HCC cells, and particularly the $CD4^-CD8^-$ cells. Our previous report revealed that $CD56^+$ NK cells and $CD14^+$ monocytes were the major effecter cells involved in the IFNα-induced cytotoxicity of PBMCs against HCC cells in this immunological process [16]. IFNα increases FasL expression on PBMCs and upregulates the immune response [42], and several investigators have reported FasL on the surface of $CD4^+$ cells, $CD8^+$ cells and NK cells. In fact, IFNα induced greater cytotoxicity in the $CD4^-CD8^-$ cells than in either the $CD4^+$ cells or $CD8^+$ cells. The data also indicated that both $CD4^+$ cells and $CD8^+$ cells reacted 2-fold to IFNα stimulation. In addition, pretreatment of HCC cells increased their sensitivity to Fas-mediated apoptosis, suggesting that (i) Fas-mediated cytotoxicity is enhanced by IFNα in innate immune effecter cells, particularly NK cells and monocytes, and (ii) Fas sensitivity of HCC cells regulated by IFNα/5-FU leads to increased cytotoxic interaction compared to regulation by either drug alone.

As described above, Fas/FasL may contribute to the anti-cancer effect of IFNα/5-FU via a tumor immune response, but we consider that such a mechanism only partially explains the anti-tumor activity of combination therapy. This is because the Fas-negative cell lines (HuH7, PLC/PRF/5 and Hep3B) showed no response to CH-11 induction, and IFNα/5-FU did not affect the Fas-mediated apoptosis. Our previous report showed some Fas-negative cell lines respond in other ways to IFNα/5-FU treatment [15–17,43]. In PLC/PRF/5, which express a high level of IFN receptor, IFNα/5-FU acted mainly through the direct cell arrest effect of IFNα [15,43]. Also, HLE, a responder to Fas-mediated apoptosis, also underwent cell death via a TRAIL/TRAIL-R pathway after IFNα/5-FU stimulation [16]. Thus, the therapeutic effect of IFNα/5-FU against HCC might be mediated via various pathways according to the specific characteristics of the cancer cells. This might also explain why the response rate to IFNα/5-FU is not complete, being almost 50% in HCC treatment [10,11,13].

Finally, the influence of IFNα/5-FU combined therapy to normal hepatocytes was evaluated in terms of apoptosis mediated by Fas/FasL system. Several investigators have reported that normal hepatocytes express Fas on their surface and that is cause of fulminant hepatitis with administration of agonistic anti-Fas antibody [35,44–47]. Although the results of in vitro assays revealed that normal hepatocyte has sensitivity to agonistic anti-Fas antibody in the present study, no enhancement was induced by the IFNα/5-FU about apoptosis in Fas/FasL system. On the other hand, the apoptotic effect of FasL was significant in the all Fas-positive HCC cells. In addition, TUNEL staining of resected human samples who received IFNα/5-FU therapy before operation also showed that apoptotic cells were only counted in the tumor site; no apoptotic cells in normal liver. These results suggested that IFN/5-FU did not enhance the cell death because of the uncertain mechanism of the escape from the apoptotic system of Fas/FasL pathway in normal hepatocyte and IFN/5-FU upregulated apoptotic effect of Fas/FasL system, showing anti-tumor activity in HCC.

In conclusion, IFNα and 5-FU synergistically enhanced the sensitivity of hepatoma cell to Fas-mediated apoptosis with an increase in caspase-3 activity. In addition, we showed that IFNα upregulates the cytotoxicity of PBMCs, and interactions between PBMCs and hepatoma cells via the Fas/FasL pathway were most enhanced when both effecter and target cells were pretreated with IFNα/5-FU. These results indicated that Fas-mediated apoptosis at least partially contributes to the beneficial effect of IFNα/5-FU therapy against HCC in the clinic.

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Cancer Cells Cause Vascular Endothelial Cell (vEC) Retraction via 12(S)HETE Secretion; The Possible Role of Cancer Cell Derived Microparticle

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Background: Cancer cell mediated vascular endothelial cell (vEC) retraction plays a pivotal role in cancer metastasis. The aim of this study is to clarify the biochemical character of vEC retraction factor derived from human breast cancer cell line, MCF-7.

Methods and Results: In order to estimate vEC retracting activity, transwell chamber assay system was employed. We first tested the effects of trypsin digestion as well as lipid extraction of culture medium (CM). Trypsin digestion of CM resulted in approximately 40% loss of vEC retracting activity and lipid extraction of CM by Brigh and Dyer methods recovered approximately 60% of vEC retracting activity, suggesting that approximately 60% of vEC retracting activity in MCF-7 derived CM is due to lipid. Although Nordihydroguaiaretic acid (NDGA), the specific lipoxygenase inhibitor, suppressed vEC retracting activity in CM, Acetyl salicylic acid (ASA), a specific cyclooxygenase inhibitor, did not affect the activity, suggesting that lipid exerting vEC retracting activity in CM belongs to lipoxygenase mediated arachidonate metabolites. Thin layer chromatography clearly demonstrated that Rf value of lipid vEC retracting factor in CM is identical to 12HETE. Authentic 12(S)HETE, but not 12(R)HETE, showed vEC retracting activity. After the ultracentrifugation of CM, most lipid vEC retracting activity was recovered from the pellet fraction, and flow cytometric analysis using specific antibody against 12(S)HETE clearly showed the association of 12(S)HETE with small particle in CM.

Conclusion: These findings suggested the principal involvement of 12(S)HETE in cancer cell derived microparticles in cancer cell mediated vEC retraction.

Key Words: Endothelial cell retraction—12(S)HETE—Cancer cell—Cancer metastasis—Microparticles.

Cancer metastasis requires several steps such as invasion through basement membrane, passage through extracellular matrix, intravasation, interaction with vascular endothelial cells (vEC), extravasation, and growth in the new site.[1,2] Among these steps, tumor invasion into the subendothelial layer has been considered one of the critical steps for the development of tumor metastasis. Since the integrity of vEC works as a barrier against cancer cell migration, cancer cell mediated retraction of vEC plays a key role in this process of cancer cell extravasation.[3]

Cancer cells are considered to induce vEC retraction through two different mechanisms; one is the direct stimulation of vEC by cell to cell contact via cell surface ligand-receptor interaction.[4,5] This mode of cancer cell-vEC interaction has been intensively studied especially in the field of cell surface adhesive ligands like E-selectin.[6] The other possible mecha-

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nism is the indirect stimulation of vECs by soluble factors secreted from cancer cells.[7] In general, soluble factors like glycoprotein or lipids are known to cause vEC retraction.[8,9] However there have been no reports investigating cancer cell derived bioactive molecules causing vEC retraction. In this report, we investigated on cancer cell secreted lipids involved in vEC retraction.

MATERIALS AND METHODS

Materials

Thin layer chromatography plate, LK6DF Silica Gel 60 å, was purchased from Whatman International Ltd (England). Monoclonal antibody against 12(S) hydroxyeicosatetraenoic acid (HETE) was purchased from Assay Designs Inc. (USA). Authentic 12(S)HETE and 12(R)HETE was purchased from Cayman Chemical (USA). Transwell chamber, Costar 3413, was purchased from Corning Incorporated (USA). Matrigel was purchased from Becton Dickinson Labware, Bredford, (MA, USA). Trypsin, acetylsalicylic acid (ASA), FITC-dextran and Nordihydroguaia-retic acid (NDGA) were purchased from Sigma Chemical Co. (USA). Other reagents were of the highest analytical grade available.

Cell Culture

Calf pulmonary arterial endothelial cells (CPAE) were obtained from Japan Cell Resources Bank (Osaka, Japan). CPAE were prepared and passaged as described previously.[10] For retraction experiments, cells were grown on the transwell chamber, Costar 3413, coated with Matrigel in medium (D-MEN) with 10% fetal bovine serum (FBS), 5 μg/ml of recombinant human basic fibroblast growth factor (b-FGF, Pepro Tech EC, London, UK), penicillin (100 units/ml), and streptomycin (100 μg/ml). Confluent cells were rinsed twice with modified HEPES Tyrode's buffer (MHTB:129.0 mM NaCl, 10.0 mM HEPES, 8.9 mM $NaHCO_3$, 5,6 mM dextrose, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, pH 7.4).

