the culture 1 h before TRAIL treatment (10 ng/ml, 4 h). The results indicated that z-VAD-fmk completely blocked TRAIL-induced apoptosis and caspase activation is a required signal event for TRAIL-induced apoptosis. As shown in Fig. 3C, the percentage of cell viability in SW1990/si-MUC5AC cells by the DMSO vehicle control was 92 and TRAIL treatment was 47% in SW1990/si-MUC5AC cells. In contrast, pretreatment of SW1990/si-MUC5AC cells with z-VAD-fmk followed by TRAIL treatment significantly increased cell viability to 93%.

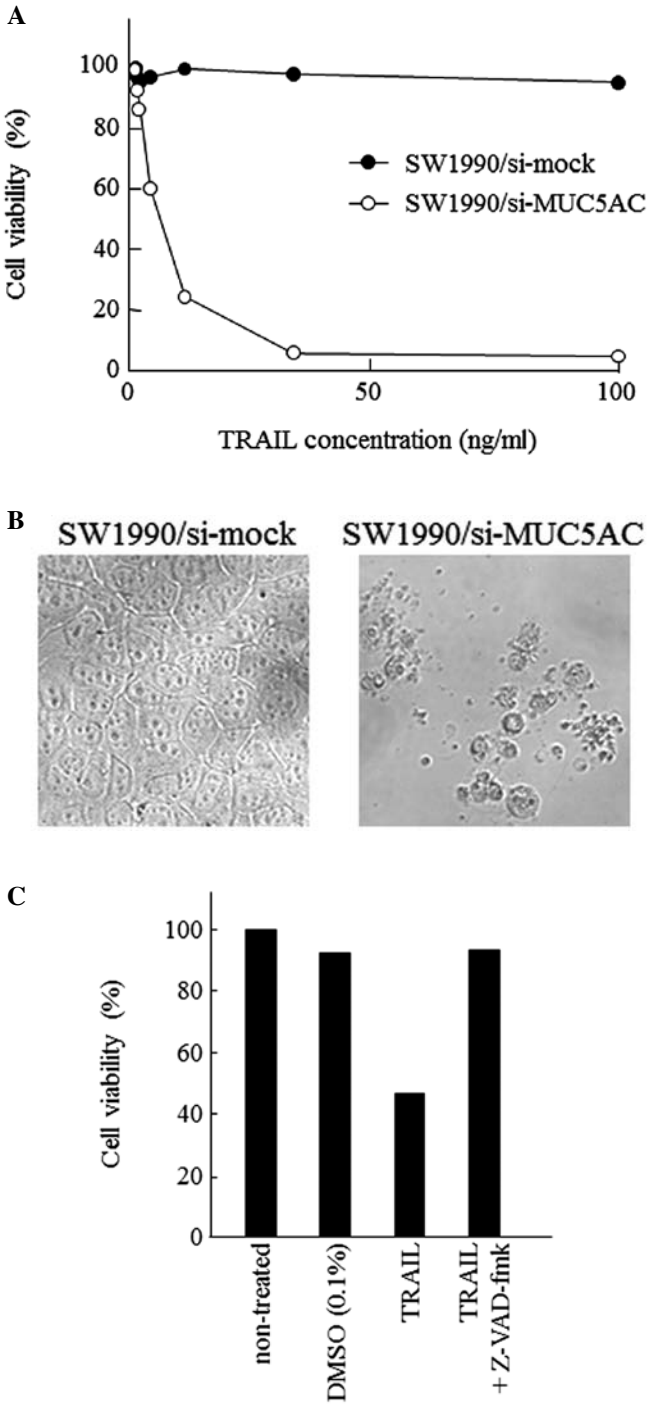

Figure 3. TRAIL induced-apoptosis is stimulated by knockdown of MUC5AC. (A) Cells ($2x10^3$ cells/100 μl) were cultured in 96-well plates in medium containing recombinant human TRAIL at 0-100 μg/ml. After 12 h, cell proliferation was evaluated by the MTT assay. (B) Cells were treated with 100 ng/ml of TRAIL for 4 h. Cells were photographed under a phase-contrast microscope. The representative of three independent experiments is shown. (C) Cells ($2x10^3$ cells/100 μl) were cultured in 96-well plates in medium containing the pan-caspase inhibitor z-VAD-fmk at a dose of 10 μM for 1 h. Twenty ng/ml of TRAIL was treated for 4 h. Cell numbers were determined by the MTT assay.

