Table 2 Cell cycle distribution by Ad-p53 and PEM treatment

Cells	*Ad*[a]	*PEM*[b]	*Cell cycle distribution (%)*[c]			
			Sub-G1	*G0/G1*	*S*	*G2/M*
NCI-H2052	(−)	(−)	0.4 ± 0.1	72.2 ± 0.3	10.7 ± 0.4[d]	17.0 ± 0.6
	Ad-LacZ	(−)	0.2 ± 0.1	70.5 ± 0.3	11.1 ± 0.2	18.6 ± 0.1
	Ad-p53	(−)	0.3 ± 0.1	74.0 ± 0.1	8.4 ± 0.1	17.6 ± 0.1
	(−)	(+)	1.5 ± 0.1[e]	24.5 ± 0.4	57.6 ± 0.1[d]	17.7 ± 0.5
	Ad-LacZ	(+)	1.4 ± 0.1[e]	21.0 ± 0.5	58.8 ± 0.2	20.2 ± 0.5
	Ad-p53	(+)	10.3 ± 0.2[e]	23.5 ± 0.3	46.8 ± 0.2	20.6 ± 0.1
MSTO-211H	(−)	(−)	1.5 ± 0.1	90.6 ± 0.1	1.4 ± 0.1[d]	6.5 ± 0.2
	Ad-LacZ	(−)	5.5 ± 0.1	86.2 ± 0.2	1.7 ± 0.1	6.7 ± 0.1
	Ad-p53	(−)	7.9 ± 0.1	86.1 ± 0.2	1.1 ± 0.1	4.8 ± 0.2
	(−)	(+)	36.9 ± 0.3[e]	44.6 ± 0.3	11.3 ± 0.2[d]	8.0 ± 0.1
	Ad-LacZ	(+)	31.9 ± 0.2[e]	46.9 ± 0.3	12.6 ± 0.2	9.2 ± 0.2
	Ad-p53	(+)	44.3 ± 0.6[e]	41.8 ± 0.5	9.6 ± 0.1	5.0 ± 0.1

Abbreviations: Ad, adenoviruses; Ad-LacZ, replication-incompetent type 5 Ad expressing β-*galactosidase* gene; Ad-p53, replication-incompetent type 5 Ad expressing the wild-type *p53* gene; h, hours; PEM, pemetrexed; vp, virus particles.

[a]Cells were infected with or without Ad (NCI-H2052: 3×10^4 vp per cell; MSTO-211H: 1×10^4 vp per cell) and cultured for further 48 h.

[b]Cells were treated with or without PEM (0.03 μg ml^{-1}) for 24 h.

[c]Cell cycle profiles were analyzed with flow cytometry and data are shown in mean percentages with s.e.'s ($n=3$).

[d]$P<0.01$, comparing between PEM-treated and -untreated populations.

[e]$P<0.01$, comparing between Ad-p53 with PEM-treated cells and corresponding PEM-treated alone populations, and between Ad-p53 with PEM-treated cells and Ad-LacZ with PEM-treated populations.

showing that the expressed p14 did not influence DNA damage-induced p53 activations.[9] Ad-p14 transduction thus did not initiate the entire p53-mediated pathways or p14 may negatively affect apoptotic pathways induced by DNA-damaging agents. In contrast, Ad-p53 transduction enhanced CDDP-induced cytotoxicity as well as PEM-mediated cell killing with a synergistic manner in most of the cells tested. Ad-p53 can thus widely activate p53 downstream pathways compared with Ad-p14.

Transduction with Ad-p16 also induced G0/G1 arrest and apoptosis in mesothelioma cells and achieved better therapeutic effects than that with Ad-p14.[5,18] Restoration of p16 expression can induce dephosphorylated pRb, but precise mechanisms of the apoptosis induction remains uncharacterized because the p16-mediated pathways are not directly linked with the p53 pathways. Moreover, Yang *et al.*[18] showed that Ad-p16 did not increase CDDP-mediated cytotoxicity as well as Ad-p14.[9] Interestingly, co-transduction with Ad-14 and Ad-p16 was less effective than Ad-p16 alone, but the underlying mechanism remains unknown.[18] A combinatorial use of Ad-p53 and Ad-p16 needs to be tested to clarify whether restoration of the p53 and the pRb pathways may compete in terms of cytotoxicity in mesothelioma. We speculate at this moment that Ad-p53 would produce better anti-tumor effects than Ad-p16 as Ad-p53 can restore the p53-mediated pathways at a full scale and the pRb-mediated pathways as well.

