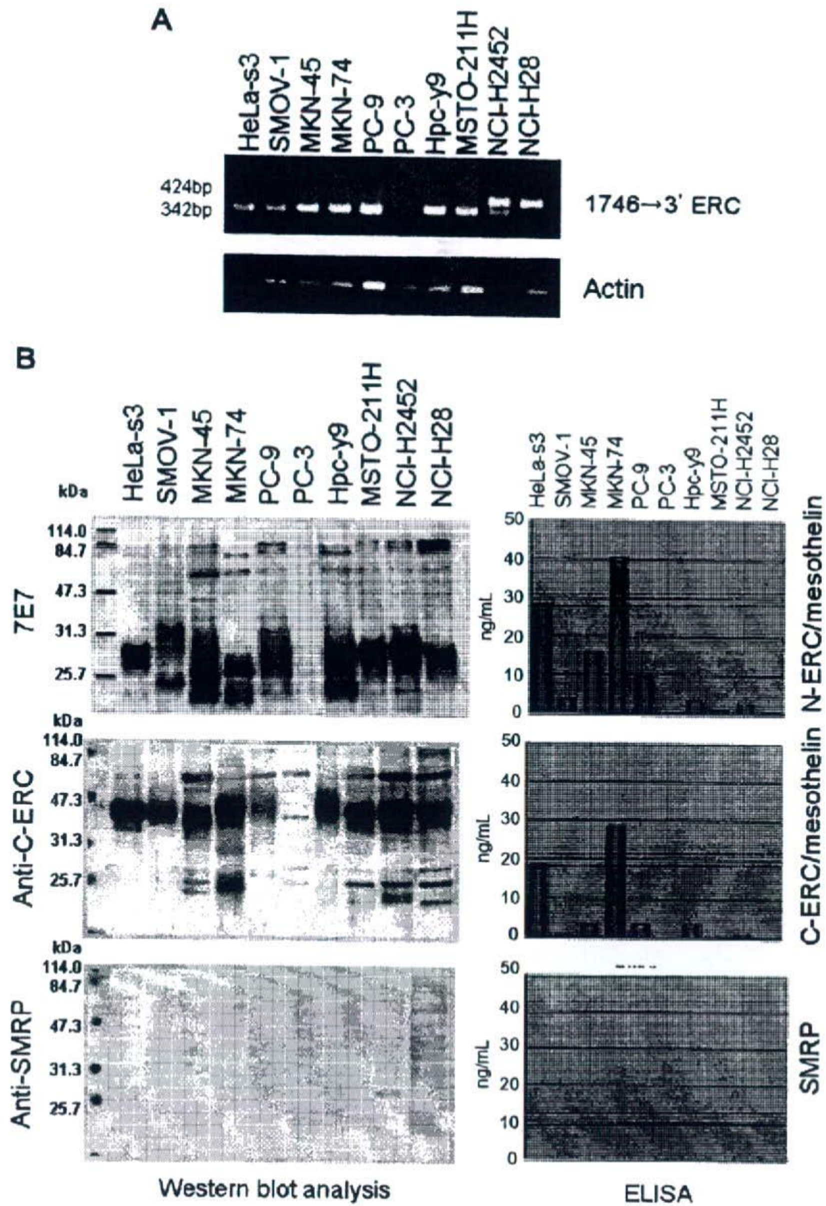

Fig. 2. Detection of N- and C-ERC/mesothelin and SMRP in various cell line cells. (A) *SMRP* and *ERC/mesothelin* mRNA expression in various cell lines were analyzed with RT-PCR using specific primers. The 424 bp product is derived from *SMRP* mRNA and the 342 bp is derived from *ERC/mesothelin* mRNA. (B) The presence of N- and C-ERC/mesothelin and SMRP protein in various cell lines were analyzed with Western blotting and ELISA systems. Total cell lysates were subjected to Western blot analysis by anti N- ERC/mesothelin (7E7), anti-C-ERC and anti-SMRP PoAb. The culture supernatants were measured with ELISA systems for N- and C-ERC/mesothelin and SMRP.

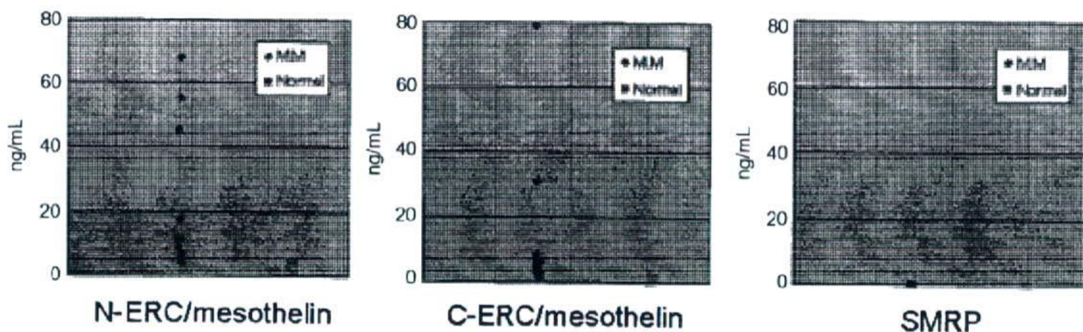

Fig. 3. Detection and quantification of ERC/mesothelin and SMRP in serum samples from malignant mesothelioma patients and healthy volunteers. The serum samples were analyzed with the specific ELISA systems for N- and C-ERC/mesothelin and SMRP.

C-ERC/mesothelin into the culture supernatant, but SMRP was not detected in any cells.

Also, the presence of ERC/mesothelin and SMRP in serum samples from mesothelioma patients was analyzed with the ELISA systems (Fig. 3). Although the serum concentrations of mesothelioma patients ranged from 3.86 to 67.97 ng/ml (average: 22.18 ng/ml) for N-ERC/mesothelin (These data were referred in previous report [12]), and from 1.59 to 79.17 ng/ml (average: 13.51 ng/ml) for C-ERC/mesothelin, SMRP was not detected in any samples. In healthy volunteers, N- and C-ERC/mesothelin were detected (1.42–4.36 ng/ml for N-ERC/mesothelin, and 0.56–2.41 ng/ml for C-ERC/mesothelin), but SMRP was not measurable.

Discussions

Although the usefulness of SMRP, which has an 82-bp insertion, has been as a diagnostic marker for mesothelioma [13,14], the expression of SMRP in the cells and body fluids from mesothelioma or other carcinoma patients has not been elucidated.

Among the tested cells in this study, cells which expressed only *ERC/mesothelin* mRNA secreted a detectable amount of N- and C-ERC/mesothelin with the ELISA system, but the SMRP molecule was not detected even in cells which expressed *SMRP* mRNA.

Even in serum samples from mesothelioma patients, N- and C-ERC/mesothelin were detected, but SMRP was not detected. These results predict that even if SMRP could be translated, it could be degraded in its C-terminal region during the expression process, be undetectable by the specific ELISA system and be indistinguishable from C-ERC/mesothelin with MESOMARK kit.

Sapede et al. [15] reported about the mechanisms involved in soluble mesothelin production by tumor cells. They explored two alternative possibilities: release of an aberrant RNA splicing product (mesothelin variant 3, *SMRP*) or enzyme-mediated shedding of membrane-bound mesothelin (phospholipases, proteases as MMP- and ADAM-family proteases).

According to the present study, accurately, not SMRP but SMRP and/or C-ERC/mesothelin without C-terminus should be considered as a marker for mesothelioma. Further study might be necessary to demonstrate the relationship between SMRP and mesothelin.

Acknowledgments

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan, and by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science. Furthermore, this study was partially supported by a consignment expense of the Molecular Imaging Program "Research Base for PET Diagnosis" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Government of Japan.

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siRNA-mediated *Erc* gene silencing suppresses tumor growth in *Tsc2* mutant renal carcinoma model

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Abstract

Silencing of gene expression by small interfering RNAs (siRNAs) is rapidly becoming a powerful tool for genetic analysis and represents a potential strategy for therapeutic product development. However, there are no reports of systemic delivery of siRNAs for stable treatment except short hairpin RNAs (shRNAs). On the other hand, there are many reports of systemic delivery of siRNAs for transient treatment using liposome carriers and others. With regard to shRNAs, a report showed fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Therefore, we decided to use original siRNA microspheres instead of shRNA for stable treatment of disease. In this study, we designed rat-specific siRNA sequences for *Erc/mesothelin*, which is a tumor-specific gene expressed in the Eker (*Tsc2* mutant) rat model of hereditary renal cancer and confirmed the efficacy of gene silencing in vitro. Then, by using siRNA microspheres, we found that the suppression of *Erc/mesothelin* caused growth inhibition of *Tsc2* mutant renal carcinoma cells in tumor implantation experiments in mice.
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Keywords: *Erc* gene; *Tsc2*; siRNA microspheres

1. Introduction

The Eker (*Tsc2*) gene mutant rat model of hereditary renal carcinoma (RC) is an example of Mendelian dominantly-inherited predisposition to a specific cancer in an experimental animal [1]. The germline mutation is like an 'initial gene' (*Tsc2* gene) of the abnormal networks of gene expression that are involved in tumor formation (federal headship of carcinogenesis) [2]. We previously isolated the *Erc* gene from the RC tissue of Eker rats by representational difference analysis [3]. The *Erc* gene (NM_031658) is more preferentially expressed in renal cell carcinoma (RCC) of the Eker rats than in normal kidney. We subsequently confirmed that *Erc* is a homolog of the human mesothelin gene (*MSLN*) because rat *Erc* and human *MSLN* are localized on chromosomes 10q12-21 and 16p13.3,

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respectively, both of which coincided with the locus of the *Tsc2/TSC2* gene [4].

Recently, Shiomi et al. already showed that N-ERC/mesothelin is a very promising tumor marker for mesothelioma, especially epithelioid mesothelioma [5]. Mesothelioma is an aggressive tumor arising from the mesothelium, and is usually associated with previous exposure to asbestos [6]. On the other hand, Hassan R. et al. reported on mesothelin as a target for cancer therapy and summarized the available pre-clinical data as well as on-going and planned clinical trials [7]. We are now characterizing the role of *Erc* in carcinogenesis.