Preparation of Culture Medium (CM) derived from MCF-7

MCF-7 cell line was obtained from the Japanese Cancer Research Bank (Tokyo, Japan). CM was prepared using MCF-7 cells, as described previously.[10] Briefly, cells were grown on culture dishes and incubated at 37°C in DMEM/F12 supplemented with 10% FBS, penicillin, and streptomycin. After incubation for 3 to 4 days to form a confluent monolayer, cells were washed three times with PBS in order to remove serum complements, and incubated with 20 ml serum free DMEM/F12. After incubation for 48 hr, CM was collected and centrifuged at 12000 g for 10 min. The collected supernatant was dialyzed against RPMI 1640 medium for 24 hr at 4°C and passed through a 0.45 μm filter to remove cells and cell debris. The filtered supernatant was concentrated by ultrafiltration membrane PM10 (Amicon, Danvers, MA, USA). The concentrate was dialyzed against PBS for 24 hr at 4°C and filtered through a 0.45 μm filter again. After protein concentration was adjusted to 1mg/ml by adding PBS, it was subjected to vEC retraction assays.

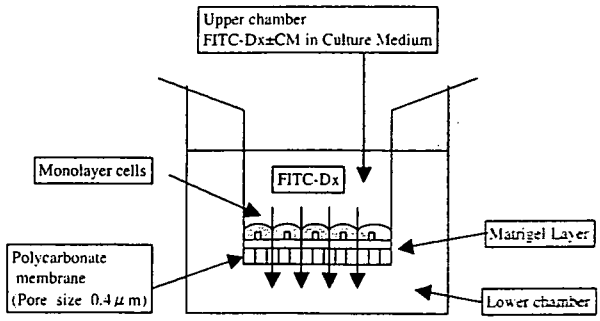

FIG. 1. CPAE-cell retraction assay. A schematic represents the transwell chamber assay for measuring the endothelial cell retraction activity. CM derived from tumor cells was added into the upper chamber to induce CPAE-cell retraction. When endothelial cells are retracted exposing inter endothelial spaces, the amount of FITC-Dx diffused from the upper to the lower chamber is increased. The activity of CPAE-cell retraction is expressed as percent activity of untreated CM, designating the difference in the amount of FITC-Dx between the absence and the presence of untreated CM as 100%.

Vascular Endothelial Cell (vEC) Retraction Assay

The extent of vEC retraction was measured as the amount of fluorescein-isothiocyanate-labeled dextran (FITC-Dx: average molecular weight, 70kDa) (Sigma) that passed across an endothelial cell monolayer. CPAE cells were cultured to form a monolayer for 3 days on the polycarbonate membranes with a 0.4 μm pore size coated with the reconstituted basement membrane MATRIGEL™ (Becton Dickinson Labware, Bredford, MA, USA) of each upper chamber of the transwell chamber (Costar, Cambridge, MA, USA) (Fig. 1). After the removal of culture medium, 200 μl of the medium containing 1mg/ml of FITC-Dx with and without crude and treated CM was added into the each upper transwell chamber. The lower

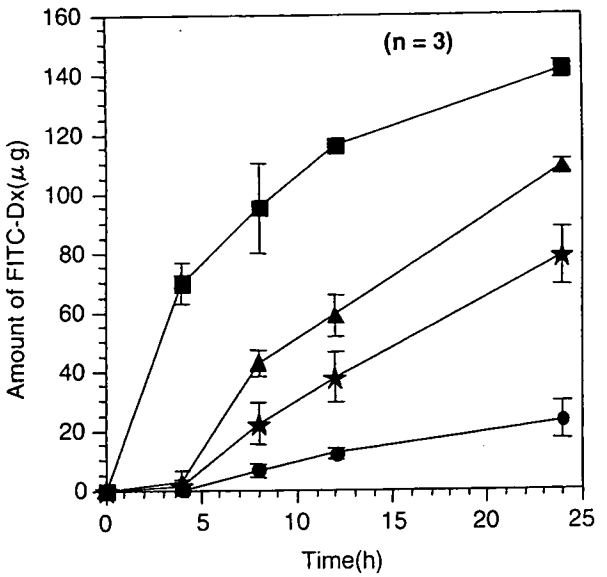

FIG. 2. Effects of MCF-7 CM on the CPAE cell retraction activity. The extent of retraction was determined by measuring the amount of FITC-Dx, which passed through a CPAE monolayer on Matrigel-coated polycarbonate. Membrane only (square). CPAE-cell monolayer on a Matrigel-coated polycarbonate membrane (circle). Addition of CM (2mg/ml) derived from MCF-7 cells on CPAE cell monolayer (triangle). Addition of CM (1 mg/ml) derived from MCF-7 cells on CPAE cell monolayer (star). Values are mean ± SD from triplicate experiments.

transwell chamber was filled with 1 ml of the same medium without FITC-Dx and these transwells were cultured at 37°C for a given period. Following culture, the amount of FITC-DX in the lower chamber was quantified by Microplate reader M-T max (Wako, Osaka, JAPAN) under excision and emission wave lengths of 490 nm and 550 nm, respectively. The concentration of FITC-Dx in the lower chamber was calculated from the relative fluorescence intensity against 1mg/ml FITC dextran in culture medium. The endothelial cell retraction activity was represented by the mean concentration of FITC-Dx in the lower chamber. Each assay was performed in triplicate. As shown in Fig. 2, our transwell vEC retraction assay clearly showed a good time- and dose-dependency, suggesting that our system is suitable for measuring vEC retracting activity in CM. We employed incubation time of 24 hours for later experiments.

Treatment of CM with Tripsin

CM was incubated with 0.1% tripsin at 37°C for 1hr. Then, the amount of sample was adjusted to be equivalent to that of the untreated, control experiment.

Lipid Extraction and Separation

Lipids were extracted from each aqueous sample according to the method of Bligh and Dyer's,[11] and samples were kept at −20°C until use. Briefly, each sample was added chloroform (250 μl) and shaken for 30 seconds, were added distilled water (300 μl), and shaken for another 30 seconds, and then, were centrifuged at 20g for 5 minutes at 4°C. After complete separation to chloroform layer, fluff layer, and alcoholic layer, chloroform layer was removed by aspiration. The chloroform layers were evaporated under nitrogen stream to form crystals. Thin layer chromatography using TLC plate, LK6DF was carried out using the solvent system (chloroform/methanol acetic acid/water = 90/8/1/0.8;v/v/v/v) at 4°C according to the method of Okuma.[12] After the visualization of separated lipids by iodine vapor, Rf value was calculated. In some experiments, separated lipid was recovered by scratching this part of the silica gel and lipid was re-extracted by Bligh and Dyer's Methods.

Flow Cytometric Analysis of 12(S)HETE

One ml of MCF-7 CM with or without addition of monoclonal antibody against 12(S)HETE were ultracentrifuged at 105000g for 2.5 hours at 4°C. The resultant pellets were incubated with 1 μg of FITC-labeled mAb against 12(S)HETE or control IgG for 30 min at 4°C. Then, samples were analyzed on a fluorescence-activated cell sorter (FACScan, Beckton Dickinson) according to the methods described by Yamamoto et al.[13] Data were processed using Cell Quest™ software (BD).

RESULTS

1. Effects of proteolytic digestion, lipid extraction, and heat treatment on CPAE-cell retraction by crude MCF-7 cell CM.

Addition of MCF-7 cell CM (0 to 2 mg/ml) enhanced CPAE-cell retraction in a dose or time related manner as shown in Fig. 1. About 75% of the cell retraction activity was obtained with 2 mg/ml of CM. In order to determine what component of CM causes CPAE-cell retraction, crude MCF-7 cell CM was subjected to trypsin digestion, lipid extraction, and heat treatment (Fig. 3). The CPAE cell retraction activity was decreased up to 38 ± 6% and 46 ± 11% by 0.1% trypsin digestion and lipid extraction, respectively. On the other hand, heat treatment at

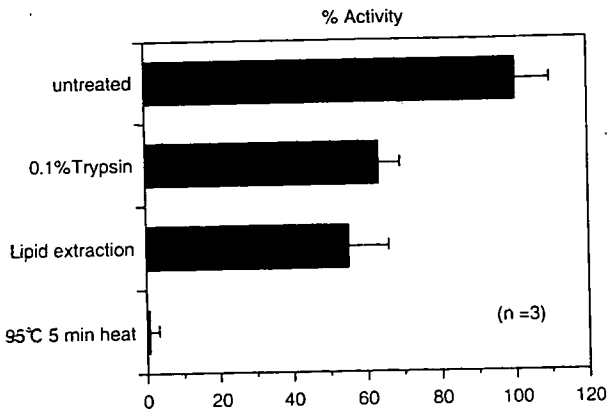

FIG. 3. Effects of trypsin digestion, lipid extraction, and heat treatment on CPAE-cell retraction activity of MCF-7 CM. Crude MCF-7 CM was treated with 0.1% trypsin at 37°C for 1 hour. Lipids in MCF-7 CM were extracted by the method of Bligh and Dyer's,[11] as described in Materials and Methods. MCF-7 CM was incubated at 95°C for 5 min. In the respective experiments, the amount of sample was adjusted to be equivalent to that of the untreated, control experiment. The activity of CPAE-cell retraction is expressed as the percent activity of untreated CM.

