has also been studied (11,12) and is considered a standard treatment for patients with a single metastasis. Radiation-induced necrosis, especially after WBRT, is a rare but irreversible complication (13), which leads to the frequent use of SRS for the treatment of BM.

Withholding WBRT, SRS alone as upfront therapy is thought to be an alternative to BM (14–17). One prospective study compared SRS alone with SRS plus WBRT (18), which did not show a statistically significant difference in terms of overall survival. A relatively small sample size, decreased local control rate and lack of difference in neurological adverse events made it difficult to conclude that SRS alone was not inferior to SRS plus WBRT (19). Although this evidence confirms WBRT as standard treatment, SRS alone is widely used in daily practice.

BM in breast cancer is unique, compared with BM in other primaries, for certain reasons. The first is the high incidence of BM in breast cancer, especially in patients with the Her2/neu subtype, which has already been mentioned. The second is, BM in breast cancer is more radiosensitive than that in other primary such as non-small cell lung cancer or renal cell carcinoma. This may lead to better local control of BM by WBRT only. The third is the better prognosis after diagnosis of BM, especially in patients with Her2/neu positive subtype (20). This may lead to increased concern about radiation necrosis and failure of local control. For these reasons, BM in breast cancer is unique in terms of risk-benefit balance. A prospective trial, ideally exclusive to breast cancer, is needed for optimal usage of SRS.

As preparation for a future prospective trial, the task force of the Japanese Breast Cancer Society made a questionnaire survey of treatment choices for breast cancer patients with BM.

PATIENTS AND METHODS

A total of 351 survey questionnaires were sent to community or academic breast oncologists who were board members of the Japanese Breast Cancer Society, in December 2005. For most institutions, one breast oncologist was selected from each institution. For some large institutions, two or more oncologists were selected, because they have multiple hospitals or divisions that may have different treatment strategies. To avoid duplicated answers from the same treatment team, we attached the statement asking to unite one answer from one hospital or divisions. The questionnaire consists of 40 multiple choice questions in about eight categories, such as characteristics of hospitals, screening for BM, operation, radiation, re-irradiation, chemotherapy, SRS and cost.

RESULTS

Of 240 institutions to which we sent survey questionnaires, 161 (67.1%) answered. More than 90% of answers were obtained from surgical oncologists; the remainders were radiation and medical oncologists, reflecting the current situation that most patients with breast cancer are treated by surgeons in Japan. The background characteristics of each institution are summarized in Table 1. Both small and large institutions were included in this survey. In many institutions, BM was a rare complication (60% of institutions answered '<5' patients with BM every year), but some institutions treat many BM patients (>20 patients per year). In 75% (125 of 155) of institutions, the treatment decision is made by a neurosurgeon and/or radiation oncologist.

More than half the institutions (83 of 161) screened for BM, although no evidence exists to support a screening strategy (Table 2). Timing of screening for BM differed, although more than half of the institutions with a screening strategy screen at disease progression. Some institutions screened before starting trastuzumab.

Table 1. Characteristics of each institution

Characteristics	Category	Number	%
Number of new patients/year	1–50	34	21
	51–100	57	35
	101–150	30	19
	151–200	15	9
	201 over	25	15
Number of new BM year	<5	95	60
	6–10	47	29
	11–20	13	8
	21 over	5	3
Radiation oncologist in your hospital?	Yes	121	75
	No	40	25
Stuff neurosurgeon in your hospital?	Yes	131	82
	No	29	18
Treatment decision mainly made by	Neurosurgeon	71	46
	Breast oncologist	40	25
	Radiation oncologist	32	21
	Conference	12	8

BM, brain metastasis

Table 2. Screening

Question	Answer	Number	%
Screening for BM	Yes	83	52
	No	78	48
If yes, when?	At systemic progression	48	58
	Routinely	18	21
	Before Trastuzumab	9	11
	Other conditions	8	10

Surgical resection was less frequently used as local therapy for BM because ~75% of the institutions (118 of 160) answered 'none or one case who received surgical resection per year' (Table 3). The infrequent choice of surgical resection might be a result of the rigid indications for surgery. More than 60% of institutions answered that no evidence of systemic disease except for BM, or controlled systemic disease by systemic therapy was crucial for surgical resection. WBRT, not SRS, was dominantly used for postoperative radiotherapy.

The indication for WBRT is summarized in Table 4. Different from surgical resection, it was not dependent on prognosis (87% of institutions answered that they considered radiotherapy regardless of the prognosis, for symptom relief). Even in patients with a poor performance status, WBRT can be used. More than 30% of institutions (52 of 161) answered that they would consider WBRT for patients with ECOG PS 4, if clinically needed. Eighty-one percent of institutions (124 of 154 institutions) interrupted chemotherapy during WBRT, although some institutions did not.

Table 3. Operation

Question	Category	Number	%
BM surgery cases/year	0–1	118	74
	2–5	37	23
	6–9	3	2
	10 or more	2	1
Indication for surgery	NED other than BM	55	32
	Stable systemic disease	53	31
	Prognosis more than 6 months	15	9
	Regardless of prognosis, if symptoms treatable only by surgery	48	28
Post-surgery radiation	WBRT	102	69
	SRS	45	31

NED, no evidence of disease; WBRT, whole-brain radiotherapy; SRS, stereotactic radiosurgery.

Table 4. Radiation

Question	Category	Number	%
Indication for RT	Prognosis	22	14
	Symptom improvement	136	84
	Upon request	3	2
PS	Only 0–2	53	33
	Only 0–3	56	35
	Regardless of PS, if communicable	39	24
	Regardless of communication, upon situation	13	8

RT, radiotherapy; PS, performance status.

Table 5. Repeat radiation

Question	Category	Number	%
Re-RT after WBRT?	Never	41	26
	Only SRS	94	58
	SRS or Local Rt	21	13
	If indicated, WBRT	5	3
For indication of repeat radiation (local RT or WBRT), does interval from first WBRT matter?	Yes (some interval needed)	16	53
	No	14	47
If you repeat radiation (SRS, local RT, WBRT), how do you tell patients about the risk of necrosis?	Will not tell	9	8
	Will tell, but not numerically	66	60
	<1%	0	0
	'a few percentage'	19	17
	'ten and a few %'	12	10
	'20–40%'	5	5

Table 5 summarizes the questions about re-irradiation for patients who had progressed to BM after WBRT. More than 80% of institutions answered that they did not repeat radiotherapy except for SRS. Interval as an indication for re-irradiation is controversial. Sixteen institutions needed an interval before re-irradiation, whereas another 14 institutions did not. Regarding the risk of re-irradiation, most surgeons estimated that the risk was greater than a few percent, but did not present their estimate to patients numerically.

Table 6 summarizes the questions about SRS and cost. Only 7% (13 of 154) of institutions gave WBRT as their first choice, although ~70% (100 of 154) answered 'depend on cases'. The indication for SRS according to the metastatic site, size and the number of BMs largely influenced the treatment decision. Concerning the indication for SRS, 98% (98 of 100) of institutions limited SRS for only small (<3 cm) lesions. Seventy-one percent (76 of 108) of institutions choose SRS only for patients with a limited number (<5 lesions) of BMs. However, 81% (117 of 144) of institutions did not limit the number of sessions as long as neurosurgeons technically permitted SRS. There was no consensus concerning prognosis and PS as indications for SRS. SRS was preferred to WBRT for both safety (less dementia) and efficacy (better BM control) reasons. The cost of SRS was not precisely estimated by the majority of surgeons.

DISCUSSION

This survey revealed that SRS is widely used as the first choice for BM treatment for patients with breast cancer in Japan. Many Japanese breast oncologists prefer SRS to WBRT as radiation therapy against BM. There are

Table 6. Stereotactic radiosurgery

Question	Category	Number	%
First choice of RT for BM	SRS	41	27
	WBRT	13	8
	Depends on cases	100	65
If you answer 'depends on cases', depends on what?	Maximum size	70	
	Number of BM	100	
	Location of BM	45	
	Control of systemic disease	18	
	PS	28	
	Financial status and others	8	
Maximum size for SRS	<2 cm	30	27
	<2.5 cm	12	11
	<3 cm	66	60
	<4 cm	2	2
Maximum number of BM for SRS	Only single	3	3
	2–4	73	68
	5–10	18	16
	No limitation in number	14	16
How control of systemic disease influences choice of RT for BM?	If good control, SRS	12	43
	If poor control, SRS	16	57
How prognosis influences choice of RT for BM?	SRS for poor prog.	7	21
	SRS for better	11	32
	Any prog. If PS is good	16	47
How many times will you repeat SRS	Only once	6	4
	Twice	15	10
	Three times	6	4
	No limitation in number	117	82
What is the main reason you avoid WBRT?	Hair loss	8	10
	Dementia	29	35
	Long treatment	16	19
	Worse BM control	30	36
Experience of neurological disturbance after WBRT	Yes	39	27
	No	107	73
Do you know the cost of WBRT exactly?	Yes	25	14
	No	120	86
Do you know the cost of SRS exactly?	Yes	27	19
	No	116	81

discrepancies between NCCN guideline recommendations and the practice in Japan. For example, for a limited number of BM, 30% of Japanese breast oncologists use SRS as adjuvant treatment although NCCN guidelines recommend WBRT as adjuvant treatment after surgery. For multiple BM, 30% of Japanese breast oncologists use SRS for patients with more than five BM, although NCCN guidelines recommend WBRT. For both a limited number of, and multiple, BM 60% of Japanese breast oncologists use SRS, although NCCN guidelines recommend WBRT for patients with systemic disease refractory to aggressive treatment. What causes these discrepancies, a preference for SRS and reluctance to use WBRT? Our survey revealed that Japanese breast oncologists believe that SRS is a safer and more effective treatment than WBRT, as shown in Table 6. Interestingly, one of the major concerns about WBRT was dementia, although 70% had not actually experienced it. Nonetheless, they did not limit the number of sessions for SRS. It seems that they believe that SRS is much safer than WBRT. Lack of recognition of the precise cost of SRS also enhances this preference for SRS, because the current national insurance system covers 70–90% of the total costs of SRS, which costs 500 000 yen per session.

The present study suggests issues for future trials. First, as shown in Table 1, the treatment decision for BM is shared by neurosurgeons and radiation oncologists, so their collaboration is essential. Another suggestion is the consideration of screening. More than half of the institutions had screened for BM although there is no supporting evidence. This should be taken into account when designing a clinical trial because screening may detect BM earlier in its clinical course, influencing the treatment choice (fewer lesions may lead to more SRS) and the survival of BM patients as a result of lead-time bias. Preference for SRS and its reasons are also important. A future trial on SRS should answer two questions: first, is limitless repetition of SRS safer than WBRT in terms of the long-term adverse effects of radiotherapy? and second, is SRS superior to WBRT in terms of local control? To answer these two questions, we need a prospective trial comparing WBRT with SRS for patients with breast cancer having limited number, and small size, of BM. This kind of randomized study would need too large a sample size to be conducted in Japan only, so international collaboration would be needed.

One limitation of the present study is that a questionnaire from one oncologist at an institution does not demonstrate the pattern of practice at the institution perfectly, because there could be many biases such as recall bias, response bias and so on. Although the background of institutions shown in Table 1 seems to show that this survey describes the current pattern of practice in Japan well, actual data from each institution are more helpful. We have therefore planned a historical cohort study to reduce these biases.

In conclusion, the present study showed that SRS alone is widely used as BM treatment for patients with breast cancer in Japan. To address the issues of both safety and efficacy, a future prospective trial studying the optimal usage of screening, SRS and WBRT is warranted.

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Conflict of interest statement

None declared.

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Radiosensitizing Effect of YM155, a Novel Small-Molecule Survivin Suppressant, in Non–Small Cell Lung Cancer Cell Lines

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Abstract

Purpose: Survivin, a member of the inhibitor of apoptosis protein family, is an attractive target for cancer therapy. We have now investigated the effect of YM155, a small-molecule inhibitor of survivin expression, on the sensitivity of human non–small cell lung cancer (NSCLC) cell lines to γ-radiation.