Analysis of the apoptosis suppression mechanism of MUC5AC using an apoptosis array. As described above, MUC5AC knockdown with siRNA induced apoptosis mediated by TRAIL. Then, to examine differences in the apoptotic signal between SW1990/si-MUC5AC and SW1990/si-mock cells, protein levels relating to apoptosis after the addition of TRAIL to the cells were analyzed using an apoptosis array. Cells were treated with 10 ng/ml of TRAIL for 4 h. The signal intensities in the apoptosis array (Fig. 4A) were digitized and normalized for the positive control value (Fig. 4B). The vehicle control

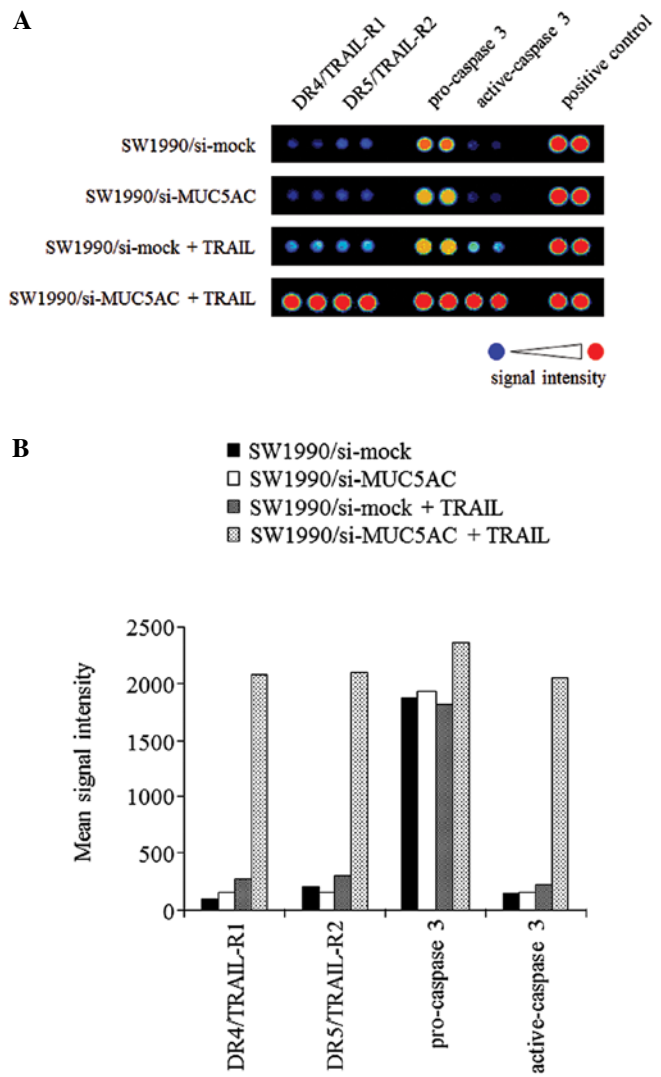

Figure 4. Apoptosis array analysis of pancreatic cancer cells upon knockdown of MUC5AC. (A) Total protein lysates (500 μg) from SW1990/si-mock cells and SW1990/si-MUC5AC cells were analyzed using Proteome Profiler™. Depicted are representative images of chemiluminescence signals with differences in signal intensities between si-MUC5AC and si-mock cells. One representative of duplicate experiments is shown. (B) Densitometric analysis of apoptosis array blots. Values relative to DR4/TRAIL-R1 expression levels of SW1990/si-mock values set to 100. Means are averaged from 2 spots.

for SW1990/si-MUC5AC cells showed weak expression of TRAIL receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2) and active-caspase 3 and strong expression of pro-caspase 3 (active-caspase 3 precursor). The same expression patterns were observed for SW1990/si-mock cells. However, the addition of TRAIL induced an enhancement in the expression of DR4/TRAIL-R1 and DR5/TRAIL-R2 in SW1990/si-MUC5AC cells. Additionally, a significant increase in active-caspase 3 expression was observed. On the other hand, TRAIL had no effect on DR4/TRAIL-R1 and DR5/TRAIL-R2 expressions in SW1990/si-mock cells.