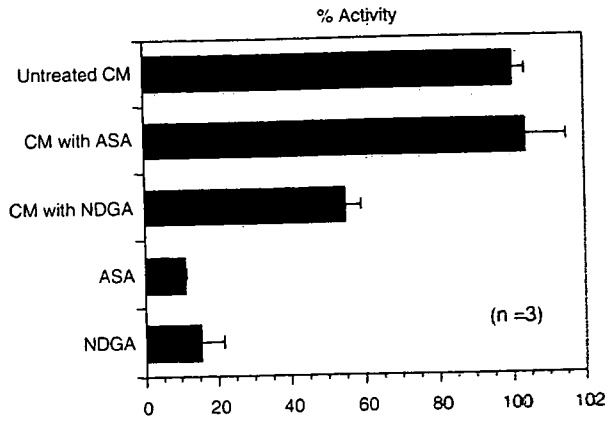

FIG. 4. Effect of acetyl salicylic acid (ASA) and nordihydroguaiaretic acid (NDGA) on CPAE cell retraction activity of MCF-7 CM. After incubation for 3 to 4 days to form a confluent monolayer, MCF-7 cells were washed three times with PBS, and incubated with 20ml serum free DMEN/F12, which added ASA (50 μM in final concentration) and NDGA (50 μM in final concentration) each. After incubation at 37°C for 48 hours. CM was made as same methods. Treated CM was added to the upper chamber of the CPAE-cell retraction assay system, and the vEC retraction activity was measured as in Materials and Methods.

95°C for 5 min completely abolished CPAE-cell retraction activity.

2. Effects of acetyl salicylic acid (ASA) and nordihydroguaiaretic acid (NDGA) on CPAE cell retraction by crude MCF-7 cell CM.

Treatment with ASA (final concentration: 50 μM), a cyclooxygenase inhibitor did not affect the secretion of CPAE-cell retraction activity from MCF-7 cells. In contrast, NDGA (final concentration: 50 μM), a lypooxgenase inhibitor markedly suppressed its activity (Fig. 4).

3. Separation of lipids extracted from MCF-7 CM by thin layer chromatography.

Lipids extracted from MCF-7 CM preserved about 64 ± 8% of the whole activity of crude CM (Fig. 6). These lipids were separated into three bands by thin layer chromatography which appeared at 0.78, 0.73, and 0.45 of Rf value (Fig. 5). The most activity (66 ± 7%) of CPAE-cell retraction was recovered in the 0.73 Rf band, corresponding to 12HETE (Fig. 6) while a total activity of 24 ± 10% was observed in the remaining two bands. To further confirm the activity of 12HETE, the stereoisomers of 12HETE. 12(S)HETE (0.3 μM: final concentration) and 12(R)HETE (0.3 μM: final concentration) were subject to endothelial-cell retraction assay. About 80% of crude MCF-7 CM activity was observed in 12(S)-HETE, but only 7% in 12(R)-HETE. (Fig. 7).

4. Localization of CPAE cell retraction activity in MCF-7 CM.

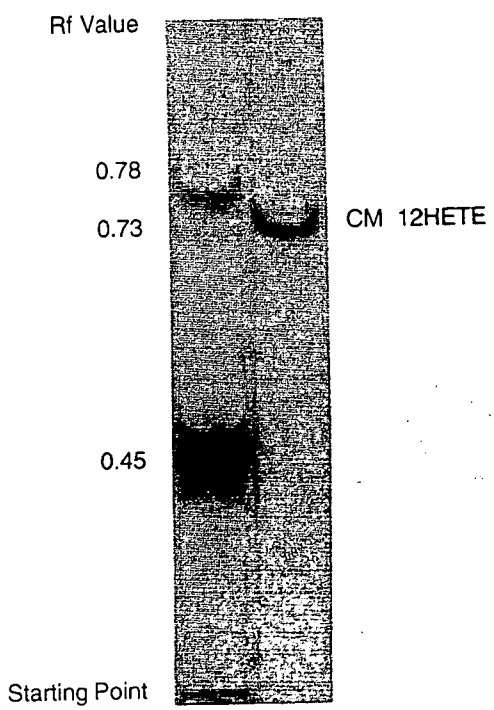

FIG. 5. Separation of lipids extracted from MCF-7 CM by thin-layer chromatography. Lipids extracted from MCF-7 CM were separated by thin-layer chromatography into three bands (0.78, 0.73, and 0.45 of Rf value). The band Rf value 0.73 was equivalent to 12 HETE.

To determine the localization of CPAE cell retraction activity, crude MCF-7 was separated into

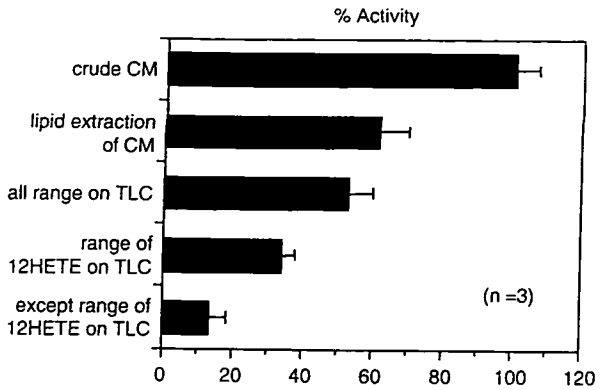

FIG. 6. Separation of lipids extracted from MCF-7 CM by thin-layer chromatography. CPAE cell retraction activity of separated bands: The lipids of these bands were extracted and subjected to CPAE, cell retraction activity was measured. The CPAE cell retraction activity of each sample is expressed as the percentage activity of crude MCF-7 CM.

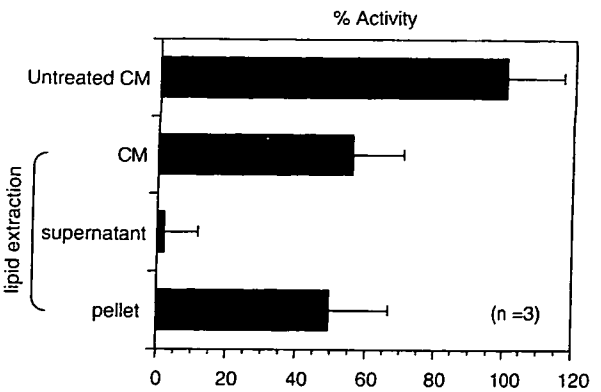

FIG. 8. The localization of CPAE-cell retraction activity in MCF-7 CM. The supernatant fraction and pellet fraction were separated from crude MCF-7 CM by ultracentrifuge (105000g, 150 min at 4°C). Lipids of the respective fraction were extracted, and subjected to endothelial-cell retraction assay as described in Materials and Methods. CPAE cell retraction activity of the respective fraction was expressed as the percent activity of untreated MCF-7 CM.

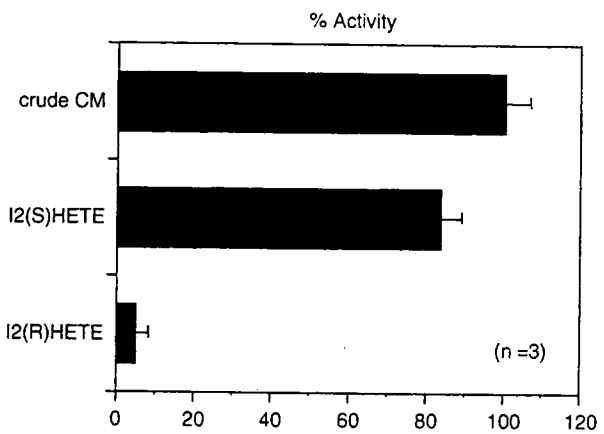

FIG. 7. 12(R)HETE and 12(S)HETE (0.3μM: final concentration) were subjected to endothelial-cell retraction assay. CPAE cell retraction activity was expressed as the percent activity of crude MCF-7 CM.

the soluble, supernatant fraction and the pellet (microparticle) fraction by ultracentrifugation and subject to endothelial-cell retraction assay. Almost all CPAE cell retraction activity was obtained in the pellet fraction (Fig. 8). Then, flowcytometric analysis of the pellet fraction was performed using monoclonal antibody against 12(S)HETE to further investigate the expression of CPAE cell retraction activity. The expression of 12(S)HETE in the surface of microparticles, as shown in Fig. 8.