Experimental Design: The radiosensitizing effect of YM155 was evaluated on the basis of cell death, clonogenic survival, and progression of tumor xenografts. Radiation-induced DNA damage was evaluated on the basis of histone H2AX phosphorylation and foci formation.

Results: YM155 induced down-regulation of survivin expression in NSCLC cells in a concentration- and time-dependent manner. A clonogenic survival assay revealed that YM155 increased the sensitivity of NSCLC cells to γ-radiation *in vitro*. The combination of YM155 and γ-radiation induced synergistic increases both in the number of apoptotic cells and in the activity of caspase-3. Immunofluorescence analysis of histone γ-H2AX also showed that YM155 delayed the repair of radiation-induced double-strand breaks in nuclear DNA. Finally, combination therapy with YM155 and γ-radiation delayed the growth of NSCLC tumor xenografts in nude mice to a greater extent than did either treatment modality alone.

Conclusions: These results suggest that YM155 sensitizes NSCLC cells to radiation both *in vitro* and *in vivo*, and that this effect of YM155 is likely attributable, at least in part, to the inhibition of DNA repair and enhancement of apoptosis that result from the down-regulation of survivin expression. Combined treatment with YM155 and radiation warrants investigation in clinical trials as a potential anticancer strategy.

Survivin is a 16.5-kDa member of the inhibitor of apoptosis protein (IAP) family. It blocks the mitochondrial pathway of apoptosis by inhibiting caspases (1, 2) and regulates cell division through interaction with the proteins INCENP and Aurora B (3). It is abundant in many types of cancer cells but not in the corresponding normal cells (4–6). High levels of survivin expression in cancer cells are associated with poor patient prognosis and survival as well as with resistance to therapy and an increased rate of cancer recurrence (7–9). Survivin has therefore become a therapeutic target and potentially important prognostic marker for many tumor types, including non–small cell lung cancer (NSCLC; refs. 7, 10). Molecular antagonists of survivin including antisense oligonucleotides, and dominant negative mutants have been shown to induce apoptosis in cancer cells *in vitro* and *in vivo* as well as to enhance chemotherapy-induced cell death (11–13). Although antisense oligonucleotides and ribozymes can be engineered to be highly specific for survivin, they may be difficult to deliver in the clinical setting.

YM155, a small imidazolium-based compound, was identified by high-throughput screening of chemical libraries for inhibitors of the activity of the survivin gene promoter in a reporter assay (14). This compound specifically inhibits the expression of survivin at both the mRNA and protein levels and exhibits pronounced anticancer activity in preclinical models (14). An advantage of YM155 compared with previously investigated suppressors of survivin expression (15–20) is that it is active in the subnanomolar range. Pharmacokinetic analysis also revealed that YM155 was highly distributed to tumor tissue in tumor xenograft models *in vivo* (14). YM155 is thus an attractive candidate drug for cancer therapy, and clinical trials of YM155 in single-agent therapy are currently under way for some types of cancer.

Glioblastoma cells that overexpress survivin were found to be less responsive to radiation than survivin-negative cells in a preclinical model (21). Clinically, high levels of survivin expression have been associated with an increased risk of local treatment failure after radiochemotherapy in patients with rectal cancer (9). These observations suggest that survivin plays

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Translational Relevance

Survivin is a potentially important molecular target for cancer therapy. Reflecting the many mechanisms that seem to regulate survivin expression, diverse approaches have been evaluated for targeting survivin in experimental models. YM155 is a novel small, imidazolium-based compound that specifically inhibits survivin expression in various types of cancer cell lines *in vitro*. In addition, YM155 has been shown to distribute preferentially to tumor tissues rather than to plasma as well as to exert pronounced antitumor activity in tumor xenograft models *in vivo*. The use of YM155 as a single agent in phase I clinical trials did not reveal significant toxicity. Although phase II studies of YM155 use as a single agent for certain types of cancer are currently under way, the effects of YM155 in combination with radiation have not been reported. We now show that inhibition of survivin expression by YM155 sensitizes tumor cells to radiation *in vitro* and *in vivo*. Therefore, our preclinical results provide a rationale for future clinical investigation of the therapeutic efficacy of YM155 in combination with radiotherapy.

a role in resistance to radiotherapy. Indeed, suppression of survivin expression with the use of antisense oligonucleotides or ribozymes has been shown to increase the radiosensitivity of cancer cells *in vitro* (20, 22–26). We have now examined the effects of the combination of YM155 and radiation on NSCLC cell lines *in vitro* and *in vivo*.

Materials and Methods

Cell culture and reagents. The human NSCLC cell lines NCI-H460 (H460) and Calu6 were obtained from the American Type Culture Collection. The cells were cultured under an atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. YM155 (Astellas Pharma, Inc.) was dissolved in DMSO.

Immunoblot analysis. Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium PPi, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE of a 15% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C with rabbit polyclonal antibodies to human survivin (1:1000 dilution; R&D Systems), to human c-IAP1 (1:1,000 dilution; MBL International), to human XIAP (1:1,000 dilution; Cell Signaling), to human STAT3 (1:1,000 dilution; Cell Signaling), or to β-actin (1:500 dilution; Sigma), or with mouse monoclonal antibodies to human p53 (1:1,000 dilution; Santa Cruz Biotechnology). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit (Sigma) or mouse (Santa Cruz Biotechnology) IgG. Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Science).

Clonogenic survival assay. Exponentially growing cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm^2 flasks containing 10 mL of complete medium in the presence of 50 nmol/L YM155 or vehicle (final DMSO concentration of 0.1%; we confirmed that this DMSO concentration did not affect the proliferation of NSCLC cell lines). After incubation for 48 h, the cells were exposed at room temperature to various doses of γ-radiation with a ^{60}Co irradiator at a rate of −0.82 Gy/min. The cells were then washed with PBS, cultured in drug-free medium for 10 to 14 d, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted. The surviving fraction was calculated as: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for nonirradiated control cells. The surviving fraction for combined treatment was corrected by that for YM155 treatment alone. Cell survival was corrected according to the equation $S = 1 - (1 - f)^{1/N}$, where S is the single-cell survival rate, f is the measured surviving fraction, and N is multiplicity, which was defined as the average number of cells per microcolony at the time of radiation and which ranged from 2.4 to 6.7 for the cell lines studied under the described conditions. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.1 for vehicle-treated cells divided by that for YM155-treated cells (after correction for drug toxicity).

Detection of apoptotic cells. Cells were fixed with 4% paraformaldehyde for 1 h at room temperature, after which a minimum of 1,000 cells per sample was evaluated for apoptosis with the use of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (*In situ* Cell Death Detection Kit; Boehringer Mannheim).

Assay of caspase-3 activity. The activity of caspase-3 in cell lysates was measured with the use of a CCP32/Caspase-3 Fluometric Protease Assay Kit (MBL). Fluorescence attributable to cleavage of the DEVD-AFC substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

Immunofluorescence staining of γ-H2AX. Cells were grown to 50% confluence in two-well Lab-Tec Chamber Slides (Nunc) and then cultured for 48 h in the presence of 50 nmol/L YM155 or vehicle before exposure to 3 Gy of γ-radiation. At various times thereafter, they were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min at 4°C, and exposed to 5% nonfat dried milk for 10 min at room temperature. The slides were washed with PBS and then incubated at room temperature first for 2 h with mouse monoclonal antibodies to histone γ-H2AX (Upstate Biotechnology) at a dilution of 1:300 and then for 1 h with Alexa 488–labeled goat antibodies to mouse IgG (Molecular Probes) at a dilution of 1:700. The slides were mounted in fluorescence mounting medium (Dako Cytomation), and fluorescence signals were visualized with a confocal laser-scanning microscope (Axiovert 200M; Carl Zeiss) equipped with the LSM5 PASCAL system (Carl Zeiss). Three random fields each containing ≈50 cells were examined at a magnification of × 100. Nuclei containing ≥10 immunoreactive foci were counted as positive for γ-H2AX, as previously described (27), and percentage of positive cells was calculated.

Evaluation of tumor growth in vivo. All animal studies were done in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. The ethical procedures followed met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines (28). Tumor cells (2×10^6) were injected s.c. into the right hind leg of 6-week-old female athymic nude mice (BALB/c nu/nu). Tumor volume was determined from caliper measurement of tumor length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when the tumors in each group of animals achieved an average volume of ~200 to 250 mm^3. Treatment groups (each containing eight mice) consisted of vehicle control (physiologic saline), YM155 alone, vehicle plus radiation, and YM155 plus radiation. Vehicle or YM155 at a dose of 5 mg/kg of body mass was administered over 7 consecutive days (days 1-7) with the use of an implanted micro-osmotic pump (Alzet model 1003D; Durect). Mice in the radiation groups received 10 Gy of γ-radiation from a cobalt irradiator either as

a single fraction on day 3 of drug treatment or fractionated over 5 consecutive days (days 3 to 7); the radiation was targeted to the tumor, with the remainder of the body shielded with lead. Growth delay (GD) was calculated as the time required to achieve a 5-fold increase in volume for treated tumors minus that for control tumors. The enhancement factor was then determined as: $(GD_{combination} - GD_{YM155})/GD_{radiation}$.

Statistical analysis. Data are presented as means ± SD or SE and were compared with the unpaired Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Inhibition of survivin expression in NSCLC cells by YM155. We first examined the effect of YM155 on survivin expression in human NSCLC cell lines by immunoblot analysis. Treatment of H460 or Calu6 cells with YM155 at 1 to 100 nmol/L for 48 hours inhibited survivin expression in a concentration-dependent manner (Fig. 1A). In contrast, YM155 had no effect on the abundance of other members of the IAP family including XIAP and c-IAP1 (Fig. 1A), suggesting that YM155 specifically inhibits survivin expression in the NSCLC cell lines. The mechanism by which YM155 inhibits survivin expression remains to be elucidated. Previous observations have shown that p53 and signal transducer and activator of transcription 3 (STAT3) regulate survivin expression at the transcriptional level (29). We therefore examined the effect of YM155 on the abundance of p53 and STAT3 in NSCLC cell lines. YM155 showed no marked effect on the amounts of p53 and STAT3 in H460 or Calu6 cells (Fig. 1A), suggesting that the inhibition of survivin expression by YM155 is independent of these transcriptional regulators. Monitoring of the time course of survivin expression in cells exposed to 50 nmol/L