Discussion

Mucins are classified generally into two groups: a secretory type and membrane-bound type. Secretory mucins include MUC2, MUC5AC, MUC5B and MUC6. These mucins are called 11p15.5 mucin because they are located on human chromosome 11p15.5 and are structurally similar. On the other hand, membrane-bound mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12 and MUC13. All these mucins have transmembrane domains. MUC3A, MUC3B, MUC11 and MUC12 are located on chromosome 7q22, while MUC4 and MUC13 are located on chromosome 3q29. MUC7 and MUC8 belong to neither of the above groups and have no mutual similarity (3,14). The core protein of a mucin molecule has a characteristic repetitive sequence. The repetitive sequence, rich in Thr and Ser, is frequently bound to a mucin-type sugar chain. Mucin-type sugar chains generally consist of N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acid. Sugar chains account for 50-80% or above of the molecular weight of mucin, giving it various properties including viscosity, a water-holding capacity and proteinase resistance. Thus, the sugar chain structure of mucin, which is varied quantitatively and qualitatively with malignant transformations, is likely to be involved in altered adhesion during metastasis and evasion from immune cells. However, the detailed mechanisms remain unclear.

We established MUC5AC-knockdown cells using siRNA and have previously reported analysis of the functions of MUC5AC in cancer cells (12). A stable human pancreatic cancer cell line, SW1990/si-MUC5AC, obtained by introducing si-MUC5AC into a parent SW1990, was almost equal to the control cells, SW1990/si-mock, regarding *in vitro* morphology, proliferation and infiltration capacity. However, an *in vivo* subcutaneously implanted model yielded results completely different between SW1990/si-MUC5AC and SW1990/si-mock cells. SW1990/si-mock cells caused continuous rapid tumor growth, whereas SW1990/si-MUC5AC cells caused only slight tumor growth when they were subcutaneously implanted into nude mice. Furthermore, the amounts of immune cells, including neutrophils and B cells, existed in the tumor derived from SW1990/si-MUC5AC cells. Tumor-specific antibodies also existed in the serum. This suggests that MUC5AC-expressing cells may suppress immune cells to evade the immune system, thereby playing an important role in creating an environment to facilitate cancer cell survival.

Neutrophils are characterized by immunity against bacteria, such as direct phagocytosis of bacteria and opsonophagocytosis of encapsulated bacteria. According to previous reports, neutrophils also have antitumor effects. The antitumor effects of neutrophils are reportedly caused by a number of mechanisms including ADCC activity through a tumor-specific antibody, apoptosis induction mediated by TRAIL (15-19). Furthermore, inhibition of tumor growth by IL-12 was reduced when neutrophils were depleted in a mouse model of prostate cancer (20). PMNs are the predominant effector cell population for the killing of breast cancer cells in the presence of HER-2/neu monoclonal antibody (6). In addition, PMN-mediated ADCC has been reported to contribute to the efficacy of the antitumoral antibody rituximab and trastuzumab (13,21,22).

Additionally, TRAIL is expressed at significantly higher levels in neutrophils than in other immune cells. TRAIL was isolated in 1995 as a cytokine that induces apoptosis and has a TNF-family analogous sequence (23). TRAIL is ~30 kDa type II cell surface protein consisting of 281 amino acids and is

about 28% homologous to the Fas ligand. TRAIL induces apoptosis in various malignant tumors, but is non-toxic to normal cells. These functions of TRAIL may lead to the promise of a molecular target agent. TRAIL receptors are classified into TRAIL-R1 (death receptor 4, DR4); TRAIL-R2 (death receptor 5, DR5); TRAIL-R3 (decoy receptor 1, DcR1); TRAIL-R4 (decoy receptor 2, DcR2) and osteoprotegerin (OPG). DRs 4 and 5 have an intracellular death domain to induce apoptosis through initiator and effector caspases, whereas DcRs 1 and 2 are decoy receptors that do not induce apoptosis (10). Differences in TRAIL receptor expressions possibly result in apoptosis induction only in malignant tumors. However, its detailed mechanism remains unclear.

