

for the HER-2 shRNA expression. It can therefore suppress the expression of HER-2 mRNA for a long period by sustained transcription of HER-2 shRNA from pDNA. This might be one of the reasons why the combination of HER-2 shRNA pDNA and PTX treatment extended the mean survival days.

Trastuzumab is widely used as a standard therapy for patients with HER-2-overexpressing metastatic breast cancer with clear evidence of clinical efficacy as a single agent or in combination with chemotherapeutic agents (29). It has also been reported that trastuzumab enhances the antitumor activity of PTX and doxorubicin against HER-2-overexpressing human breast cancer xenografts (27); however, in our results, trastuzumab was not effective for KB culture cells (data not shown) and KB tumor xenografts as a single agent. Moreover, trastuzumab did not increase the sensitivity in combination with PTX (Fig. 6) like HER-2 siRNA and HER-2 shRNA pDNA pretreatment (Figs. 7A and 8A). This finding correspond well with the result that 60-70% metastatic patients with HER-2-positive tumors appear intrinsically resistant to trastuzumab as a sole therapy (30,31). Furthermore, trastuzumab alone or in combination with docetaxel or PTX was not effective in clinical trials for HER-2-positive prostate tumors (32,33). A cell line developed from a patient resistant to trastuzumab treatment was found to maintain high levels of HER-2 expression without identifiable mutations (34). These findings might explain why trastuzumab was not effective for KB tumor xenografts. In this study, combination therapy with HER-2 siRNA or HER-2 shRNA pDNA increased the inhibitory effect of PTX and mean survival days in KB tumor xenografts, suggesting that HER-2 RNAi therapy might be effective for trastuzumab-resistant cells in combination with chemotherapy.

In our study, the mechanism of the enhanced antitumor effect for chemotherapy by HER-2 suppression remains unclear. We speculated that HER-2 suppression contributed to the reduction of antiapoptotic signaling and then increased the sensitivity to chemotherapy. As another mechanism, PTX increased the liposome-delivered transfection efficiency by inhibiting targeting endosomes to lysosomes (35,36). We confirmed that PTX treatment increased 2-fold in gene expression by NP in KB cells (data not shown). Therefore, using the combined lipid-mediated transfection of genes with PTX might be a powerful technique due to the effect of enhanced gene therapy. Combination therapy has the potential to reduce the side effects of PTX.

In conclusion, we demonstrated the potential of HER-2 siRNA and HER-2 shRNA pDNA to increase the sensitivity of PTX in HER-2-positive KB cells and tumor xenografts using NP delivery. Suppression of HER-2 expression significantly inhibited cell growth and induced caspase 3/7 activity in KB cells. Thus, the combination of HER-2 siRNA and PTX may serve as a novel tool for gene therapy with fewer adverse effects.

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# Combination of RET siRNA and irinotecan inhibited the growth of medullary thyroid carcinoma TT cells and xenografts *via* apoptosis

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Medullary thyroid carcinoma (MTC) is a rare endocrine tumor that frequently metastasizes, and treatment with irinotecan (CPT-11) is limited because of side effects. Mutations in the Rearranged during transfection (RET) proto-oncogene are considered the causative event of MTC. The objective of this study was to examine whether small interfering RNA (siRNA) and its combined treatment with CPT-11 could inhibit MTC cell growth *in vitro* and *in vivo*. The transfection of RET siRNA suppressed RET expression, reduced proliferation, and increased caspase-3/7 activity *via* the down-regulation of Bcl-2 expression. Combined treatments with CPT-11 or SN-38 significantly increased caspase 3/7 activity compared with RET siRNA, CPT-11 or SN-38 treatment alone. Importantly, intratumoral injection of RET siRNA along with intravenous injection of CPT-11 significantly inhibited the tumor growth of MTC xenografts *via* an increased apoptotic effect. These findings that RET siRNA enhanced sensitivity for CPT-11 will provide a novel strategy for the treatment of MTC with RET mutation. (*Cancer Sci* 2010; 101: 941–947)

Medullary thyroid carcinoma (MTC) is a rare endocrine tumor originating from calcitonin-secreting C cells. MTC may be sporadic or hereditary, including multiple endocrine neoplasia (MEN) type 2A, MEN type 2B and familial medullary thyroid carcinoma (FMTC).<sup>(1)</sup> MTC has an overall 10-year cause-specific survival of 60–70%, with a particularly poor prognosis for patients with MEN2B and sporadic MTC, who have a 5-year mortality of 30–50%.<sup>(2)</sup> Hereditary MTC is caused by a germline mutation in the Rearranged during transfection (RET) gene. About 40% of sporadic MTCs harbor a somatic RET mutation.<sup>(1)</sup> The RET gene encodes a receptor tyrosine kinase with an extracellular ligand-binding domain and a cysteine-rich domain, a signal transmembrane domain and an intracellular domain containing the catalytic tyrosine kinase domain.<sup>(3)</sup> RET has a glial cell line-derived neurotrophic factor as a ligand,<sup>(4)</sup> but mutated RET leads to a constitutive active RET tyrosine kinase, causing malignant behavior of C cells.<sup>(5)</sup>

Surgery is currently the only effective treatment for primary MTC.<sup>(6)</sup> Radioactive iodine has no benefit since C cells do not take up iodine. The role of chemotherapeutic regimens is also limited.<sup>(7)</sup> Imatinib specifically inhibits the tyrosine kinase activity of Abl, Kit and platelet-derived growth factor receptor (PDGFR).<sup>(8)</sup> However, imatinib therapy for MTC yielded no objective responses and induced considerable toxicity in patients.<sup>(9)</sup> CPT-11 injected intraperitoneally was relatively effective for TT tumor xenografts.<sup>(10)</sup> There is, therefore, no curative therapy for patients with metastatic MTC, which is responsible for many of the deaths caused by MTC. Gene therapy is a potentially useful alternative treatment for MTC. Specific inhibition of RET signaling using the dominant-negative RET mutant and ribozyme resulted in both growth suppression and subsequently the death of MTC cells.<sup>(11–13)</sup> Expressions of

thymidine kinase, interleukin (IL-2) and IL-12 by adenoviral vectors have resulted in tumor suppression in MTC animal models,<sup>(14)</sup> however, the observed antitumor effect *in vivo* was mostly transient.

New technology based on specific inhibition of the RET gene may be useful for MTC treatment to improve safety. RNA interference (RNAi) is a powerful gene-silencing process that holds great promise in the field of cancer therapy.<sup>(15)</sup> Small interfering RNAs (siRNAs) are expected to have a medicinal application in human gene therapy as drugs with high specificity for molecular targeting. RET siRNA can inhibit the expression of RET tyrosine kinase receptor, and it is expected to inhibit all signals following RET tyrosine kinase; however, gene therapy with RET siRNA has not been reported *in vivo* for thyroid cells endogenously expressing mutated RET.

It is rational to select tyrosine kinase inhibitors for the treatment of MTC, although a selective drug that targets RET has not yet been found. Therefore, in this study, we selected RET siRNA as potentially therapeutic molecules. Human MTC (TT) cells endogenously expressed RET bearing the C634W mutation associated with MEN2A mutations, which have been identified mainly in one of six cysteine residues (codon 609, 611, 618, 620 in exon 10, and codon 630, 634 in exon 11) in the RET extracellular domain;<sup>(1,16)</sup> therefore, TT cells are useful as a model for studying MTC.<sup>(16)</sup>

In this study, we investigated whether the transfection of RET siRNA alone and in combination with CPT-11 could induce the growth inhibition of TT cells and tumor xenografts. Combination therapy with RET siRNA and CPT-11 may be effective for MTC.

## Materials and Methods

**Cell culture.** TT cells were obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). The cells were grown in Ham's F-12 culture medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

**siRNA.** The stealth RNA interference duplex-targeting nucleotides of RET (RET-1 siRNA, RET 2-siRNA and RET-3 siRNA) and stealth RNAi Negative Control Low GC Duplex and Medium GC Duplex as control siRNAs (Cont-L siRNA and Cont-M siRNA, respectively) were synthesized by Invitrogen (Carlsbad, CA, USA). The stealth siRNA sequences of RET were RET-1 sense: 5'-AAAUCCGAAAUCUUCUCCG-CC-3' and antisense strand 5'-GGCGGAAGAUGAAGAUUUC-GGAUUU-3', RET-2: sense 5'-AAUCCUCUUCUAAACAUCUCGGGA-3' and antisense 5'-UCCCGAGAUGUUUAU-

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GAAGAGGAUU-3', RET-3: sense 5'-AAAUCAGGGAGUCA-GAUGGAGUGGA-3' and antisense 5'-UCCACUCCAUC-UGACUCCUGAUUU-3'. RET siRNA cocktail is used as a cocktail of three duplexes (100 nmol of RET stealth siRNA cocktail containing 33.3 nmol of each individual siRNA).