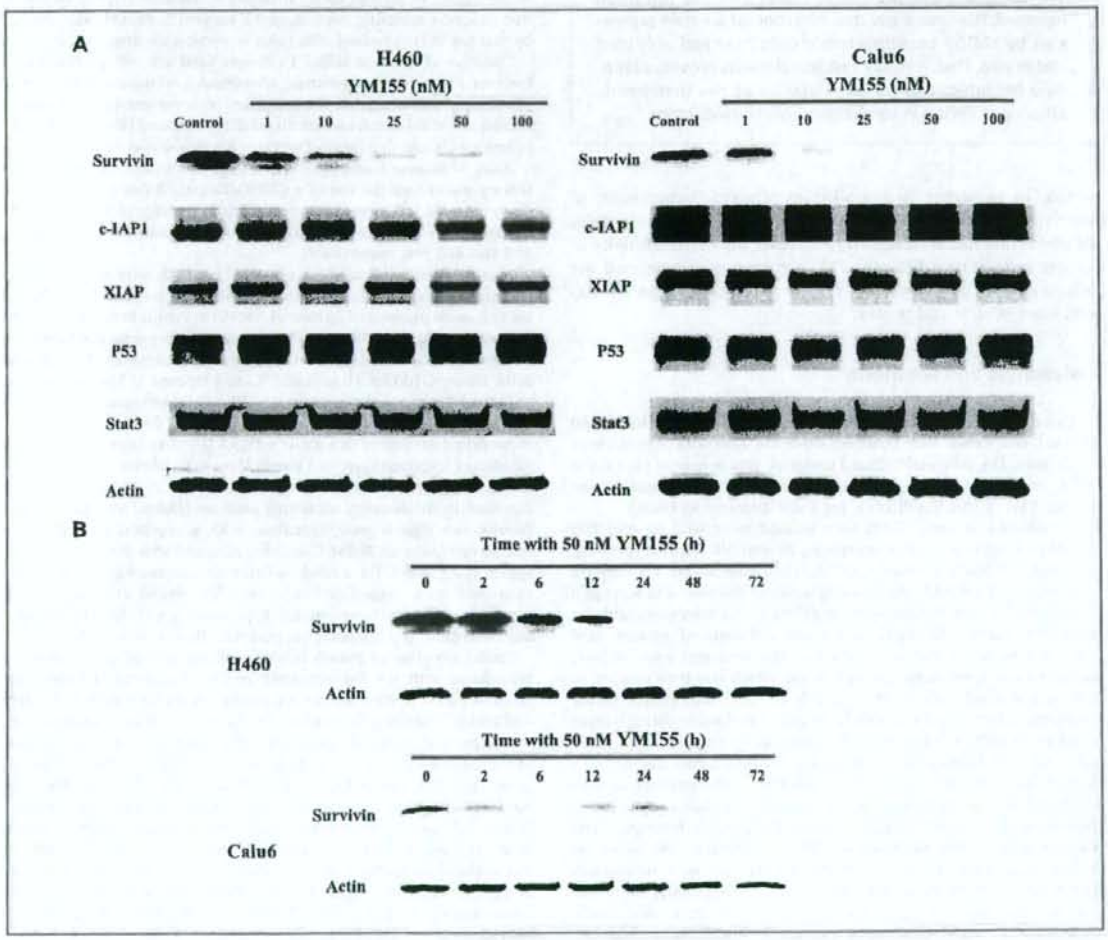

Fig. 1. Effect of YM155 on survivin expression in human NSCLC cells. *A*, H460 or Calu6 cells were incubated in the absence (control, 0.1% DMSO) or presence of various concentrations (1, 10, 25, 50, or 100 nmol/L) of YM155 for 48 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to survivin, to c-IAP1, to XIAP, to p53, to STAT3, or to β-actin (loading control). *B*, H460 or Calu6 cells were incubated with 50 nmol/L YM155 for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to survivin or to β-actin.

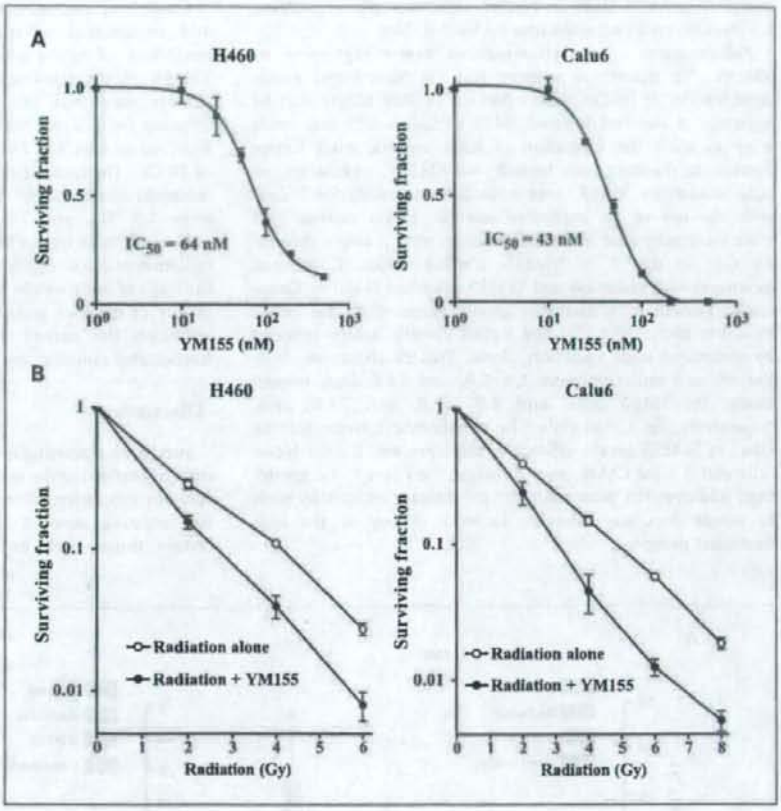

Fig. 2. Effect of YM155 on the sensitivity of H460 or Calu6 cells to γ-radiation. *A*, cells were incubated with the indicated concentrations of YM155 for 48 h and then assayed for clonogenic survival. Points represent means from three independent experiments; bars represent SD. *B*, cells were incubated with 50 nmol/L YM155 or vehicle (control, 0.1% DMSO) for 48 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium for 10 to 14 d for determination of colony-forming ability. Colonies were counted and the surviving fraction was calculated. Plating efficiency for nonirradiated H460 cells was 77.0% and 38.8% for vehicle-treated and YM155-treated cells, respectively; that for nonirradiated Calu6 cells was 57.0% and 23.5%, respectively. All surviving fractions with radiation were corrected for these baseline plating efficiencies. Points represent means from three independent experiments; bars represent SD.

YM155 for up to 72 hours revealed that the abundance of survivin in Calu6 cells had decreased by 2 hours and that survivin was virtually undetectable in H460 cells after 24 hours (Fig. 1B). In both cell lines, treatment with 50 nmol/L YM155 resulted in time-dependent inhibition of survivin expression.

YM155-induced sensitization of NSCLC cells to radiation. To examine the effect of YM155 on cell survival, we first did a clonogenic survival assay. Exposure to the drug at concentrations of 1 to 500 nmol/L for 48 hours revealed that YM155 inhibited the survival of H460 cells with a median inhibitory concentration (IC_{50}) of 64 nmol/L and that of Calu6 cells with an IC_{50} of 43 nmol/L (Fig. 2A). On the basis of these data, we adopted treatment with 50 nmol/L YM155 for 48 hours as the standard protocol for radiation experiments. We next examined whether YM155 might affect the sensitivity of NSCLC cell lines to radiation. Treatment with 50 nmol/L YM155 for 48 hours shifted the survival curves for both H460 and Calu6 cells to the left (Fig. 2B), with a dose enhancement factor of 1.57 and 1.61, respectively, suggesting that YM155 increased the radiosensitivity of both cell lines.

Enhancement of radiation-induced apoptosis in NSCLC cells by YM155. We next examined the effect of YM155 on radiation-induced apoptosis in H460 or Calu6 cells with the use of the TUNEL assay. Combined treatment of either cell line with YM155 and γ-radiation resulted in an increase in the number of apoptotic cells at 24 and 48 hours that was greater than the sum of the increases induced by YM155 or radiation alone (Fig. 3A). To confirm the results of the TUNEL assay, we measured the activity of caspase-3 in cell lysates. Again, the combined treatment of H460 or Calu6 cells with YM155 and γ-radiation induced a synergistic increase in caspase-3 activity (Fig. 3B). These data thus suggested that YM155 promotes radiation-induced apoptosis in NSCLC cell lines.

Inhibition of DNA repair in irradiated NSCLC cells by YM155. Defects in DNA repair have been associated with enhanced sensitivity of cells to radiation (30, 31), and survivin is thought to play a direct or indirect role in DNA repair (21). We therefore next investigated the effect of YM155 on DNA repair by immunostaining of cells with antibodies to the phosphorylated form (γ-H2AX) of histone H2AX, foci of which form at DNA double-strand breaks (DSBs). The formation of γ-H2AX foci in H460 cells was apparent between 30 minutes and 6 hours after γ-irradiation (Fig. 4A). In the presence of YM155, however, these foci persisted for at least 24 hours after irradiation. Evaluation of the percentage of H460 or Calu6 cells with γ-H2AX foci at 24 hours after irradiation revealed that YM155 significantly inhibited the repair of DSBs (Fig. 4B). These results thus suggested that down-regulation of survivin expression by YM155 results in the inhibition of the repair of

radiation-induced DSBs in NSCLC cells, possibly accounting for the observed radiosensitization by this drug.

Enhancement of radiation-induced tumor regression by YM155. To determine whether the YM155-induced radiosensitization of NSCLC cells observed *in vitro* might also be apparent *in vivo*, we injected H460 or Calu6 cells into nude mice to elicit the formation of solid tumors. After tumor formation, the mice were treated with YM155, γ-radiation, or both modalities. YM155 was infused continuously for 7 days with the use of an implanted osmotic pump system, and mice were subjected to local irradiation with a single dose of 10 Gy on day 3 of YM155 administration. Combined treatment with radiation and YM155 inhibited H460 or Calu6 tumor growth to a markedly greater extent than did either modality alone (Fig. 5). The tumor growth delays induced by treatment with radiation alone, YM155 alone, or both YM155 and radiation were 2.9, 5.6, and 14.8 days, respectively, for H460 cells, and 8.9, 41.0, and 76.0 days, respectively, for Calu6 cells. The enhancement factor for the effect of YM155 on the efficacy of radiation was 3.3 for H460 cells and 3.5 for Calu6 cells, revealing the effect to be greater than additive. No pronounced tissue damage or toxicity such as weight loss was observed in mice in any of the four treatment groups.

Finally, we evaluated whether the combination of YM155 and fractionated radiation treatment would result in the inhibition of tumor growth similar to that observed with YM155 plus single-fraction radiation. Mice bearing H460 tumors were thus again subjected to continuous YM155 infusion for 7 days, but local irradiation was done in 2-Gy fractions on days 3 to 7 of drug administration (for a total dose of 10 Gy). The tumor growth delays induced by treatment with radiation alone, YM155 alone, or both YM155 and radiation were 3.8, 5.3, and 16.6 days, respectively (Fig. 6). The enhancement factor for the effect of YM155 on the efficacy of radiation was 3.0. Again, there was no evidence of toxicity on the basis of body weight loss, and there were no animal deaths in any of the four groups. These data suggested that YM155 enhances the tumor response to both single-dose and fractionated radiotherapy *in vivo*.

Discussion

Survivin is a potentially important molecular target for cancer therapy. Reflecting the many mechanisms that seem to regulate survivin expression, diverse approaches have been evaluated for targeting survivin in experimental models. Although certain drugs, such as inhibitors of histone deacetylases,

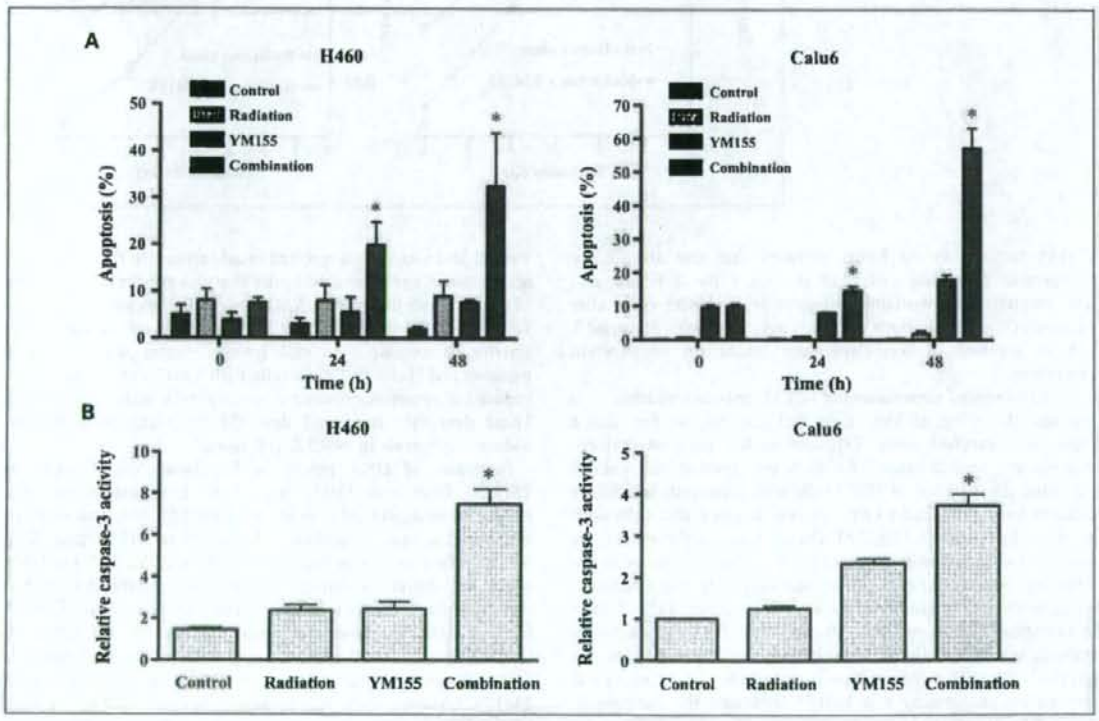

Fig. 3. Effect of YM155 on radiation-induced apoptosis and caspase-3 activity in H460 or Calu6 cells. *A*, cells were incubated with 50 nmol/L YM155 or vehicle (0.1% DMSO) for 48 h, exposed (or not) to 3 Gy of γ-radiation, and then incubated in drug-free medium for 24 or 48 h, at which times the percentage of apoptotic cells was determined by TUNEL staining. *B*, lysates of cells treated as in *A* were assayed for caspase-3 activity 24 h after irradiation. Columns represent means from three independent experiments; bars represent SD; those in *B* are expressed relative to the corresponding value for the control condition. * $P < 0.01$ versus the corresponding value for treatment with radiation or YM155 alone.