The present study aimed to identify the immune cells on which MUC5AC acts using an immune cell-depleted animal model and analyze the suppression mechanism. Then, we investigated the tumor growth of MUC5AC-knockdown cells using nude mice depleted of neutrophils with an anti-Gr-1 antibody and SCID mice depleted of B and T cells.

To examine whether B cells act directly on the rejection of implanted MUC5AC-knockdown cells, we investigated the tumor growth of MUC5AC-knockdown and MUC5AC-expressing cells in SCID mice. The growth of MUC5AC-knockdown cells did not recover (Fig. 1A). In SCID mice, an investigation in the double implantation system demonstrated that the tumor growth of MUC5AC-expressing cells was increased irrespective of whether a primary tumor was present or absent (unpublished observation). However, such growth was not observed in nude mice (unpublished observation). This suggests that MUC5AC suppresses memory B-cell immune reactions that function during prolonged antitumor reactions or cell metastasis.

In an experiment with neutrophil-depleted model mice, the tumor growth of MUC5AC-knockdown cells, barely observed in the presence of neutrophils, was recovered to the same level as MUC5AC-expressing cells when neutrophils were depleted (Fig. 1C). This suggests that MUC5AC plays an important role in directly suppressing the antitumor effects of neutrophils. Additionally, IL-8 production, barely observed in MUC5AC-expressing cells, was significantly increased in MUC5AC-knockdown cells (Fig. 2). According to these results, neutrophil infiltration into a tumor inducing by IL-8 was found to exert antitumor effects. However, the mechanism by which MUC5AC-knockdown increases IL-8 production by tumor cells still remains unknown and should be investigated in a future study.

An apoptosis array was employed to examine why MUC5AC-expressing cells differed from MUC5AC-knockdown cells in their susceptibilities to apoptosis mediated by TRAIL. DR4/TRAIL-R1 and DR5/TRAIL-R2 were weakly expressed in the routine cultures of both cells. However, the addition of TRAIL increased the expressions of DR4/TRAIL-R1, DR5/TRAIL-R2 and active-caspase 3 (effector caspase) in MUC5AC-knockdown cells (Fig. 4). Future studies will be aimed at investigating how TRAIL increases death receptor expressions in MUC5AC-knockdown cells.

The caspase family of proteases is the ultimate effector of programmed cell death. Under ordinary circumstances, caspases are kept in check by the inhibitor of apoptosis proteins (IAPs) such as cIAP1, cIAP2, XIAP, NAIP, livin/ML-IAP, BRUCE/Apollon and survivin, which bind to and inactive caspases until they are needed. Caspases are overexpressed in tumors, but IAPs likewise are overexpressed. Therefore, failure to activate caspases could create resistance to apoptosis (24,25). Several studies show that MUC4 and ErbB2 are coexpressed in some tumor types such as the breast, non-small cell lung cancer, and pancreas. The possibility that MUC4 could elicit its anti-apoptotic effects by ErbB2, engaging a variety of signaling cascades to elicit cellular responses, such as proliferation and survival, has been suggested (26,27). Prostaglandin E_2 (PGE_2) synthesized by cyclooxygenase-2 (COX-2), overproduced in various malignancies, has also been reported to be associated with anti-apoptotic effects and increase survivin expression (28,29). It is believed that MUC5AC may have promoted the expression or action of IAPs in some way or MUC5AC may have formed a complex with a certain molecule and promoted tumor growth via suppression of tumor cell apoptosis.