**Western blotting.** TT cells were transfected at a concentration of 100 nM siRNA by Lipofectamine 2000 (Invitrogen Corp.), and then incubated for 72 h. Cell protein extracts were prepared with sampling buffer containing 1% Triton X-100 in phosphate-buffered saline pH 7.4 (PBS), and then Western blotting was performed as previously reported.<sup>(17)</sup> Expression of RET protein was identified using rabbit anti-RET polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat anti-rabbit IgG peroxidase conjugate (Santa Cruz Biotechnology) as the secondary antibody. Expression of Bcl-2 and  $\beta$ -actin protein was identified as previously described.<sup>(17)</sup>

**Antiproliferative activity.** TT cells at 50% confluence in 96-well plates were transfected with 100 nM siRNA by Lipofectamine 2000, and then incubated for 72 h. In combination with CPT-11 or SN-38, the culture medium was exchanged for medium containing CPT-11 (Irinotecan; Yakuruto, Tokyo, Japan) ranging from 0.1 to 1000  $\mu$ g/mL or SN-38 (ethyl-10-hydroxycamptothecin; Wako, Osaka, Japan) ranging from 0.001 to 10  $\mu$ g/mL 48 h after transfection of siRNA, and then incubated for another 48 h. After incubation, cell viability was measured by a cell proliferation assay kit (Dojindo, Kumamoto, Japan) as previously reported.<sup>(17)</sup>

The numbers of dead cells after treatment with RET siRNA, CPT-11, SN-38 or their combination were measured by propidium iodide (PI) staining. The cells were transfected with 100 nM siRNA and then incubated for 48 h. The culture medium was exchanged for medium containing 10  $\mu$ g/mL CPT-11 or 0.2  $\mu$ g/mL SN-38, and then incubated for another 48 h. The percentage of PI-positive cells was determined by examining fluorescence intensity on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described.<sup>(17)</sup>

**Real-time RT-PCR for quantification of RET and Bcl-2 mRNA.** TT cells at 50% confluence in 6-well plates were transfected with 100 nM siRNA with Lipofectamine 2000 and then incubated for 72 h. Total RNA was isolated from transfected cells using the NucleoSpin RNA II (Macherey-Nagel, Dueren, Germany). SYBR green quantitative RT-PCR amplification for Bcl-2 and  $\beta$ -actin cDNA was carried out by MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as previously reported.<sup>(17)</sup> For the amplifi-

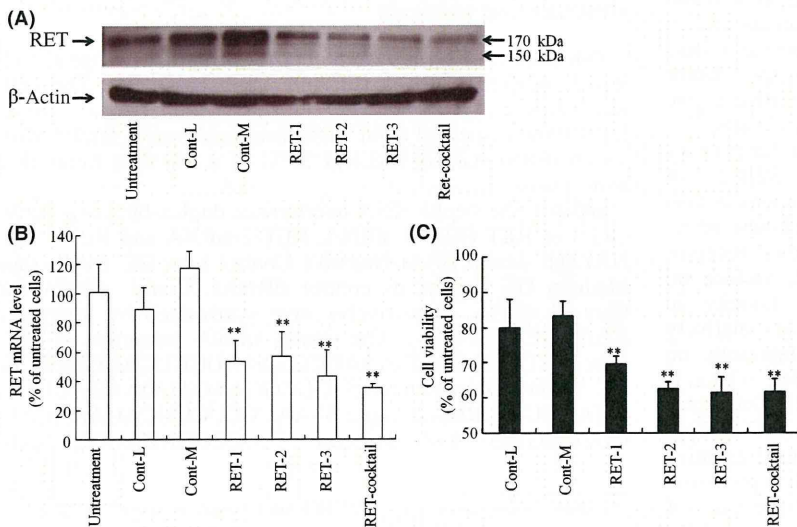
cation of human RET cDNA, primers RET-FW, 5'-GGCAGC-CAGAAACATCCT-3', and RET-RW, 5'-ACTTTGCGTGGTG-TAGAT-3', were used.

**Caspase 3/7 assay.** TT cells at 50% confluence in 12-well plates were transfected with 100 nM siRNA using Lipofectamine 2000 and then incubated for 48 h. The culture medium was then exchanged for medium containing 10  $\mu$ g/mL CPT-11 or 0.2  $\mu$ g/mL SN-38 and then incubated for another 48 h. After the incubation, caspase 3/7 activity was measured by Caspase3/7 assay system (Caspase-Glo® 3/7 assay; Promega, Madison, WI, USA) as previously reported.<sup>(18)</sup>

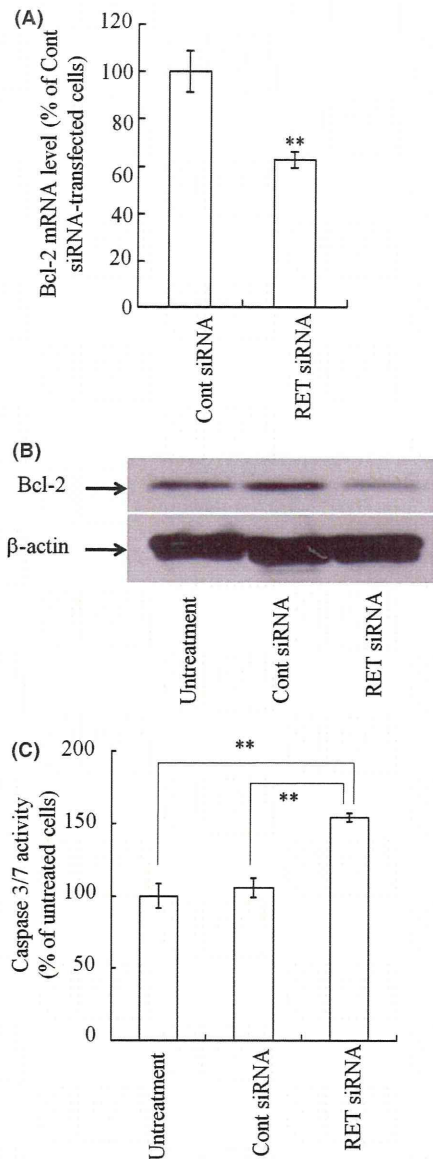
**Assessment of TT tumor growth.** Female ICR nu/nu mice (6 weeks of age) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). To generate TT tumor xenografts,  $1 \times 10^7$  TT cells suspended in 50  $\mu$ L PBS containing 50% Matrigel (Collaborative Research, Bedford, MA, USA) were inoculated subcutaneously into the flank region of mice. When the average volume of xenograft tumors reached about 100 mm<sup>3</sup>, therapy was started (day 0). For siRNA transfection *in vivo*, we used cationic nanoparticles (NP) consisting of cholesteryl-3 $\beta$ -carboxyamido-ethylene-*N*-hydroxyethylamine as a cationic lipid, and 5 mol% Tween-80, as previously reported.<sup>(19)</sup> For transfection into tumors, 10  $\mu$ g siRNA was mixed with NP at a charge ratio (+/-) of cationic lipid/siRNA of 1/1 with gentle shaking and standing for 15 min at room temperature. The complex of NP and siRNA was directly injected into the xenografts on days 0, 3, and 6, and CPT-11 was intravenously injected at a dose of 30 mg/kg on day 2, 5, and 8. On 24 days, blood was collected into tubes and centrifuged to separate plasma from blood cells. Serum concentrations of calcitonin were measured using a human calcitonin ELISA kit (Biosource International, Inc., CA, USA). Animal experiments were conducted with ethics approval from our institutional animal care and use committee.

**Preparation of probes for *in situ* hybridization.** The RET cDNA fragment (873 bp) was amplified from TT cells cDNA. The RET primers used for RET cDNA amplification were: forward primer, 5'-TCCTGGGAGAAGCTCAGTGT-3' and reverse primer, 5'-CACGTTGAAGTGGAGCAAGA-3'. The fragments were cloned into pGEM-T vector (Promega), and <sup>35</sup>S-labeled cRNA probes were prepared as previously reported.<sup>(20)</sup>

***In situ* hybridization.** For transfection into tumors, the complex of NP and 10  $\mu$ g siRNA per tumor was directly injected into TT tumor xenografts on days 0, 3, and 6. At 48 h after final injection the tumors were frozen in powdered dry ice, and



**Fig. 1.** Inhibition of RET expression by transfection of RET siRNA with Lipofectamine 2000 into TT cells. Expressions of RET protein (170 KDa) and mRNA were detected by Western blot (A) and quantitative RT-PCR (B) analyses, respectively, 72 h after transfection with 100 nM Cont-L and -M siRNA, RET-1, -2 and -3 siRNA and their cocktail. The number of viable cells was determined by WST-8 assay 72 h after transfection (C).  $n = 4$  for each sample. \*\* $P < 0.01$ , compared with Cont-L siRNA transfected cells.



**Fig. 2.** Effect of transfection with RET siRNA with Lipofectamine 2000 on Bcl-2 expression and caspase 3/7 activity in TT cells. Cells were incubated for 72 h after transfection with 100 nM RET siRNA or Cont siRNA. Expressions of Bcl-2 mRNA and protein were detected by quantitative RT-PCR (A) and Western blot (B). Caspase 3/7 activity was measured by caspase 3/7 luminometric assay (C).  $n = 4$  for each sample. \*\* $P < 0.01$ .

coronal sections were cut 16  $\mu$ m thick with a cryostat, thaw-mounted onto gelatin and poly-lysine-coated slides. *In situ* hybridization was carried out as previously described.<sup>(20)</sup> All sections were counterstained with hematoxylin and eosin.

***In vivo* detection of apoptosis.** For siRNA transfection into tumors, 10  $\mu$ g siRNA per tumor was directly injected by NP into TT tumor xenografts on days 0, 3, and 6. CPT-11 was intravenously injected at a dose of 30 mg/kg on day 2, 5, and 8. On day 11, the Red *in vivo* FLIVO™ apoptosis Kit (Immunochemistry Technologies, Bloomington, MN, USA) was injected *i.v.* and allowed to circulate for 30 min. The tumors were frozen in powdered dry ice, and coronal sections were cut 20  $\mu$ m thick with a cryostat, thaw-mounted onto poly-lysine-coated slides. The

specimens were examined microscopically using an Eclipse TS100-F microscope (Nikon, Tokyo, Japan).

**Statistical analysis.** The statistical significance of the data was evaluated with Student's *t*-test.  $P < 0.05$  was considered significant.