The siRNA technology has rapidly become an important tool for gene therapy to target several pathological conditions such as cancer and viral infectious diseases [8]. Many researchers have examined how to effectively deliver siRNA to target cells and tissues using gene carriers such as cationic liposomes, polymers and lipids [8–16]. It was reported that the cationized-gelatin microspheres containing siRNA for VEGF could inhibit tumor growth in mice [17]. However, a clinically viable delivery system for siRNA has not been developed yet. The effects of siRNA are transient even though they are highly suppressive. Therefore, it is necessary to develop methods to sustainably deliver siRNA to target cells for continuous cleavage of the target mRNA.

In the present study, we prepared biodegradable microspheres for long-term sustained release of siRNA that target and continuously suppress the production of rat *Erc*. We used poly (DL-lactic/glycolic acid, PLGA) as a biodegradable and biocompatible polymer [18–22]. The gene carrier is important to deliver siRNA into the cells, therefore arginine and polyethyleneimine were used as carriers. We examined the efficacy of the siRNA in vitro as well as the physical properties of microspheres [20,21]. Then, we injected microspheres carrying *Erc* siRNA into rat *Tsc2* mutant tumors in tumor implantation experiments in mice to determine their effects upon tumor growth.

2. Materials and methods

2.1. Cell culture and siRNA transfection for Erc silencing in vitro

Anti-rat *Erc* siRNA (siRNAE244) and (siRNAE1393) was purchased from Ambion Inc. (Austin, TX). The sense and antisense sequences of siRNAE244 were 5′-GAC CGG CAC CGU UGA CUU UGC-3′ and 5′-CAC UGG CCG UGG CAA CUG AAA-3′, respectively. The sense and antisense sequence of siRNAE1393 were 5′-GGACAACAUCCCCUUAAGUUA-3′ and 5′-ACU UAAGGGGAUGUUGUCCAG-3′, respectively. A non-silencing siRNA (sense, 5′-CAG UUC CGC CAC UUG CCA A dTdT-3′; antisense, 5′-UUG GCA AGU GGC GGA ACU G dTdT-3′) served as a mock double-stranded RNA (dsRNA) control. Mouse MKOC1-277 and rat ERC33 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Co., USA) containing 10% FBS (Invitrogen, Carlsbad, California) and 1% penicillin/streptomycin (stock 10,000 U/mL, 10,000 μg/mL, Invitrogen, Carlsbad, CA). Cells were seeded into 12-well plates at a density of 6×10^4 cells per well with 1 mL of FBS(+) DMEM and incubated at 37 °C in a humidified 5% CO_2 chamber for 24 h before the transfection study. Then the medium was discarded and replaced with antibiotic-free medium. After 12 h, cells were transfected with 10 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent as per manufacturer's instructions.

2.2. Reverse transcription polymerase chain reaction and quantitative real-time PCR

Total RNA was extracted by using Absolutely RNA Reverse Transcription-PCR Miniprep kits (Stratagene, La Jolla, CA). The concentration of total RNA was adjusted to 200 μg/mL using a spectrophotometer. For quantitative reverse transcription-PCR, the following reagents were used according to the manufacturer's instructions: One Tube Reverse transcription-PCR system (Roche Applied Science, Alameda, CA) and SYBR Green PCR Core Reagents. Primer sequences used were as follows: ERC13930, sense; 5′-ATGCTCAACTGTGACATCTC-3′, antisense; 5′-TGTGACAAATTAGCGCT TCC-3′, ERC2440, sense; 5′-CCTAGAAGGACAGATGTTCC-3′, antisense; 5′-TAGCAACAGAAAT AGGCCCC-3′.

For real-time PCR, conversion of RNA to cDNA was performed first, by incubating 50 ng of total RNA, random hexamer primers (Roche Applied Science, Alameda, CA), and SuperScript II enzyme (Invitrogen, Carlsbad, CA) at 48 °C for 60 min and 95 °C for 5 min. TaqMan probes for rat (Rn00581388_m1, ABI) and mouse *Erc/mesothelin* (Mm00450770_m1, ABI), and SYBR Green PCR Core Reagents were purchased from ABI. Quantitative real-time PCR amplification of the cDNA template corresponding to 50 ng total RNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and ABI PRISM 7500 (Applied Biosystems, Foster City, CA). PCR conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The glyceraldehyde-3-phosphate dehydrogenase gene (*G3pdh*) transcripts served as the internal control. The primer and probe mixtures for ZIP6, ZIP10 and G3pdh were pur-

chased from Applied Biosystems. Primer sequences for *G3pdh* used were as follows: sense; 5′-AACGGCACAGT CAAGGCTGAGAACG-3′ antisense; 5′-CAACATACT CGGCACCGGCATCG-3′.

2.3. Soft agar assay

Rat ERC33 cells were treated with 10 nM siRNAE244 or scramble siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, California) reagent as per manufacturer's instructions. After 24 h, cells were trypsinized, mixed with agar (final concentration, 0.3%; Becton–Dickinson and Company, Franklin Lakes, New Jersey) and RPMI 1640 containing 10% fetal bovine serum, and overlaid onto an underlayer of 0.53% Difco agar containing the same medium in 6-well plates. To assist *Erc* suppression, siRNAs (10 nM) were included in top gel. Cells (3000 per well) were seeded and allowed to grow for >10 days before counting the number of colonies [23].

2.4. Preparation of siRNA microspheres

PLGA with a 75:25 lactic/glycolic acid ratio and a molecular weight of 14,400 was purchased from WAKO (Japan). Branched polyethylenimine (PEI, Mw 25,000, Sigma–Aldrich, St. Louis, MO) and arginine (WAKO, Japan) were used as gene carriers.

As shown in Fig. 1, we prepared PLGA microspheres containing siRNA using a w/o/w in-water drying method [18,19]. Briefly, 100 μL of 0.4% polyvinyl alcohol (PVA) (W_1), containing 0.13 μg of siRNA and 5 μg of PEI carrier, and 0.5 g of PLGA was dissolved in 3 mL of CH_2Cl_2 (O) and then homogenized (Polytron PT3100, Kinematica AG, Littau Lucerne, Switzerland) at 10,000 rpm for 2 min. The resulting W_1/O emulsion was homogenized with 500 mL of 0.25% PVA (W_2) [21,22]. The resulting $W_1/O/W_2$ emulsion was stirred gently for 3 h to evaporate the organic solvent. The microspheres were passed through a 75-μm sieved to remove large particles and then sedimented by centrifugation at 3000 rpm for 15 min. The PLGA microspheres containing siRNA encapsulated in PEI were collected by centrifugation, rinsed with distilled water three times, and then lyophilized [21,22].

2.5. Physicochemical characterization of siRNA microspheres

Microspheres were observed using an S-2250N scanning electron microscope (Hitachi, Tokyo, Japan). The samples were coated with 25-nm-thick gold using a quick carbon coater (SC-701, Sanyu Electronics, Tokyo, Japan). The particle diameter (the horizontal fret diameter) and size distribution of the microspheres were determined by image analysis of 1000 particles using WinROOF image analysis software (Mitani, Tokyo, Japan). The mean particle diameter was defined as the arithmetic mean of the number based on particle size distribution.

The siRNA encapsulation efficiency into microspheres was determined by HPLC. In brief, microspheres (25 mg) were dissolved in 0.5 mL of acetonitrile and then 0.5 mL of phosphate buffer (pH 6.0) was added. After vigorous shaking for 2 h, the supernatants were collected by centrifugation (5000 rpm, 20 min) and then analyzed using HPLC (Prominence, SHIMADZU, Kyoto, Japan) with an ultraviolet (UV) detector under the following conditions: column, TSKgel Oligo DNA RP (4.6 mm × 15 cm, TOSOH, Japan); mobile phases, (A) 0.1 M triethylamine–acetic acid and (B) acetonitrile [and] 10–30% B (45 min, linear); flow rate, 1.0 mL/min; wave length, 260 nm; and

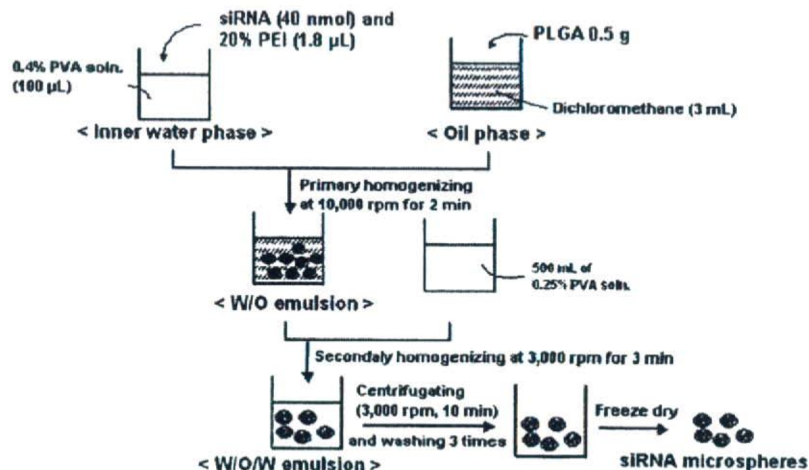

Fig. 1. We prepared biodegradable microspheres for long-term sustained release of siRNA that target and continuously suppress the production of rat *Erc/mesothelin*. We used poly (DL-lactic/glycolic acid, PLGA) as a biodegradable and biocompatible polymer. We prepared PLGA microspheres containing siRNA using a w/o/w in-water drying method.

injection volume, 10 μL. The ratio (%) of the measured to the formulated amount of siRNA was defined as the encapsulation efficiency of siRNA into microspheres.

2.6. Injection of tumor cells into mice

For implantation, a cocktail of ERC33 cells (1×10^6 cells per mice) suspended in 0.5 mL of serum-free medium and 10 mg of microspheres containing siRNA were used as carrier. It was injected into the flank of BALB/c nude mice. Tumor volume was calculated as length × width × height in mm^3 every week after injection [24]. ERC33-bearing mice were followed up for 6 weeks after implantation.

2.7. Statistical analysis

We analyzed all of the data using the Statcel statistical program (OMC, Tokyo, Japan), then evaluated the statistical significance using Mann–Whitney's *U* test. We considered $P < 0.05$ to be statistically significant.