DISCUSSION

It is well known that cancer cell causes the retraction of vascular endothelial cell, one of the key steps for the establishment of tumor metastasis.[3] Several molecules, such as vasoactive substance,[14] lipid mediator,[9,15] thrombin,[16] or cytokines,[17–20] have been reported to increase the permeability of vasculature, but the molecular mechanisms of vEC retraction by cancer cells has not been fully understood. One of coauthors, Nakamori, previously reported a cancer cell derived, heat resistant glycoprotein with MW 10-15kDa as vEC retracting factor.[7] In this study, we demonstrated that approximately 60% of heat labile, vEC retracting activity of MCF-7 CM was derived from the lipid fraction, where 12(s)HETE played a major role in this process.

Since culture medium of cancer cells were considered to contain multiple vEC retracting factors, we first estimated what component was responsible for the vEC retracting activity of MCF-7 CM. As shown in Fig. 3, vEC retracting activity was heat labile. Trypsin digestion of CM abolished approximately 40% of vEC retracting activity, suggesting the some involvement of peptide molecules. By lipid extraction, approximately 60% of vEC retracting activity was recovered. These results suggest that a major vEC retracting activity in MCF-7 CM belonged to lipid component. Since several arachidonate metabolites have been reported to show vasoactive activity,[21] we next tested the effects of inhibitors of arachidonate metabolism. As shown in Fig. 4, treatment of cancer cells with a cyclooxygenase inhibitor, acetylsalicylic acid (ASA), did not affect the secretion of vEC retracting activity, whereas, a lypooxgenase inhibitor, nordihydroguaiaretic acid (NDGA), markedly suppressed the generation of vEC retracting activity from

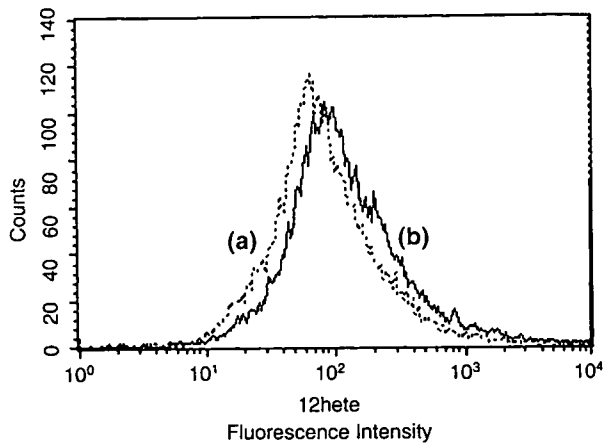

FIG. 9. Flow cytometric histogram of 12(S)HETE expression on ultracentrifugated pellet of CM. The pellet fraction of MCF-7 CM (1 ml) was incubated with 1μg of FITC-labeled mAb against 12(S)HETE or control IgG for 30min at 4°C, as described in Materials and Methods. Then, samples were analyzed on a fluorescence-activated cell sorter (FACScan). Dotted line: second antibody alone; Solid line: anti-12(S)HETE antibody plus second antibody.

cancer cells. These findings strongly suggested that vEC retracting activity of lipid component resulted from arachidonate metabolites via lipoxygenase.

To further investigate the lipid component responsible for vEC retracting activity, we separated the lipids extract from MCF-7 CM by thin layer chromatography. As shown in Fig. 6, most, 60% of vEC retracting activity was recovered from the lipid at Rf value 0.73, which corresponded to 12-HETE. There have been several reports describing the natural existence of two isoforms of 12HETE,[22] 12(S)-HETE and 12(R)-HETE. Kenneth et al. reported the roles of 12(S)-HETE in the signal transduction system of vECs integral to retraction.[21] Therefore, we tested vEC retracting activity of these isomers. Consistent with the previous report by Kenneth et al., 12(S)-HETE, but not 12(R)-HETE showed any vEC retracting activity, as shown in Fig. 7. From these observations, it is suggested that major vEC retracting activity derived from cancer cells is due to 12(S)-HETE.

It is quite interesting to know how cancer cells generate and secrete hydrophobic 12(S)-HETE, which was demonstrated to be present in culture medium of cancer cells. One possibility is that 12(S)-HETE might be present in microparticles, formed by shedding from cancer cells. Actually, several reports including ours[23] demonstrated the important role of micropartcles as a carrier for hydrophobic lipids in blood stream. In order to test this hypothesis, we separated vEC retracting activity of MCF-7 CM by ultracentrifugation. As shown in Fig. 8, almost all vEC retracting activity was recovered from pellet fraction. The association of 12(s)-HETE with microparticles was further confirmed by flow cytometric analysis with monoclonal antibody against 12(S)HETE. As shown in Fig. 9, the expression of 12(S)HETE was confirmed in MCF-7 cells derived microparticles.

Cancer cells are activated and shed microparticles following various humoral or mechanical stimuli. 12(S)-HETE is generated during this process because intracellular biochemical pathways including arachidonate metabolism are also activated. Therefore, it is quite possible that 12HETE generated and present in microparticles plays an important role in vEC retraction and the subsequent establishment of cancer metastasis.

In summary, cancer cells secrete vEC retraction activity. 12(S)-HETE generated and present in microparticles play a major role in cancer cells induced vEC retraction.

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Human Equilibrative Nucleoside Transporter 1, as a Predictor of 5-Fluorouracil Resistance in Human Pancreatic Cancer

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Abstract. *Background: The purpose of this study was to find a novel biomarker to predict 5-fluorouracil (5-FU) or gemcitabine (2',2'-difluoro-deoxycytidine) sensitivity in pancreatic cancer. Materials and Methods: The relationship between 5-FU and gemcitabine sensitivity and the mRNA levels of human equilibrative nucleoside transporter 1 (hENT1), thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) was investigated using seven types of human pancreatic carcinoma cell line (AsPC1, BxPC3, MiaPaCa-2, PSN1, Panc1, PCI6, and KMP-4). Quantitative mRNA expression was measured by LightCycler. A [^{3}H] gemcitabine cellular uptake assay was performed to examine the inhibition of hENT1 by nitrobenzylmercaptoprine ribonucleoside (NBMPR). Results: The expression levels of hENT1 mRNA significantly correlated with the IC_{50} value of 5-FU in all seven lines and also correlated with gemcitabine resistance in six lines (except AsPC1). No significant association was observed between TS or DPD mRNA levels and 5-FU sensitivity. In the PSN1 cells, [^{3}H] gemcitabine uptake via hENT1 was significantly inhibited by NBMPR, and 5-FU sensitivity was significantly increased when the cells were pretreated with NBMPR. Conclusion: Our results suggest that hENT1 plays an important role in 5-FU resistance and that hENT1 mRNA levels might be a useful marker to predict5-FU sensitivity in pancreatic cancer.*

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Key Words: Human equilibrative nucleoside transporter 1, pancreatic cancer, 5-fluorouracil, gemcitabine, thymidylate synthase, dihydropyrimidine dehydrogenase.

Pancreatic cancer is one of the most lethal of all the common gastrointestinal malignancies. This disease carries a dismal prognosis with a 5-year survival rate of less than 5% (1). In advanced disease where surgery is not an option, other therapeutic options including 5-fluorouracil (5-FU) chemoradiation and gemcitabine (2',2'-difluoro-deoxycytidine) chemotherapy are considered (2). 5-FU, first synthesized by Heidelberger *et al.* in 1957 (3), is one of the most commonly used chemotherapeutic reagents in digestive carcinoma including pancreatic cancer. According to several randomized trials, 5-FU chemotherapy combined with external beam radiation therapy has become a frequently employed therapy for patients with locally advanced pancreatic carcinomas (1). Gemcitabine, a novel pyrimidine nucleoside analogue, has been reported to improve the survival and clinical benefit responses compared to 5-FU in patients with advanced, symptomatic pancreatic cancer (4). Although single-agent gemcitabine is currently the standard first-line treatment for the patients with metastatic pancreatic cancer (2), it provides only limited benefit because of the endogenous or acquired resistance of tumor cells, and 5-FU or its derivatives still play a key role in combination with gemcitabine (5, 6).

