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4-[3,5-Bis(trimethylsilyl)benzamido] Benzoic Acid (TAC-101) Induces Apoptosis in Colon Cancer Partially Through the Induction of Fas Expression

TATSUHIKO SAKO[1,4], YOSHIFUMI NAKAYAMA[1], NORITAKA MINAGAWA[1], YUZURU INOUE[1], KOJI ONITSUKA[1], TAKEFUMI KATSUKI[1], YOSUKE TSURUDOME[1], KAZUNORI SHIBAO[1], KEIJI HIRATA[1], NAOKI NAGATA[1], SHINJI OHIE[3], KIMITOSHI KOHNO[2] and HIDEAKI ITOH[1]

[1]Department of Surgery 1 and [2]Department of Molecular Biology, University of Occupational and Environmental Health, School of Medicine, 1-1 Iseigaoka, Yahatanisi-ku, Kitakyushu 807-8555;
[3]Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co., Ltd., 1-27 Misugidai, Hanno, Saitama 357-8527;
[4]Department of Surgery, Kyushu Rosai Hospital, 1-3-1 Kashihara-Takamatsu, Kokura-minami-ku, Kitakyushu 800-0296, Japan

Abstract. *Background: 4-[3,5-Bis (trimethylsilyl) benzamido] benzoic acid (TAC-101) is a novel retinobenzoic acid derivative, which has a specific binding affinity to the retinoic acid receptors (RAR)-α and -β. Apoptotic induction by TAC-101 was investigated using a rat hepatic metastatic model of rat RCN-9 colon cancer cells in vivo and FACScan analysis with the DLD-1 human colon cancer cell line in vitro. Materials and Methods: Hepatic metastatic tumors were induced using intra-portal injection of RCN-9 cells into F344 rats in vivo. TAC-101 (8 mg/kg) was orally administered for 5 consecutive days a week for 4 weeks. Subsequently, hepatic tumors were counted after laparotomy. Apoptotic index (A.I.) in the hepatic tumors was evaluated using immunohistochemistry for single-stranded DNA. The proliferative index (P.I.), Fas and Fas ligand were also immunohistochemically evaluated. Moreover, evaluation of apoptosis by TAC-101 in vitro using FACScan analysis was performed in the DLD-1 human colon cancer cell line. Results: Oral administration of TAC-101 resulted in a significant inhibition of hepatic metastasis without weight loss of the rats. TAC-101 significantly decreased P. I. but increased A. I. in the hepatic metastatic tumors. TAC-101 did not affect the expression of Fas ligand, but obviously increased the expression of Fas in the metastatic tumors. Moreover, TAC-101 induced early apoptosis in DLD-1 cells in a time-dependent manner in vitro. Conclusion: These findings suggest that TAC-101 inhibits hepatic metastasis of colon cancer and induces apoptosis partially through enhanced Fas expression.*

Correspondence to: Y. Nakayama, Department of Surgery 1, University of Occupational Environmental Health, 1-1 Iseigaoka, Yahata-nishi-ku, Kita-kyushu 807-8555, Japan. Tel: (+81) 93 691 7441, Fax: (+81) 93 603 2361, e-mail: nakayama@med.uoeh-u.ac.jp

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In colon cancer, hepatic metastasis is a critical problem, which affects mortality rate. A reduction in hepatic metastasis would lead to an improved prognosis for patients with advanced colon cancer.

Recently, retinoic acid (RA) has shown to be a potentially powerful anti-cancer agent, which acts in several types of cancer therapy. RA has been shown to have clinical efficacy as a chemotherapeutic agent against selected malignancies (1,2). RA is a multifunctional drug that is particularly effective at preventing the development of many primary tumors (3-5). The mechanism of RA's effect on tumors appears to be related to its effects on proliferation and differentiation of tumor cells (6-8). Retinoids can also inhibit angiogenesis induced by tumor cells (9). Moreover, RA has an anti-invasive effect and anti-metastatic effect through the modulation of urokinase-type plasminogen activator activity (10,11), the suppression of collagenase expression and activity such as matrix metalloproteinase-1, -2, -7 and -9 (12-14), and the activation of the E-cadherin/catenin complex (15).