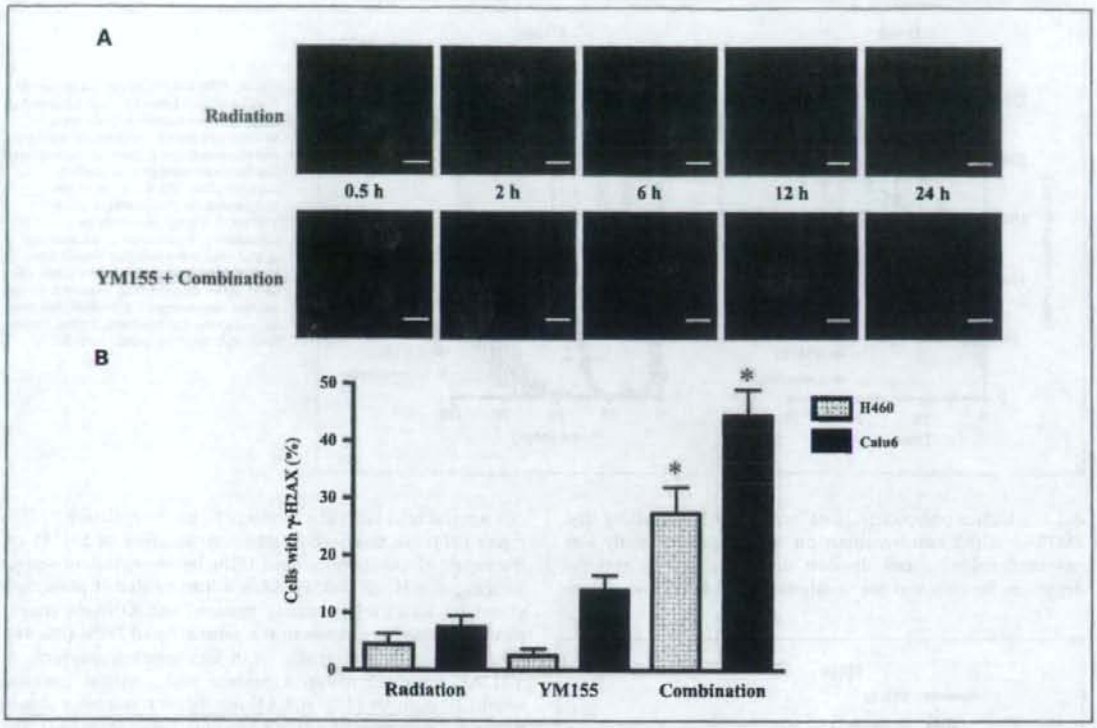

Fig. 4. Effect of YM155 on the radiation-induced formation of γ-H2AX foci in NSCLC cells. *A*, H460 cells were incubated with vehicle (0.1% DMSO) or 50 nmol/L YM155 for 48 h and then exposed to 3 Gy of γ-radiation. After incubation for the indicated times in drug-free medium, the cells were fixed and subjected to immunofluorescence staining for γ-H2AX (green fluorescence). Scale bar, 10 μm. *B*, H460 or Calu6 cells were incubated with vehicle or YM155 and then exposed (or not) to γ-radiation as in *A*. They were fixed at 24 h after irradiation and the percentage of cells containing γ-H2AX foci was determined. Columns represent means from three independent experiments; bars represent SD. $^*P < 0.05$ versus the corresponding value for radiation or YM155 alone.

mitogen-activated protein kinases, and cyclin-dependent kinases, have been shown to suppress survivin expression by targeting various signaling pathways, these drugs inhibit survivin expression nonspecifically (15–17, 19, 32). Gene therapy strategies based on small interfering RNA or other antisense oligonucleotides are specific for survivin, but the effective delivery of these molecules remains a challenge for the transition to the clinic (33). YM155 is a small-molecule agent that specifically inhibits survivin expression in various types of cancer cell lines *in vitro* (14). In addition, YM155 has been shown both to distribute preferentially to tumor tissues rather than to plasma as well as to exert pronounced antitumor activity in tumor xenograft models *in vivo* (14). The use of YM155 as a single agent in phase I clinical trials did not reveal significant toxicity (34). Although phase II studies of YM155 use as a single agent for certain types of cancer are currently under way, the effects of YM155 in combination with radiation have not been reported. We now show that YM155 increased the sensitivity of tumor cells to radiation *in vitro* and *in vivo*.

Clonogenic survival analysis, the most reliable approach for assessing the ability of genotoxic agents to induce cell death (35), revealed that YM155 markedly potentiated the decrease in NSCLC cell survival induced by γ-radiation. Given that induction of apoptosis is a key mechanism of cytotoxicity for most antitumor agents, including γ-radiation, defects in apoptotic signaling may underlie resistance to such agents (36). Radiation-sensitive tumors undergo radiation-induced apoptosis *in vitro* more readily than do radiation-resistant tumors (37–40). Treatment with caspase inhibitors has been shown to protect tumor cells against radiation-induced apoptosis and to increase their radioresistance (21, 41, 42), suggesting that radiation-induced apoptosis is caspase-dependent and that caspases contribute to radiosensitivity. The antiapoptotic activity of survivin is mostly attributable to inhibition of the activation of downstream effectors of apoptosis such as caspase-3 and caspase-7 (25). We have now shown that radiosensitization of NSCLC cells by YM155 was associated with increases both in the activity of caspase-3 and in the proportion of apoptotic cells. Our findings thus suggest that YM155 sensitized tumor cells to radiation at least in part by enhancing radiation-induced apoptosis.

We examined further the mechanism by which YM155 induces radiosensitization. Survivin is essential for the proper execution of mitosis and cell division, with disruption of survivin expression resulting in cell division defects that can lead to polyploidy and the formation of multinucleated cells (43, 44). Although treatment with 50 nmol/L YM155 for 48 hours inhibited survivin expression in NSCLC cells, it

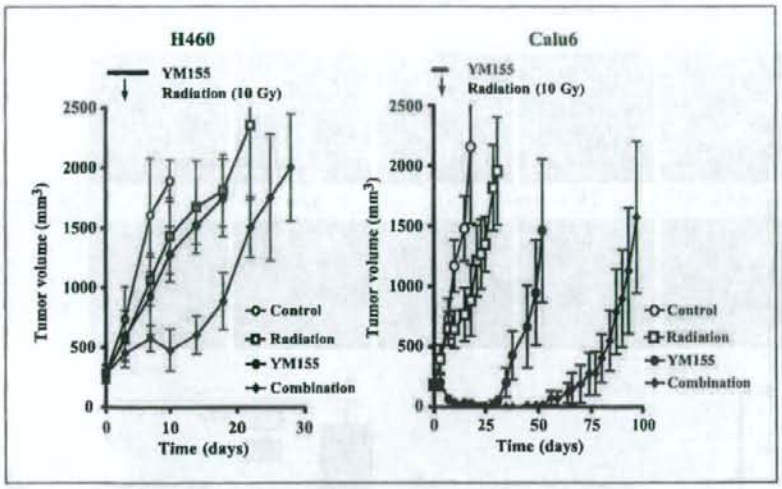

Fig. 5. Effect of YM155 on the growth of H460 or Calu6 tumors in mice subjected to single-dose radiotherapy. Cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 and radiation. YM155 (5 mg/kg) or vehicle was administered by continuous infusion over 7 d, and mice in the radiation groups were subjected to γ-irradiation with a single dose of 10 Gy on day 3 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Points, means from eight mice per group; bars, SE.

did not induce polyploidy (data not shown), suggesting that YM155-induced radiosensitization in the present study was not attributable to cell division defects caused by survivin depletion. Survivin was previously suggested to enhance tumor cell survival after radiation exposure through regulation of DSB repair (21). We therefore investigated the effect of YM155 on the repair of radiation-induced DSBs by immunofluorescence imaging of γ-H2AX foci. H2AX is a histone that is phosphorylated by ataxia telangiectasia mutated and DNA-dependent protein kinase in response to the generation of DSBs (45, 46). This reaction occurs rapidly, with half-maximal amounts of γ-H2AX generated within 1 minute and maximal amounts within 10 minutes (47), and a linear relation has been shown between the number of γ-H2AX foci and that of DSBs (48). The number of γ-H2AX foci is thus a sensitive and specific indicator of the existence of DSBs, with a decrease in this number reflecting DSB repair. We found that YM155 inhibited the repair of radiation-induced DSBs in NSCLC cells. If left unrepaired, DSBs can result in chromosome loss or cell death; agents that inhibit such repair thus increase the sensitivity of cells to ionizing radiation (49, 50). Our results therefore suggest that inhibition of DSB repair by YM155 contributes to the radiosensitization induced by this drug. Given that suppression of survivin expression impairs the repair of radiation-induced DNA damage (9, 21), our results further suggest that inhibition of DNA repair by YM155 is attributable to down-regulation of survivin expression.

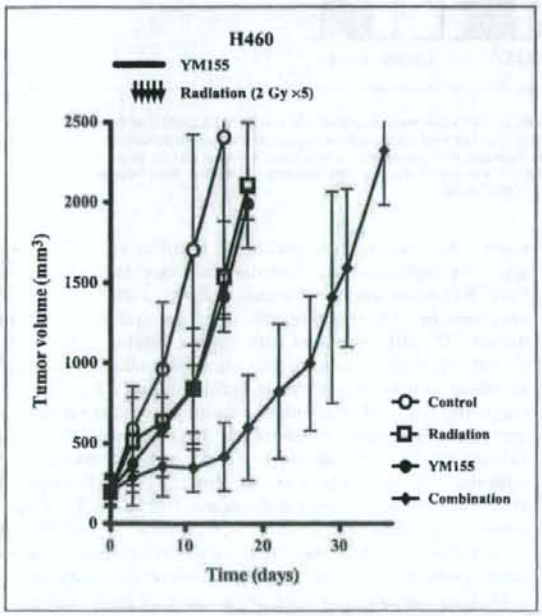

Fig. 6. Effect of YM155 on the growth of H460 tumors in mice subjected to fractionated radiotherapy. H460 cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 and radiation. YM155 (5 mg/kg) or vehicle was administered by continuous infusion over 7 d, and mice in the radiation groups were subjected to γ-irradiation with a daily dose of 2 Gy on days 3 to 7 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Points represent means from eight mice per group; bars represent SE.