## Results

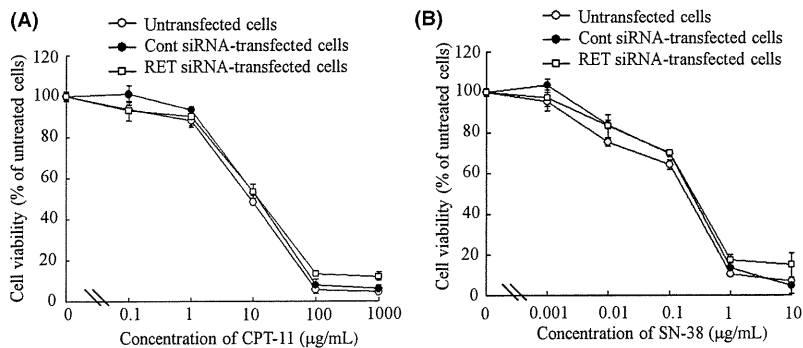
***In vitro* growth inhibitory effect of RET siRNA.** We initially characterized the expression of RET protein in TT cells after transfection of RET siRNA with Lipofectamine 2000. Here, we used RET-1, RET-2, RET-3 and their cocktail siRNAs as RET siRNAs (40%, 44%, 48%, and 44% in GC contents of the siRNA sequence, respectively), and Cont-L and -M siRNAs as negative control siRNAs (36% and 48% in GC contents of the siRNA sequence, respectively). Expression of RET protein was strongly suppressed in TT cells 48 h after transfection of RET-1, RET-2, RET-3 siRNA or their siRNA cocktail, but unaffected by the transfection of Cont-L or -M siRNA (Fig. 1A). Furthermore, the transfection of RET siRNA suppressed the expression of RET mRNA to about 40–50% of untransfected cells (Fig. 1B).

To investigate whether the suppression of RET expression induced the inhibition of cell growth, we examined cell viability 72 h after transfection of RET siRNA at 100 nM. After transfection, the cell viabilities of all RET siRNAs were 60–70%, but those of Cont-L and -M were 80 and 83%, respectively (Fig. 1C). When transfected at a concentration of 12.5 nM siRNA, the RET siRNA cocktail showed a stronger decrease of cell viability (67%) than RET-1, RET-2 and RET-3 siRNA (76, 72, and 75%, respectively) (data not shown). The slight inhibition of cell growth by transfection with Cont-L and -M siRNA may be due to the toxicity of Lipofectamine 2000. In subsequent studies, we used the RET siRNA cocktail as a RET siRNA, and Cont-L siRNA as a control siRNA.

**Bcl-2 expression and caspase 3/7 activity after transfection with RET siRNA into TT cells.** It has been reported that the expression of a dominant-negative RET down-regulated the expression of Bcl-2.<sup>(21)</sup> Therefore, we investigated whether transfection of RET siRNA affected Bcl-2 expression in TT cells (Fig. 2A,B). Transfection of RET siRNA decreased the expression of Bcl-2 mRNA and protein (Fig. 2A,B) and furthermore, significantly increased caspase 3/7 activity in cells compared with Cont siRNA (Fig. 2C). These findings suggested that the suppression of RET expression may increase caspase 3/7 activity *via* the down-regulation of Bcl-2 expression.

***In vitro* sensitivity of CPT-11 or SN-38.** Here, we used CPT-11 as a potent antitumor drug for TT tumor xenografts, because it has been reported that MTC cell lines were sensitive to a topoisomerase I inhibitor, camptothecin (CPT).<sup>(22)</sup> To investigate whether the transfection of RET siRNA enhanced sensitivity for chemotherapy, we incubated CPT-11 or its active metabolite SN-38 for 48 h in the cells 48 h after transfection of RET siRNA. In cells transfected with RET siRNA, cytotoxicities by CPT-11 and SN-38 were similar with those in cells transfected with Cont siRNA (Fig. 3A,B), indicating that their activities were at least additive for tumor suppression by RET siRNA.

We next investigated whether RET siRNA transfection affected the number of cell deaths and caspase 3/7 activity by CPT-11 or SN-38 treatment. The cells were transfected with RET or Cont siRNA for 48 h, and then treated with 10  $\mu$ g/mL CPT-11 or 0.2  $\mu$ g/mL SN-38 for 48 h, the concentrations of which were around the IC<sub>50</sub> of CPT-11 and SN-38 in untransfected cells, respectively. To measure the number of cell deaths, PI staining of the cellular nucleus was used as a marker. RET siRNA transfection increased the number of PI-staining cells and caspase 3/7 activity compared with Cont siRNA



**Fig. 3.** Concentration-dependent effect of CPT-11 or SN-38 on cytotoxicity after transfection with RET siRNA. Cells were transfected with 100 nM RET siRNA or Cont siRNA with Lipofectamine 2000 and incubated for 48 h. After incubation, the cells were treated with various concentrations of CPT-11 (A) or SN-38 (B) and incubated for another 48 h. The number of viable cells was determined by WST-8 assay.  $n = 4$  for each sample.

transfection (Fig. 4A,B). Among treatments, the combination of RET siRNA plus SN-38 significantly increased activity and cells compared to RET siRNA or SN-38 alone (Fig. 4A,B) ( $P < 0.01$ ).

**Suppression of RET expression in TT tumor xenografts.** Next, we investigated whether the expression of RET mRNA was decreased by intratumoral injection of RET siRNA. Here, we used NP as a siRNA transfection vector for *in vivo* experiments.<sup>(19)</sup> Cont or RET siRNA was directly injected with NP into TT tumor xenografts 3 times at 3-day intervals and then tumors were excised 2 days after the final injection. The sense probe for the RET gene was used as a negative control probe. Silver grain distribution in the dark field and black grain in the

bright field following *in situ* hybridization exhibiting RET mRNA expression (Fig. 5A,B, supplemental Figs S1 and S2). Positive cells for RET mRNA were strongly detected in the tumor cells, but not stroma cells with the antisense probe for the RET gene (Fig. 5B). Most of the tumor cells were positive for the expression of RET mRNA in saline-injected and Cont siRNA-transfected cells. In contrast, RET siRNA transfection decreased the number of silver grains in the dark field (Fig. 5A) and black grains in the bright field (Fig. 5B) in some tumor sections, indicated that the expression of RET mRNA was suppressed by RET siRNA injection.

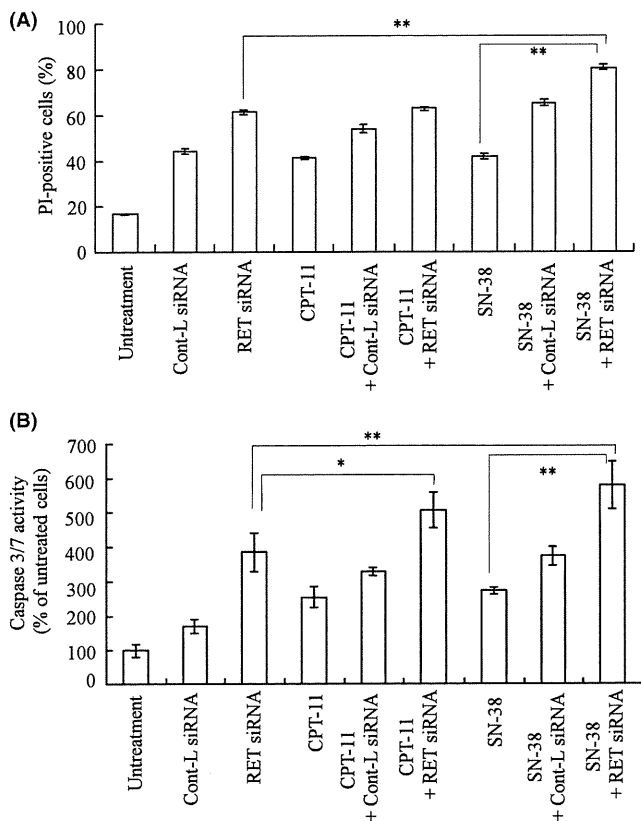
**Synergistic inhibition of the growth of TT tumor xenografts.**

We evaluated the efficacy of combination therapy of RET siRNA and CPT-11 in inhibiting the growth of subcutaneous TT tumors. The anti-tumor effect was evaluated by direct injection of Cont or RET siRNA with NP into xenografts once a day three times (day 0, 3, and 6) following three *i.v.* injections of CPT-11 (day 2, 5, and 8). No significant growth inhibitory effect was observed in mice treated with RET siRNA, CPT-11 or CPT-11 plus Cont siRNA compared with mice injected with saline (Fig. 6A). CPT-11 plus Cont siRNA exhibited a similar tumor suppressive effect with CPT-11 alone. A significant growth inhibitory effect was observed in combination therapy with CPT-11 and RET siRNA compared with CPT-11 alone. All treatments, the transfection of RET siRNA, the injection of CPT-11 alone, and their combination did not significantly change body weight during 3 weeks of treatment (data not shown). In this study, the effect of the combination on tumor growth was significantly higher than that of CPT-11 alone at lower doses that did not exhibit adverse effects.