3. Results

3.1. Physicochemical characterization of siRNA microspheres

The siRNA encapsulation efficiency in microspheres was shown by using HPLC (data not shown). We confirmed that the formulated amount of siRNA; the encapsulation efficiencies of siRNA were 64.3% when PEI were used as carrier.

3.2. Efficacy of specific rat Erc gene silencing in Tsc2 mutant renal carcinoma cell

To establish a system to specifically suppress rat *Erc*, without affecting the endogenous mouse homolog in the tumor implantation experiments in nude mice, we designed rat-specific siRNAs. We confirmed which siRNA sequence, siRNAE244 or siIRNAE1393, was the most effective for rat *Erc* gene silencing ($n = 5$, Fig. 2) by performing quantitative real-time PCR analysis using Taqman probes. *Erc* gene silencing using siRNAE244 showed approximately 80% reduction in the mRNA levels in rat ERC 33 cells (Fig. 3A). On the contrary, siRNAE244 exhibited minimal suppression in mouse MKOC1-277 cells, indicating that it could act as a rat-specific siRNA (Fig. 3B). Therefore, we selected siRNAE244 for our investigation in vivo ($P = 0.02$). So far, no apparent effect of siRNAE244 on cellular proliferation of ERC33 was detected in soft agar assay (data not shown).

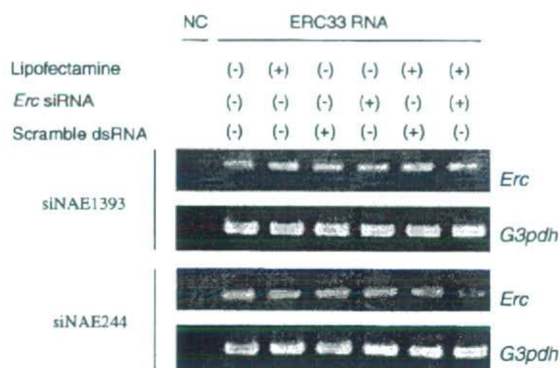

Fig. 2. Evaluation of Erc gene silencing in rat ERC33 cell line by using reverse transcription-PCR. Cells were transfected with siRNA for Erc (siRNAE244 or siRNAE1393) or scramble dsRNA. After 72 h, total cellular RNAs was subjected to RT-PCR for Erc or G3pdh. Lane NC: no template cDNA.

3.3. Erc gene silencing suppressed tumor growth of rat renal carcinoma cells in vivo

To investigate the efficacy of specific siRNAE244 microspheres in a *Tsc2* mutant renal carcinoma model, we simultaneously injected ERC33 cells and siRNA containing microspheres into nude mice, and then measured tumor volumes. As shown in (Fig. 4), siRNAE244 microspheres suppressed tumor growth relative to scramble dsRNA microspheres from the 4th week (42.7 mm^3 vs. 66.3 mm^3 $P = 0.018$) to 5th week (49.8 mm^3 vs. 181.0 mm^3, $P = 0.018$) after injection. At the 6th week, considerable growth of siRNAE244-injected ERC33 cells was observed, although there was a significant difference in volume compared with control (170.0 mm^3 vs. 290.0 mm^3, $P = 0.018$). Such a growth might reflect an exhaustion of siRNAE244. We did observe a difference between the tumor volume in mice after treatment with scramble dsRNA microspheres and siRNAE244, as shown in (Fig. 5A and B).

4. Discussion

We are exploring the role of *Erc* during tumorigenesis. As an approach to characterize the effect of *Erc* suppression on tumor growth in vivo, we aimed to establish an in vivo knockdown system. For this purpose, we selected rat-specific sequence for siRNA against *Erc* and successfully achieved rat-specific *Erc* gene silencing in vitro. We then performed a trial for siRNA-mediated rat *Erc* gene silencing in vivo by using the *Tsc2* mutant RC model and we observed a reduction in tumor growth following treatment. In this study, for implantation, we used a cocktail of ERC33 cells and microspheres containing siRNA. The implantation by using cocktail can provide excellent exposure of necessary drug delivery for tumor cells. On the other hand, it is difficult for us to provide rapid, excellent microspheres containing siRNA in implanted tumor cells because

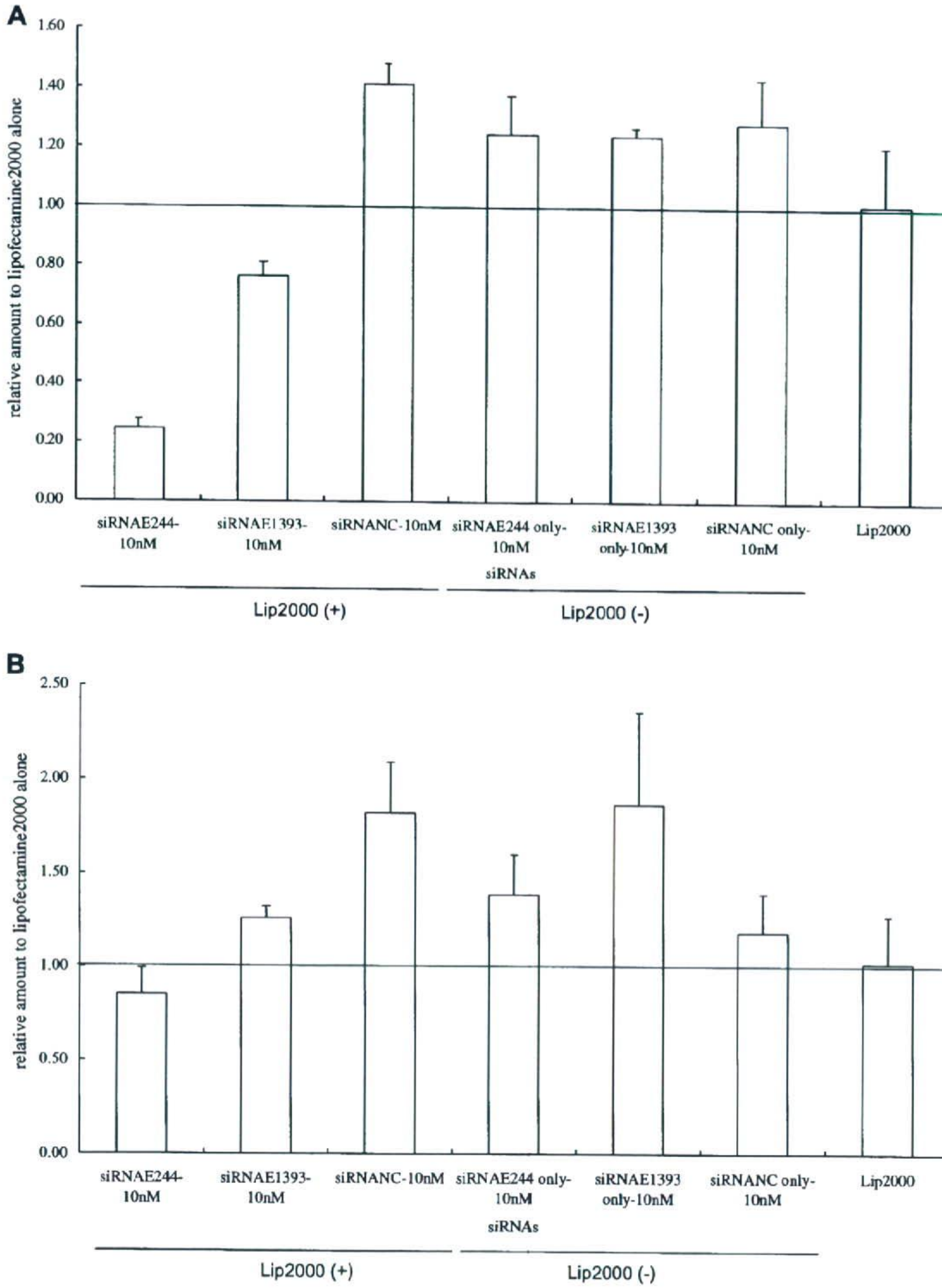

Fig. 3. Evaluation of mRNA levels of Erc by real-time PCR. (A) Rat ERC33. (B) Mouse MKOC1-277. Cells were treated either with Lipofetcamine only (Lipo2000), with control dsRNA (NC), or siRNAs for Erc (siRNAE244 or siRNAE1393) in the presence (+) or absence (−) of Lipofectamine.

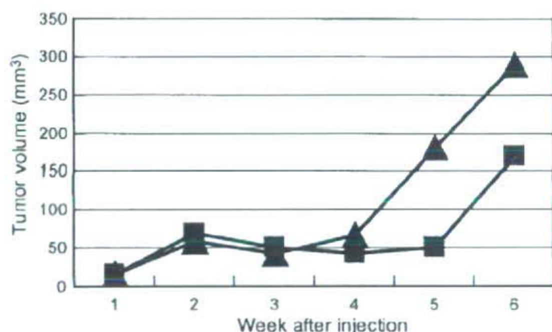

Fig. 4. Evaluation of tumor volume after *Erc* gene silencing in vivo. ERC33 cells were injected with microspheres containing either scramble dsRNA (triangle) or siRNAE244 (square) into nude mice ($n = 5$).

we are in preliminary stage to need experience of using microspheres containing siRNA.