TAC-101 is a novel benzoic acid derivative, one of the synthetic retinoids, which has a binding affinity for the retinoic acid receptors (RAR)-α and -β (16). Several reports have documented anti-tumor activity and anti-metastatic activity by TAC-101 in nude mice in *in vivo* and *in vitro* experiments (16-24). These actions of TAC-101 have been

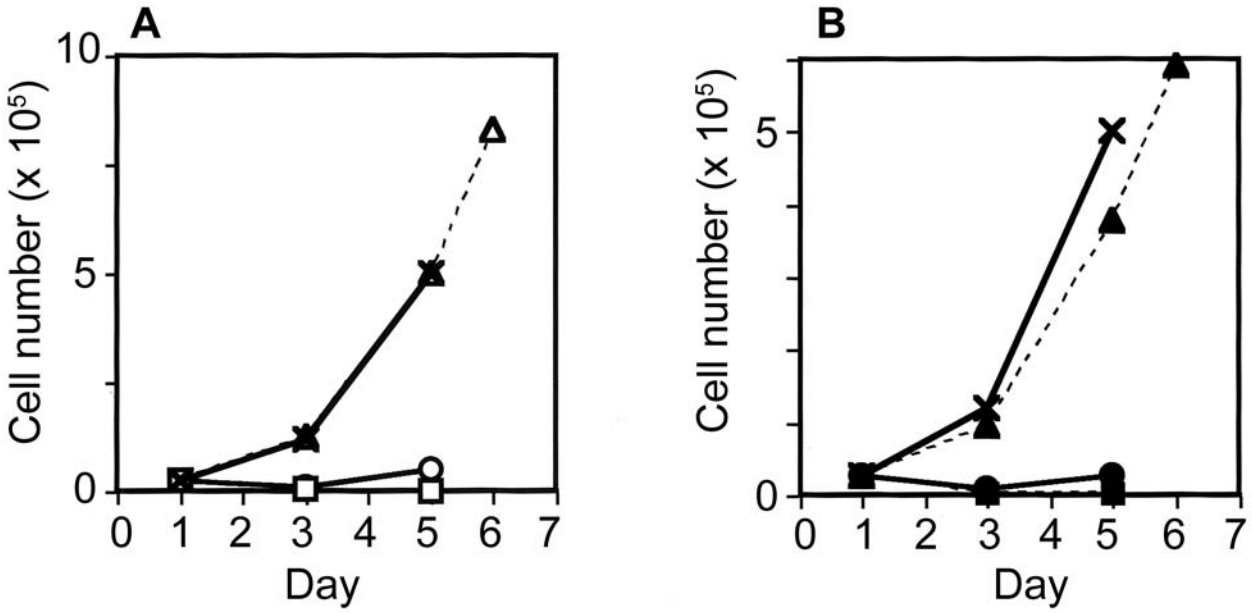

Figure 1. *Dose-response effects of TAC-101 (A) or ATRA (B) on the cell growth of RCN-9 cells. RCN-9 cells were seeded at 2 x 10^4 cell/ dish in RPMI-1640 with 10% FCS and, after 24-h incubation, the cells were treated with DMSO (control) (-×-), 1 μM (-△-), 10 μM (-○-) and 100 μM (-□-) TAC-101 or 1 μM (-▲-), 10 μM (-●-) and 100 μM (-■-)ATRA.*

evaluated with metastatic models of nude mice and human cancer cell lines. As hepatic metastasis of cancer cells is influenced by immunity, metastasis should be examined using experimental models conserving the immune system, such as rat models. Almost no data is available about TAC-101 using the conserved immune system model.