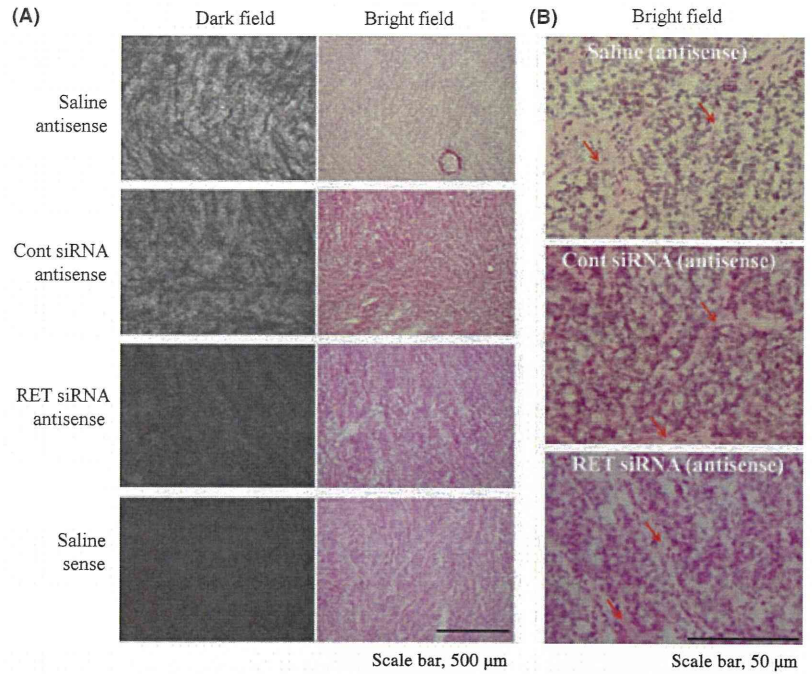
RET siRNA transfection alone could not significantly suppress tumor growth. The results of *in situ* hybridization showed that the suppression of RET expression in the tumors was restricted to the tumor in the vicinity of the injection site (data not shown). Strong inhibition of *in vivo* tumor growth by RET siRNA depends on the development of a gene vector having the ability to introduce siRNA widely into tumors.

**Serum calcitonin concentration and *in vivo* apoptosis.** C cells of the thyroid gland secrete calcitonin, the level of which is elevated in patients with C cell hyperplasia and MTC; therefore, calcitonin is a very sensitive and reliable marker for the presence of MTC, and the level of calcitonin correlates with tumor burden and the response to therapy.<sup>(23,24)</sup> On day 24 in Fig. 6A, all mice were sacrificed, and serum calcitonin concentration was measured. Serum calcitonin level in TT tumor xenografts showed a 21-fold increase compared with that in normal mice (Fig. 6B). RET siRNA or CPT-11 alone decreased serum calcitonin levels to 39% and 80% compared with saline (Fig. 6B). Corresponding to the highest growth inhibition, the combination of CPT-11 plus RET siRNA markedly decreased the serum calcitonin level to 30%.

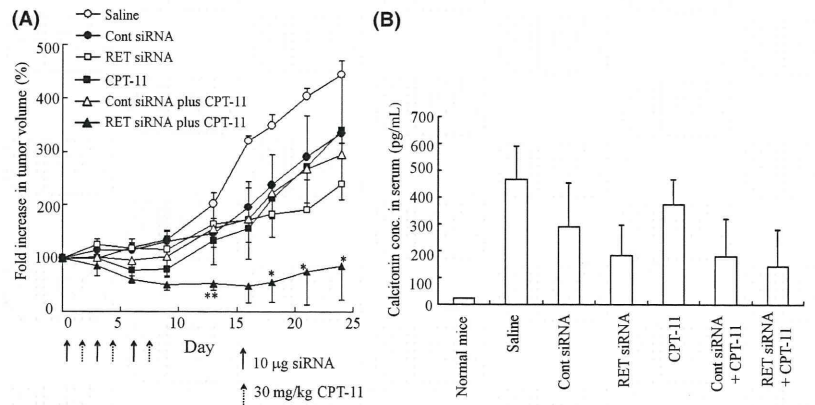
To confirm the effectiveness *in vivo*, we detected apoptotic cells 48 h after combination therapy with RET siRNA and



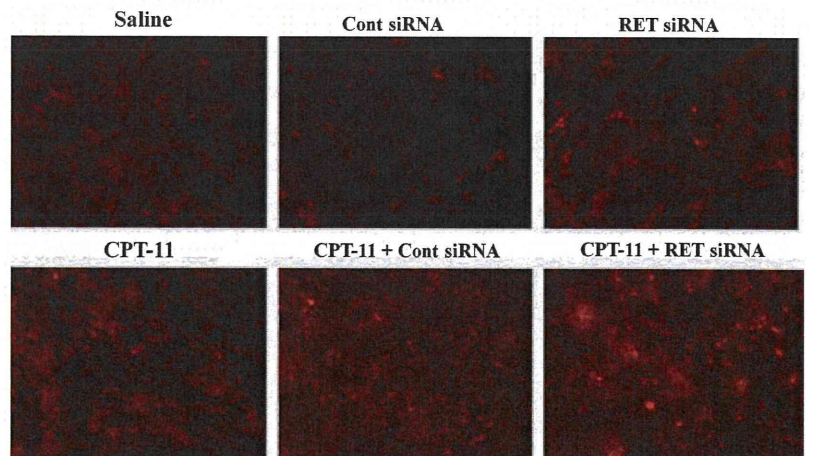
**Fig. 4.** Dead cells (A) and caspase-3/7 activity (B) by combined treatment with RET siRNA plus CPT-11 or SN-38 in TT cells. The number of dead cells was measured by a FACSCalibur flow cytometer after staining with PI (A). Caspase-3/7 activity was measured by caspase-3/7 luminometric assay (B).  $n = 3$  for each sample. \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with RET siRNA transfected cells.



**Fig. 5.** *In situ* hybridization of a  $^{35}\text{S}$ -labeled antisense or sense probe generated against RET mRNA. An antisense probe was used to detect RET mRNA. The sense probe for the RET gene was used as a negative control probe. In (A), silver grains in a dark field indicate RET mRNA expression. Scale bar: 500  $\mu\text{m}$ . In (B), microautoradiographs in bright fields in Fig. 5(A) were enlarged. Black grains indicate RET mRNA expression. Red arrows indicate stroma cells. Scale bar: 50  $\mu\text{m}$ .



**Fig. 6.** *In vivo* combination therapy of RET siRNA and CPT-11 for TT tumor xenografts. Ten microgram of RET or Cont siRNA per tumor was directly injected with NP into the tumor on days 0, 3, and 6. CPT-11 at a dose of 30 mg/kg was injected *i.v.* on days 2, 5, and 8. Tumor volume was measured for 24 days (A).  $*P < 0.05$ ,  $***P < 0.01$ ; compared with the group injected with CPT-11. All mice were sacrificed on day 24 and the concentration of calcitonin in blood was measured (B). Data in A and B are shown as the mean  $\pm$  SE.  $n = 3$  for each group.



**Fig. 7.** Distribution of apoptotic cells in TT tumor xenografts after injection of RET siRNA and CPT-11. Experimental conditions were the same as in Fig. 6. On day 11, all mice were sacrificed and apoptosis cells were detected by the Red *in vivo* FLIVO<sup>TM</sup> apoptosis Kit. Red signals indicate apoptotic cells. Magnification  $\times 100$ .

CPT-11. In tumor sections after injections of RET siRNA or CPT-11 alone, red, indicating apoptotic cells, was slightly observed, but not in saline or Cont siRNA (Fig. 7). On the other hand, combination treatment with RET siRNA plus CPT-11 strongly induced apoptotic cells. These data also indicated that combination therapy was effective for MTC.

## Discussion

In this study, we demonstrated that the combination with RET siRNA and CPT-11 could induce the growth inhibition of TT cells and tumor xenografts. Recently, RET siRNA delivered by chitosan-coated poly(isobutylcyanoacrylate) nanoparticles led to tumor growth inhibition after intratumoral administration in mice transplanted with NIH3T3 cells transformed with the *ret/PTC1* gene;<sup>(25)</sup> however, it has been reported that the *ret/PTC-1* signaling pathway was different between thyroid and fibroblast cells;<sup>(26)</sup> therefore, this is the first report that RET siRNA transfected with NP inhibited tumor growth in mice bearing TT cells.

The combination therapy *in vivo* revealed greater than additive effects in cytotoxicity *in vitro*. CPT-11 is known as an effective inhibitor of angiogenesis for endothelial cells in tumor as well as cytotoxic drug.<sup>(27)</sup> The mechanism of increasing growth inhibition for TT tumor xenografts by RET siRNA and CPT-11 was not clear, although inhibition of receptor tyrosine kinase in tumor cells could sensitize cells to CPT-11.<sup>(28,29)</sup> Overexpression of Bcl-2 has been observed in MTC,<sup>(30,31)</sup> and is associated with the poor response of MTC to chemotherapy.

The expression of dominant-negative RET in TT cells decreased the expression of Bcl-2 and could reduce cell viability,<sup>(21)</sup> and the antitumor effect by CPT-11 was enhanced by the combination with RET inhibitor, CEP-751, for TT tumor xenografts.<sup>(10)</sup> As another possibility, RET has been shown to activate nuclear factor  $\kappa$ B (NF $\kappa$ B) *via* the phosphatidylinositol-3-kinase/Akt pathway.<sup>(1)</sup> Akt mediates multiple cellular responses, such as survival signaling by NF $\kappa$ B activation and BAD inactivation, and cell cycle progression; therefore, inhibition of RET expression might modulate cellular sensitivity through the survival signaling pathway.<sup>(32,33)</sup> From these findings, the combination of RET siRNA and CPT-11 will have great potential for gene therapy in MTC.

In conclusion, we demonstrated that combining RET siRNA with CPT-11 resulted in significant greater growth suppression of TT cells and tumor xenografts *via* increased apoptosis. As the thyroid gland is anatomically accessible, future treatment of primary MTC tumors by direct intratumoral injection of siRNA/NP complex expressing mutated genes is possible. Thus, the combination of RET siRNA and CPT-11 may serve as a novel tool for gene therapy.

## Acknowledgments

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** *In situ* hybridization of a <sup>35</sup>S-labeled antisense or sense probe generated against RET mRNA. Microautoradiographs in bright fields of Fig. 5A were enlarged. Black grains indicate RET mRNA expression. Scale bar: 500 μm.

**Fig. S2.** *In situ* hybridization of a <sup>35</sup>S-labeled antisense or sense probe generated against RET mRNA. Microautoradiographs in bright fields of Fig. 5B were enlarged. Black grains indicate RET mRNA expression. Scale bar: 50 μm.