With regard to siRNA delivery systems in vivo, at present, the main obstacle to the development of therapeutic products using RNAi technologies is the absence of a suitable delivery method. We are interested in a stable treatment regimen because in transient treatment, lipid delivery of synthetic siRNAs can induce immune activation in vivo [25,26]. On the other hand, using viral delivery systems for stable treatment, are efficient but cause concerns over serious side effects [26]. A side effect was associated with the downregulation of microRNAs (miRNAs), indicating possible competition of the latter with shRNAs for limiting cellular factors required for the processing of various small RNAs. In vitro and in vivo shRNA transfection studies suggest that one such factor, shared by both shRNA and miRNA pathways, which is readily saturated, is the nuclear karyopherin exportin-5 [26]. Therefore, shRNA-induced toxicity requires global miRNA inhibition [26]. For effective siRNA delivery systems, monitoring and controlling intracellular shRNAs are imperative for achieving stable in vivo gene silencing while mitigating adverse effects. However, it is very difficult to optimize shRNA dosage for minimizing the risk of oversaturation of the endogenous small RNA pathways [26]. On the other hand, Okada et al. confirmed to release of siRNA with PEI from microspheres in vitro instead of using shRNA [20]. Besides, Okada et al. showed anti-tumor effects of anti-VEGF siRNA encapsulated with PLGA microspheres in mice [21]. Therefore, we prepared long-term sustained release biodegradable microspheres encapsulating siRNA that target and continuously suppress the production of rat *Erc/mesothelin* [21,22]. We confirmed that siRNAE244-induced Erc gene silencing suppressed tumor growth of rat renal carcinoma cells in vivo, as shown in Fig. 5. Therefore, we are very interested in *Erc/mesothelin* function which may play a role in tumor growth. Although we have not observed specific effects of siRNAE244 on cellular phenotype in vitro, several functions have been ascribed to mesothelin [28–30]. CA125 is a tumor antigen originally defined by the monoclonal antibody OC125 that is routinely used for diagnosis of ovarian cancer and to monitor the recurrence after therapy [27]. Miyajima et al. show CA125 and mesothelin are co-expressed in advanced grade ovarian adenocarcinoma. Taken together, their data indicate that mesothelin is a novel CA125-binding protein and that CA125 might contribute to the metastasis of ovarian cancer to the peritoneum by initiating cell attachment to the mesothelial epithelium via binding to mesothelin [27]. Chang et al. reported that mesothelin may play a role in cellular

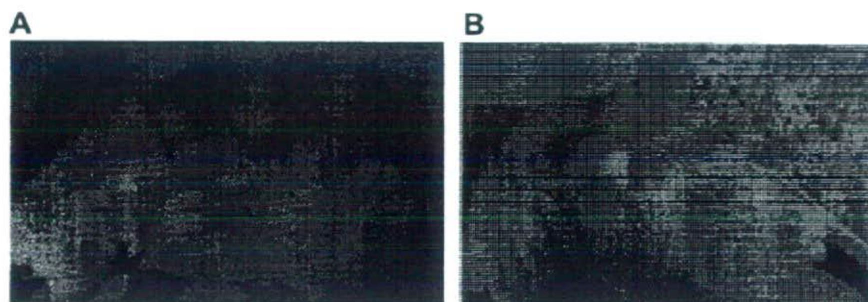

Fig. 5. Macroscopic appearance of tumors. Representative tumors (5 week) treated with siRNAE244 (A) or scramble dsRNA (B) are shown. Tumor positive areas are highlighted by black dotted lines. Pale-colored mass is less apparent in (A) indicating that remarkable attenuation of tumor growth was caused by using siRNAE244.

adhesion [28]. A recent report indicated that mesothelin expression modulates cellular anoikis [23]. The mucin MUC16 carries the peptide epitope CA125, which is a prominent molecular marker for monitoring the progression and recurrence of epithelial ovarian cancer [29]. Gubbels et al. reported the strong binding kinetics of the mesothelin–MUC16 interaction and the cell adhesion between ovarian tumor cells [29]. Frierson et al. reported mesothelin expression in endometrioid and undifferentiated ovarian carcinoma in a large scale tissue microarray study [30]. Hino et al. established the rat ELISA system to detect the soluble form of *Erc/MPF/Mesothelin*, and demonstrated the high concentration of this form in the sera of rats bearing mesohelioma. [3]. These reports in correlation with our results suggest that *Erc/mesothelin* may have a role in tumor growth. Further investigation with respect to the role of *Erc/mesothelin* in new anti-cancer therapy is currently in progress in our laboratory.

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Secretion of N-ERC/mesothelin and expression of C-ERC/mesothelin in human pancreatic ductal carcinoma

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Abstract. ERC/mesothelin gene *(MSLN)* encodes a precursor protein, which is cleaved by proteases to generate N-ERC/mesothelin and C-ERC/mesothelin. N-ERC/mesothelin is a soluble protein, also known as megakaryocyte-potentiating factor, which is released into extracellular space. N-ERC/mesothelin is known to be a serum marker of mesothelioma. We have previously developed an enzyme-linked immunosorbent assay system for N-ERC/mesothelin, which can detect mesothelioma. C-ERC/mesothelin is expressed in normal mesothelial cell, pancreatic cancers, ovarian cancers, mesotheliomas and some other cancers. Pancreatic ductal carcinoma remains a fatal disease because its diagnosis often occurs very late. In this study, we examined ERC/mesothelin expression in human pancreatic cancer cell lines (MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H) by reverse transcription-polymerase chain reaction and immunoblotting and N-ERC/mesothelin concentration in the supernatant of cultured cancer cells by the ELISA system. We also investigated C-ERC/mesothlein expression in human pancreatic ductal carcinoma tissues by immunostaining using 5B2 anti-mesothelin monoclonal antibody and N-ERC/mesothelin concentration in sera obtained from patients with pancreatic ductal carcinoma via ELISA. *In vitro*, N-ERC/mesothelin concentration in cell culture medium nearly correlated with the expression level of C-ERC/mesothelin. Although C-ERC/mesothelin was frequently expressed in human pancreatic ductal carcinoma, serum N-ERC/mesothelin concentration of cancer patients was equivalent to healthy controls. N-ERC/mesothelin was not useful as a serum marker of pancreatic ductal carcinoma, but because of frequent expression, C-ERC/mesothelin might be useful as a target of molecular imaging and immunotherapy.

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Key words: ERC/mesothelin, pancreatic ductal carcinoma, ELISA, immunohistochemistry

Introduction

ERC/mesothelin gene (*MSLN*) encodes a 71 kDa precursor protein, which is cleaved by proteases to yield 31 kDa N-terminal (N-ERC/mesothelin) and 40 kDa C-terminal (C-ERC/mesothelin) proteins (1,2). N-ERC/mesothelin, originally identified as megakaryocyte-potentiating factor (MPF), is soluble and released into extracellular space (1-9). C-ERC/mesothelin is a glycoprotein tethered to the cell surface by glycosyl-phosphatidyl-inositol (GPI) anchor. Some forms of C-ERC/mesothelin are released into extra-cellular space by aberrant splicing or proteases (1,2,10-13).

N-ERC/mesothelin/MPF was isolated from the medium of cultured pancreatic cancer cells (3,4) and is known to be a serum marker of mesothelioma (5-9). C-ERC/mesothelin is expressed not only in normal mesothelial cells of the pleura, pericardium and peritoneum, but also in malignant cells of pancreatic ductal carcinomas, ovarian cancers, mesotheliomas and some other cancers (1,14-17). C-ERC/mesothelin can be detected in the sera of patients with ovarian carcinoma and mesothelioma (10-12,18). Previously, we discovered *Erc*, which is expressed in renal cell cancers of Eker rats. We also confirmed that *Erc* is a homolog of human *MSLN* (19-21).

Pancreatic ductal carcinoma remains a fatal disease because of its poor prognosis. Unfortunately, the diagnosis of pancreatic ductal carcinoma often occurs very late and consequently, $<40\%$ of patients are candidates for tumor resection (22-24). Thus, the overall 5-year survival rate of these patients is $<10\%$ (23,24). On the other hand, those of patients with stage I disease is 58.1% (25). Novel strategy for early diagnosis of pancreatic ductal carcinoma is warranted.

ERC/mesothelin is expressed in human pancreatic ductal carcinoma and not expressed in normal pancreatic tissue (15,16). Previous studies showed the usefulness of N-ERC/mesothelin and C-ERC/mesothelin as diagnostic markers for C-ERC/mesothelin expressing tumors (5-12). To date, there has been no report about the effectiveness of N-ERC/Meosthelin and C-ERC/mesothelin as serum markers of pancreatic ductal carcinoma. We have previously devised a novel enzyme-linked immunosorbent assay (ELISA) system for N-ERC/mesothelin and showed that it is useful for diagnosis of human mesothelioma (5-7). In this study, we

examined the expression of C- and N-ERC/mesothelin in cultured pancreatic cancer cell lines and human pancreatic ductal carcinomas and investigated the usefulness of our ELISA system as a diagnostic procedure of human pancreatic ductal carcinoma.

In the cultured cells, the concentration of N-ERC/mesothelin in the medium nearly correlated with the expression of C-ERC/mesothelin. C-ERC/mesothelin was frequently expressed in human pancreatic ductal carcinoma. There was, however, no increase in N-ERC/mesothelin concentration in the sera of pancreatic cancer patients compared with that of normal controls. Although N-ERC/mesothelin is established as a reliable marker for mesothelioma, N-ERC/mesothelin is not useful as a diagnostic marker of pancreatic ductal carcinoma. As for C-ERC/mesothelin, it might be useful as a target of molecular imaging and immunotherapy, because of its frequent expression.

Materials and methods

Pancreatic cancer cell lines. MIA-PaCa2 and PK-1 were provided by Cell Resource Center for Biomedical Research, Tohoku University, Sendai-shi, Miyagi, Japan. KP-3 and TCC-PAN2 were provided by Health Science Research Resources Bank, Sennan-shi, Osaka, Japan. PK-59 and PK-45H were provided by RIKEN CELL BANK, Tsukuba-shi, Ibaraki, Japan. MIA-PaCa2 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Culture supernatants and cells were harvested 48 h after culturing at 37˚C and 5% CO_2 atmosphere, upon reaching >80% confluency.