Previous studies have shown that TAC-101 possesses various biological activities, including differentiation-induction (25), apoptosis induction in various cancer cells in DNA ladder fragmentation (17-23), anti-angiogenesis (21,22), and life-prolonging effects on experimental liver metastasis of cancer cells (16,17,19,24). However, the mechanisms by which TAC-101 acts remain unclear. Therefore, we investigated the inhibitory effects on hepatic metastasis of TAC-101 in the rat model, focusing on apoptosis induction.

Materials and Methods

Chemicals. TAC-101, 4-[3,5-bis (trimethylsilyl) benzamido] benzoic acid, was synthesized and kindly donated by Taiho (Hannou, Japan) (16,25). ATRA was purchased from Sigma (St. Louis, MO, USA).

Tumor cell line and animals. The RCN-9 rat colon cancer cell line was purchased from Riken cell bank (Tsukuba, Japan), the cells of which were originally established from a 1, 2-dimethyl-hydrazine(DMH)-induced colon adenocarcinoma in male F344/DuCrj rats (26). The cultures were incubated in RPMI-1640 (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% FCS, 0.05% L-glutamine, 100 IU/ml penicillin and 60 μg/ml kanamycin, at 37˚C in a humidified 5% CO_2/ 95% air atmosphere. Male, 7-week-old, F344/DuCrj rats were purchased from Charles River Japan Inc. (Atsugi, Japan)

The DLD-1 human colon cancer cell line was kindly supplied by Prof. Kohno (University of Occupational and Environmental Health, Japan). Cells were maintained in RPMI-1640 medium containing 10% FCS and antibiotics at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2.

Cell growth experiments of RCN-9 with or without TAC-101 or ATRA. RCN-9 cells were seeded at 2 x 10^4 cells per 35-mm dish and, after incubation in RPMI-1640 with 10% FCS for 24 h, the cells were treated with DMSO (control), TAC-101 at 1, 10 and 100 μM or ATRA at 1, 10 and 100 μM for up to 5 days. The medium containing DMSO, TAC-101 or ATRA was changed every 2 days. The final concentration of DMSO in all the cultures was 0.1%. Cell numbers were measured using an electronic Coulter counter (Coulter, Hialeah, FL, USA).

Hepatic metastatic model. Multiple hepatic metastatic tumors were induced using intra-portal inoculation of RCN-9 cells into F344/DuCrj rats (27). After the rats had been anesthetized using nembutal, a laparotomy was performed through the midline incision. The superior mesenteric or portal vein was identified and 0.05 ml of tumor cell suspension (5x10^6 cells) was slowly injected into these veins using a 27-gauge needle. TAC-101 was orally administered for 5 consecutive days a week for 4 weeks, starting on day 1. After 4 weeks, hepatic metastatic tumors were identified and counted after laparotomy, and these livers were resected, fixed in