In the present study, we established MUC5AC-knockdown cells using siRNA to elucidate the functions of MUC5AC, whose expression is increased in pancreatic cancer. IL-8 production was promoted in MUC5AC-knockdown cells. In addition, cell death was induced by TRAIL through the apoptosis pathway, suggesting reduced tumorigenicity *in vivo*. It was suggested that induction of neutrophil migration is weak in normal MUC5AC-producing pancreatic cancer cells because IL-8 production is low and apoptosis induction by neutrophil-derived TRAIL is blocked due to the presence of MUC5AC, and these conditions promote pancreatic cancer cell proliferation and growth *in vivo*, being involved in aggressive tumor formation. Our observations suggest that the very potent anti-apoptotic effects of MUC5AC allow tumor cells to escape key barriers to tumor progression. These studies add to the knowledge on the significance of MUC5AC expression in cancer cells.

Acknowledgements

This study was conducted at Kureha Corporation, Tokyo and Osaka City University, Osaka, Japan. Hirotaka Hoshi, Motoyuki Uchida and Hiroko Iijima are employees of Kureha Corporation, but the study presents no conflict of interest.

References

1. Wang Z, Song W, Aboukameel A, Mohammad M, Wang G, Banerjee S, Kong D, Wang S, Sarkar FH and Mohammad RM: TW-37, a small-molecule inhibitor of Bcl-2, inhibits cell growth and invasion in pancreatic cancer. Int J Cancer 123: 958-966, 2008.
2. Sheehan JK, Brazeau C, Kutay S, Pigeon H, Kirkham S, Howard M and Thornton DJ: Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions. Biochem J 347: 37-44, 2000.
3. Hollingsworth MA and Swanson BJ: Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 4: 45-60, 2004.
4. Ho JJL, Crawley S, Pan PL, Farrelly ER and Kim YS: Secretion of MUC5AC mucin from pancreatic cancer cells in response to forskolin and VIP. Biochem Biophys Res Commun 294: 680-686, 2002.
5. Tecchio C, Huber V, Scapini P, Calzetti F, Margotto D, Todeschini G, Pilla L, Martinelli G, Pizzolo G, Rivoltini L and Cassatella MA: IFNalpha-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. Blood 103: 3837-3844, 2004.