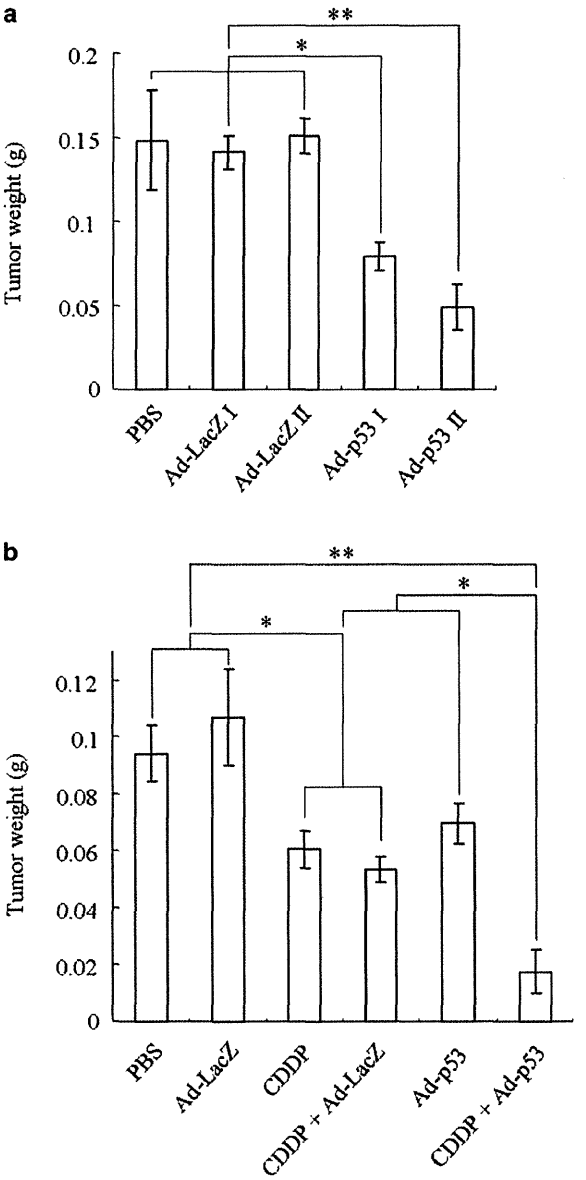

Figure 5 Anti-tumor effects *in vivo* produced by adenoviruses bearing the *p53* gene (Ad-p53) with cisplatin (CDDP). MSTO-211H cells (1×10^6) were injected into the intrapleural cavity of nude mice ($n=6$) (day 1) and they were treated as indicated on day 3. (**a**) Intrapleural injection of Ad-p53 or Ad-LacZ with two different doses (I: 1×10^{10}; II: 1×10^{11} viral particles (vp) per mouse), or phosphate buffered-saline (PBS) as a control. (**b**) Intraperitoneal injection of CDDP (0.12 mg per mouse) and intrapleural injection of Ad-p53 or Ad-LacZ (1×10^{10} vp per mouse). The tumor weights were measured on day 28. Means and s.e. bars are shown. *$P<0.05$; **$P<0.01$.

Previous studies showed that combination of Ad-p53 and a DNA-damaging agent was greater in the cytotoxicity than Ad-p53 alone,[19] although contradictory studies were also reported.[20] This study firstly demonstrated in *p53*-wild-type mesothelioma cells that augmented p53

expressions produced synergistic anti-tumor effects with CDDP or PEM in most of the cases and even in an orthotopic animal model. Notably, NCI-H2052 cells, insensitive to Ad-p53-mediated cytotoxicity, increased the sensitivity to CDDP and to PEM with Ad-p53 transduction, which verified the benefit of the combination. In addition, the combinatory effects of Ad-p53 with PEM, an novel anticancer agent, is firstly demonstrated in this study, although a possible combination of Ad-p53 and DNA/RNA synthesis inhibitors such as 5-fluorouracil has been reported.[21] Activities of enzymes involved in PEM metabolisms may play a crucial role in the anti-tumor effects, but the relationship between the *p53* status and PEM sensitivity has been unclear.[22,23] An administration schedule of an anticancer agent and Ad-p53 might be influential to the cytotoxic activity, but our preliminary data did not show any significant difference in the cytotoxicity irrespective of the administration order (data not shown).