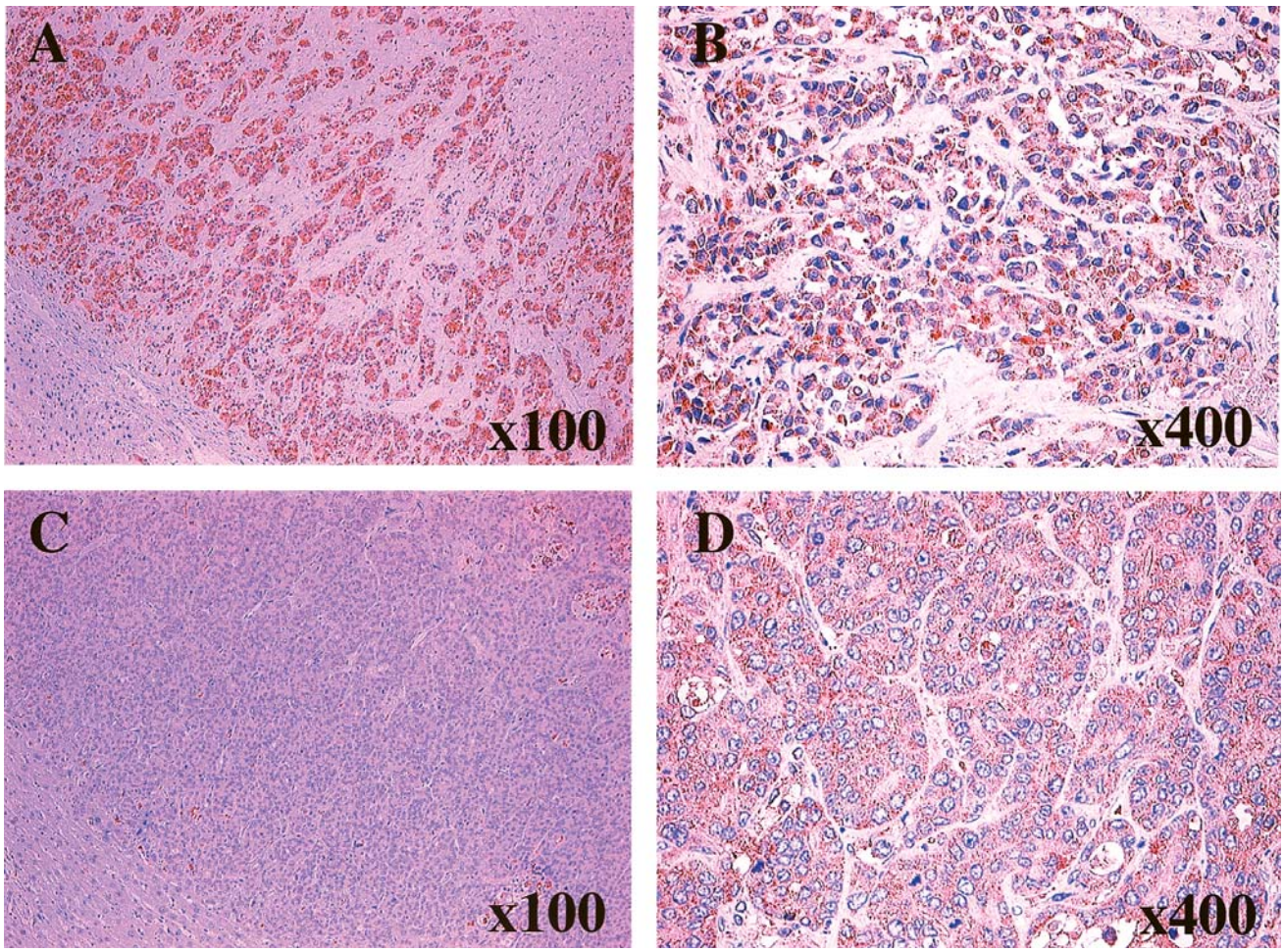

Figure 2. *Immunohistochemical expression of Fas (A, C) and Fas ligand (B, D). Fas ligand expression was similar in both treated (B) and control rats (D). On the other hand, the Fas expression of the metastatic tumors in the TAC-101-treated rats (A) was higher than that of the control rats (C).*

10% neutral buffered formalin and embedded in paraffin for immunohistochemical examination. For *in vivo* experiments, TAC-101 was suspended in 0.5% hydroxypropoxyl methyl cellulose.

Immunohistochemical staining for single-stranded DNA and determination of apoptotic index (A.I.). Apoptotic cells in the metastatic tumors were stained by immunohistochemistry for single-stranded (ss) DNA (28). Two-μm sections were dewaxed in xylene, dehydrated in ethanol and then incubated with 3% hydrogen peroxidase for 20 min to block endogeneous peroxidase activity. After washing with PBS, the sections were incubated in 10% normal bovine serum for 5 min, followed by incubation for 1 h with a rabbit polyclonal antibody against ssDNA at a 1:100 dilution. Biotinylated goat anti-rabbit IgG (Dako LSAB kit; Dako Japan Co., Ltd., Kyoto, Japan) was used at a dilution of 1:500. Finally, 0.02% diaminobenzidine and 1% hydrogen peroxidase (Dako Japan) in PBS were used as the substrate in the development of color. The sections were then counterstained with hematoxylin.