Cells can synthesize nucleotides not only through the *de novo* synthesis pathway but also the salvage pathway. In the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by nucleoside transporter (NT) proteins. In addition to nucleosides, nucleoside analogues, such as gemcitabine, are also taken up into the cell *via* these specific transporters (7). Gemcitabine is a substrate for five of the NTs found in humans (8). Human equilibrative nucleoside transporter 1 (hENT1) is one of those NTs, and gemcitabine is transported into cells mostly by hENT1 (8). It has been reported that the sensitivity to nucleoside analogues correlates with the expression of hENT1, and that cells lacking hENT1 are highly resistant to gemcitabine (8). On the other hand, 5-FU

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is known as a *de novo* synthesis inhibitor, and there is a possibility that the cellular uptake and supply of nucleosides and nucleobases through hENT1 (*via* the salvage pathway) would interfere with the effect of 5-FU in the cells. Recently, Kubota reported that high mRNA expression of hENT1 might result in low sensitivity to 5-FU in colorectal cancer (9). However, it remains to be elucidated whether or not the expression level of hENT1 influences the sensitivity to 5-FU or gemcitabine in human pancreatic cancer.

5-FU is one of the thymidylate synthase (TS) inhibitors (10), and multiple clinical investigations have suggested that high TS expression resulted in 5-FU resistance in colorectal and gastric cancers (11-13). The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU) (14). High levels of DPD mRNA expression in colorectal tumors have been shown to correlate with resistance to 5-FU (15). However, in pancreatic cancer, the relationship between expression levels of TS or DPD and 5-FU resistance is still obscure.

Understanding the mechanism by which pancreatic carcinomas become resistant to chemotherapy is an essential step towards predicting or overcoming that resistance. Although several molecular markers have been reported to determine the sensitivity/resistance to 5-FU or gemcitabine in human pancreatic cancer (16-26), these markers have not yet been useful in clinical settings. In this study, in order to find a novel biomarker to predict chemosensitivity, the mRNA expression of the genes related to metabolism and nucleoside transport which may affect the efficacy of 5-FU or gemcitabine were focused upon.

Materials and Methods

Cell cultures. Seven types of human pancreatic carcinoma cell line were used in the present study. The AsPC1, MiaPaCa-2 and PSN1 cell lines were obtained from the Japanese Collection of Research Bioresources (JCRB, Japan). The BxPC3 and Panc1 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The PCI6 cell line was a gift from Dr. H. Ishikawa (Hokkaido University, Sapporo, Japan) and the KMP-4 cell line was a gift from Professor Imamura (Kyoto University, Kyoto, Japan). All the cell lines were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Nikken Biomedical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Rockville, MD, USA) and 100 units/ml each of penicillin and streptomycin.

Chemicals. 5-FU was purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). Gemcitabine was kindly provided by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). [^{3}H] gemcitabine was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). Nitrobenzylmercaptopurine ribonucleoside (NBMPR) was purchased from Sigma (St. Louis, MO, USA). 5-FU, gemcitabine, and NBMPR were dissolved in distilled water and applied to the cells at a volume less than 0.1% of the medium volume.

Table I. *Pairs of primers for detecting hENT1, TS, or DPD gene by (quantitative) RT-PCR.*

Gene		Primers
hENT1	Forward	5'-AATATCTTTGACTGGTTGGG-3'
	Reverse	5'-CAGCCATGAAGAAAATGAAC-3'
TS	Forward	5'-TCTGCTGACAACCAAACGTG-3'
	Reverse	5'-GTTCACCACATAGAACTGGC-3'
DPD	Forward	5'-GAGAAGCAATGAGATGCCTG-3'
	Reverse	5'-CAGCCGGAACTGAGGAATTT-3'

RT-PCR, reverse transcription-polymerase chain reaction; hENT1, human equilibrative nucleoside transporter 1; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction was performed with Trizol Reagent (Life Technologies, Inc., Grand Island, NY, USA) in a single-step method and complementary DNA (cDNA) was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). PCR was performed in a 25 μl reaction mixture containing 2 μl of cDNA template, 1x Perkin Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.5 pmol of primers, and 1 unit of Taq DNA Polymerase (AmpliTaq Gold, Roche Molecular Systems Inc., Branchburg, NJ, USA). The PCR primers used for detection of hENT1, TS and DPD are shown in Table I. The primers for porphobilinogen deaminase (PBGD) were synthesized as described previously (27). Following PCR (hENT1, 35 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min; TS, DPD and PBGD, 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min), the products were run on 2 % agarose gel and visualized by ethidium bromide staining.

Quantification of hENT1, TS, and DPD gene expression. A quantitative gene expression assay was performed using a LightCycler (Idaho Technology, USA), as described previously (28). The PCR primers used for detection of hENT1, TS and DPD were the same as used for RT-PCR. The PCR conditions were set up as follows: hENT1, one cycle of denaturing at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec; TS, DPD and PBGD, one cycle of denaturing at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. In each assay, a house-keeping gene, PBGD was employed as an internal standard. The quantification data from each sample were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. The relative gene expression levels were shown as the quantified gene expression divided by the quantified PBGD levels.

Growth inhibitory effect of 5-FU, gemcitabine or combination of NBMPR and 5-FU. The cell viability was determined by MTT (3- [4,5-dimethylthiazol-2-yl] -2,5- diphenyltetrazolium bromide) assay (Sigma). The cells were seeded in 96-well culture plates in culture medium at an optimal density. After 24 h, the medium was exchanged with fresh culture medium containing either vehicle only or the indicated dose of the test chemicals. To assess the effect of NBMPR on the 5-FU sensitivity, the cells were treated with 1 μM NBMPR for 3 min before and during 5-FU exposure. After a 2-day

Table II. *IC_{50} values for 5-FU and gemcitabine in seven types of human pancreatic cancer cell line.*

	IC_{50}	
	5-FU (μg/ml)	Gemcitabine (ng/ml)
AsPC1	33.2±2.4	129.0±10.4
BxPC3	1.7±0.2	50.0±7.1
MiaPaCa-2	2.3±0.2	27.1±4.3
PSN1	1.7±0.3	1.4±0.2
Panc1	3.7±0.4	178.3±23.6
PCI6	6.3±0.3	217.4±20.1
KMP-4	2.0±0.3	32.2±3.5

5-FU: 5-fluorouracil.

incubation, the MTT assay was performed according to the instructions provided by the manufacturer. Absorbance was measured at 560 nm using a microtiter plate reader (Wako, Osaka, Japan). With this method, the results only correlated with the viable cell number. The IC_{50} values for each chemical were estimated by plotting the rate of growth inhibition *versus* the drug concentration.

[^{3}H] gemcitabine cellular uptake assay. To examine the inhibition of hENT1 function by NBMPR, a [^{3}H] gemcitabine cellular uptake assay was performed as described previously (29). The cells were seeded in a flat-bottomed 24-well microplate ($1x10^4$ / well) and incubated for 24 h. The cells were treated with 1 μM NBMPR for 3 min, and then exposed to [^{3}H] gemcitabine at a concentration of 23.9 ng/ml (1.0 μCi/ml). After 1 h exposure, the cells were washed three times in 1 ml of ice cold phosphate-buffered saline (PBS). The cells were then re-suspended in 0.5 ml of 0.5% Triton X-100, and 0.4 ml aliquots were sampled for radioactivity counting. Aliquots of 20 μl were also sampled for protein determination. The uptake level of [^{3}H] gemcitabine was expressed as the radioactivity level divided by protein concentration measured by the Bradford method (Bio-Rad Laboratories, Madrid, Spain).

Statistical analysis. All data were expressed as mean ± SD. The differences between groups were examined using the Student's *t*-test or Mann-Whitney *U*-test. Correlation between the level of gene expression and extent of sensitivity to each reagent was examined by Pearson's correlation analysis. A *p*-value less than 0.05 denoted the presence of a statistically significant difference.

Results

Chemosensitivity of human pancreatic cancer cells to 5-FU and gemctabine. The IC_{50} values for 5-FU and gemcitabine in the seven types of human pancreatic cancer cell line are shown in Table II. The AsPC1 cells (IC_{50}: 33.2 mg/ml) were much more resistant to 5-FU than the other six lines (range of IC_{50}: 1.7-6.3 mg/ml). The IC_{50} values for gemcitabine were spread from the lowest at 1.43 ng/ml to the highest at 217.4 ng/ml.