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## Research Paper

# Accelerated Blood Clearance Was Not Induced for a Gadolinium-Containing PEG-poly(L-lysine)-Based Polymeric Micelle in Mice

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**Purpose.** Accelerated blood clearance (ABC) is induced by repeated injections of PEGylated liposomes. In this study, the ABC was investigated for a gadolinium-containing PEG-poly(L-lysine)-based polymeric micelle (Gd-micelle) and PEGylated liposome (Gd-liposome) in mice.

**Materials and Methods.** Effects of the first injection of Gd-micelle on the tissue distribution of the second dose of Gd-micelle were studied. Additionally, effects of the first injection of Gd-micelle, Gd-liposome, empty liposome, polyethyleneglycol (PEG<sub>500,000</sub>), and PEG-lipid on the distribution of the second dose of the Gd-liposome were evaluated.

**Results.** Results indicated that the tissue distribution of the second injection of the Gd-micelle at a dose of 33, 5, or 2 μmol Gd/kg was not affected by the first injection of the Gd-micelle at different doses and time intervals or of the empty PEGylated liposome 7 days before. ABC of Gd-liposome at a dose of 2.3 μmol Gd/kg (corresponding to 10 μmol lipids/kg) was observed when the empty PEGylated liposome or Gd-liposome, but not the Gd-micelle, PEG<sub>500,000</sub> or PEG-lipid, was pre-administered.

**Conclusions.** The hydrophobic core of the micelle or lipid bilayer of PEGylated liposome has a major effect on this phenomenon. These studies have significant implications for the evaluation of PEG-poly(L-lysine)-based micellar formulation of Gd-based contrast agents.

**KEY WORDS:** accelerated blood clearance; gadolinium; PEGylated liposome; polyethylene glycol (PEG); polymeric micelle.

## INTRODUCTION

Long-circulating liposomes with surface-modified polyethyleneglycol (PEG) are often used as carriers of therapeutic agents, since they avoid capture by the reticuloendothelial system (RES) and can extend the systemic circulation time of agents, thereby improving drug delivery (1,2). It was hypothesized that PEG on the surface of liposomes forms a water shell, resulting in decreased adsorption of opsonins and subsequent phagocytosis by cells of the RES (3,4). However, PEGylated liposomes are known to lose their long-circulating property with multiple dosing. Recently, it has been reported that the first dose of PEGylated liposomes injected intravenously caused a loss of the long-circulating property and extensive accumulation in the liver at the second dose injected several days later in mice, rats, rabbit, and rhesus monkeys (5–11), a phenomenon known as accelerated blood

clearance (ABC). Besides PEGylated liposomes, other nanocarriers, such as nanoparticles containing PEG, also produced this phenomenon (12). Therefore, ABC would have a significant impact on the application of long-circulating liposomes and nanoparticles with multiple administrations. In clinical applications of liposomal carriers, Gabizon *et al.* reported a reduced clearance of doxorubicin-containing PEGylated liposome in the repeated injections. This opposite behavior to the ABC phenomenon resulted from toxic activity of the encapsulated doxorubicin against the RES (13). Presently, the ABC phenomenon is not a problem in a cancer chemotherapy by the use of a PEG-liposomal carrier, whereas the ABC phenomenon in human clinics must be important for less toxic drug or gene delivery applications of the PEGylated liposomes.

To date, studies of ABC have focused mainly on PEGylated liposomes. Many factors can affect the extent to which ABC is induced by PEGylated liposomes. First of all, the dose of lipid plays an important role, with ABC enhanced at lower concentrations of lipid (6,7,12). Second, ABC occurs in a time-dependent manner (5,7). The time interval between the first and second doses is a key factor. Third, when the amount of PEGylated lipid in the first injection was ≤5 mol%, the second dose of PEGylated liposomes was eliminated more quickly from plasma than liposomes containing >10 mol% PEGylated lipid injected as a first dose (7,8). In addition, the ABC phenomenon

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was reported to be independent of liposomal size, surface charge, and PEG molecular weight (5,7,8).

During the past decade, polymeric micelles, supramolecular assemblies of block copolymers, have demonstrated their utility in drug delivery systems and are currently recognized as promising nanocarriers for enhancing the efficacy of drugs and genes (14–16). Since ABC has a considerable impact on the multiple drug administration, it is necessary to study whether the phenomenon is induced by repeated injections of polymeric micelles. Gadolinium (Gd)-based contrast agents are widely used in magnetic resonance imaging (MRI) to improve the conspicuity of lesions or visualization of blood vessels (17). However, these agents are rapidly cleared from the circulation. To overcome this problem, nanocarriers, such as liposomes and polymeric micelles, are used to encapsulate the agents so as to prolong their circulation and allow them to accumulate in tumors for diagnosis (18–20). If polymeric micelles containing a diagnostic agent cause the ABC phenomenon, then circulation time will be reduced after a second dose and the accuracy of the diagnosis will be affected. Furthermore, polymeric micelles containing MRI agents or drugs administered during diagnosis and treatment will lose some of their drug efficacy because of the accelerated clearance. Hence, it is of great importance to know whether the ABC phenomenon can be induced by polymeric micelles or not. Recently, the accelerated clearance of [<sup>3</sup>H]-labeled PEGylated liposomes was observed in mice pre-administered with an empty polymeric micelle composed of poly(ethylene glycol)-*b*-poly( $\beta$ -benzyl L-aspartate) (PEG-PBLA) 50 nm in diameter (16).

In this study, we first investigated whether the ABC effect was caused by repeated injections of a polymeric micelle encapsulating Gd-DOTA (Gd-micelle) and of a PEGylated liposome encapsulating Gd-DTPA (Gd-liposome) as a positive control. Concentrations of Gd ions were measured for this investigation. Furthermore, we examined the effect of a PEG homopolymer on the tissue distribution of Gd-liposomes.

## MATERIALS AND METHODS

### Materials

Magnevist® (Gd-DTPA) was purchased from Bayer Schering Pharma (Berlin, Germany). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] (mPEG<sub>2000</sub>-DSPE), hydrogenated soy bean phosphatidylcholine (HSPC), and egg phosphatidylcholine (EPC) were purchased from the NOF Corporation (Tokyo, Japan). Cholesterol and polyethylene glycol 500,000 (PEG<sub>500,000</sub>) were of analytical

grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

### Animals

Four-week-old female ddY mice were purchased from Sankyo Lab Service Corp. (Tokyo, Japan). All care and handling of animals were performed with the approval of the Animal and Ethics Review Committee of Hoshi University and of Principles of Laboratory Animal Care (NIH #publication 85-23, revised in 1985).

### Preparation of the Gd-micelle

Synthesis of a chelate moiety-binding block copolymer was performed as reported in our previous paper (19). Briefly, a poly(ethylene glycol)-*b*-poly(L-lysine) block copolymer (PEG-P(Lys)) was prepared through acid hydrolysis of a poly(ethylene glycol)-*b*-poly[ $\epsilon$ -(benzyloxycarbonyl)-L-lysine] (PEG-P(Lys(Z))) block copolymer (Fig. 1). We synthesized PEG-P(Lys(Z)) with polymerization of a Lys(Z) *N*-carboxy anhydride monomer from PEG-NH<sub>2</sub> (molecular weight of PEG-NH<sub>2</sub> = 5,200). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (*N*-hydroxysuccinimide ester) was fully conjugated to lysine residues of PEG-P(Lys).

The composition of PEG-P(Lys-DOTA) was determined by means of <sup>1</sup>H-NMR spectroscopy in D<sub>2</sub>O under alkali conditions (pH > 10). GdCl<sub>3</sub>·6H<sub>2</sub>O was added to PEG-P(Lys-DOTA) at pH 6.0 to 6.5 for 3 hr at 50°C. Gd content was determined using inductively coupled plasma (ICP) (SPS7800, SII Nano Technology Inc., Tokyo, Japan). We obtained the block copolymer as PEG-P(Lys-DOTA-Gd) (Gd content = 7.7 wt%, the number average of Gd is 8.2). The block copolymer formed a polymeric micelle spontaneously in an aqueous solution (Gd-micelle). The size and zeta-potential of the Gd-micelle diluted with saline for three independent preparations was 84.5 ± 6.0 nm and -1.70 ± 0.80 mV, respectively, at 25°C as determined by dynamic light scattering (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan).

### Preparation of the Empty Liposome and Gd-liposome

First, an empty liposome, which induced the ABC phenomenon, was prepared by the lipid film hydration method as described previously (21). Briefly, a mixture of HSPC, cholesterol, and mPEG<sub>2000</sub>-DSPE in a molar ratio of 1.85:1.0:0.15 was dissolved in chloroform. The solution was

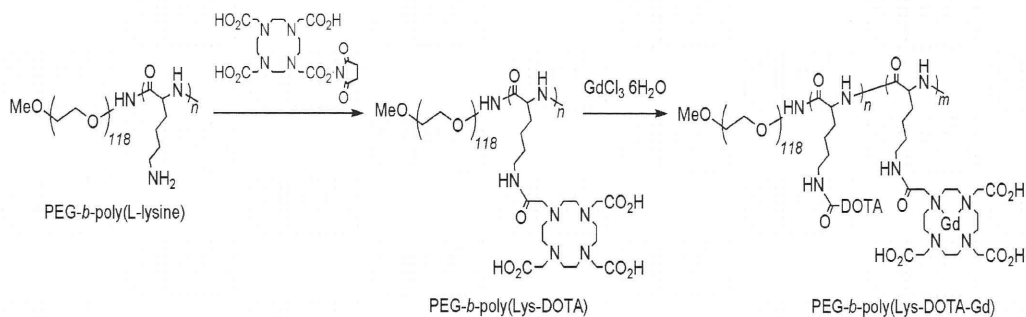


Fig. 1. Synthesis of PEG-P(Lys-DOTA-Gd).

evaporated dry to form the lipid film. Then, the liposome was produced by hydration of the lipid film with saline, followed by size reduction with sonication. The size and zeta-potential of the liposome diluted with saline were 178.5 nm and  $-22.1$  mV, respectively.