The antitumor activity of YM155 has previously been shown to be time-dependent, with continuous infusion of the drug resulting in greater antitumor activity and less systemic toxicity compared with bolus injection in tumor xenograft models *in vivo* (14). Ongoing clinical trials of YM155 are thus being done with the drug administered on a continuous schedule. We also administered YM155 by continuous infusion in our *in vivo* experiments. The combination of YM155 with single-dose radiotherapy resulted in a marked increase in tumor growth delay compared with that apparent with either radiation or YM155 alone, indicating that YM155 enhanced the antitumor effect of ionizing radiation *in vivo*. Given that standard radiation therapy in the clinic is delivered according to a fractionated schedule, we also examined whether YM155 enhanced the tumor response to clinically relevant fractionated doses (2 Gy) of radiation. Indeed, YM155 was also effective in

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Meeting Report

Report from the second Japanese Urological Association–Japanese Society of Medical Oncology joint conference, 2007: 'Diagnosis and treatment of urological malignant tumors: How can we promote subspecialists?'

Nagahiro Saijo,* Tsuneharu Miki,* Yoshinobu Kubota,* Seiji Naito,* Hideyuki Akaza,* Shunji Takahashi* and Hironobu Minami*

Preface: The second Japanese Urological Association–Japanese Society of Medical Oncology joint conference was held on 25 October 2007. The theme of this year's conference was 'Diagnosis and treatment of urological malignant tumors: How can we promote subspecialists?' This Meeting Report briefly discusses the themes of uro-oncology education; collaboration of urologists and medical oncologists for treatment of advanced renal cell carcinoma; the role of urologists in treatment of urological cancer; the role of the medical oncologist in therapy, and collaboration between the JUA and the JSMO.

Program

Moderators:	**Nagahiro Saijo**	Deputy Director, National Cancer Center Hospital East
	Tsuneharu Miki	Professor, Kyoto Prefectural University of Medicine
1. EDUCATION OF URO-ONCOLOGY IN THE JAPANESE UROLOGICAL ASSOCIATION		
Presenter:	**Yoshinobu Kubota**	Professor, Yokohama City University
2. INVOLVEMENT OF UROLOGISTS AND MEDICAL ONCOLOGISTS IN THE TREATMENT OF ADVANCED RENAL CELL CARCINOMA		
Presenter:	**Seiji Naito**	Professor, Kyushu University
3. THE POSITION OF THE UROLOGIST IN THE TREATMENT OF UROLOGICAL CANCER		
Presenter:	**Hideyuki Akaza**	Professor, University of Tsukuba
4. THE ROLE OF THE MEDICAL ONCOLOGIST IN THERAPY FOR UROLOGICAL MALIGNANCY IN JAPAN		
Presenter:	**Shunji Takahashi**	Chief, Cancer Institute Hospital
5. COLLABORATION BETWEEN THE JAPANESE SOCIETY OF MEDICAL ONCOLOGY AND THE JAPANESE UROLOGICAL ASSOCIATION FOR DEVELOPING TRAINING SYSTEMS FOR MEDICAL ONCOLOGISTS		
Presenter:	**Hironobu Minami**	Professor, Kobe University
DISCUSSION		

Summary of second Japanese Urological Association–Japanese Society of Medical Oncology joint conference

Moderators

Nagahiro Saijo MD
Deputy Director
National Cancer Center Hospital East
Tsuneharu Miki MD
Professor
Department of Urology
Kyoto Prefectural University of Medicine

The second Japanese Urological Association–Japanese Society of Medical Oncology (JUA–JSMO) joint conference was held on 25 October 2007, at the National Kyoto International Congress Center from 18:00 to 20:00 hours. The meeting was sponsored by the JUA and the JSMO and was cosponsored by Takada Pharmaceutical Company. The theme of this year's conference was 'Diagnosis and treatment of urological malignant tumors: How can we promote subspecialists?' The session was chaired by Tsuneharu Miki, a professor at Kyoto Prefectural University of Medicine, and Nagahiro Saijo, Deputy Director of the National Cancer Center Hospital East. Three and two speakers were invited from the JUA and the JSMO, respectively.

Yoshinobu Kubota, a professor at Yokohama City University School of Medicine, talked about current educational programs in urology at universities and through the Japanese Urology Association. He reported that the study of urology in Japan covers molecular biology, diagnosis, surgery and chemotherapy, including palliative care of the kidneys, urinary tract and male genital organs. He stressed the need for a Society of Uro-oncology as a subspecialty of urology and bidirectional communication between the JUA–JSMO and the Japanese Association of Radiation Oncology (JASTRO) through joint symposiums and educational seminars.

Seiji Naito, a professor at the Graduate School of Medical Sciences, Kyushu University, talked about the recent development of new molecular target drugs in the field of urology. The development of new drugs like Sorafenib and Sunitinib in Japan has depended solely on clinical trials

* Presenters in order of Program.

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conducted by urologists. He stressed that the quality of these clinical trials for oncology drugs was high, even though the trials were conducted by urologists. He also mentioned that surgery will remain an important treatment modality in the field of uro-oncology and emphasized the importance of collaborations between the JUA and the JSMO, the JASTRO and the Japanese Society of Palliative Care.

Hideyuki Akaza, a professor at the University of Tsukuba, criticized the situation of Gan Shinryo Renkei Kyoten Byoin, nominated by the Ministry of Health, Labour and Welfare, because it lacks fundamental functions requested by the government. He also criticized the functions of two National Cancer Center Hospitals, Tsukiji and Kashiwa, because essential key elements do not exist for the integration of Gan Shinryo Renkei Kyoten Byoin. He stressed that neither of the National Cancer Center Hospitals function as medical centers, since Cancer Centers should be connected with all other branches of medicine. He concluded that bidirectional education and efforts will be essential to establish the field of uro-oncology in Japan.

Shunji Takahashi, Chief of Ariake Ganken Hospital, talked about the current situation of the Cancer Board for the treatment of urogenital tumors at his hospital. Ariake Ganken Hospital organizes a Cancer Board for each tumor type and cares for their cancer patients using a multidisciplinary specialist team consisting of surgeons, medical oncologists, radiation oncologists and nurses. At this moment, their model represents the ideal situation for taking care of cancer patients in Japan.

Hironobu Minami, a professor at Kobe University Graduate School of Medicine and the executive director of the Japanese Society of Medical Oncology, talked about the missions, strategic plans and visions of the JSMO. The JSMO began certifying medical oncologists in 2005 and presently has 205 certified medical oncologists. They have subspecialties for thoracic oncology, hematology/oncology, gastrointestinal oncology, breast cancer, and other areas. Unfortunately, there is only one specialist in uro-oncology. More specialists are essential for optimizing the care of patients with urogenital tumors. The JUA and the JSMO should collaborate with regard to the education of urologists and medical oncologists.

Although the meeting was scheduled to last from 18:00 to 20:00 hours, more than 100 participants attended and many productive discussions were held. The next joint meeting will be scheduled in conjunction with the JUA or the JSMO Annual Meeting.

Education of uro-oncology in Japanese Urological Association

Presenter

Yoshinobu Kubota MD
Professor
Department of Urology, Yokohama City University School of Medicine

Urology in Japan covers diagnoses and treatments including surgeries and molecular therapeutics of a broad range of diseases in urogenital organs. This wide field of urology is different from the urology in the USA which is mainly urological surgery. Thus, Japanese urologists have required wide knowledge, technical skills and experience in several therapeutic modalities including chemotherapy for urogenital cancers. Chemotherapy for urological cancers is therefore a familiar subject of urology in Japan.

Recently, cancer chemotherapy has progressed to be one of the key tools of the treatment for solid cancers such as urogenital cancer. And the JUA has been involved in developing several subspecialties in collaboration with other associated scientific and medical societies.

Considering these situations, there are two key issues for the JUA regarding education on chemotherapy for urogenital cancers. One is education on cancer chemotherapy for urologists and trainees. The other is to train uro-oncologists, especially specialists in chemotherapy for urogenital cancer. In each educational issue, collaboration with medical oncology and urology is essential.

The establishment of a society for the subspecialty of urological oncology, in collaboration with both the JSMO and the JUA is important (Fig. 1). Also, closer communication between the JSMO and the JUA is recommended for further developing and promoting new chemotherapies which are effective against urogenital cancer.

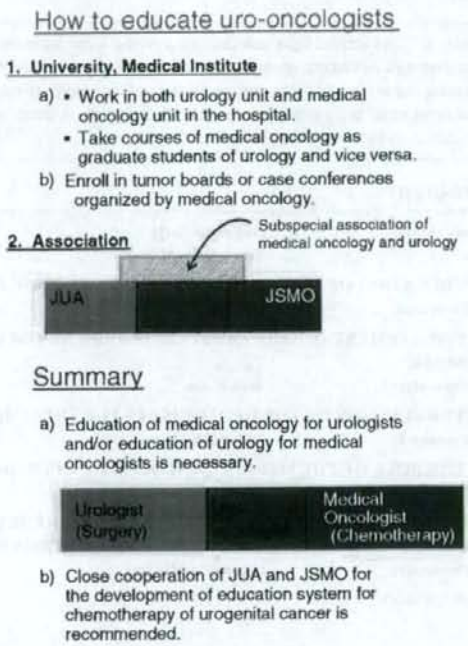

Fig. 1 How to educate the uro-oncologist.

Involvement of urologists and medical oncologists in the treatment of advanced renal cell carcinoma

Presenter

Seiji Naito MD
Professor
Department of Urology, Graduate School of Medical Sciences, Kyushu University

Cytokine therapy, mainly using IFN-α or IL2, has been conventionally adopted as a drug therapy for advanced renal cell carcinoma. Cytokine therapy has demonstrated a low response rate (approximately 15%) and a limited duration of response (6–10 months) so it is not considered to be a satisfactory treatment. Recently, several molecular-targeted drugs, which exert their therapeutic effects by inhibiting intracellular signal transduction involved in tumor cell proliferation or angiogenesis, have been developed. The beneficial effects of these drugs on renal cell

carcinoma have been reported and so the therapeutic strategy has dramatically changed recently. In Europe and the USA, sunitinib, which is an orally available, multitargeted receptor tyrosine kinase inhibitor of vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), is now being positioned as a first line treatment for metastatic renal cell carcinoma. Furthermore, sorafenib, which is an orally available multikinase inhibitor active on Raf-1, and receptor tyrosine kinases including VEGFR-1, -2, -3, PDGFR-β, c-Kit, Flt-3 and RET, are being positioned as second line treatment for cytokine refractory metastatic renal cell carcinoma. Temsirolimus, which is an inhibitor of the kinase m-TOR (mammalian target of rapamycin), is being positioned as a first line treatment for poor-risk patients with metastatic renal cell carcinoma. In Japan, the phase II studies of sunitinib and sorafenib have been completed and applications for the approval of these drugs have been submitted to the Health, Labour and Welfare Ministry. They will soon be approved for use in the clinical setting. The dosage of sorafenib used in the Japanese phase II study was equivalent to the dosage used in the clinical studies in Europe and the USA. However, the response rate and the incidence of adverse drug reactions such as hand–foot skin reaction and hypertension obtained in the Japanese study were higher than those observed in other clinical studies in Europe and the USA. It is difficult to make an accurate comparison without careful consideration, but the possible effect of ethnicity on the response and adverse drug reactions can not be ruled out. Considering the possibility of the long-term administration of these drugs, adverse drug reactions may be encountered which have not yet been predicted. The postmarketing collection and broad distribution of data on adverse drug reactions and therapeutic effects from registered patients treated at specified medical facilities, at least for some time, are indispensable to preserve patient safety, validate the efficacy of these drugs and educate urologists in charge of treatment. In order to deal with such a situation, urologists should promote close cooperation with physicians who specialize in medical oncology, radiation oncology, psychotherapy, palliative therapy, dermatology, cardiovascular diseases, respiratory diseases, etc. (Fig. 2).