Reverse transcription-polymerase chain reaction (RT-PCR). mRNA levels of ERC/mesothelin in the cultured cells (MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H) were analyzed by RT-PCR. Cells in petri dishes were lysed by the acid guanidinium thiocyanate-phenol-chloroform extraction method (26) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNAs were extracted from these lysates following manufacturer's instructions. Total RNA (1 μg) was reverse transcribed for 30 min at 50˚C and subjected to polymerase chain reaction amplification. The primers used to amplify the ERC/mesothelin were: sense 5'-CAAGAA GTGGGAGCTGGAAG-3' and antisense 5'-GTCTCCAGG GACGTCACATT-3'. As a control for RT-PCR, ß-actin mRNA was amplified using the following ß-actin-specific primers: sense 5'-CCGCGAGAAGATGACCCAGA-3'; and antisense 5'-CAGGAGGAGCAATGATCTTG-3'. All primers were purchased from Operon (Tokyo, Japan). RT-PCR was carried out in an MBS Satellite 0.2 (Thermo Fisher Scientific, Kanagawa, Japan), using Titan RT-PCR System (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. After an initial denaturation step of 4 min at 94˚C, each sample was subjected to 25 cycles of amplification (denaturation, 30 sec at 94˚C; annealing, 30 sec at 50˚C; and elongation, 1 min at 68˚C) followed by a final elongation step for 10 min at 68˚C. PCR product (10 μl) was analyzed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Immunoblotting. MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H cells in petri dishes were lysed in a solution containing 2% sodium dodecylsulfate, 10% glycerol, 50 mM Tris-HCl (pH 6.8) and 100 mM dithiothreitol, followed by boiling for 2 min. These lysates were electrophoresed in 10% Laemmli gels and transferred onto nitrocellulose membranes. Membranes were blocked in 1% skim milk in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) for 1 h at room temperature. Next, membranes were incubated with 5B2 anti mesothelin antibody (Novocastra Laboratory Vision BioSystems, Boston, MA, USA, 1:100 dilution) or AC15 anti ß-actin antibody (Sigma, St. Louis, MO, USA, 1:5000 dilution) in PBS-T with 1% skim milk for 1 h at room temperature. EnVision+ system labeled polymer-horseradish peroxidase (HRP) (K4000 or K4001 purchased from Dako, Glostrup, Denmark) at a 100-fold dilution in PBS-T with 1% skim milk was added and allowed to react with the membrane at room temperature for 1 h. ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to visualize ERC/mesothelin on the membrane.

Human subjects. Patients with ductal pancreatic carcinoma, treated in Juntendo hospital between April 1, 2006 and November 30, 2007, were evaluated in this study. Pathological diagnosis was based on the histological analysis of tissue samples obtained from pancrearectomy or endoscopic ultrasonography guided fine needle aspiration biopsy (FNA), using sterile 21-gauge needles. This study was approved by the Institutional Review Board of Juntendo University School of Medicine and its hospital. Patients gave their signed informed consent. Nineteen healthy controls were sampled at random from a database, as described (6), with an age range of 50-79 years.

Immunohistochemistry. Tissue sections, 3 μm thick, were prepared from archival formalin-fixed, paraffin-embedded specimens. After deparaffinization, the tissue sections were heated in 10 mM citrate buffer (pH 6.0) for antigen retrieval and then treated with 3% hydrogen peroxide. Next, the sections were incubated with primary antibody solutions diluted in Tris-buffered saline with 0.1% Tween-20 (TBS-T) overnight at 4˚C. We used mouse monoclonal anti human C-ERC/mesothelin antibody 5B2 (1:50 dilution) as the primary antibody and EnVision+ system labeled with polymer-HRP (Dako) as the secondary antibody. Diaminobenzidine was used as the substrate for peroxidase. For immunostained slides, the intensity of staining was semiquantitatively graded on a scale of 1+ to 3+ and the proportion of stained ducts of cancer gland was graded as 0%, 1 to <10%, 10-50% and >50%.

ELISA. N-ERC/mesothelin concentration in sera and cell culture supernatants (MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H) were analyzed by sandwich ELISA method. Sandwich ELISA method was performed as

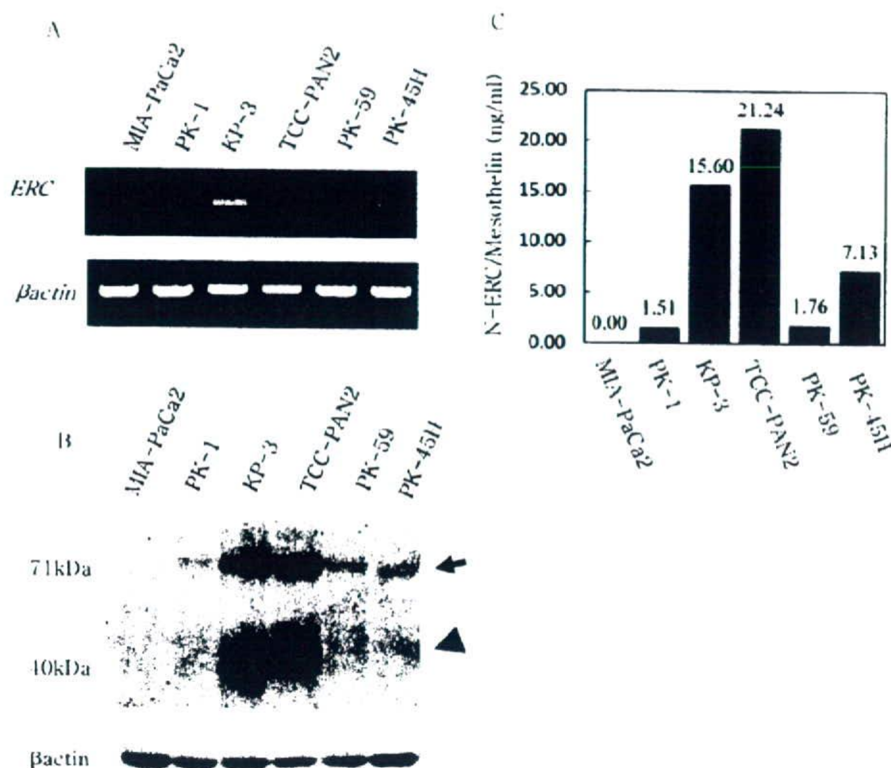

Figure 1. ERC/mesothelin expression in human pancreatic cancer cell lines. (A) ERC/mesothelin transcript in human pancreatic cancer cell lines detected by RT-PCR. (B) ERC/mesothelin protein in human pancreatic cancer cell lines detected by immunoblotting. Arrow, precursor ERC/mesothelin (71 kDa); Arrowhead, C-ERC/mesothelin (40 kDa). (C) Secreted N-ERC/mesothelin in the cell culture medium of human pancreatic cancer cell lines detected by ELISA.

previously described (5,6), using 7E7 monoclonal antibody and HRP-conjugated polyclonal antibody-282. Absorbance at 450 nm was measured in an ELISA reader (E-MAX; Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis. We analyzed ELISA data using JMP and SAS version 8.1.3 (SAS Institute, Cary, CA, USA). To compare serum concentration between groups, the Mann-Whitney test was used. $P<0.05$ was considered statistically significant.

Results

ERC/mesothelin expression in human pancreatic cancer cell lines. RT-PCR revealed ERC/mesothelin mRNA expression in most of the investigated pancreatic cancer cell lines, except for MIA-PaCa2 (Fig. 1A). Immunoblotting showed strong ERC/mesothelin and C-ERC/mesothelin expression in 2 of 6 cell lines (KP-3 and TCC-PAN2) and weak expression in PK-1, PK-59 and PK-45H. MIA-PaCa2 did not demonstrate any ERC/mesothelin and C-ERC/mesothelin expression (Fig. 1B). N-ERC/mesothelin was secreted into the culture supernatants of 5 cell lines, of which 2 (KP-3 and TCC-PAN2) showed high N-ERC/mesothelin concentration. PK-1, PK-59 and PK-45H secreted N-ERC/mesothelin moderately. MIA-PaCa2 did not secrete N-ERC/mesothelin at all (Fig. 1C).

C-ERC/mesothlein expression in human pancreatic ductal carcinoma. Of 19 tissue samples, 8 were obtained from pancrearectomy and 11 from FNA. They included 10 men and 9 women with age range of 40-78 years (mean 69.8) and consisted of one stage I, 14 stage III and 4 stage IV patients (the International Union against Cancer classification). The immunostaining results are shown in Table I. Positive staining for C-ERC/mesothelin was seen in 14 of the 19 samples. Six of 11 samples from FNA and all of the samples from pancreatic resections showed positive staining. The staining pattern was often focal and cytoplasmic with polarity to apical membrane (Fig. 2A and B). In some cases, polarity of the signal was weak or none (Fig. 2C).

Serum N-ERC/mesothelin levels in patients with pancreatic ductal carcinoma. Serum samples from 19 patients were obtained before surgery, chemotherapy, or any other therapies. The sera were evaluated for N-ERC/mesothelin. The 19 age-matched healthy control samples, from the database as described (6), included 10 men and 9 women with age range of 50-79 years (mean 65). There was no significant difference in serum N-ERC/mesothelin

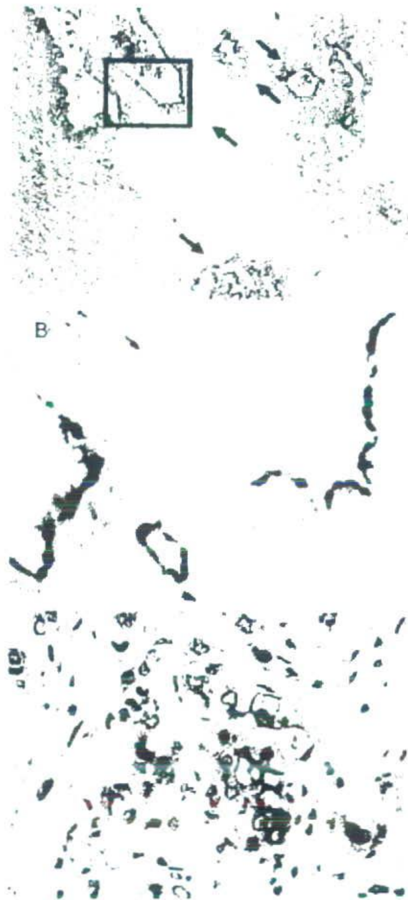

Figure 2. C-ERC/mesothelin expression in human pancreatic cancer tissue. (A) Immunohistochemical staining of C-ERC/mesothelin using 5B2 antibody, magnification (x40). Positive staining is shown by arrows. (B) Same sample as (A), respectively, with higher magnification (x200). An area covered by a square frame in (A) is expanded. (C) Another 5B2 stained sample with magnification (x200).

Table I. C-ERC/mesothelin immunostaining results of human pancreatic ductal carcinoma.