The evaluation of immunohistochemical staining for ssDNA was as previously described (28). The A.I. was defined as the number of positively-staining tumor cells among 1000 tumor cells. Five representative areas without necrosis in a section were selected by light microscopy using 200-fold magnification. Positively-staining tumor cells with the morphological characteristics of apoptosis were identified using standard criteria (29). Positively-staining cells located in the stroma and lumen were excluded because these apoptotic cells may have originated from other cell types.

Immunohistochemical staining of Fas and Fas ligand. After deparaffinization, dehydration and blocking endogeneous peroxidase activity, sections were incubated in 10% normal goat serum to reduce non-specific antibody binding.

For Fas staining, specimens were incubated in a 1:500 dilution of FAS(C-20)-G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) rabbit polyclonal antibody (30) for 60 min at room temperature. After washing with PBS, the slides were then treated with EnVision+ reagent (EnVision+ system, Dako,

Table I. *Anti-metastatic effect of TAC-101 on experimental hepatic metastasis.*

	Hepatic metastasis	
Group	Tumor incidence	No. of metastasis
Control (n=6)	5/6	3.67±2.58
TAC-101 (n=6)	1/6*	0.33±0.82**

Experimental hepatic metastasis of RCN-9 was induced by portal injection. TAC-101 was administered *p.o.* for five consecutive days per week for 4 weeks. Laparotomy was performed on day 30.
*p=0.021 **p=0.013

Table II. *Effect of TAC-101 on apoptotic index (A. I.) and proliferative index (P. I.).*

Group	A. I.	P. I.
Control (n=6)	2.80±2.01	219.8±34.9
TAC-101 (n=6)	6.84±2.62*	30.0±8.1**

The A. I. was defined as the number of ssDNA positively-staining tumor cells among 1000 tumor cells. Five representative areas without necrosis in a section were selected by light microscopy using 200-fold magnification. The P. I. of each tumor was determined by averaging the number of PCNA-positive cells in 5 random high-power fields (x 400).
*p=0.0001 **p=0.0001

Copenhagen, Denmark) for 30 min and were washed with PBS 3 times (31).

For Fas ligand staining, specimens were incubated in a 1:500 dilution of NCL-FAS-L (clone 5D1, Novo Castra Laboratories Ltd., UK), mouse monoclonal antibody (32) for 60 min at room temperature. After washing with PBS, sections were then incubated with biotinylated goat anti-mouse immunogloblin G (LSAB kit/HRP, Nichirei Corporation, Tokyo, Japan) at a dilution of 1:100 for 60 min at room temperature. Sections were then incubated with peroxidase-labelled streptoavidin reagent (LSAB kit/HRP, Nichirei Corporation) at a dilution of 1:500 for 60 min, followed by 3 washes with PBS.

Finally, the slides were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxidase for 10 min, counterstained with hematoxylin and mounted.

Immunohistochemical staining for PCNA and determination of proliferative index (P. I.). After deparaffinization, dehydration and blocking endogeneous peroxidase activity, sections were then washed in PBS and incubated in 10% normal goat serum to reduce non-specific antibody binding. Mouse anti-PCNA antibody (1:200) (PC-10, DAKO Japan) was used for 60 min at room temperature as the primary antibody (33). After washing with PBS, the slides were treated with EnVision+ reagent for 30 min and washed with PBS 3 times (31). Finally, the slides were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxidase for 10 min, counterstained with hematoxylin and mounted. Then, the P. I. of each tumor was determined by averaging the number of PCNA-positive cells in 5 random high-power fields (x 400), counted in a blinded fashion by a single observer with the aid of an optical grid (33).