Expression of hENT1, TS and DPD in human pancreatic cancer cells. The expression of hENT1, TS and DPD mRNAs were detected in all seven cell lines tested by RT-PCR analysis (Figure 1).

Correlation between hENT1 levels and the sensitivity to 5-FU or gemcitabine. As shown in Figure 2A, there was a significant correlation between hENT1 mRNA level and the sensitivity to 5-FU, with a coefficient of correlation of 0.989, which was statistically significant at $p<0.001$. That is, the expression of hENT1 was higher in the cells which were less sensitive to 5-FU. Because the AsPC1 cells showed much higher values in both hENT1 mRNA level and IC_{50} of 5-FU than the other six cell lines, the coefficient of correlation was re-analyzed using the cell lines except AsPC1, giving a value of 0.872 which was again statistically significant at $p<0.03$ (Figure 2B). Although no significant association was observed between hENT1 mRNA level and sensitivity to gemcitabine in the 7 lines ($R=0.414$, $p=0.3787$, Figure 3A), a strong correlation was observed between hENT1 mRNA level and the IC_{50} value of gemcitabine ($R=0.877$, $p<0.02$, Figure 3B) when the data for AsPC1 were excluded because the hENT1 expression of this cell line was much higher than that of the other lines.

Correlation between TS or DPD levels and the sensitivity to 5-FU. The relationships between the TS and DPD mRNA expression level and sensitivity to 5-FU are shown in Figures 4 and 5. No statistically significant trend was observed between TS and DPD mRNA expression levels and response to 5-FU in the seven types of human pancreatic cancer cell line (Figures 4A and 5A). Only when the data of the AsPC1 cells were excluded because of its extremely low sensitivity to 5-FU compared with the other six lines, was a moderate correlation coefficient observed between the IC_{50} value of 5-FU and TS or DPD expression levels (0.637 or 0.600, respectively) (Figures 4B and 5B).

Enhanced growth inhibitory effect of 5-FU by nucleoside transporter inhibitor. We hypothesized that the uptake of nucleosides or nucleobases through hENT1 might prevent 5-FU from inhibiting *de novo* DNA synthesis in pancreatic cancer cells. To clarify this hypothesis, we examined whether the inhibition of hENT1 could enhance the 5-FU cytotoxicity *in vitro*. NBMPR, known as an hENT1 inhibitor, was used for the inhibition of hENT1.

Firstly, in order to examine the effect of single-agent NBMPR in pancreatic cancer cells, PSN1 cells, which showed high sensitivity to 5-FU and gemcitabine, were treated with increasing doses of NBMPR in the range of 0.1-10 μM. No growth inhibition was observed in PSN1 cells treated with NBMPR alone up to 10 μM (data not shown). However, the uptake of [^{3}H] gemcitabine was significantly

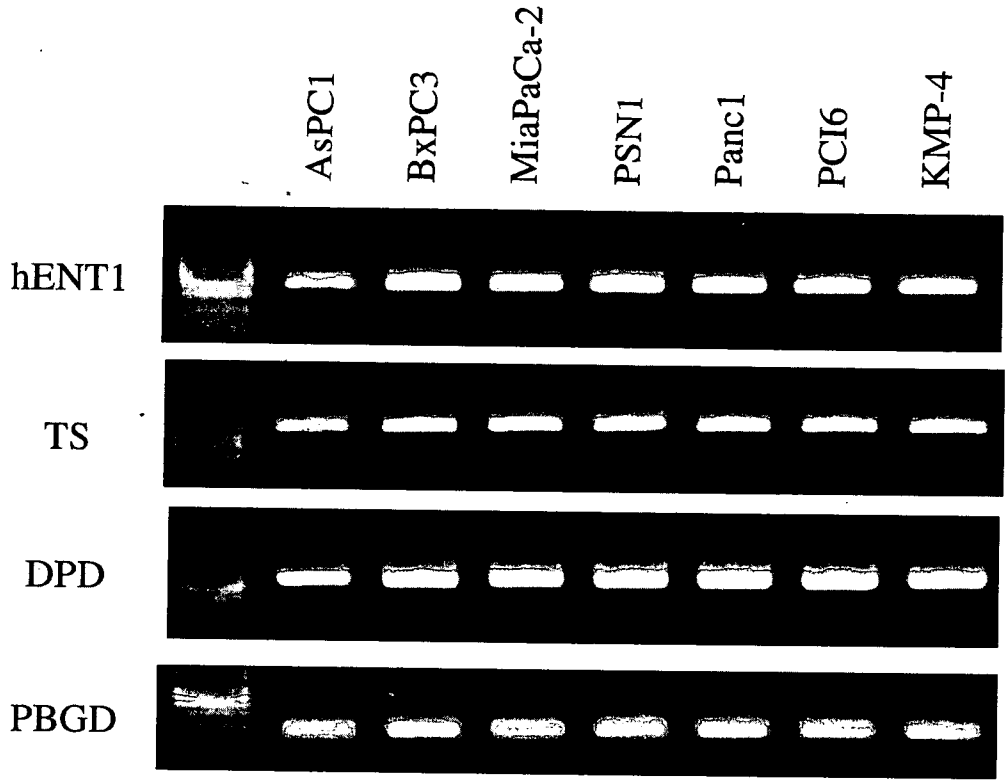

Figure 1. *Expression of hENT1, TS and DPD mRNA in seven human pancreatic cancer cell lines.*

inhibited when the cells were treated with 1 μM NBMPR ($p<0.05$, Figure 6).

To examine whether the hENT1 inhibitor enhanced the cytotoxic effect of 5-FU in pancreatic cancer cells, PSN1 cells were treated with a combination of NBMPR and 5-FU. As shown in Figure 7, the IC_{50} value of 5-FU in the cells pretreated with 1 μM NBMPR was significantly lower than that in the control cells pretreated with PBS ($p<0.05$). The IC_{50} values of NBMPR-pretreated cells and PBS-pretreated cells were 0.91±0.12 and 1.88±0.18, respectively.

Discussion

In this study, the basal mRNA level of hENT1 expression significantly correlated with the IC_{50} value of 5-FU in human pancreatic cancer cell lines. That is, the cells with higher mRNA expression of hENT1 were less sensitive to 5-FU. 5-FU, one of the TS inhibitors, is known to block *de novo* DNA synthesis and deplete intracellular nucleotide pools so that cells depend on the salvage pathway to get preformed nucleosides from the extracellular fluid. The lower sensitivity to 5-FU in the cells with higher hENT1 expression might be due to the better supplies of preformed nucleosides *via* hENT1. To prove this hypothesis, we next examined the 5-FU sensitivity in pancreatic cancer cells pretreated with NBMPR, which is a specific inhibitor of hENT1. Treatment with 1 μM NBMPR resulted in significant inhibition of the uptake of nucleoside analogues, but single-agent NBMPR (up to 10 μM) did not show any growth inhibitory effect on the pancreatic cancer cells tested. The sensitivity to 5-FU was significantly increased (IC_{50} value was decreased 52±11%) when the cells were pretreated with 1 μM NBMPR. Our results suggest that hENT1 plays an important role in regulating the mechanism of 5-FU resistance in human pancreatic cancer cells.