In conclusion, this study analyzed the anti-tumor effects of Ad-p53 to mesothelioma cells and demonstrated combinatory effects with CDDP or PEM, which are the first-line agents for mesothelioma. These data indicate a clinical feasibility of Ad-p53 and moreover there are several advantages of intrapleural administration of Ad-p53. Negative intrapleural pressure facilitates Ad distributions into mesothelioma that spreads along the pleura. The administration diminishes rapid Ad uptake in the liver and subsequently decreases Ad-induced acute hepatoxicity. In addition, a previous study revealed that administration of Ad into the pleural cavity did not induce any serious adverse reaction.[24] We propose based on this study that intrapleural injection of Ad-p53 with systemic administration of the first-line agents can be a therapeutic strategy for intractable mesothelioma.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Survival of Japanese Patients with Pulmonary Arterial Hypertension after the Introduction of Endothelin Receptor Antagonists and/or Phosphodiesterase Type-5 Inhibitors

Seiichiro Sakao, Nobuhiro Tanabe, Yasunori Kasahara and Koichiro Tatsumi

Abstract

Objective Although endothelin receptor antagonists (ERAs) and phosphodiesterase type 5 (PDE5) inhibitors have become the most commonly used treatments for pulmonary arterial hypertension (PAH) since their introduction in 2005, it remains unknown whether these medications play a significant role in the survival of Japanese patients with PAH.

Methods The cardiac catheterization and survival data of 103 PAH patients were retrospectively reviewed. A comparison of survival benefits with regard to the type of PAH was completed in PAH patients diagnosed between 2005 and 2012 and those diagnosed between 1983 and 2004 and in patients undergoing treatment with ERAs and/or PDE5 inhibitors and those being treated with conventional therapy and/or oral beraprost. Although pulmonary vascular resistance (PVR) at baseline differed, the more recent group showed better survival rates compared with those observed in the early group (5-year survival: 70.1% vs. 44.8) ($p<0.05$). In addition, the survival of PAH patients treated with ERAs and/or PDE5 inhibitors was superior to that of the patients treated without these medications (5- and 8-year survival: 77.8% and 66.7% vs. 39.0% and 37.0%, respectively) ($p<0.05$), especially in patient with idiopathic and heritable PAH.

Conclusion Superior survival rates are observed in patients with idiopathic and heritable PAH after introduction of ERAs and PDE5 inhibitors, and the use of these drugs provides benefits for survival.

Key words: pulmonary arterial hypertension (PAH), endothelin receptor antagonists (ERAs), phosphodiesterase type 5 (PDE5) inhibitors

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Introduction

An important pathological feature of pulmonary arterial hypertension (PAH) is pulmonary vascular remodeling associated with marked proliferation of pulmonary artery endothelial cells (ECs) and/or smooth muscle cells (SMCs) as well as components of the extracellular matrix that results in the obstruction of blood flow in resistant pulmonary arteries (1, 2). Moreover, it appears that all of these conditions ultimately lead to signaling imbalances between vasoconstrictive (e.g., endothelin) and vasodilatory (e.g., prostacyclin and nitric oxide) compounds (3).

There are three classes of drugs approved for the evidence-based treatment of PAH (4): prostacyclin analogues (e.g., epoprostenol, beraprost, treprostinil and iloprost [treprostinil and iloprost are approved outside Japan]), endothelin receptor antagonists (ERAs) (5, 6) (e.g., ambrisentan and bosentan) and phosphodiesterase type 5 (PDE5) inhibitors (7) (e.g., sildenafil and tadalafil). These drugs, which are currently used for the treatment of PAH, act not only by opposing any abnormal vasoconstriction, but also by inhibiting the growth of normal SMC (2). Because the drugs currently approved to treat PAH are not curative, patients require long-term therapy. In addition, long-term use of these drugs may provide sustained benefits in terms of exercise capacity and pulmonary hemodynamics in comparison to placebos or historical controls in patients with PAH (3).

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Because of their availability and convenience, oral drugs (e.g., ambrisentan, bosentan, sildenafil and tadalafil) have recently become common treatments for PAH in Japan after being introduced in 2005. However, it remains to be elucidated whether these oral medications significantly improve survival in Japanese patients with PAH in comparison to conventional therapy.

Historically, the management of patients with PAH has been limited to conventional therapies such as anticoagulants, calcium channel blockers, diuretics, digoxin and supplemental oxygen. Beraprost was the first orally active and chemically stable prostacyclin analog to be developed and has been available for the treatment of PAH in Japan since 1992. Patients treated with beraprost demonstrate improvements in exercise capacity and symptoms within short-term durations (8). However, no beneficial effects of oral beraprost are observed on exercise capacity at nine or 12 months (9). This indicates that oral beraprost may not have sustained long-term effects. Therefore, this drug has a weak recommendation in the PAH evidence-based treatment algorithm (4) and has been approved only in Japan and Korea (10). In fact, because of its cost and availability, oral beraprost was the first-line therapy for PAH in Japan before the approval of ERAs and PDE5 inhibitors for the treatment of PAH. Therefore, in this study, the beneficial effects of ERAs and PDE5 inhibitors on survival were evaluated without regard to oral beraprost therapy.