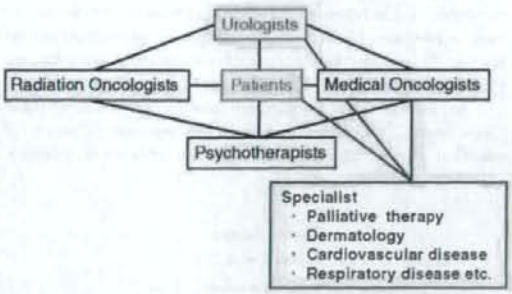

Fig. 2 Holistic medical care for patients with renal cell carcinoma by cooperation among urologists and other relevant physicians including medical oncologists

Other promising new molecular-targeted drugs for renal cell carcinoma are also in the development phase. Various combination therapies, such as the concomitant use of molecular-targeted drugs, molecular-targeted drugs and cytokines, or the sequential use of molecular-targeted drugs are now being investigated, so the strategy of drug therapy for renal cell carcinoma may change in the near future. In principle, a nephrectomy has been recommended prior to cytokine therapy for patients with metastatic renal cell carcinoma. Whether or not a nephrectomy should also be recommended prior to molecular-targeted therapy still remains unknown. The timing of surgical treatment for metastatic sites during molecular-targeted therapy may also be an important issue in the future.

Urologists should be specialists that provide holistic medical care throughout the course of renal cell carcinoma. In the practical treatment of renal cell carcinoma, urologists are expected to organize a medical team, usually consisting of radiologists, physicians specializing in palliative therapy and those specializing in psychosomatic internal medicine, as well as medical oncologists depending on the patient's pathological condition.

In conclusion, since the advent of effective molecular-targeted drugs, the treatment strategy for metastatic renal cell carcinoma is dramatically changing. In order to practice effective holistic medical care, opportunities for exchanging expertise among physicians including urologists, medical oncologists and other relevant clinicians should be encouraged and increased to promote the development of urologists with comprehensive knowledge and experience regarding the treatment of renal cell carcinoma.

The position of the urologist in the treatment of urological cancer

Presenter

Hideyuki Akaza MD
Professor
Department of Urology, University of Tsukuba, Ibaraki, Japan

A significant number of patients with malignancies including urological cancer have systemic involvement such as metastasis at either a macroscopic or microscopic level. In addition, some patients have various complications at diagnosis. Thus, a treatment strategy should be developed not only for the cancer lesion itself but for other systemic conditions. In a total care system like this the role of the urologist is very important.

It is important in cancer care not to treat only cancer lesions but to care for the patient according to the comorbidity and complications that may occur during cancer treatment (Fig. 3).

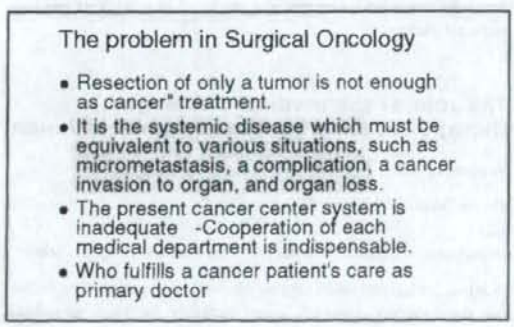

Fig. 3 The problems in surgical oncology

It is crucial for cancer care to be carried out at an institution where all medical departments are ready, or in an environment where hospital to hospital cooperation is possible. A cancer control program act (Gann taisaku kihon-hou) was enacted in 2007 and the cancer base hospital

design (Gann kyoten byouinn) is progressing as one of the policies for the realization of 'the standardization of cancer therapy'.

The following provision is in guidelines for the maintenance of the cancer base hospital delivered by the Ministry of Health, Labour and Welfare on 1 February 2007:

> The National Cancer Center Central Hospital and Hospital East, regarded as the cancer base hospital, set these guidelines and decide to bear roles, such as support to other cancer base hospitals, particularly the training of a specialty medical practitioner.[1]

Are these organizations fully endowed with a central mechanism that unifies a cancer base hospital? Have they got the mechanism fully established to respond to a subspecialty (such as cardio-vascular, respiratory, or renal function, which affect cancer therapy occasionally and are indispensable to it in these institutions) a complication, or a multiorgan operation?

What has caused the 'cancer refugee' who has become the center of attention these days? For example, the extirpation of renal cell carcinoma with a tumor thrombus in the vena cava inferior or further upstream can not be done without cardio-vascular surgery or vascular surgery. In what kind of institution is this possible? Can the complications accompanying urinary dysfunction or sexual dysfunction associated with prostate cancer, or various problems during endocrine therapy be dealt with appropriately?

At the first JUA-JSMO joint conference during the 44th Annual meeting of the Japanese Society of Clinical Cancer Oncology, the previous chairman of the European Organization of Research and Treatment of Cancer, Louis Denis made the following comments: 'In Europe most of the cancer centers are attached to hospitals as a separate section where they can be connected with all the other branches of medicine. Clinical trials of the last 30 years have forced cancer specialists into close collaboration'.[1] Is this what is lacking in cancer center design in Japan today? It is important in the treatment of urological cancer (at least for the time being until an ideal structure is established) to have efficient cooperation between the urological discipline and medical oncology.

Urologists should study medical oncology in general and medical oncologists should study urological oncology in order to become urological oncologists. These efforts should be bidirectional. Urological oncology in the USA is a specialty produced as a result of this bidirectional study.

The role of the medical oncologist in therapy for urological malignancy in Japan

Presenter

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In Japan, urological tumors have been treated by surgeons alone, including chemotherapy. However, recent demands for more specialized cancer treatment from patients and the development of molecular target therapy in the urological area have necessitated medical oncologists specializing in the urological tumor. There are very few medical oncologists participating in the treatment of urological tumors in Japan right now. How medical oncologists should participate in the treatment of urological tumors in Japan is discussed from the standpoint of our hospital.

The Cancer Institute Hospital moved to new buildings at Ariake, Koto-ku in Tokyo and the Department of Medical Oncology opened a new ward in June 2006. We started to participate in chemotherapy for urological tumors since the summer of 2006. First, at the 'Urological Cancer Board' once a week, surgeons, medical oncologists, pathologists, and nurses discussed cases of urological tumors treated with chemotherapy (Fig. 4). We are learning the diagnosis, standard treatment including surgery, and clinical courses of urological malignancies in the cancer board. In turn, we provided advice on oncological emergencies such as severe bone marrow suppression and electrolyte disturbances, or the new therapies for resistant cases.

During September 2006 we started to treat urological malignancy with second or third line chemotherapies, or phase I study in the new ward. We use irinotecan, taxanes, or other drugs for urothelial cancers or germ cell tumors, and started phase I study of new tyrosine kinase inhibitors for renal cell carcinoma (RCC) or urothelial cancer. Around five patients are always treated in the ward.

Most of the chemotherapy for urothelial cancer or germ cell tumor includes cisplatin, such as methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) and bleomycin, etoposide and cisplatin (BEP). Most patients are hospitalized because of nausea and renal toxicity. Furthermore, MVAC is often associated with severe mucositis, and BEP is associated with severe bone marrow suppression, so many patients have been hospitalized for a long time. We first tried to decrease the length of hospitalization during cisplatin-containing therapy to 3 or 4 days by mucositis treatment and infection prevention. Then we started an outpatient treatment program of cisplatin-containing chemotherapy by clinical path including pre- and post chemotherapeutic hydration.

Molecular target therapy has recently been introduced into urological tumor treatment, especially for RCC. Molecular target drugs such as sunitinib, sorafenib, and bevacizumab are associated with a diverse range of adverse effects compared with conventional cytotoxic drugs. Bevacizumab (Avastin) was approved for colon cancer in Japan in April 2007. Grade 3–4 adverse effects with bevacizumab include hypertension (8–25%), bleeding (2–9%), arterial or venous thrombosis (1–20%), gastrointestinal perforation (1–2%), and proteinuria (1%). In the several months following the approval of bevacizumab, we experienced a few cases of deep vein thrombosis, GI perforation, and GI bleeding. To manage those adverse effects efficiently and safely, we needed a multidisciplinary approach (Fig. 5).

A few months before bevacizumab went on the market, we made 'Team Avastin', which consisted of doctors, pharmacists, nurses (of outpatient clinics, inpatient units, and an ambulatory treatment

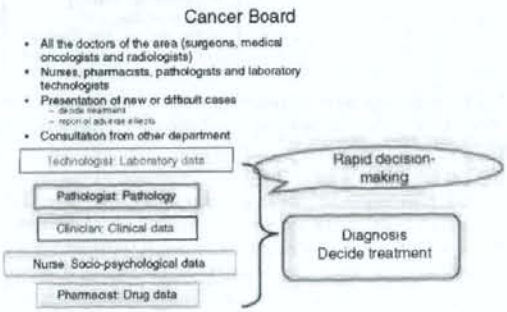

Fig. 4 Members and purposes of the 'Cancer board'

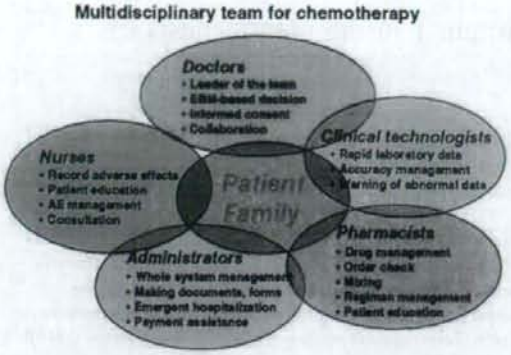

Fig. 5 Members of the multidisciplinary team for chemotherapy and their roles.

center), and medical collaboration officers in order to manage those adverse effects. The team made the clinical path for the initiation of bevacizumab therapy, the manual for managing the adverse effects of bevacizumab including consultation to gastrointestinal or respiratory surgeons, and made close liaison with cardiologists and neural surgeons in other hospitals. The team has revised the manual frequently, and solved the many problems associated with bevacizumab treatment.

In Japan, molecular target drugs such as sorafenib and sunitinib might be approved for RCC treatment in 2008. We are planning to make a new professional team consisting of medical oncologists, urological surgeons, nurses, pharmacists, and medical collaboration officers. The team will simulate the management of the severe adverse effects of these drugs, and make clinical paths and new manuals. We are also planning to start translational research such as a biomarker study.

In conclusion, in the area of urological malignancy, medical oncologists can participate in (i) some part of chemotherapy, (ii) care of complications with chemotherapy, (iii) experimental therapy such as phase I study, (iv) facilitating multidisciplinary care of patients, and (v) facilitating translational research.

Collaboration between the Japanese Society of Medical Oncology and the Japanese Urological Association for developing training systems for medical oncologists

Presenter

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Professor
Medical Oncology, Kobe University School of Medicine and Kobe University Hospital

It is well recognized that the quality of chemotherapy in oncology practice in Japan is lower than that in the United States or European countries. Patients with cancers are treated in an organ-specific medical system. Specifically, lung cancer is treated by chest physicians, gastrointestinal cancers by gastroenterologists or sometimes by surgeons, and genitourinary cancers by urologists. However, the organ-specific system often yields inadequate care. Patients with primary peritoneal adenocarcinoma presenting with ascites represent a subset of advanced cancers that are potentially curable by chemotherapy and surgical procedures for epithelial ovarian cancer. They often visit gastroenterologists with symptoms of abdominal fullness. Unfortunately, however, gastroenterologists often inadequately treat such patients with palliative chemotherapy for gastroenterological cancers without curative intent because they have undergone training for gastroenterological malignancies but not gynecological cancers. Similarly, patients with lung metastases from RCC sometimes visit thoracic oncologists who are not trained in immunotherapy, and patients are often inadequately treated.

Systemic chemotherapy for cancers should be performed by medical oncologists who have undergone a training program that includes all malignancies. However, training systems for medical oncologists including genitourinary cancers are currently under development in Japan. It is highly recommended that the JSMO and the JUA collaborate to establish such training systems for medical oncologists.