Detection of apoptosis using FACScan. Evaluation of apoptosis was performed by staining cells with annexin V and propidium iodide (PI), since annexin V can identify the externalization of phosphatidylserine during the apoptotic process and, therefore, can detect early apoptotic cells (34, 35). Cells (1 x 10^5) were plated in 60-mm dishes and exposed for 10 and 20 h to 20 μM TAC-101 or DMSO alone (as a control), then harvested and labelled with annexin V and PI using the Apoptosis kit (Medical & Biological Laboratories Co., Ltd. Nagoya, Japan), according to the manufacturer's instructions. These cells were then analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Fluorescence data were displayed as dot plots using Cell Quest software (Becton Dickinson).

Statistical methods. Data represent mean±SD, and statistical significance was determined using the Student's *t*-test and regression theory, as appropriate. The association between TAC-101 administration and tumor incidence was analyzed using the χ^2 test. Statistical significance was established at the $p<0.05$ level.

Results

Growth inhibition of RCN-9 cells caused by TAC-101. Growth curves of the RCN-9 cells with TAC-101 or ATRA are shown in Figure 1. The concentration of TAC-101 required for 50% inhibition of growth (IC_{50}) in RCN-9 cells was 5.0 μM, while the IC_{50} of ATRA was 6.0 μM. Morphological changes in RCN-9 cells were observed in those treated with 10 μM and 100 μM TAC-101 and ATRA (data not shown).

Anti-hepatic-metastatic activity of TAC-101. Intra-portal injection of RCN-9 cells ($5x10^6$ cells) into F344 rats produced multiple hepatic metastatic tumors after 4 weeks. Oral administration of TAC-101 (8 mg/kg) for 5 consecutive days a week for 4 weeks resulted in a significant inhibition of hepatic metastasis without weight loss of the rats and did not markedly affect the tissues of the liver, kidney, lung or heart in histological examination (data not shown). Morphological changes in RCN-9 cells at the hepatic metastatic area were observed in the treated rats, with the cells showing a smaller nucleus and cytoplasm (Figure 2). Four weeks later, the incidence of hepatic metastasis in rats treated with TAC-101 was 17% (1/6), while that in the control rats was 83% (5/6). TAC-101 significantly reduced the incidence of hepatic metastasis in rats (p=0.021) (Table I). The number of hepatic metastases in the rats treated with TAC-101 was 0.33±0.82, while that in the control rats was 3.67±2.58. The number of