Sample	Intensity			Proportion			
	1+	2+	3+	0	<10	10-50	>50
FNA	3	1	2	5	3	1	2
Operation	2	4	2	0	1	4	3
Total	5	5	4	5	4	5	5

The intensity of staining was semiquantitatively graded on a scale of 1+ to 3+ and the proportion of stained ducts of cancer gland was graded as 0%, 1 to <10%, 10-50%, >50%.

concentration between cancer patients and the healthy control group (P=0.569) (Fig. 3A). Between patients with resectable tumor and those with far advanced unresectable tumor, there was no significant difference in serum N-ERC/mesothelin concentration (P=0.710) (Fig. 3B).

Discussion

In the present study, we examined C- and N-ERC/mesothelin expression in the two patients with pancreatic ductal carcinoma and cultured pancreatic cancer cell lines. The expression of C-ERC/mesothelin was studied by immunoblotting of cultured cell lysates or by immunohistochemical staining of carcinoma tissue. The concentration of N-ERC/mesothelin in the supernatant of cultured cells or in sera of patients was measured by the ELISA system established by us (5-7). Our study indicated that N-ERC/mesothelin concentration in supernatants correlated with the expression levels of C-ERC/mesothelin in cultured cells. Human pancreatic ductal carcinoma frequently expressed C-ERC/mesothelin. Contrary to our initial expectation, we did not find any significant difference in the

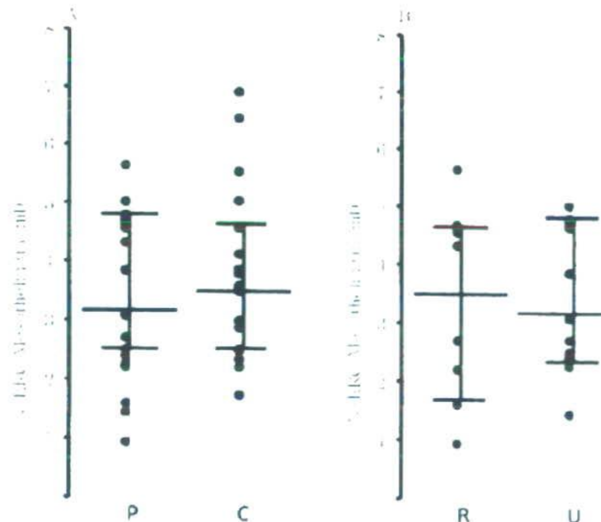

Figure 3. Scatter plots of serum N-ERC/mesothelin concentration. (A) Comparison of N-ERC/mesothelin concentration in sera from patients with pancreatic ductal carcinoma (P) and healthy controls (C). (B) Comparison of patients with resectable tumor (R) and unresectable far advanced tumor (U).

serum concen-tration of N-ERC/mesothelin between patients with pancreatic ductal carcinoma and normal controls.

Almost all human pancreatic cancer cell lines, except for MIA-PaCa2, expressed mRNA of ERC/mesothelin. KP-3 and TCC-PAN2 strongly expressed C-ERC/mesothelin protein and secreted high amounts of N-ERC/mesothelin, while MIA-PaCa2 did not express C-ERC/mesothelin nor secrete N-ERC/mesothelin at all. N-ERC/mesothelin concentration in the supernatants of cultured cells almost correlated with C-ERC/mesothelin expression.

All surgically resected samples showed positive staining for C-ERC/mesothelin. However, five of 11 FNA cases showed negative staining. Baruch *et al* reported that >50% of samples from FNA are negative for C-ERC/mesothelin because the staining pattern is most often focal and unevenly distributed (16). Argani *et al* reported that in tissue sections of pancreatic adenocarcinomas, diffuse staining was seen in only 30% of the mesothelin positive tumors (15). There is the possibility that our staining data of FNA samples, as shown in Table I, may have underestimated the actual expression of C-ERC/mesothelin because of the small and limited volume of FNA samples. Either way, we and others have shown that human pancreatic ductal carcinoma frequently expressed C-ERC/mesothelin. The staining pattern of pancreatic ductal carcinoma was cytoplasmic with or without polarity to apical membrane, while that of mesothelioma was membranous (1,5,7,17,20).

Based on these results, we investigated whether the serum level of N-ERC/mesothelin could be a novel diagnostic marker of human pancreatic ductal carcinoma, using our previously reported ELISA system for detection (5-7). We age-matched the patients and healthy controls, because N-ERC/mesothelin in the sera has a tendency to elevate as people get older. Unexpectedly, we found that N-ERC/mesothelin in sera of patients with pancreatic ductal carcinoma was comparable to those of healthy controls.

Other ERC/mesothelin expressing tumors, including ovarian cancers and mesotheliomas, can be detected by measurement of serum C- or N-ERC/mesothelin concentration (5-12). We have shown that our ELISA system for N-ERC/mesothelin also detects mesotheliomas (5-7). Presently, the reason why N-ERC/mesothelin was not increased in the sera of patients with pancreatic ductal carcinoma while C-ERC/mesothelin was frequently expressed in the carcinoma tissues is unknown. In the far advanced pancreatic ductal carcinoma patients with stage IV disease, N-ERC/mesothelin concentration was not higher than in the patients with stage I-III diseases (data not shown). Thus, it appears that the proportion of C-ERC/mesothelin expressing ducts of the gland was not the reason. It was considered that differences between *in vitro* and *in vivo* conditions, for example changes in the micro-environments such as differential expression of proteases and their inhibitors, or tumor vascularity, may contribute to this discrepancy. High concentration of serum N-ERC/mesothelin indicates a patient that does not have pancreatic ductal carcinoma, but other C-ERC/mesothelin expressing tumor, perhaps mesothelioma. In this case, examination for pancreatic ductal carcinoma could be omitted.

In conclusion, N-ERC/mesothelin concentration in supernatants correlated with the expression levels of C-ERC/mesothelin in cultured cells. Human pancreatic ductal carcinoma frequently expressed C-ERC/mesothelin. However, serum N-ERC/mesothelin concentration of cancer patients was equivalent to healthy controls. N-ERC/mesothelin was not useful as a serum marker of pancreatic ductal carcinoma. Because of frequent expression of C-ERC/mesothelin in pancreatic ductal carcinoma tissues, there is a possibility that imaging detection system or immunotherapy, using C-ERC/mesothelin, will be developed in the future.

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Establishment of a novel specific ELISA system for rat N- and C-ERC/mesothelin. Rat ERC/mesothelin in the body fluids of mice bearing mesothelioma

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Mesothelioma is a type of malignant tumor that most commonly arises from the pleural or peritoneal membrane and is usually associated with previous exposure to asbestos. In humans, ERC/mesothelin is expressed on the normal mesothelium and in some cancers such as mesothelioma or ovarian carcinoma. Recently, several enzyme-linked immunosorbent assay (ELISA) systems for ERC/mesothelin have been developed, the reported usefulness of which has been assessed and demonstrated as a diagnostic tool. However, the basic roles or physiological functions of, and relationship between, ERC/mesothelin and asbestos exposure-mediated carcinogenesis remain to be resolved. In order to elucidate the precise mechanism, animal models of mesothelioma are desperately needed. In this study, we consider the development of a novel specific ELISA system for not only rat N-ERC/mesothelin but also C-ERC/mesothelin, and the first data on the presence of rat ERC/mesothelin in the body fluids of rat malignant mesothelioma-bearing nude mice. The transplanted mice have revealed the higher concentrations of rat N-ERC/mesothelin in the blood and ascites than C-ERC/mesothelin. We hope these novel ELISA systems are useful in the rat model system to clarify the mechanism of asbestos-induced carcinogenesis and to develop new effective drugs for mesothelioma. (*Cancer Sci* 2008)

The number of cases of mesothelioma caused by exposure to asbestos is growing, and as exemplified by the shocking events in 2005 and highlighted in TV and newspaper reports every day in Japan, this condition represents an enormous social problem.

Mesothelioma is a type of malignant tumor that most commonly arises from the pleural or peritoneal membrane. It is believed that mesothelioma is caused by the inhalation of asbestos fibers. However, how the aspirated asbestos in the lung might provoke tumor formation in the pleural membrane that covers the lungs, or the peritoneal membrane, has not yet been clarified.[1,2]

In regard to human mesothelioma, a group from Australia has reported that soluble mesothelin-related protein (SMRP), the C-terminal fragment of mesothelin, may be a tumor marker. Another group from National Institute of Health has reported that the N-terminal fragment of mesothelin may be a mesothelioma marker.[3–11] Also, Shiomi *et al*. reported the usefulness of the N-ERC/mesothelin as a serum marker for mesothelioma.[9]

The mesothelin protein is present in the normal mesothelium, a membrane lining several body cavities including the pleura, peritoneum, and pericardium. It could be speculated that mesothelioma derived from the mesothelium should demonstrate overexpression of mesothelin. This protein, 622 amino acids in length, is expressed as a GPI anchor–type membranous protein of with a molecular weight of about 71 kDa, that is cleaved by a furin-like protease into the 31 kDa N-terminal fragment and 40 kDa C-terminal fragment.

On the other hand, we have conducted research on the Eker model rat, a rat model of renal cell calcinoma, and cloned the *Erc* gene, which is expressed in abundance in the kidney of Eker rats. The expected amino acid sequence of the rat *Erc* cDNA exhibits 87.4% similarity to the mouse protein and 56.1% similarity to human MPF/mesothelin. Although the similarity to the human protein is not so high, both the sequences possess two hydrophobic regions, secretory signal sequences at the N terminus, signal sequences characteristic of a GPI anchor-type protein at the C terminal, secretory recognition sequences for a furin-like protease, and are located in the same chromosome as the tumor suppresser *Tsc2/TSC* gene. Based on the above, it is considered that the rat ERC may be a functional orthologue of human mesothelin; therefore, we call this gene product ERC/mesothelin.

We attempted to develop a rat ERC/mesothelin ELISA assay kit following the idea that ERC/mesothelin may be a potentially useful marker of malignant mesothelioma in humans, since the possible existence of a strong relationship between ERC/mesothelin and mesothelioma in humans has been suggested.