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Role of multidrug resistance-associated protein 1 in the pathogenesis of allergic airway inflammation

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Yoshioka M, Sagara H, Takahashi F, Harada N, Nishio K, Mori A, Ushio H, Okada KS, Ota M, Ito YM, Nagashima O, Atsuta R, Suzuki T, Fukuda T, Fukuchi Y, Takahashi K. Role of multidrug resistance-associated protein 1 in the pathogenesis of allergic airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 296: L30–L36, 2009. First published October 17, 2008; doi:10.1152/ajplung.00026.2008.—Multidrug resistance-associated protein 1 (MRP1) is a cysteinyl leukotriene (CysLT) export pump expressed on mast cells. CysLTs are crucial mediators in allergic airway disease. However, biological significance of MRP1 in allergic airway inflammation has not yet been elucidated. In this study, we sensitized wild-type control mice (*mrp1*$^{+/+}$) and MRP1-deficient mice (*mrp1*$^{-/-}$) to ovalbumin (OVA) and challenged them with OVA by aerosol. Airway inflammation and goblet cell hyperplasia after OVA exposure were reduced in *mrp1*$^{-/-}$ mice compared with *mrp1*$^{+/+}$ mice. Furthermore, CysLT levels in bronchoalveolar lavage fluid (BALF) from OVA-exposed *mrp1*$^{-/-}$ mice were significantly lower than those from OVA-exposed *mrp1*$^{+/+}$ mice. Levels of OVA-specific IgE, IL-4, and IL-13 in BALF were also decreased in OVA-exposed *mrp1*$^{-/-}$ mice. IgE-mediated release of CysLTs from murine bone marrow-derived mast cells was markedly impaired by MRP1 deficiency. Our results indicate that MRP1 plays an important role in the development of allergic airway inflammation through regulation of IgE-mediated CysLT export from mast cells.

cysteinyl leukotrienes; mast cell

MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 1 (MRP1) is a 190-kDa transmembrane protein belonging to the ATP-binding cassette transporter superfamily (15). The MRP1 gene was isolated from the doxorubicin-resistant human small cell lung cancer cell line H69AR (6), and subsequent in vitro studies established that MRP1 mediates the cellular excretion of many drugs and confers multidrug resistance of cancer cells (3). MRP1 has been shown to be expressed in various human tissues and cells, including mast cells (10). MRP1 transports glutathione *S*-conjugates of endogenous and xenobiotic lipophilic compounds across the cellular membrane into the extracellular space (15). Among these transport substrates, leukotriene C4 (LTC4) is a high-affinity endogenous glutathione *S*-conjugate substrate for the MRP1 (19) and is excreted from mast cells, which play an important role in the pathogenesis of allergy and asthma (2, 20).

Bronchial asthma is a common disorder in adults and children and remains poorly understood and difficult to manage (4). Airway inflammation is a hallmark of this disease (4). Previous studies have indicated that cysteinyl leukotrienes (CysLTs) such as LTC4, LTD4, and LTE4, originally termed slow-reacting substance of anaphylaxis, are crucial mediators in the pathogenesis of allergic asthma (8). LTC4 is synthesized by and excreted from mast cells and is rapidly converted to LTD4 and then to LTE4 (5). CysLTs induce airway smooth muscle contraction, increase vascular permeability and mucus secretion, and may recruit more inflammatory cells to the airway in allergic asthma (13). However, the importance of MRP1, which is the LTC4 export pump on mast cells, for allergic airway inflammation remains poorly defined.

To elucidate the role of MRP1 in the pathogenesis of allergic airway inflammation in vivo, we used an ovalbumin (OVA) sensitization and airway challenge protocol and compared MRP1-deficient mice (*mrp1*$^{-/-}$) with wild-type control mice (*mrp1*$^{+/+}$) in a well-established model. We also cultured bone marrow-derived mast cells (BMMCs) from *mrp1*$^{-/-}$ and *mrp1*$^{+/+}$ mice and stimulated them with IgE and anti-IgE antibody. The biological significance of MRP1 involvement in allergic airway inflammation and IgE-dependent export of CysLTs from mast cells is discussed.

MATERIALS AND METHODS

Animals. MRP1-deficient *mrp*$^{-/-}$ mice were generated by gene targeting in embryonic stem cells as described previously (31). *Mrp*$^{-/-}$ mice originally on the genetic background (129/Ola)/FVB (50:50) were backcrossed 12 times with FVB mice to obtain >99% FVB genetic background. Normal FVB mice were used as wild-type controls (*mrp*$^{+/+}$). *Mrp*$^{+/+}$ and *mrp*$^{-/-}$ mice (male, 6–8 wk of age) were purchased from Taconic Laboratories (Germantown, NY). Mice were maintained in a limited access barrier and housed in a humidity (55 ± 10%)- and temperature (24 ± 2°C)-controlled room under a

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12:12-h light-dark cycle. The study protocol was reviewed and approved by the Juntendo University and Dokkyo University School of Medicine Committee on Animal Care and complies with National Institutes of Health guidelines for animal care.

Sensitization and airway challenge. Mice were sensitized on *days 0* and *14* by an intraperitoneal injection of 50 μg of OVA (Sigma, St. Louis, MO) and 2 mg of aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan) in 200 μl of PBS. Nonsensitized mice received only aluminum hydroxide in PBS. On *days 22*, *24*, *26*, and *28*, the sensitized mice were challenged with aerosolized 1% OVA 30 ml for 30 min. The nonsensitized mice received PBS only. Bronchoalveolar lavage and histological analysis of the lungs were performed 48 h after the last aerosol challenge.

Histological analysis of lung. The murine lungs were infused and fixed with 10% formalin and then embedded in paraffin. Sections of 2.5-μm thicknesses were stained with either hematoxylin and eosin or periodic acid-Schiff (PAS). Semiquantitative scoring systems were used to grade the extent of lung inflammation and goblet cell hyperplasia as previously described (9). Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a five-point scoring system: 0, no cell; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; and 4, a ring of cells >4 cells deep. To determine the extent of mucus production, we quantified goblet cell hyperplasia in the airway epithelium using a five-point grading system: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Scoring of inflammatory cells and goblet cells was performed in at least 15 different fields for each lung section. Mean scores were obtained from six animals.

Bronchoalveolar lavage fluid and serum analyses. Mice were killed with an overdose of pentobarbital sodium. Blood was drawn, and bronchoalveolar lavage fluid (BALF) was collected with twice repeated washes of excised lungs using 0.7 ml of PBS. Total cell counts and differential cell counts were performed. Cytokine, OVA-specific IgE, and CysLT levels were measured using enzyme-linked immunosorbent assay (ELISA). Mouse IL-4 and IL-13 ELISA were purchased from R&D Systems (Minneapolis, MN). Mouse OVA-specific IgE ELISA was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). CysLT ELISA was purchased from Cayman Chemicals (Ann Arbor, MI).

Generation of murine BMMCs. BMMCs were generated from the femoral bone marrow cells of $mrp^{+/+}$ and $mrp^{-/-}$ mice and maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FCS, 100 μM 2-mercaptoethanol, 10 μM MEN-nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described (11). After 4 wk of culture, >98% of the cells were identifiable as mast cells as determined by toluidine blue staining and fluorescence-activated cell sorting analysis of cell surface expression of c-*kit* and FcεRI.

β-Hexosaminidase release assay. In vitro degranulation of mast cells was determined by β-hexosaminidase release assay as described previously (11, 26). Briefly, BMMCs were incubated with trinitrophenyl (TNP)-specific mouse IgE (BD Pharmingen, San Diego, CA) at the concentration of 1 μg/5 × 10^6 cells for 1 h on ice. BMMCs (1×10^6 cells/ml) were then resuspended in Tyrode's buffer (10 mM HEPES buffer, pH 7.4, 130 mM NaCl, 5 mM KCl, and 5.6 mM glucose) containing 1 mM $CaCl_2$ and 0.6 mM $MgCl_2$ and stimulated with 0.5 μg/ml anti-mouse IgE (BD Pharmingen) for 45 min at 37°C. Cell supernatants and total cell lysate solubilized by sonication were collected, and β-hexosaminidase in the supernatants and cell lysate was quantified by spectrophotometrical measurement of the hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-hexosaminidase (Sigma-Aldrich Japan, Tokyo, Japan) in 0.1 M sodium citrate buffer (pH 4.5). The reaction was terminated by the addition of 0.2 M glycine (pH 10.7). The percentage of β-hexosaminidase release was calculated using the following formula: percent release = (OD of the stimulated supernatant × 100)/(OD of the total cell lysate), where OD is optical density.

IgE-mediated CysLT export from murine BMMCs. BMMCs of $mrp^{+/+}$ and $mrp^{-/-}$ mice were prepared, cultured, and incubated with TNP-IgE and anti-IgE antibody as described above. The cells were separated from the medium by centrifugation. The amount of CysLT secreted into the supernatant was quantitated using the ELISA kit (Cayman Chemicals). The cells were resuspended in lysis buffer, homogenized, centrifuged, and then collected for determination of intracellular CysLT. Each experiment was performed in triplicate.

Statistics. Data are means ± SD and were analyzed using the unpaired *t*-test. Differences between means were considered statistically significant at $P < 0.05$.

RESULTS

Histopathology of the lungs of $mrp1^{-/-}$ and $mrp1^{+/+}$ mice. To investigate the biological significance of MRP1 in vivo in allergic airway inflammation, we sensitized $mrp1^{-/-}$ and $mrp1^{+/+}$ mice to OVA and challenged them with OVA by aerosol. Control mice received PBS. The lungs from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice exposed to PBS aerosol showed normal lung histology in both groups (Fig. 1, *A* and *B*). Sensitization and subsequent exposure to OVA resulted in peribronchial and perivascular inflammation both in the $mrp1^{-/-}$ and $mrp1^{+/+}$ mice, and excessive production of airway mucus glycoproteins by goblet cells in airway epithelium was observed (Fig. 1, *C–F*). However, this inflammation following OVA exposure was reduced in $mrp1^{-/-}$ mice compared with $mrp1^{+/+}$ mice (Fig. 1, *C* and *D*). To evaluate the extent of inflammation, we employed a semiquantitative scoring system as described previously (9). As shown in Fig. 1*G*, blinded semiquantitative grading of the lung sections revealed a statistically significant difference in the degree of airway inflammation between the $mrp1^{-/-}$ and $mrp1^{+/+}$ mice ($P = 0.0143$). In addition, blinded semiquantification of goblet cell staining with PAS also revealed attenuated mucus scores in OVA-exposed $mrp1^{-/-}$ mice compared with OVA-exposed $mrp1^{+/+}$ mice (Fig. 1*H*) ($P = 0.0431$). These data indicate that airway inflammation and goblet cell hyperplasia are reduced in OVA-exposed $mrp1^{-/-}$ mice compared with OVA-exposed $mrp1^{+/+}$ mice.

Inflammatory cell recruitment in BALF. The recovery of cells from the BALF of PBS-exposed $mrp1^{-/-}$ and $mrp1^{+/+}$ mice revealed a predominance of alveolar macrophages in both groups, without any significant differences (data not shown). Aerosol challenge of mice with OVA induced a marked increase in the total cell numbers compared with control groups with PBS (Fig. 2). However, the total cell numbers in BALF were significantly decreased in OVA-exposed $mrp1^{-/-}$ mice compared with OVA-exposed $mrp1^{+/+}$ mice ($P = 0.0243$). Differential cell counts revealed the predominant recruitment of eosinophils into BALF of both OVA-exposed $mrp1^{-/-}$ and $mrp1^{+/+}$ mice. However, OVA-exposed $mrp1^{-/-}$ mice had significantly lower numbers of eosinophils and lymphocytes than the $mrp1^{+/+}$ mice ($P = 0.0243$ and 0.0187, respectively). The numbers of macrophages and neutrophils were not significantly different between groups. These results imply that $mrp1^{-/-}$ mice show reduced recruitment of inflammatory cells, especially eosinophils and lymphocytes, into the airway lumen after OVA challenge compared with $mrp1^{+/+}$ mice.