6. Stockmeyer B, Beyer T, Neuhuber W, Repp R, Kalden JR, Valerius T and Herrmann M: Polymorphonuclear granulocytes induce antibody-dependent apoptosis in human breast cancer cells. J Immunol 171: 5124-5129, 2003.
7. Challacombe JM, Suhrbier A, Parsons PG, Jones B, Hampson P, Kavanagh D, Rainger GE, Morris M, Lord JM, Le TT, Hoang-Le D and Ogbourne SM: Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. J Immunol 177: 8123-8132, 2006.
8. Koga Y, Matsuzaki A, Suminoe A, Hattori H and Hara T: Neutrophil-derived TNF-related apoptosis-inducing ligand (TRAIL): a novel mechanism of antitumor effect by neutrophils. Cancer Res 64: 1037-1043, 2004.
9. Kemp TJ, Ludwig AT, Earel JK, Moore JM, Vanoosten RL, Moses B, Leidal K, Nauseef WM and Griffith TS: Neutrophil stimulation with Mycobacterium bovis bacillus Calmette-Guérin (BCG) results in the release of functional soluble TRAIL/Apo-2L. Blood 106: 3474-3482, 2005.
10. de Vries EG, Gietema JA and de Jong S: Tumor necrosis factor-related apoptosis-inducing ligand pathway and its therapeutic implications. Clin Cancer Res 12: 2390-2393, 2006.
11. Voortman J, Resende TP, Abou El Hassan MA, Giaccone G and Kruyt FA: TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol Cancer Ther 6: 2103-2112, 2007.
12. Hoshi H, Sawada T, Uchida M, Saito H, Iijima H, Toda-Agetsuma M, Wada T, Yamazoe S, Tanaka H, Kimura K, Kakehashi A, Wei M, Hirakawa K and Wanibuchi H: Tumor-associated MUC5AC stimulates *in vivo* tumorigenicity of human pancreatic cancer. Int J Oncol 38: 619-627, 2011.
13. Hernandez-Ilizaliturri FJ, Jupudy V, Ostberg J, Oflazoglu E, Huberman A, Repasky E and Czuczman MS: Neutrophils contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. Clin Cancer Res 9: 5866-5873, 2003.
14. Singh PK and Hollingsworth MA: Cell surface-associated mucins in signal transduction. Trends Cell Biol 16: 467-476, 2006.
15. Lane D, Côté M, Grondin R, Couture MC and Piché A: Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3. Mol Cancer Ther 5: 509-521, 2006.
16. Lane D, Robert V, Grondin R, Rancourt C and Piché A: Malignant ascites protect against TRAIL-induced apoptosis by activating the PI3K/Akt pathway in human ovarian carcinoma cells. Int J Cancer 121: 1227-1237, 2007.
17. Di Carlo E, Forni G, Lollini P, Colombo MP, Modesti A and Musiani P: The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97: 339-345, 2001.
18. Alvarez MJ, Prada F, Salvatierra E, Bravo AI, Lutzky VP, Carbone C, Pitossi FJ, Chuluyan HE and Podhajcer OL: Secreted protein acidic and rich in cysteine produced by human melanoma cells modulates polymorphonuclear leukocyte recruitment and antitumor cytotoxic capacity. Cancer Res 65: 5123-5132, 2005.
19. Chen YL, Chen SH, Wang JY and Yang BC: Fas ligand on tumor cells mediates inactivation of neutrophils. J Immunol 171: 1183-1191, 2003.
20. Sanford MA, Yan Y, Canfield SE, Hassan W, Selleck WA, Atkinson G, Chen SH and Hall SJ: Independent contributions of GR-1+ leukocytes and Fas/FasL interactions to induce apoptosis following interleukin-12 gene therapy in a metastatic model of prostate cancer. Hum Gene Ther 12: 1485-1498, 2001.
21. Niitsu N, Khori M, Hayama M, Kajiwara K, Higashihara M and Tamaru J: Phase I/II study of the rituximab-EPOCT regimen in combination with granulocyte colony-stimulating factor in patients with relapsed or refractory follicular lymphoma including evaluation of its cardiotoxicity using B-type natriuretic peptide and troponin T levels. Clin Cancer Res 11: 697-702, 2005.
22. Otten MA, Rudolph E, Dechant M, Tuk CW, Reijmers RM, Beelen RH, van de Winkel JG and van Egmond M: Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors. J Immunol 174: 5472-5480, 2005.
23. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA and Goodwin RG: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673-682, 1995.
24. Schimmer AD: Inhibition of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res 64: 7183-7190, 2004.
25. Wei Y, Fan T and Yu M: Inhibitor of apoptosis proteins and apoptosis. Acta Biochim Biophys Sin (Shanghai) 40: 278-288, 2009.
26. Komatsu M, Jepson S, Arango ME, Carraway CAC and Carraway KL: Muc4/sialomucin complex, an intramembrane modulator of ErbB2/HER2/Neu, potentiates primary tumor growth and suppresses apoptosis in a xenotransplanted tumor. Oncogene 20: 461-470, 2001.
27. Workman HC, Sweeney C, Carraway KL III: The membrane mucin Muc4 inhibits apoptosis induced by multiple insults via ErbB2-dependent and ErbB2-independent mechanisms. Cancer Res 69: 2845-2852, 2009.
28. Baratelli F, Krysan K, Heuzé-Vourc'h N, Zhu L, Escuadro B, Sharma S, Reckamp K, Dohadwala M and Dubinett SM: PGE2 confers survivin-dependent apoptosis resistance in human monocyte-derived dendritic cells. J Leukoc Biol 78: 555-564, 2005.
29. Leone V, di Palma A, Ricchi P, Acquaviva F, Giannouli M, Di Prisco AM, Iuliano F and Acquaviva AM: PGE2 inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras-PI3K association and cAMP-dependent kinase A activation. Am J Physiol Gastrointest Liver Physiol 293: G673-G681, 2007.