Shiomi *et al*. have developed a human ERC/mesothelin ELISA assay kit and reported that ERC/mesothelin is a useful marker for malignant mesothelioma.[9]

Nakaishi *et al*. established an assay system using mouse monoclonal antibody (MoAb) 30F2 as the solid antibody and a polyclonal antibody of N-ERC (150–160) as the enzyme-labeled antibody, for detecting the N-terminal fragment, N-ERC, of ERC/mesothelin. They measured the blood concentrations of N-ERC/mesothelin in the Eker rat, a model of renal cell carcinoma using this assay system.[12]

In this study, to investigate whether ERC/mesothelin could be a mesothelioma marker in the rat and to investigate the functions of ERC/mesothelin in relation to the development and progression of mesothelioma, we developed a novel highly specific and sensitive sandwich ELISA system for not only rat N-ERC/mesothelin but also C-ERC/mesothelin. Moreover, a Xenograft mouse model for mesothelioma has been developed.

Using these novel ELISA systems, we determined the presence of ERC/mesothelin in the body fluids of tumor-bearing nude mice, in order to explore the potential usefulness of this protein as a marker of mesothelioma.

Materials and Methods

Production of antibodies. ***Production of rabbit PoAb.*** We synthesized synthetic peptides corresponding to the peptide sequences 150–166, 259–280, 306–327, and 549–566 of full-length rat ERC/

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mesothelin and immunized rabbits. Each antiserum was purified by affinity column chromatography using an antigen peptide-coupled solid-phase column.

Production of mouse MoAb. MoAb 30F2[12] was purified from the resultant ascitic fluid of transplanted mice with hybridoma cells with a protein A column.

Western blotting. The full-length rat ERC/mesothelin cDNA was transfected into CHO-K1 cells and the cell culture supernatant was collected. The supernatant was subjected to Western blot analysis with N-ERC (150–166) and N-ERC (259–280) PoAb, and rat C-ERC (549–566) and rat C-ERC (306–327) PoAb. Secondary antibodies in the form of goat antirabbit IgG conjugated to peroxidase (No. 17502; Immuno-Biological Laboratories Co. Ltd. (IBL), Gumna, Japan) were then added and allowed to react with the membranes at room temperature for 1 h. ERC/mesothelin on the membranes was visualized by the ECL detection system (Amersham Biosciences, GE Healthcare UK Ltd., Buckinghamshire, England).

Epitope Mapping of MoAb. We produced four different molecular sizes of the GST-fusion protein by cutting the rat ERC/mesothelin gene into four 0.2 kb fragments.

The samples were electrophoresed on 12.5% Laemmli gel, and transferred to a polyvinylidene difluoride memblane.

The membrane was incubated with 1 µg/mL of the first group of antibodies (30F2 MoAb; N-ERC [259–280] PoAb) in phosphate-buffered saline (PBS) for 1 h, incubated with the second group of antibodies, and then detected as described in the section above.

Establishment of the ELISA assay system

Production of standard rat ERC/mesothelin protein for the ELISA system. The full-length cDNA of the rat ERC/mesothelin coding region was inserted into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) and transfected into CHO-K1 cells. The culture supernatant of the stable transfectant was collected and applied on affinity column chromatography using 30F2 MoAb. The eluate was additionally purified with affinity column chromatography using N-ERC (259–280) PoAb. The concentration of purified protein was measured by protein assay (Bio-Rad, Tokyo, Japan). The purity of the protein was demonstrated densitometrically by using a densitograph (ATTO, Tokyo, Japan) and gel-filtration chromatography (data not shown). This affinity-purified rat N-ERC/mesothelin was used as the ELISA standard.

Establishment of the ELISA system. An Immuno Module Plate (Nalge Nunc, Rochester, NY, USA) was coated with the 30F2 MoAb (in 0.1 mol carbonate buffer, pH 9.5) and incubated at 4°C overnight, then blocked with 1% bovine serum albumin in PBS. The sample and standard N-ERC/mesothelin proteins were diluted with 0.05% Tween 20 in PBS and added to each well, followed by incubation of the well at 37°C for 1 h. After nine washes with the washing buffer, 100 µL of horseradish peroxidase-labeled N-ERC (259–280) PoAb was added to each well, followed by incubation for 30 min at 4°C. After nine washes with the washing buffer, 100 µL of tetramethyl benzidine buffer was added as the substrate to each well, followed by incubation for 30 min at room temperature in the dark. Color development was stopped by the addition of 100 µL of stop solution (1 N H_2SO_4). The optical density of each sample at 450 nm was then measured.

The same protocol was employed for the assay of rat C-ERC/mesothelin.

For this assay, the Immuno Module Plate was coated with rat C-ERC (549–566) PoAb, and rat C-ERC (306–327) PoAb was used as a horseradish peroxidase-labeled antibody.

Immunohistochemistry of rat ERC/mesothelin in Wistar rats. Four-micrometer-thick tissue sections were prepared from the formalin-fixed, paraffin-embedded tissue of Wistar rats. After deparaffinization, the tissue sections were heated in 10 mmol citrate buffer (pH 6.0) for antigen retrieval, and treated with 3% hydrogen peroxide. Then, the sections were incubated with 1 µg/mL of a primary rat C-ERC (549–566) PoAb in PBS-T at room temperature overnight. Envision K4002 (Dako Japan Co. Ltd., Kyoto, Japan) was applied to the tissue sections, without dilution, as the secondary antibody. Diaminobenzidine (DAB) was used as the substrate for peroxidase.

FACS analysis. We confirmed the expressions of rat ERC/mesothelin in the cultured cells of the rat mesothelioma cell line (MeET-4)[13] and Yoshida salcoma (YS),[14] using rat C-ERC/mesothelin antibodies.

Cells were cultured in TIL medium (No. 33612; IBL, Gunma, Japan) containing 10% FCS, washed, and susupended to obtain 2×10^6 cells/mL/50 µL/tube.

The cells were incubated with 5 µg/mL of the primary antibody (Rat C-ERC [549–566] and [306–327] PoAb) for 1 h at 4°C.

Goat antirabbit IgG conjugated to fluorescein-isothiocyanate (No. 17522, IBL) was used as the secondary antibody; it was applied at 50 µg/mL or 20 µL to each well, followed by incubation at 4°C for 20 min. FACS analysis was then conducted.

Intraperitoneal transplantation of mesothelioma cells in nude mice and measurement of rat ERC/mesothelin in the body fluids. MeET-4 and YS cells were cultured in TIL medium containing 10% FCS, washed, and suspended with physiological saline solution to obtain 1×10^7/0.5 mL.

The cell suspensions were injected hypodermically into the abdominal cavity of nude mice (Balb/c 6–8 week old females; Charles River Laboratories, Kanagawa, Japan).

The ascitic fluid and serum specimens were collected 8 and 12 days after transplantation and the concentration of N- and C-ERC/mesothelin was measured using the specific ELISA systems.

Results

Specificity of the antibodies. The specificities of the antibodies were confirmed by Western blot analysis of the culture supernatant.

The 30-kDa N-ERC/mesothelin fragment was detected using rat N-ERC (150–166) or N-ERC (259–280) antibodies, and the 40-kDa C-ERC/mesothelin fragment was detected using rat C-ERC (549–566) and rat C-ERC (306–327) antibodies (Fig. 1a).

Epitope mapping of 30F2 MoAb. Antirat ERC/mesothelin (30F2) mouse MoAb reacted with all the GST-fusion proteins prepared with peptide fragments cut from the rat ERC/mesothelin.

We confirmed that the epitope of 30F2 MoAb was on the N-terminal portion of ERC/mesothelin (Fig. 1b).

Establishment of the ELISA system. We used mouse 30F2 MoAb as the solid antibody and the HRP-conjugated rat N-ERC (259–280) rabbit PoAb as the enzyme-labeled antibody in the novel N-ERC/mesothelin assay kit (No. 27765; IBL, Gunma, Japan) (Fig. 2).

For the rat C-ERC/mesothelin assay kit, we used rat C-ERC (549–566) rabbit PoAb as the solid antibody and HRP-conjugated rat C-ERC (306–327) rabbit PoAb as the enzyme-labeled antibody (No. 27761; IBL). The results exhibited a linear log/log plot for the recombinant rat N-ERC/mesothelin concentration range between 0.078 ng/mL and 5.0 ng/mL by N-ERC/mesothelin assay kit, and the recombinant rat C-ERC/mesothelin range between 0.1 ng/mL and 7.0 ng/mL by C-ERC/mesothelin assay kit (Fig. 3).

Determination of the presense of rat ERC/mesothelin in the cell culture supernatant by ELISA system. We determined the presence of rat ERC/mesothelin in the cell culture supernatant using the following method. MeET-4, YS, and rat ERC/mesothelin/CHO cells (5×10^4) were seeded into 24-well culture plates. Culture supernatants were then collected from four wells at various time-points.

The concentration of rat ERC/mesothelin was increased with the increasing proliferative activity of the MeET-4 cells.

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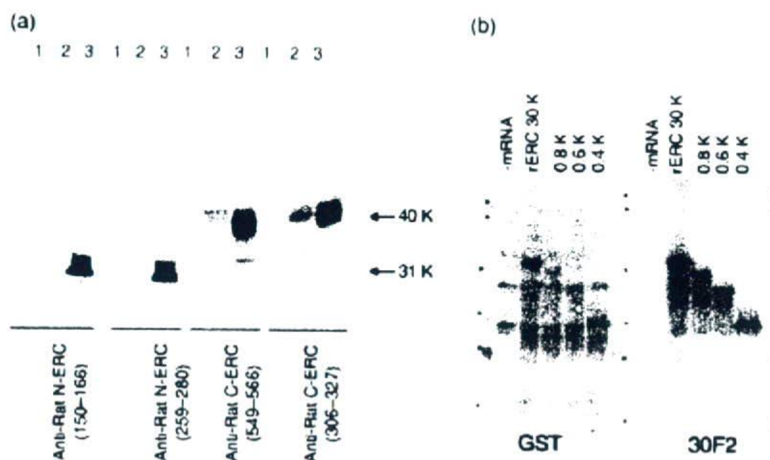

Fig. 1. Characterization of anti-ERC/mesothelin antibodies. (a) Western blot analysis lane 1, MeET-4 cell lysate; lane 2, lysates of MeET-4 subcutaneously transplanted into nude mouse; and lane 3, culture supernatant of rat ERC/CHO transfectant. (b) The Epitope mapping of antirat ERC/mesothelin MoAb (30F2). Four different molecular sizes of the rat ERC/mesothelin fused with GST protein were produced and underwent Western blot analysis by 30F2 MoAb. 30F2MoAb reacted with all the GST-fusion proteins.