CysLT levels in BALF. To investigate the role of MRP1 as a CysLT export pump in vivo, we measured total CysLT levels in BALF from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice exposed to PBS or OVA aerosol. As shown in Fig. 3, levels of CysLTs in BALF

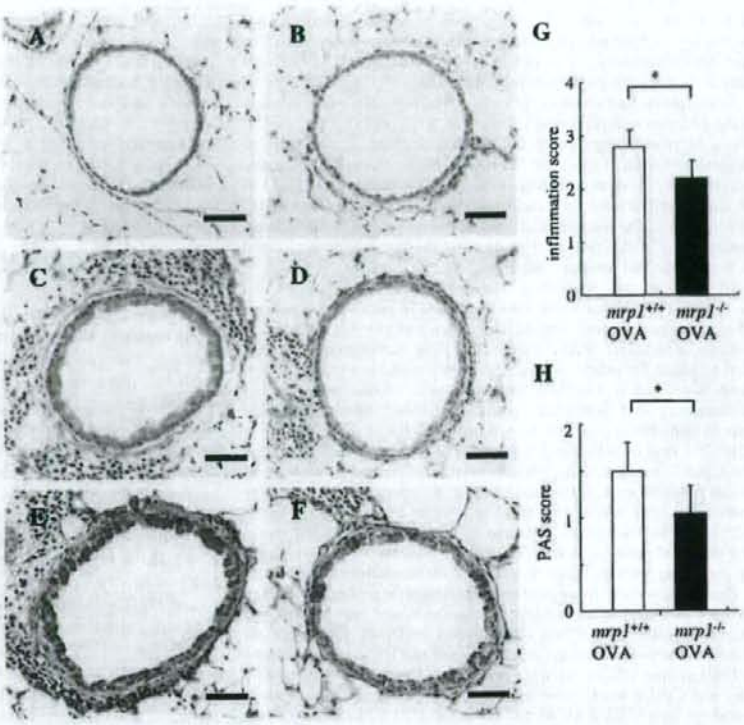

Fig. 1. Histological analysis of lung sections of multidrug resistance-associated protein 1 (MRP1)-deficient (*mrp1*$^{-/-}$) mice and wild-type *mrp1*$^{+/+}$ mice. Representative photomicrographs of hematoxylin- and eosin-stained (*A–D*) and periodic acid-Schiff-stained lung sections (*E* and *F*). Scale bar, 50 μm. Lung tissues were obtained 48 h after the last challenge of PBS or ovalbumin (OVA) aerosol. *A*: *mrp1*$^{+/+}$ mice exposed to PBS aerosol. *B*: *mrp1*$^{-/-}$ mice exposed to PBS. *C* and *E*: *mrp1*$^{+/+}$ mice exposed to OVA. *D* and *F*: *mrp1*$^{-/-}$ mice exposed to OVA. Semiquantitative analyses of inflammatory cell infiltration (*G*) and mucus production (*H*) in lung sections were performed as previously described (14). Scoring of inflammatory cells and goblet cells was performed in at least 15 different fields for each lung section. To prevent observer bias, samples were coded and examined in a blind manner. Mean scores were obtained from 6 animals. *$P < 0.05$.

from OVA-exposed *mrp1*$^{-/-}$ mice were significantly lower than those from OVA-exposed *mrp1*$^{+/+}$ mice ($P = 0.0082$).

OVA-specific IgE and cytokine levels. To further assess the mechanism for the reduced airway inflammation in OVA-exposed *mrp1*$^{-/-}$ mice, we measured OVA-specific IgE levels in BALF and serum samples. As shown in Fig. 4*A*, OVA-specific IgE levels in BALF from OVA-exposed *mrp1*$^{-/-}$ mice were significantly lower than those from OVA-exposed *mrp1*$^{+/+}$ mice ($P = 0.025$). OVA-specific IgE levels in serum were also decreased in OVA-exposed *mrp1*$^{-/-}$ mice (Fig. 4*B*) ($P = 0.0285$). We next measured Th2 cytokines IL-4 and IL-13 in each BALF sample. In PBS-exposed mice of both groups, the levels of IL-4 and IL-13 were below the lower limit of detection (data not shown). As shown in Fig. 4, *C* and *D*, levels of both IL-4 and IL-13 in BALF from OVA-exposed *mrp1*$^{-/-}$ mice were significantly lower than those from OVA-exposed *mrp1*$^{+/+}$ mice ($P = 0.0361$ and 0.0101, respectively).

IgE-mediated CysLT export from BMMCs. To examine the role of MRP1 in IgE-mediated CysLT export from mast cells, we cultured BMMCs from *mrp1*$^{-/-}$ and *mrp1*$^{+/+}$ mice and stimulated them with TNP-IgE and anti-IgE antibody. Subsequently, the amounts of released CysLTs in the cell-free culture media and the intracellular contents of CysLTs were separately analyzed. MRP1 deficiency did not affect the degranulation as determined by β-hexosaminidase release (Fig. 5*A*) and IgE receptor expression of BMMCs (data not shown). However, CysLT levels in culture media from BMMCs of *mrp1*$^{-/-}$ mice were significantly lower than those from *mrp1*$^{+/+}$ mice (Fig. 5*B*) ($P = 0.0017$). In contrast, intracellular CysLT levels in BMMCs of *mrp1*$^{-/-}$ mice were significantly higher than those of *mrp1*$^{+/+}$ mice (Fig. 5*C*) ($P = 0.0003$). These results indicate that MRP1 plays a crucial role in IgE-mediated export of CysLTs from activated mast cells.

DISCUSSION

Recent generation of *mrp1*$^{-/-}$ mice has enabled investigation of the biological function of MRP1 in vivo (30). Wijnholds et al. (31) demonstrated that ear swelling induced by topical application of arachidonic acid was dramatically reduced in the *mrp1*$^{-/-}$ mice. Importantly, they also revealed that BMMCs from *mrp1*$^{-/-}$ mice had a reduced capacity to excrete LTC4 after stimulation with calcium ionophore (31). Their report strongly suggests that MRP1 plays an important role in allergic inflammatory response in vivo. However, little is known of the implication of MRP1 in IgE-mediated transport of CysLTs from mast cells and the biological significance of MRP1 in allergic airway disease, including asthma.

In our study, we developed a murine allergic airway inflammation model by intraperitoneal OVA sensitization and airway challenge. We revealed that *mrp1*$^{-/-}$ mice showed decreased airway inflammation and goblet cell hyperplasia after OVA exposure. CysLT levels in BALF from OVA-exposed *mrp1*$^{-/-}$ mice were significantly lower than those from *mrp1*$^{+/+}$ mice.

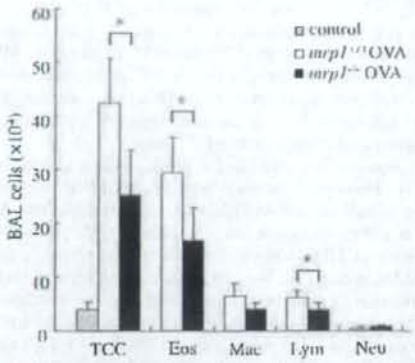

Fig. 2. Bronchoalveolar lavage fluid (BALF) cell counts. BALF were collected from *mrp1*$^{+/+}$ and *mrp1*$^{-/-}$ mice 48 h after the last PBS aerosol or OVA aerosol challenge. Total cell counts (TCC) were assessed with a standard hemocytometer. Cell populations were identified on air-dried cytocentrifuged smears (800 rpm for 5 min) after staining with Diff-Quick stain. Differential cell counts were performed on a minimum of 500 cells to identify eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu). Data are means + SD of 5 mice per group. *$P < 0.05$.

In addition, OVA-specific IgE, IL-4, and IL-13 levels in BALF were also decreased in OVA-exposed *mrp1*$^{-/-}$ mice. IgE-dependent release of CysLTs from murine BMMC's was markedly impaired due to MRP1 deficiency. These findings strongly imply that MRP1 plays a key role in the development of allergic airway disease through regulation of IgE-mediated CysLT export from mast cells. To our knowledge, our study is the first report to reveal that *mrp1*$^{-/-}$ mice are less sensitive to asthmatic response to allergen exposure by using a murine model.

IgE-mediated activation of mast cells in the airway leads to oxygenation of arachidonic acid by 5-lipoxygenase (5-LO) and generation of LTs (12). Among them, secreted CysLTs bind to CysLT receptors and induce bronchoconstriction, mucus hypersecretion, and eosinophil chemotaxis (12, 17). Therefore, inhibition of CysLT biosynthesis or receptor-mediated action is beneficial for patients with bronchial asthma (8). In our murine allergic airway inflammation model, CysLT-synthesizing cells

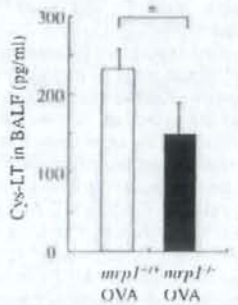

Fig. 3. Measurement of cysteinyl leukotriene (CysLT) levels in BALF. The levels of total CysLTs in the BALF were determined by EIA. Data are mean + SD of 5 mice per group. Similar results were obtained in 2 independent experiments. *$P < 0.05$.

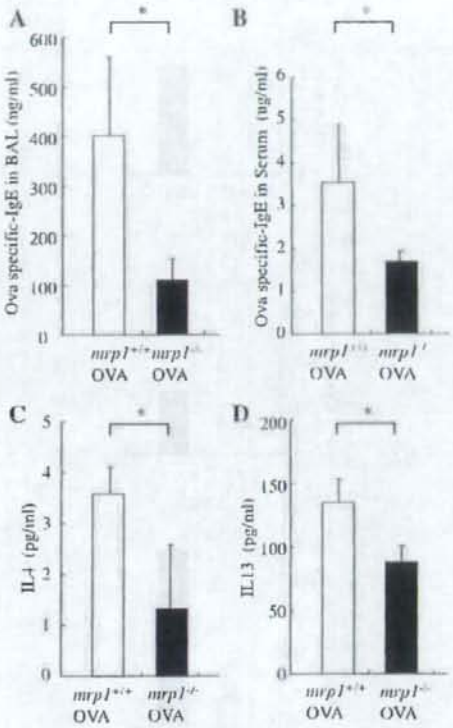

Fig. 4. Measurement of OVA-specific IgE and Th2 cytokine levels. OVA-specific IgE levels in the BALF (*A*) and serum (*B*) were measured by EIA. The levels of IL-4 (*C*) and IL-13 (*D*) in the BALF were also measured. Data are means + SD of 4–6 mice per group. Similar results were obtained in 2 independent experiments. *$P < 0.05$.

including mast cells in *mrp1*$^{-/-}$ mice had a reduced capacity to secrete CysLTs, resulting in decreased CysLT levels in BALF. Suppression of CysLT production due to MRP1 deficiency reduced recruitment of eosinophils and mononuclear cells in the lungs. These findings suggest the possibility that MRP1 inhibitor may be useful as an anti-asthma drug to attenuate airway inflammation to allergen exposure by suppressing IgE-mediated CysLT production.

Th2 inflammatory response is a central component of allergic airway inflammation. In our murine model, Th2 cytokine IL-4 and IL-13 production and lymphocyte recruitment in the lungs were significantly decreased in OVA-exposed *mrp1*$^{-/-}$ mice, resulting in decreased antigen-specific IgE production. Previous studies have demonstrated that OVA-induced airway eosinophil infiltration and goblet cell hyperplasia were markedly reduced in LTC4 synthase (LTC4S)-deficient mice compared with wild-type control mice (18). Importantly, antigen-specific IgE and Th2 cytokine expression in the lungs were also significantly reduced in OVA-exposed LTC4S-deficient mice, although delayed-type cutaneous hypersensitivity (Th1 cell-dependent response) was intact (18). Others have demonstrated that blockade of $CysLT_1$ receptor reduced elevation of IL-4