Two kinds of Gd-liposomes were prepared because Gd-DTPA content was dependent on the preparation methods. One kind of Gd-liposome was prepared by an ethanol injection method (GdL-E). In brief, a mixture of EPC, cholesterol, and mPEG<sub>2000</sub>-DSPE in a molar ratio of 2.15:0.88:0.15 was dissolved in ethanol and then hydrated with Gd-DTPA at 50°C. The resulting liposomes were sonicated for 10 min, then subjected to exhaustive dialysis against phosphate-buffered saline (PBS, 137 mM NaCl, 8.10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) with a dialysis membrane having a 2,000 molecular-weight cutoff for 24 hr. The size and zeta-potential of the liposome were  $150.1 \pm 18.8$  nm and  $-0.94 \pm 6.78$  mV, respectively, for three independent preparations. As a control of GdL-E, an empty liposome not including Gd-DTPA (empty GdL-E) was prepared by the same method as the Gd-liposome (GdL-E), except that saline was used to hydrate the ethanol solution of lipid. The particle size of empty GdL-E was 139.5 nm. Another kind of Gd-liposome was prepared by reverse phase evaporation (GdL-R) to encapsulate a larger amount of Gd-DTPA. The lipid was the same as GdL-E described above and dissolved in 4 mL of chloroform and 2 mL of diethyl ether. Gd-DTPA was added to the lipid solution. The mixture was sonicated to form an emulsion, which was evaporated to produce the liposome. Finally, the resulting liposome was sized at 60°C on an extruder (Avanti Polar Lipids, Inc., AL, USA) with three passes through a 0.4  $\mu$ m Nuclepore membrane (Waterman, Maidstone, UK) and five passes through a 0.2  $\mu$ m Nuclepore membrane, followed by exhaustive dialysis as described above. The particle size and zeta-potential of the liposome were  $140.9 \pm 13.5$  nm and  $-2.52 \pm 5.18$  mV, respectively, for three independent preparations. The phospholipid concentration of the liposome including HSPC or EPC was measured with the Phospholipids C-test Wako (Wako Pure Chemical Industries, Ltd.). GdL-E contained 2.26  $\mu$ mol Gd per 10  $\mu$ mol lipids, and GdL-R contained 2.29  $\mu$ mol Gd per 5  $\mu$ mol lipids.

### Release Studies of Gd-micelle and Gd-liposomes

The release of Gd-DTPA from Gd-liposome (GdL-E or GdL-R) and Gd from Gd-micelle was evaluated by dialysis method using a Spectrapor 6 tubing with molecular weight cut-off of 1,000 Da (Spectrum Laboratories Inc., Tokyo, Japan). Briefly, the sample of Gd-micelle containing 1.2 mM Gd and Gd-liposomes of GdL-E containing 0.96 mM Gd-DTPA or GdL-R containing 0.96 mM Gd-DTPA (1 mL) were dialyzed against PBS (pH 7.4, 200 mL) at 37°C. At the indicated time points (10 min, 1, 3, 6, 24 h), 1 mL aliquots of the medium were withdrawn, and the same volume of fresh medium was added. The Gd concentration was analyzed by ICP. The accumulative release of Gd or Gd-DTPA released from the Gd-micelle or Gd-liposome, respectively was expressed as a percentage of the released Gd or Gd-DTPA and plotted as a function of time.

### Pharmacokinetics and Tissue Distribution of the Gd-micelle and Gd-liposome

For pharmacokinetics study, the mice were intravenously injected with the Gd-micelle at a dose of 33  $\mu$ mol Gd/kg (67.3 mg polymer/kg) or the Gd-liposomes including GdL-E at a dose of 6.75  $\mu$ mol Gd/kg and 10  $\mu$ mol lipids/kg and GdL-R at 2.65  $\mu$ mol Gd/kg and 5  $\mu$ mol lipids/kg. About 30 to 100  $\mu$ L of blood were taken from a tail vein with a quantitative capillary at 10 min, 1 h, 3 h, 6 h, and 24 h after the injection. The Gd-micelle or the Gd-liposome was injected into a lower part of a tail vein, and blood sample was taken at a certain time point described above from an upper part of the tail vein at the other side of the injected vein. Therefore, this experiment was free from the sample pollution problem. The blood samples were added to saline and centrifuged at 3,000 rpm for 15 min, and the supernatant was used to measure Gd content by ICP. The elimination half-life ( $T_{1/2}$ ) was calculated based on a single compartment model. For the tissue distribution of Gd-micelles and Gd-liposomes study, the second dose of Gd-micelles or Gd-liposomes was injected intravenously through the tail vein at a certain time interval after the first injection. Samples of blood were taken from the hepatic portal vein 6 h after the second injection, and tissues of liver, spleen, and kidney were removed at the same time. The plasma and blood volume were calculated as 0.0488 mL/g body weight for plasma and 0.0778 mL/g body weight for blood, respectively (19).

### Measurement of Gd Content

For the quantitative determination of Gd content, blood samples were centrifuged at 3,000 rpm for 15 min, and then plasma was taken out and diluted with 0.1% HNO<sub>3</sub> for ICP. Tissue samples of the liver, spleen, and kidney were digested with a mixture of 98% H<sub>2</sub>SO<sub>4</sub> and 62% HNO<sub>3</sub> (1:2, v:v) and then subjected to ICP.

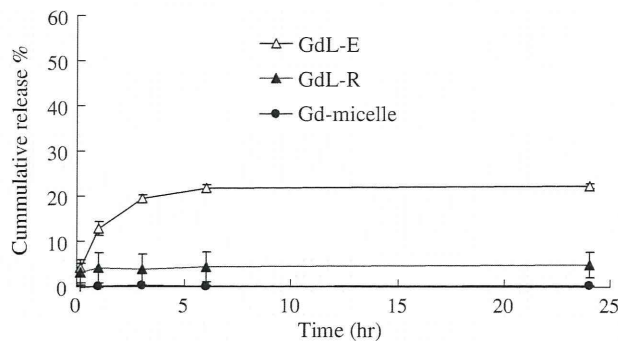
### Statistical Analysis

The statistical analysis was performed with the Dunnett's multiple comparison test. The level of significance was set at  $p < 0.05$  or  $p < 0.01$ .

## RESULTS

### Release Behavior of Gd-micelle and Gd-liposomes

Gd or Gd-DTPA release behavior from Gd-micelle or Gd-liposomes was studied by the dialysis method. As shown in Fig. 2, only 0.2% of Gd leaked from the Gd-micelle at 37°C in PBS (pH 7.4) for 24 h. On the other hand, 4.8% of entrapped Gd-DTPA leaked from the Gd-liposome prepared by reverse phase evaporation method (GdL-R) and 22.4% for 24 h from the Gd-liposome prepared by ethanol injection method (GdL-E). Hence, it is obvious that Gd-micelle has hardly release behavior of Gd, and GdL-R showed much slower release than GdL-E. The results indicated that the leakage of Gd or Gd-DTPA from nanocarriers was greatly affected by the preparation methods.



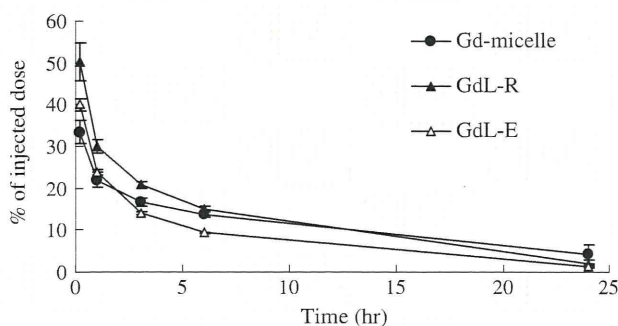
**Fig. 2.** Release profiles of Gd from Gd-micelle or Gd-DTPA from Gd-liposomes prepared by ethanol injection method (GdL-E) and reverse phase evaporation method (GdL-R) in PBS (pH 7.4) at 37°C. Data represent mean $\pm$ S.D. ( $n=3$ ).

### Pharmacokinetics of the Gd-micelle and Gd-liposome

As shown in Fig. 3, at 10 min after the intravenous injection, 33.3% of the injected dose was found in blood for the Gd-micelle, and 40.0% and 50.3% for the Gd-liposome of GdL-E and GdL-R, respectively. At a dose of 33  $\mu\text{mol Gd/kg}$ , the  $T_{1/2}$  of the Gd-micelle was  $10.2\pm 3.9$  h. Besides, the  $T_{1/2}$  of GdL-E at a dose of 6.75  $\mu\text{mol Gd/kg}$  and GdL-R at a dose of 2.65  $\mu\text{mol Gd/kg}$  were  $5.9\pm 0.5$  h and  $6.0\pm 1.0$  h, respectively. In a previous study, we showed that Gd-DTPA was very rapidly cleared from the bloodstream with a minute's order half-life (19). Therefore, the detected Gd in blood is considered to be Gd-DTPA encapsulated in the liposome in a quantitative manner for measurements 6 h post intravenous injection. On the other hand, the main purpose of this study is the ABC phenomenon of a polymeric micelle MRI contrast agent, and PEGylated liposome is used as a positive control for the ABC phenomenon. Therefore, detection of liposome with Gd measurements is appropriate for the present purpose.