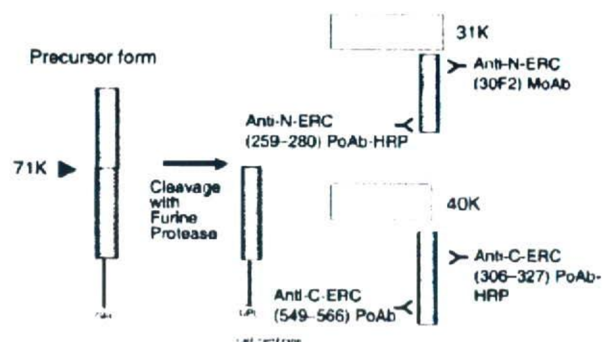

Fig. 2. Products of ERC/mesothelin gene and enzyme-linked immunosorbent assay (ELISA) system. We used mouse 30F2 MoAb as the solid antibody and the HRP-conjugated rat N-ERC (259–280) rabbit PoAb as the enzyme-labeled antibody in the novel N-ERC/mesothelin assay kit. For the Rat C-ERC/mesothelin assay kit, we used rat C-ERC (549–566) rabbit PoAb as the solid antibody and HRP-conjugated rat C-ERC (306–327) rabbit PoAb as the enzyme-labeled antibody.

However, N- and C-ERC/mesothelin were not detected in the YS cells (Fig. 4). These results corresponded with the mRNA expression of ERC/mesothelin by reverse transcription–polymerase chain reaction method (data not shown).

Intraperitoneal transplantation of mesothelioma cells in nude mice and measurement of rat ERC/mesothelin in the body fluids. The concentrations of rat N-ERC and C-ERC/mesothelin in the blood and ascitic fluid of nude mice transplanted intraperitoneally with MeET-4 and YS cells were measured by the specific ELISA systems.

As a result, the ascites with the transplantation of MeET-4 into abdominal cavity showed quite high concentrations of N- and C-ERC/mesothelin (727.5 ng/mL to 2680.5 ng/mL of N-ERC/mesothelin, 108 ng/mL to 251.5 ng/mL of C-ERC/mesothelin). The serum harvested showed a relatively high concentration (511.2 ng/mL to 1075.8 ng/mL of N-ERC/mesothelin, 32.7 ng/mL to 60.8 ng/mL of C-ERC/mesothelin). The serum harvested from mice that underwent subcutaneous transplantation of MeET-4 showed a low concentration of N- and C-ERC/mesothelin (357.6 ng/mL to 605.8 ng/mL of N-ERC/mesothelin, 6.3 ng/mL to 10.4 ng/mL of C-ERC/mesothelin). (Fig. 5). The ERC/mesothelin in mouse body fluids detected by each ELISA system should be of rat MeET-4 origin, because of tjhe absence of detectable amounts of rat ERC/mesothelin concentrations without transplantation.

The N- or C-ERC/mesothelin was not detected in the ascites and serum with the transplantation of YS cells.

In almost all cases when ERC/mesothelin was detected, the concentrations of N-ERC/mesothelin were higher than the concentrations of C-ERC/mesothelin. N-ERC/mesothelin might thus be considered a good marker compared to C-ERC/mesothelin.

Immunohistochemistry. The results of immunohistochemistry confirmed the expression of rat ERC/mesothelin in the lung (Fig. 6a).

FACS analysis. Cell surface expression of the ERC/mesothelin was confirmed using the specific antibody against rat C-ERC/mesothelin, in both MeET-4 and YS1567 cells (Fig. 6b).

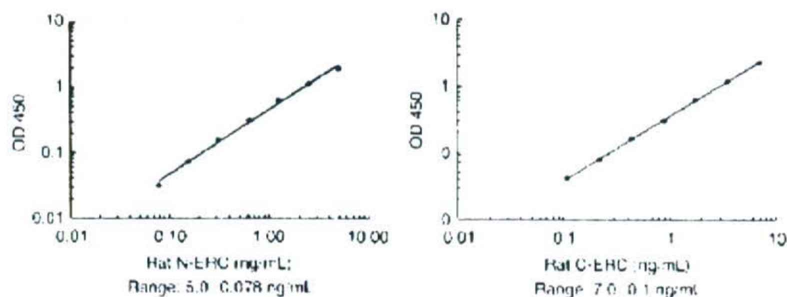

Fig. 3. Typical standard curves of rat ERC/mesothelin enzyme-linked immunosorbent assay (ELISA) systems. The typical standard curves for rat N-ERC/mesothelin and C-ERC/mesothelin are shown.

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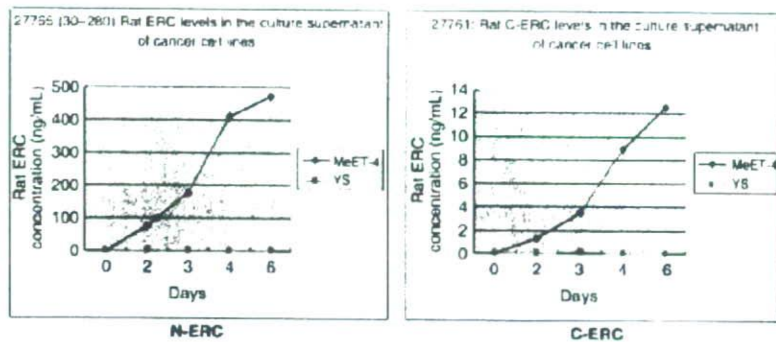

Fig. 4. Rat ERC levels in the culture supernatant of cancer cell lines. On day 0, rat mesothelioma cells (MeET-4) (◆) or Yoshida sarcoma (YS) (■) cells (5×10^4) were seeded in 24-well culture plates. At each time point, culture supernatants were taken from four wells. The concentration of rat ERC/mesothelin was determined using sandwich enzyme-linked immunosorbent assay (ELISA).

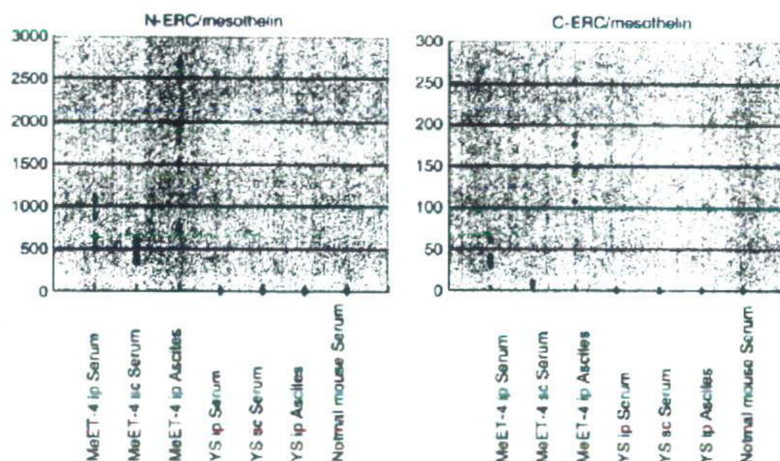

Fig. 5. Rat N- and C-ERC/mesothelin concentrations in body fluids of tumor-bearing nude mouse. The ascites with the transplantation of rat mesothelioma cells (MeET-4) or Yoshida sarcoma (YS) into the abdominal cavity (MeET-4 or YS ip ascites), serum harvested as same manner (MeET-4 or YS ip Serum), and serum harvested from mice with subcutaneous transplantations of MeET-4 or YS (MeET-4 or YS sc Serum) were measured for N- and C-ERC/mesothelin concentrations by specific enzyme-linked immunosorbent assay (ELISA) systems.

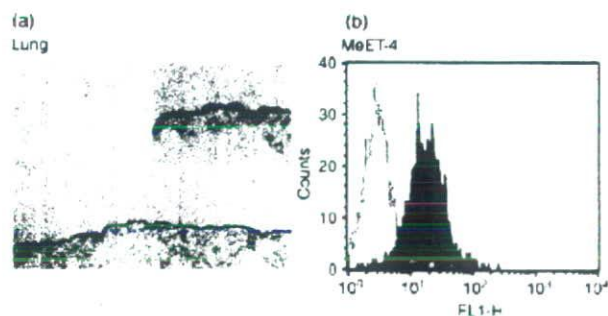

Fig. 6. The cell surface expression of rat C-ERC/mesothelin using anti-C-ERC/mesothelin antibody (306). (a) Immunohistochemical analysis in a normal rat lung. An enlarged view of a portion of stained epithelial cells was presented in upper right. (b) FACS analysis of MeET-4 mesothelioma cells.

C-ERC/mesothelin was detected on the cell surface, but N-ERC/mesothelin was not detected. This results showed that N-ERC/mesothelin fragments should be secreted into the blood or body fluid immediately after protease digestion. The mechanism of releasing C-ERC/mesothelin from the cell surface has not yet been fully elucidated. However, C-ERC/mesothelin seems to be attached to the cell surface by phosphatidylinositol. It is released when cells are treated with phosphatidylinositol-specific phospholipase C.[15]

Discussion

As mentioned earlier, there appears to be a strong relationship between ERC/mesothelin and mesothelioma caused by asbestos exposure in humans; however, the nature of the relationship or the functions of the protein have not yet been clarified at present. The rat ERC/mesothelin was detected in the blood and ascitic fluid of the nude mice transplanted with MeET-4 cells, using a specific ELISA system. This results demonstrated that the rat ERC/mesothelin is of malignant MeET-4 cell origin. In the future, we would like to clarify the relationship between the rat ERC/mesothelin found in the body fluids of MeET-4 tumor-bearing mice and mesothelioma.

The measurement range of the ELISA system for N-ERC/mesothel described by Nakaishi *et al.*[12] was between 1.25 ng/mL and 80 ng/mL. However, in this study, the novel ELISA system indicated an improved measurement range between 0.078 ng/mL and 5.0 ng/mL by using new antibodies. This improvement could achieve precise measurements in the blood and body fluids of mice.

This system should be a useful tool for elucidating the functions of ERC/mesothelin and its role in the development of mesothelioma, as well as screening and the evaluation of antimesothelioma drugs. Also, we hope these ELISA and mouse model systems will be valuable in evaluating new antimesothelioma therapies.

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