Recently, Kubota *et al.* analyzed the relationship between the mRNA levels of several types of genes including hENT1, TS and DPD and chemosensitivity to 5-FU using surgically obtained colorectal cancer specimens and reported that the quantified gene expression levels correlated with the sensitivity to 5-FU (9). Both TS and DPD are known to be key enzymes for treatment with 5-FU (14). Several studies using human tumor cell lines and clinical samples have reported that TS and DPD mRNA levels are correlated with the response to 5-FU in several types of carcinoma (11-15). In our study using pancreatic cancer cell lines, however, the correlation between TS and DPD mRNA levels and 5-FU antitumor activity was not evident. Although showing very

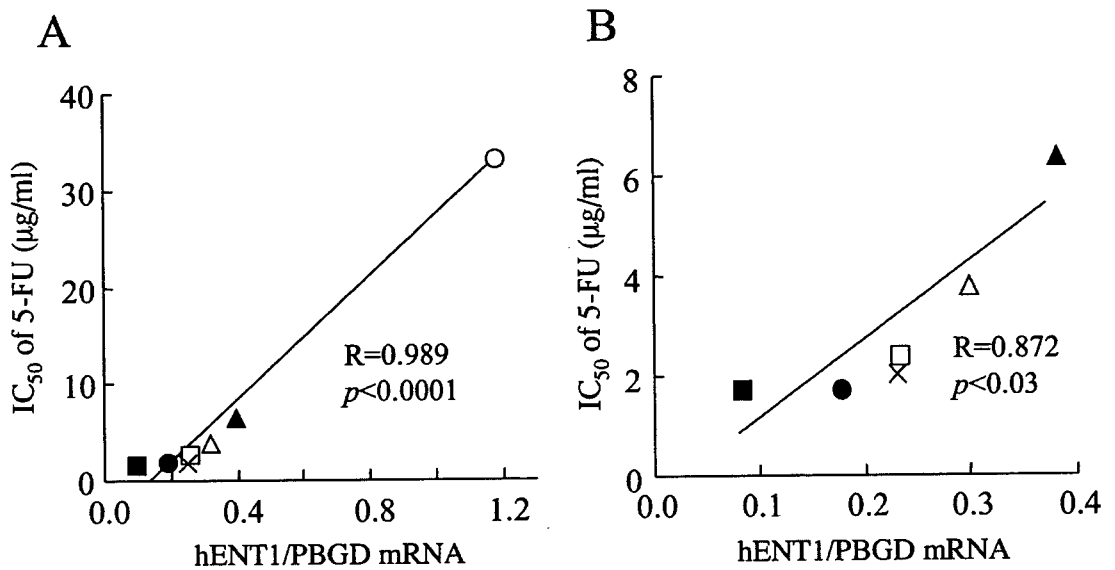

Figure 2. *Correlation between hENT1 mRNA levels and IC_{50} values of 5-FU in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). hENT1 mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

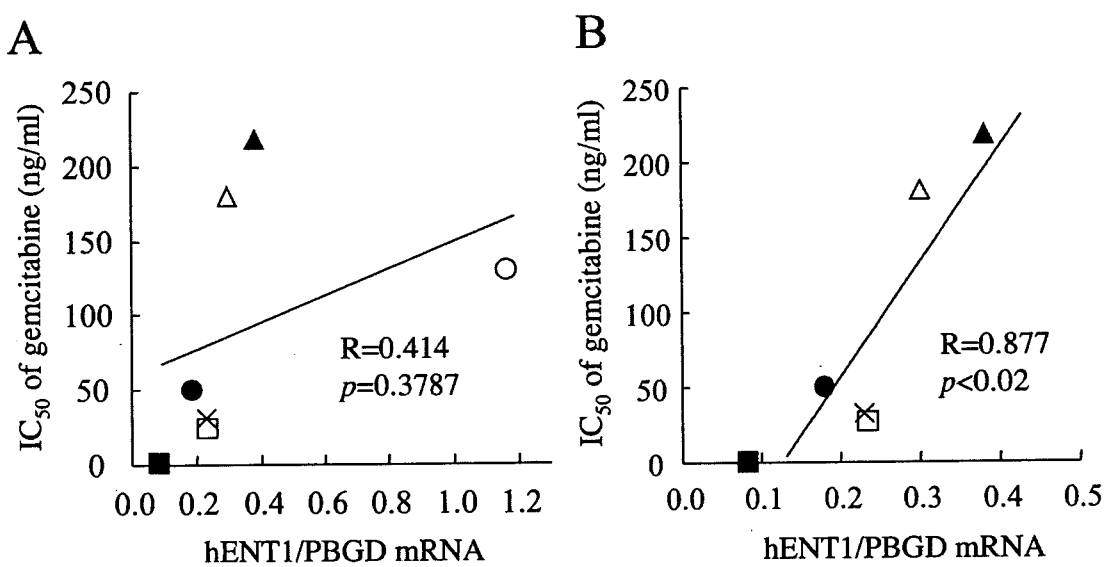

Figure 3. *Correlation between hENT1 mRNA levels and IC_{50} values of gemcitabine in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). hENT1 mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

low mRNA expression of TS and DPD, the AsPC1 cells were the most resistant to 5-FU of the seven lines tested. Interestingly, the highest expression of hENT1 was observed in the AsPC1 cells. There is a possibility that better supplies of nucleosides and nucleobases through highly-expressed hENT1 might interfere with the 5-FU function that blocks *de novo* DNA synthesis. The re-analysis of the correlation coefficient using six lines (excluding AsPC1) still showed only moderate correlations between IC_{50} values of 5-FU and TS or DPD expression, and they were not statistically

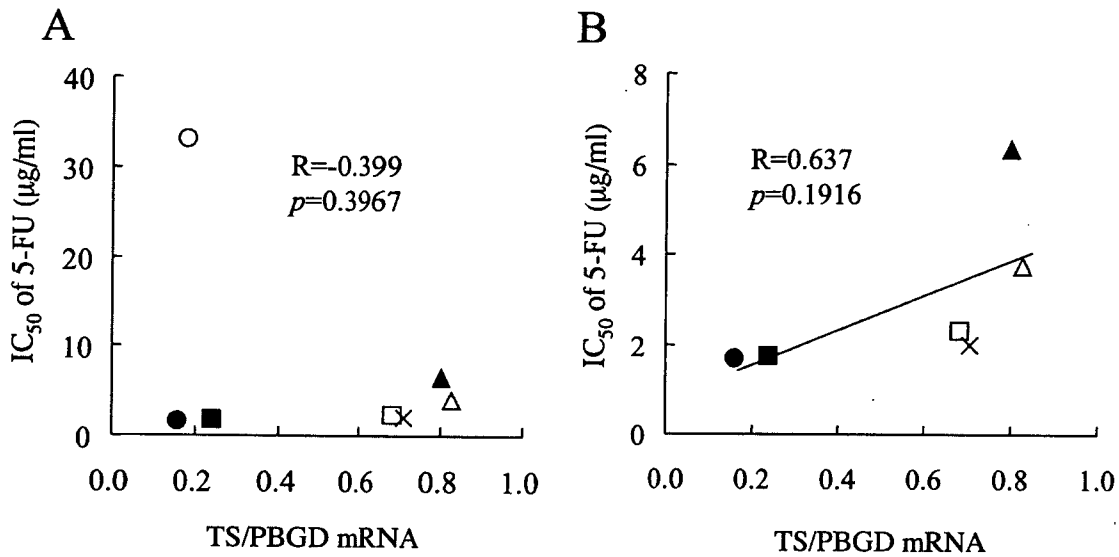

Figure 4. *Correlation between TS mRNA levels and IC_{50} value of 5-FU in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). TS mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

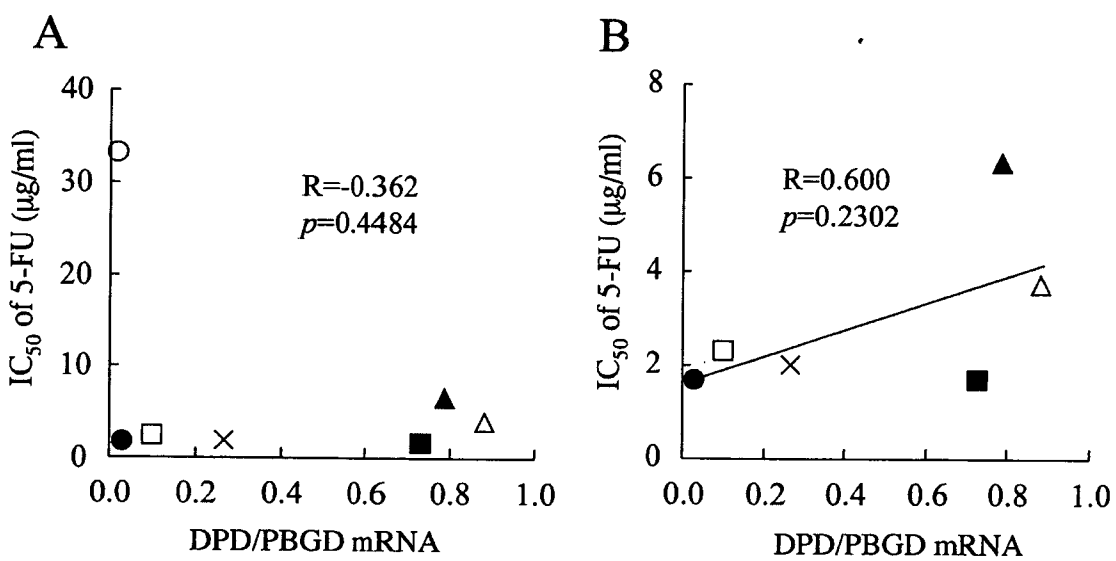

Figure 5. *Correlation between DPD mRNA levels and IC50 value of 5-FU in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). DPD mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

significant. These results suggested that the contribution of TS and DPD mRNA levels to 5-FU sensitivity might not be essential in human pancreatic cancer.