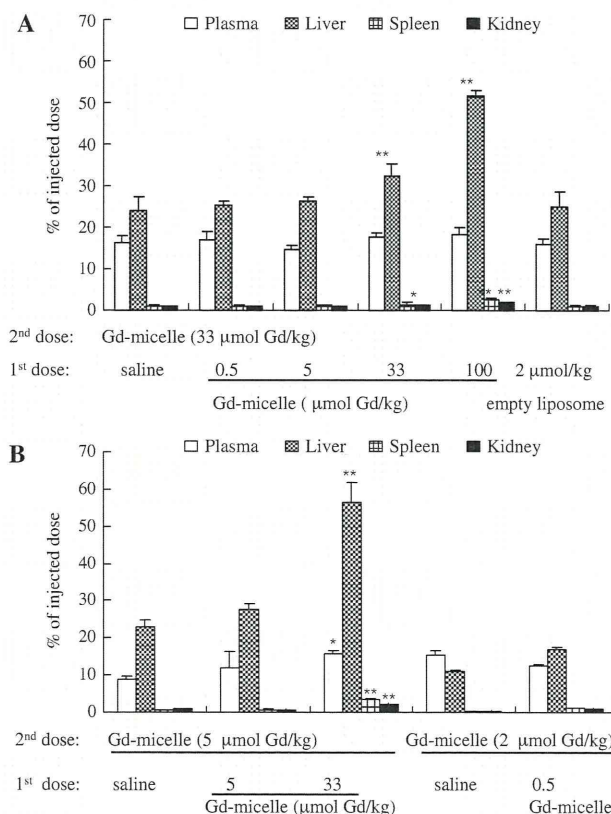
### Effect of the First Dose on the Distribution of the Gd-micelle

The effects of the first dose on the distribution of the Gd-micelle injected a second time were evaluated. When the second dose of Gd-micelle was fixed at 33  $\mu\text{mol/kg}$ , there was no significant difference of percent injected doses in plasma,



**Fig. 3.** Plasma elimination profiles of Gd following a single intravenous injection of Gd-micelle at a dose of 33  $\mu\text{mol Gd/kg}$  and Gd-liposomes including Gd-liposome prepared by ethanol injection method (GdL-E) at a dose of 6.75  $\mu\text{mol Gd/kg}$  and Gd-liposome prepared by reverse phase evaporation method (GdL-R) at a dose of 2.65  $\mu\text{mol Gd/kg}$ . Data represent mean $\pm$ S.D. ( $n=3-4$ ).

kidney, and spleen between various first doses of the Gd-micelle from 0 to 100  $\mu\text{mol/kg}$  (Fig. 4A). A dose of 100  $\mu\text{mol Gd/kg}$  is the clinical dose of Gd-DTPA (17). Interestingly, the distribution of Gd-micelles in plasma, kidney, spleen and liver with the first injection of the empty liposome was similar to that with the first injection of saline. For the liver, the percent injected dose after a first dose of 33  $\mu\text{mol/kg}$  and 100  $\mu\text{mol/kg}$  was significantly higher than in the control saline group, possibly due to the incomplete elimination of the first dose of the Gd-micelle in liver at day 7 because of high doses of polymeric micelles (67.3 mg  $\sim$  203.9 mg polymer/kg). The dose of 2  $\mu\text{mol Gd/kg}$  of the Gd-micelle was the minimum at which Gd was detectable by means of ICP 6 h after injection. As shown in Fig. 4B, when the second dose of the Gd-micelle was decreased to 5  $\mu\text{mol/kg}$  and 2  $\mu\text{mol/kg}$ , the distribution was similar to that of 33  $\mu\text{mol/kg}$  (Fig. 4A). Hence, the results showed that the tissue distribution of the Gd-micelle at the second dose of 33, 5, or 2  $\mu\text{mol/kg}$  was not affected significantly except in liver by pre-administration of the Gd-micelle or the empty liposome. Although Gd in the first dose may interfere with the Gd accumulation in liver following the second dose injection, Gd-micelle as the first dose for micelle-forming properties are



**Fig. 4.** Effect of the first dose on the tissue distribution of Gd-micelle. The second dose of Gd-micelle with 33  $\mu\text{mol/kg}$  (A) or 5  $\mu\text{mol/kg}$  or 2  $\mu\text{mol/kg}$  (B) was intravenously injected at day 7 after the first injection of 0.5, 5, 33, 100  $\mu\text{mol/kg}$  of Gd-micelle or the empty liposome at a dose of 2  $\mu\text{mol lipid/kg}$ . Tissues including blood, liver, spleen, and kidney were taken out 6 h after the second injection of Gd-micelle. Data represent mean $\pm$ S.D. ( $n=3, 6$ ).  $P$  values apply to differences between the saline group and Gd-micelle or liposome treated group. \* $p<0.05$ , \*\* $p<0.01$ .

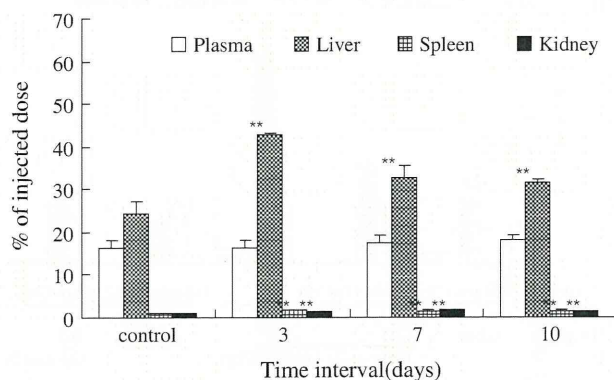
Gd-content-dependent, and Gd-free polymeric micelle is different from the Gd-containing micelle in size and micelle forming characteristics.

#### Effect of Time Interval Between the Two Injections on the Distribution of the Gd-micelle

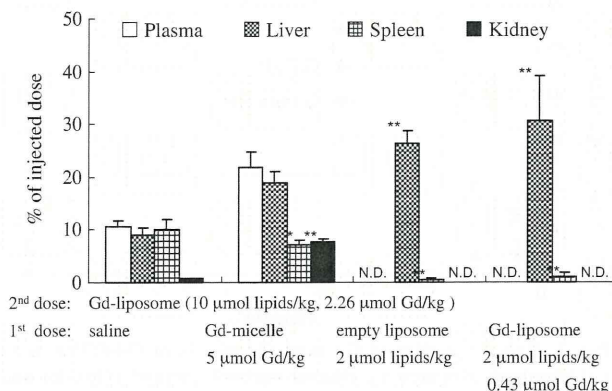
Since it was reported that the ABC effect was maximized when the interval between the two injections of liposome was 10 days in mice (7,12), we changed the time interval for the injection of Gd-micelle at a dose of 33  $\mu\text{mol/kg}$  from 3 days to 10 days. No significant difference in plasma Gd levels (15 ~ 18% dose) was observed between the control group and the groups with different time intervals 6 h after the second injection (Fig. 5). The control group was given the Gd-micelle at 33  $\mu\text{mol/kg}$  after a first injection of saline. The Gd% of injected dose in the liver was much higher at day 3 after the second injection than that on other days, probably due to the incomplete elimination of the first dose of the Gd-micelle.

#### Effect of the First Dose on the Distribution of the Gd-liposome

Since a lower dose of lipid in the first injection results in a more significant ABC, the effects of dose were investigated. The first dose of the Gd-micelle (5  $\mu\text{mol Gd/kg}$ ), empty GdL-E (2  $\mu\text{mol lipids/kg}$ ), or GdL-E (2  $\mu\text{mol lipids/kg}$  corresponding to 0.45  $\mu\text{mol Gd/kg}$ ) was given with a second dose of GdL-E at 10  $\mu\text{mol lipids/kg}$  corresponding to 2.26  $\mu\text{mol Gd/kg}$ . As shown in Fig. 6, the first injection of the Gd-micelle resulted in a similar percentage of the injected dose of the Gd-liposome in plasma, liver, spleen, and kidney in comparison with the saline group. On the other hand, after the second injection of GdL-E, the Gd concentrations in plasma and kidney were too low to be detected, with the first injection of the empty GdL-E and the GdL-E. At that time, the %dose in the liver significantly increased, but that in spleen significantly decreased as compared to saline ( $p < 0.05$ ).



**Fig. 5.** Effect of the time intervals on the tissue distribution of Gd-micelle. The second dose of Gd-micelle at 33  $\mu\text{mol/kg}$  was intravenously injected at day 3, day 7, or day 10 after the first injection of the same micelle at 33  $\mu\text{mol/kg}$ . The control group was referred to the second dose of Gd-micelle at a dose of 33  $\mu\text{mol/kg}$  with the first injection of saline. Tissues including blood, liver, spleen, and kidney were taken out at 6 h after the second injection of Gd-micelle. Data represent mean  $\pm$  S.D. ( $n=3$ ).  $P$  values apply to differences between the control group and treated group. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Fig. 6.** Effect of the first dose on the tissue distribution of Gd-liposome (GdL-E). The second dose of GdL-E with 10  $\mu\text{mol lipids/kg}$  and 2.26  $\mu\text{mol Gd/kg}$  was intravenously injected at day 7 after the first injection of Gd-micelle (5  $\mu\text{mol Gd/kg}$ ), empty GdL-E (2  $\mu\text{mol lipids/kg}$ ), and GdL-E (2  $\mu\text{mol lipids/kg}$  and 0.43  $\mu\text{mol Gd/kg}$ ). Tissues of blood, liver, spleen, and kidney were removed 6 h after the second injection of GdL-E liposome. Data represent mean  $\pm$  S.D. ( $n=3$ ).  $P$  values apply to differences between the saline group and Gd-micelle or liposome treated group. \* $p < 0.05$ , \*\* $p < 0.01$ . N.D. The Gd concentration was too low to be detected by ICP.