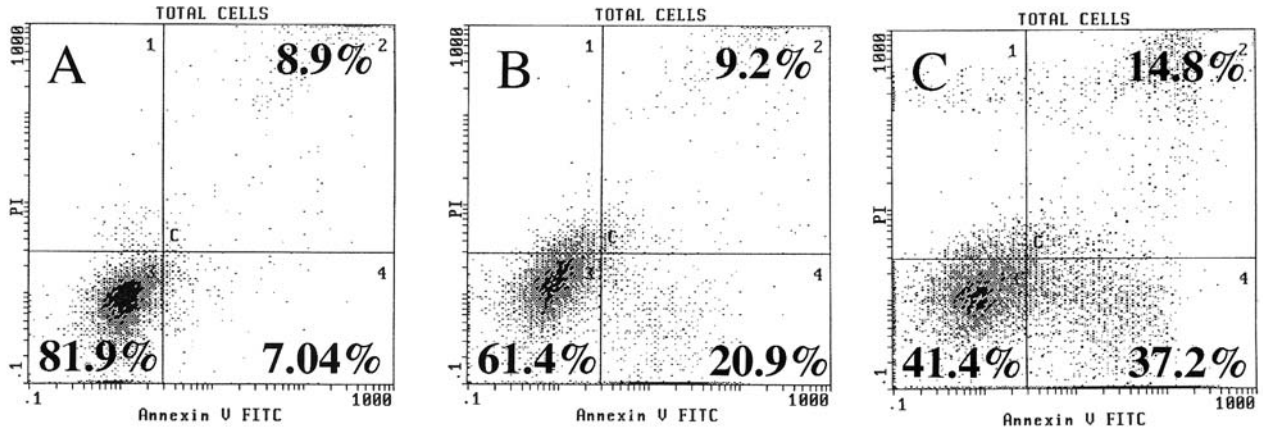

Figure 3. *Detection of apoptosis using FACScan analysis. DLD-1 cells (1 x 10^5 cells) were treated with DMSO alone for 20 h (A), or exposed to 20 μM TAC-101 for 10 h (B) or 20 h (C). Cells were stained with PI and annexin V and analyzed by flow cytometry. In the figure, each dot represents one cell. Annexin V-positive and PI-negative cells (lower right quadrant) are in an early stage of apoptosis and are still viable. Annexin V- and PI-positive cells (upper right quadrant) are in a later stage of apoptosis and necrosis and are no longer viable. Annexin V- and PI-negative cells (lower left quadrant) are intact cells. The percentage of cells of each quadrant is shown. The experiment was repeated three times, with similar results.*

hepatic metastases in the rats treated with TAC-101 was significantly lower than that of the control rats ($p=0.013$) (Table I).

P. I. in hepatic metastatic tumors. Oral administration of TAC-101 significantly decreased the P.I. in the metastatic hepatic tumors as compared with control rats ($p<0.0001$). P. I. was 219.8 ± 34.9 in the control group and 30.0±8.1 in the TAC-101 group (Table II).

Apoptosis in hepatic metastatic tumors. Apoptotic cells of the metastatic tumor were stained by immunohistochemistry for single-stranded DNA. The A.I. in the hepatic metastatic tumors of rats treated with TAC-101 was significantly higher (6.84 ± 2.62) than that of the control rats (2.80±2.01) ($p<0.0001$) (Table II).

Expression of Fas and Fas ligand in hepatic metastatic tumors. Immunohistochemistry was used to analyze the pattern and distribution of Fas (Figure 2A and C) and Fas ligand (Figure 2B and D). Fas ligand expression was similar in both treated and control rats. On the other hand, the Fas expression of metastatic tumors in the TAC-101-treated rat (Fas. 2A) was higher than that in the control rats (Figure 2C).

Detection of apoptosis using FACScan. The mean fraction of early apoptotic cells (lower right quadrant) was 7.0% for the control cultures (Figure 3A), 20.9% for the cultures exposed to TAC-101 for 10 h (Figure 3B) and 37.2% for the cultures exposed to TAC-101 for 20 h (Figure 3C). The mean fraction of late apoptotic and necrotic cells (upper right quadrant) was 8.9% for the control cultures (Figure 3A), 9.2% for the cultures exposed to TAC-101 for 10 h (Figure 3B), and 14.8% for the cultures exposed to TAC-101 for 20 h (Figure 3C). The mean fraction of intact cells (lower left quadrant) was 81.9% for the control cultures (Figure 3A), 61.4% for the cultures exposed to TAC-101 for 10 h (Figure 3B) and 41.4% for the cultures exposed to TAC-101 for 20 h (Figure 3C). The number of cells in early apoptosis obviously increased as the duration of exposure to TAC-101 increased. On the other hand, the number of intact cells decreased as the duration of exposure to TAC-101 increased. These data indicate that TAC-101 induces apoptosis in DLD-1 cells in a time-dependent manner.

Discussion

The formation of cancer metastasis requires overcoming the host's immunity. Investigators generally use metastatic models of nude mice to investigate the anti-metastatic effects of many compounds, due to the lack of a T cell immune response to cancer cells in nude mice. The hepatic immune system may interact with cancer cells in the sinusoids as well as when the tumor is proliferating in the hepatic parenchyma (27). The hepatic metastatic model in rats, used in the present study, maintains the hepatic immunity, in contrast to models of nude rats and mice. TAC-101 has already been evaluated with metastatic models of nude mice and human cancer cell lines (16-24). Thus, in the present study, we chose a rat model of hepatic metastasis with a rat colon cancer cell line to evaluate the anti-metastatic effect of TAC-101 under conserved immune system conditions.