Gemcitabine is a cell cycle-dependent (S-phase specific) deoxycytidine analogue of the antimetabolite class. It has also been reported that the expression levels of hENT1 mRNA were significantly correlated with IC_{50} values for gemcitabine in human non-small cell cancer *in vitro* (30). Moreover, the expression of hENT1 is also reported to be associated with survival in patients with gemcitabine-treated pancreatic cancer (31, 32). Based on those reports, higher expression of hENT1 should result in higher

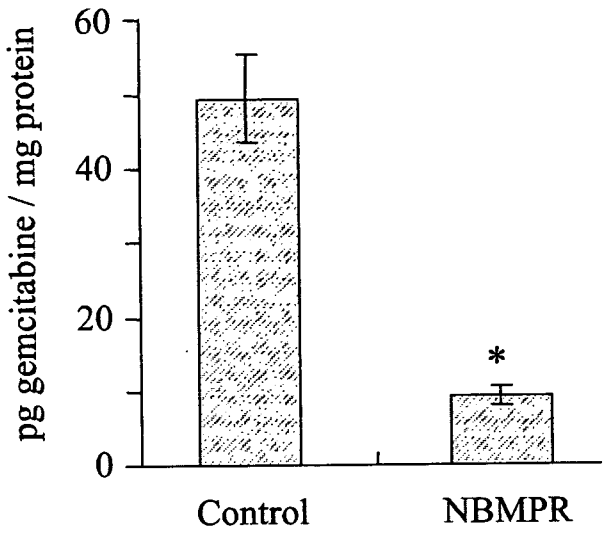

Figure 6. *Effect of NBMPR on the uptake of gemcitabine in PSN1 cells. PSN1 cells were treated with NBMPR or control PBS, and then exposed to [3H] gemcitabine. The uptake level of [^{3}H] gemcitabine was expressed as radioactivity levels divided by protein concentrations. Columns, mean of results in triplicate; bars, SD; *p<0.05.*

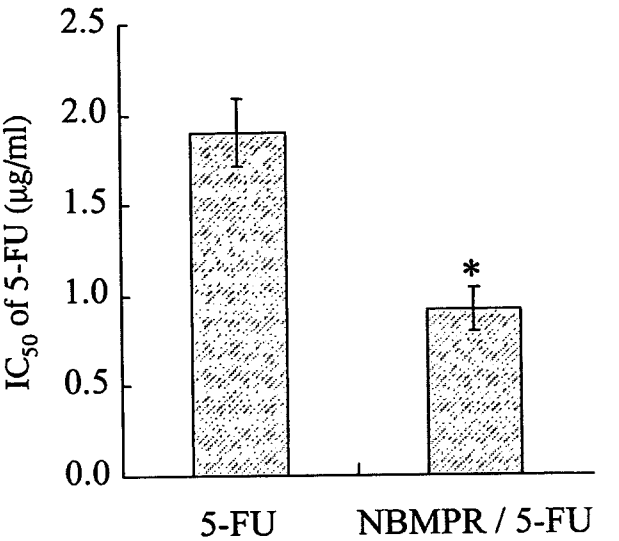

Figure 7. *Effect of NBMPR on the antitumor effect of 5-FU in PSN1 cells. PSN1 cells were pretreated with NBMPR or PBS (control), and then treated with 5-FU. Columns, mean of results in three independent experiments; bars, SD; *p<0.05.*

sensitivity to gemcitabine. In our current study, however, the expression levels of hENT1 mRNA were higher in the cell lines which were less sensitive to gemcitabine when the data of the AsPC1 cell line were excluded. Nakano *et al.* have reported that the expression of hENT1 mRNA was increased in the development of gemcitabine resistance, and they did not find any correlations between IC_{50} value of gemcitabine and hENT1 expression in pancreatic cancer cell lines (33). Once transported inside the cell, gemcitabine is converted by deoxycytidine kinase (dCK) to its triphosphate, the active form, which is incorporated into DNA and causes apoptosis. Deficiency in dCK activity has been considered to be one of the main mechanisms responsible for the development of gemcitabine resistance. Moreover, ribonucleotide reductase (RR) is also reported to be another factor in gemcitabine resistance (34-36). Therefore, although hENT1 plays an important role in gemcitabine uptake and subsequent cytotoxicity (37), other factors (such as dCK, RR, or apoptosis-related genes) or the balance between hENT1 and those factors might be more important in determining gemcitabine sensitivity in pancreatic cancer cells (18, 19, 38, 39).

In conclusion, in the present study, it was demonstrated that poor 5-FU sensitivity might be strongly influenced by the hENT1 mRNA level in human pancreatic cancer cells. The measurement of mRNA expression level using the quantitative RT-PCR method could easily be applied to clinical specimens. Further studies are needed to determine whether hENT1 would be useful as a predictive marker of resistance to 5-FU in pancreatic cancer patients.

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膵癌化学療法における gemcitabine 有効性向上のための基礎的・臨床的検討

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〔特　集〕膵癌化学療法の最前線

膵癌化学療法におけるgemcitabine有効性向上のための基礎的・臨床的検討

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要　旨：膵癌に対する第1選択化学療法剤として広く使用されているgemcitabine(GEM)の作用機序解析に基づいた治療効果増強のための基礎的・臨床的検討を行った．ヒト膵癌細胞株およびヌードマウス移植ヒト膵癌モデルにおいて行い，5-fluorouracil (5-FU) によるGEM取込み増強効果および5-FU系薬剤によるGEMとの至適併用スケジュールを検討した．その結果，5-FU剤先行投与によってGEM取込みが増強し，先行投与後GEM施行が最も抗腫瘍効果が高いことが明らかになった．さらに，切除不能進行・再発膵癌に対して，至適併用スケジュール（UFT先行GEM投与：UFT250mg/m^2：day1～6, day8～13, GEM 800mg/m^2：day7, 14, 休薬：day15～21）による第II相試験を行い，重篤な有害事象は認めず，奏功率25%，50%生存期間7ヶ月，1年生存率25%と言う成績を得た．また，GEM耐性ヒト膵癌細胞株の網羅的遺伝子発現解析から，GEM代謝に関連するRibonucleotide reductase M1 subunit（RRM1）発現昂進がGEM耐性に最も関連することを明らかにし，RRM1 RNAiを用いたRRM1発現抑制によってGEM感受性が向上することを検証した．さらに，臨床例の検討から，RRM1がGEM感受性にも関与している可能性を示した．

索引用語：膵癌　化学療法　ジェムシタビン　薬剤感受性

はじめに

わが国において膵癌は増加傾向にあり，早期発見の困難さから，診断時には，その7～8割は切除不能の進行癌であると考えられている．さらに切除可能であった場合でも，切除後の再発率は高く，治療成績の向上のためには，切除不能進行膵癌や再発膵癌に対する有効な化学療法の確立が必須と考えられる．一方，膵癌に対する化学療法は，従来は5-fluorouracil (5-FU) を中心とした治療が広く行われてきたが，その治療成績は良好とは言えず，膵癌は抗癌剤の効きにくい代表的な腫瘍であった．このような状況の中，Burrisら[1)]によって，1997年に塩酸gemcitabine（以下GEM）と5-FUを比較した大規模第III相試験で，GEMが症状緩和効果や1年生存率で5-FUに比べ有意に優れていることが報告され，それ以来，欧米ではGEMが膵癌化学療法の第1選択薬とされてきた．遅ればせながらわが国でも，2001年4月に膵癌においてもGEMが保険適応とされ，標準的な第1選択薬として利用できるようになった．しかしながら，GEMを用いた膵癌の治療成績は未だ満足できるものでなく，膵癌同様に5-FUを中心に化学療法が行われ抗癌剤の効きにくい腫瘍とされてきた大腸癌が近年のさまざまな治療方法の工夫から良好な治療成績が得られるようになった状況からはほど遠いものがある．膵癌治療成績向上のためには，GEM治療の有効性を図っていくことが，重要課題の一つと考えられ，本稿では，GEM

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