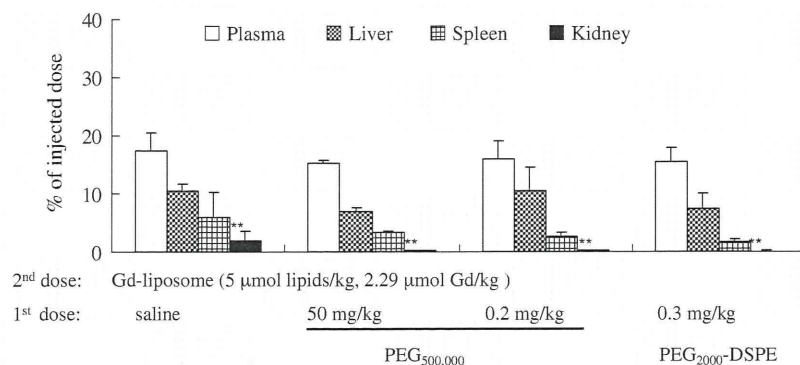
Therefore, the data herein show that the accelerated clearance of Gd-liposome at 10  $\mu\text{mol lipids/kg}$  corresponding to 2.26  $\mu\text{mol Gd/kg}$  was induced by both the Gd-liposome and empty liposome, but not by the Gd-micelle. This finding indicates that Gd ions at the first dose of 0.45  $\mu\text{mol/kg}$  did not affect the induction of ABC caused by liposomes.

#### Effect of PEG on the Distribution of Gd-liposomes

Next, the effect of injecting a PEG homopolymer and PEG<sub>2000</sub>-DSPE on the distribution of Gd-liposomes was examined. Since the encapsulation efficiency of Gd was low with the ethanol injection method, we prepared another Gd-liposome by the reverse phase evaporation method (GdL-R). The tissue distribution of GdL-R at 6 h after injection at a dose of 5  $\mu\text{mol lipids/kg}$  was not significantly influenced by the pre-administration of 50 mg/kg PEG<sub>500,000</sub>, 0.2 mg/kg PEG<sub>500,000</sub>, or 0.3 mg/kg PEG<sub>2000</sub>-DSPE 7 days before (Fig. 7). The dose of 0.2 mg/kg PEG<sub>500,000</sub> and 0.3 mg/kg PEG<sub>2000</sub>-DSPE with the concentration of 0.04 mg/ml is similar to that of the 5 mol% PEGylated liposome (0.3 mg/kg PEG<sub>2000</sub>-DSPE), which could produce the ABC phenomenon (Fig. 6). Hence, the first injection of PEG<sub>500,000</sub> saline or PEG<sub>2000</sub>-DSPE saline failed to cause the ABC phenomenon after the second administration of Gd-liposome. Hence, only injections of PEG macromolecules did not induce the ABC effect.

#### DISCUSSION

In the present study, the influence of dose on the tissue distribution of Gd-micelles after repeated administrations was investigated. Many studies have found that a lower dose of lipid in liposomes or nanoparticles results in a greater ABC effect (6–8,12), and the magnitude of the ABC phenomenon reached a maximum when the time interval between two



**Fig. 7.** Effect of PEG<sub>500,000</sub> and PEG<sub>2000</sub>-DSPE on the tissue distribution of Gd-liposome (GdL-R). The second dose of Gd-liposome with 5  $\mu\text{mol}$  lipids/kg and 2.29  $\mu\text{mol}$  Gd/kg was intravenously injected at day 7 after the first injection of PEG<sub>500,000</sub> saline at a dose of 50 mg/kg or 0.2 mg/kg, and PEG<sub>2000</sub>-DSPE saline at 0.3 mg/kg. The control group of GdL-R was injected at a dose of 10  $\mu\text{mol}$  lipids/kg with the first injection of saline. Tissues of blood, liver, spleen, and kidney were removed at 6 h after the second injection of GdL-R. Data represent mean  $\pm$  S.D. ( $n=3-5$ ).  $P$  values apply to differences between the saline group and PEG<sub>500,000</sub> or PEG<sub>2000</sub>-DSPE treated group. \* $p < 0.05$ , \*\* $p < 0.01$ .

injections was 5–7 days in rats (5) and 10 days in mice (7,12). Hence, we investigated the distribution of the Gd-micelle at various doses and an interval of 3, 7, or 10 days between injections. We found that repeated injections of the Gd-micelle, even with the second dose reduced to 2  $\mu\text{mol}$  Gd/kg (corresponding to 4 mg polymer/kg) and at different time intervals at a dose of 33  $\mu\text{mol}$  Gd/kg (corresponding to 67.3 mg polymer/kg), did not result in an accelerated clearance. ABC of the second injection of the Gd-liposome was induced by the first injection of both the Gd-liposome and the empty PEGylated liposome, but not by the first injection of the Gd-micelle (Fig. 6). Gd-DTPA encapsulated in liposomes would not affect the ABC phenomenon. Therefore, our observation that the ABC phenomenon did not occur with the Gd-micelle is important as it means that injections of the Gd-micelle will not change the biodistribution of a second administration of diagnostic or therapeutic agents.

For Gd-micelle, Gd was chelated to the micelle and thus existed in the form of micelle as shown in Fig. 1, which was consistent with the release results in Fig. 2 that Gd did not leak from the Gd-micelle in PBS (pH 7.4). Gd concentration in plasma, therefore, will reflect the pharmacokinetic behavior of the Gd-micelle. For Gd-liposomes, although the Gd concentration in plasma contained both the leaked Gd-DTPA from the Gd-liposome and the encapsulated Gd-DTPA in the Gd-liposome, the leaked-free Gd-DTPA is reported to be very rapidly cleared from the bloodstream with a minute's order half-life (19), and thus the detected Gd in blood is considered to be only the Gd-DTPA encapsulated in the liposome 6 h after intravenous injection in this study. Compared to GdL-R, the leakage of Gd-DTPA from GdL-E was faster, resulting in the lower Gd concentration (the encapsulated Gd) of GdL-E in blood in Fig. 3. Most importantly, the purpose of this study is to investigate if the distribution for the second dose of the Gd-liposomes or Gd-micelle was affected after pre-administered with the first dose or not. Therefore, the leakage of Gd-DTPA from the Gd-liposomes will not influence this study. In addition, many studies demonstrated that ABC phenomenon for empty liposome was observed determined by [<sup>3</sup>H]-labelled or <sup>99m</sup>Tc-labelled method (5–9).

It is believed that macrophages in the RES play an important role in ABC, and liposomes were mainly located in Kupffer cells after a second injection (5,8). When hepatosplenic macrophages were depleted, no enhanced clearance of liposomes was observed (6). The induction of ABC with liposomes could be attributable to a 150 kDa serum factor (5), anti-PEG IgM (9,11,12,22,23), anti-PEG antibody (10), or anti-PEG IgG antibody (24).

Whereas the mechanism of the immune response on repeated injections of liposomes has not been fully elucidated yet, the enhanced clearance effect can still be divided into two phases: the induction phase following the first injection and the effectuation phase following the second injection (6). According to this theory, there are two very important factors: one is the biological material (e.g. antibody) produced in the induction phase, the other is the recognition of the antibody by the second dose. For the effectuation phase, it was reported that the ABC phenomenon was induced by the second dose of a PEGylated liposome, but not of a liposome lacking a PEG-coating (23). This indicates that PEG is essential for the nanocarrier to recognize the antibody in the effectuation phase. In this study, the ABC phenomenon was not observed after repeated injections of the Gd-micelle at different doses and time intervals. This ABC failure of Gd-micelle may be caused by the failure for the production of biological material in the induction phase (data not shown) or/and for the recognition by the antibody in the effectuation phase. Even if the first injection was of empty liposome, the second injection of the Gd-micelle did not produce the ABC phenomenon either. This suggests that the antibody produced by the empty liposome in the induction phase is not recognized by the PEG moiety of the Gd-micelle. Therefore, not only PEG, but also other factors such as structure and hydrophobic character affect recognition.

For the induction phase, the ABC phenomenon was not observed when the amount of PEGylated lipid of liposomes in the first injection was more than 10 mol% (7,8). We have previously reported the accelerated clearance of [<sup>3</sup>H]-labelled PEGylated liposomes in mice pre-administered empty PEG-PBLA polymeric micelles (16). Furthermore, repeated

injections of PEG-PLA nanoparticles also produced the ABC phenomenon (12). Hence, the structure and component of nanocarriers has a considerable impact on the induction phase of ABC. From a structural perspective, the Gd-micelle formed through ionic interactions; therefore, it does not have any hydrophobic part (Fig. 1). In contrast, the PEG-PBLA micelle is composed of both a hydrophilic part, PEG, and a hydrophobic part, PBLA. Similarly, PEGylated liposomes possess a hydrophilic PEG chain and a hydrophobic bilayer membrane. The immunogenicity of an antigen can be affected by factors such as the physical and chemical properties of the antigen, its dose, and so on (25). The reasons why the Gd-micelle evaded the ABC phenomenon have not yet been elucidated at the present stage. The absence of a hydrophobic part may be a key for this elucidation because the other ABC-phenomenon-positive PEG-based carrier systems possess hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. We are currently investigating the ABC phenomenon induced by other kinds of polymeric micelles and nanoparticles. It is hoped that these experiments will provide more evidence for the mechanism of the ABC phenomenon.

## CONCLUSIONS

The Gd-micelle did not induce ABC following its pre-administration at various doses and time intervals. In contrast, the Gd-liposome induced the phenomenon when it or an empty PEGylated liposome, but not the PEG<sub>500,000</sub> macromolecule or PEG<sub>2000</sub>-DSPE, was pre-administered. ABC-phenomenon-positive PEG-based carrier systems possess a hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. The absence of a hydrophobic part of Gd-micelle may be a key factor for not producing the ABC phenomenon.

## ACKNOWLEDGMENTS

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