As TAC-101 is an RA, the inhibitory mechanism of hepatic metastasis by this compound is thought to involve several actions, such as an anti-proliferative effect (18,19,21,24), anti-angiogenic effect (21,24), anti-invasive effect (18,20,22) and induction of apoptosis (17-19,22,23), similar to RA. RARs are known to form a complex with AP-1 and to interfere with the transactivation of AP-1-mediated molecules, including matrix metalloproteinase (MMP)-1, MMP-3 and MMP-9 (36,37), urokinase-type plasminogen activator (u-PA), VEGF (38), HGF (39), TGF-β (40) and PDGF (41). TAC-101 also has a marked inhibitory effect on AP-1 binding to DNA (16,20) and inhibits the production of u-PA and MMP-9 (18). These actions might contribute to the inhibition of hepatic metastasis. In the present study, we determined that TAC-101 inhibits the cell growth of RCN-9 at the same concentration as ATRA (Figure 1). Moreover, TAC-101 significantly inhibited hepatic metastasis *in vivo* (Table I) and suppressed the proliferation of tumor cells, as defined by P. I. (Table II). These effects are thought to be caused *via* RARs.

FACScan analysis demonstrated that TAC-101 induces apoptosis in the DLD-1 cell line in a time-dependent manner. Previous studies of apoptosis induced by TAC-101 have mostly monitored apoptosis by DNA ladder fragmentation. Therefore, they were unable to accurately distinguish apoptotic cells from necrotic cells. In the present study, we used double stain analysis (annexin V and PI) and FACScan for apoptosis detection (Figure 4). Using this method, which is more sensitive and easier than DNA ladder fragmentation, we were able to distinguish early apoptosis from necrosis and late apoptosis, and obtained qualitative data. Some retinoic acids have been reported to induce apoptosis mediated by RAR-α (42-45). Therefore, we postulate that apoptosis induction by TAC-101 may function *via* RAR-α-mediated signaling.

The Fas and Fas ligand are involved in apoptosis mediated by the immune system. Fas is a cysteine-rich type-I membrane protein belonging to the tumor necrosis factor (TNF) receptor family. After contraction with Fas ligand, cells expressing Fas undergo apoptosis (46) through an intracellular signaling pathway dependent on a cytoplasmic motif on Fas called the death domain (47). Möller *et al.* (48) demonstrated the expression of Fas in normal colonic epithelium in the cytoplasm and on the basolateral surface of epithelial cells, irrespective of its localization in the crypt or mucosal surface. They suggested that the Fas system may be involved in normal regulation of cell turnover and colonic tissue homeostasis. On the other hand, previous studies have indicated that Fas expression is reduced in colorectal carcinomas *in vivo* and *in vitro* (48,49), and that the Fas ligand is strongly expressed in hepatic metastatic tumors of colonic adenocarcinoma (32). Expression of Fas ligand on colon cancer cells may play an important role in establishing immunologically privileged environments that allow these cells to escape host immune surveillance (50). In the present study, we demonstrated that TAC-101 significantly increased the apoptotic index of the hepatic metastatic tumors as compared to that of the control rats (Table II). Although Fas ligand expression of both treated and control rats was of similar intensity (Figure 2), TAC-101 obviously increased Fas expression of the metastatic tumors compared with the control rats (Figure 2). Thus, increased Fas expression by TAC-101 may contribute to increasing apoptotic index.

In the present study, we determined two pathways of hepatic metastatic inhibition by TAC-101, namely growth inhibition of colon cancer cells and induction of apoptosis *via* the Fas-Fas ligand system on cancer cells. More detailed evaluation will be required to clarify the mechanism of the inhibitory effects of TAC-101.

Acknowledgements

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