The aim of this study was to investigate cumulative survival benefits with regard to types of PAH in patients with PAH after the introduction of ERAs and PDE5 inhibitors in comparison to those observed in patients treated with conventional therapy and/or oral beraprost.

Materials and Methods

Study subjects

From June 1983 to February 2012, 103 patients older than 15 years with PAH were treated at Chiba University Hospital. In all patients, the diagnosis of PAH was established using cardiac catheterization and based on a documented mean pulmonary arterial pressure ≥25 mmHg and a pulmonary capillary wedge pressure or left ventricular end-diastolic pressure ≤15 mmHg. All patients were classified as Group 1.1 to 1.4 on the current Dana Point classification (11). The study exclusion criteria were: 1) distal chronic thromboembolic pulmonary hypertension; 2) pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH); 3) left heart disease; and 4) chronic pulmonary disease (11). Although six of the 103 patients were classified as being in WHO function class IV, only four patients were treated with intravenous infusion of epoprostenol. Three of these patients were treated with ERAs and/or PDE5 inhibitors before the introduction of epoprostenol. According to Japanese legislation, informed consent is not required for retrospective collection of data corresponding to current practice. However, the database was anonymized and complied with the restrictive requirements of the Ministry of Health, Labor and Welfare dedicated to privacy, information technology and civil rights in Japan. The Ethics Committees of Chiba University Hospital approved the study protocol.

Efficacy measurements

The subjects were retrospectively divided into two groups: those diagnosed between 1983 and 2004 (n=66) and those diagnosed between 2005 and 2012 (n=37), since the introduction of ERAs and PDE5 inhibitors in Japan occurred in 2005. The subjects were further separated into two groups: those treated with ERAs and/or PDE5 inhibitors (n=36) and those treated with conventional therapy, including anticoagulants, calcium channel blockers, diuretics, digoxin, supplemental oxygen and/or oral beraprost and epoprostenol (n=67). In this analysis, the most recent hemodynamic data obtained before treatment were investigated. For more detailed analyses, the subjects were divided into subgroups according to types of PAH. The survival status of all patients was followed on a yearly basis and at the end of the study. Five of the 103 patients were lost to follow-up. The date of initiation of ERAs and PDE5 inhibitors was selected as the starting point to determine the survival period for assessing the effects of the drugs. The survival rates were calculated for all patients and by subgroups using Kaplan-Meier estimates. The baseline demographic and hemodynamic data were investigated in all patients and by subgroups.

Statistical analysis

The data were analyzed using JMP 9.0.0 (Japanese version, SAS Institute Inc., Tokyo, Japan) and the Excel-Toukei 2010 software program (Social Survey Research Information Co., Ltd., Tokyo, Japan). All results are expressed as the mean ± the SD for continuous variables and as the number or percentage for categorical variables. The baseline demographic and hemodynamic data were compared using unpaired Student's *t*-tests. The survival from all-cause death was estimated using the Kaplan-Meier method, and differences between groups were examined for significance using the log-rank test. Univariate and multivariate cox proportional hazards models were used to investigate the independent effects of the factors on survival. A p value of 0.05 was considered to be statistically significant.

Results

Baseline characteristics

One hundred and three patients with PAH were initially enrolled in this study (Table 1). The mean patient age was 46.9±15.0 years (range: 15 to 75) with a 4:1 female to male ratio. Forty-four patients (42.7%) were identified as having idiopathic and heritable PAH. The patients were followed for a mean period of 58.3±61.3 months and a median of

Table 1. Baseline Clinical Characteristics and Hemodynamics in the Patients with PAH

	1983-2012, n=103	1983-2004, n=66	2005-2012, n=37
Female/male	86/17	53/13	33/4
Age(yrs)	46.9±15.0	46.0±15.4	48.3±14.4
Survivor, n (%)	50 (47.6%)	21 (31.8%)	29 (78.4%)
Type of PAH			
Idiopathic and heritable PAH, n (%)	44 (42.7%)	31 (47.0%)	13 (35.1%)
PAH associated with connective tissue disease, n (%)	39 (37.9%)	25 (37.9%)	14 (37.8%)
PAH associated with congenital heart disease, n (%)	8 (7.8%)	3 (4.5%)	5 (13.5%)
PAH associated with portal hypertension, n (%)	12 (11.7%)	7 (10.6%)	5 (13.5%)
Hemodynamics			
mRAP, mmHg	5.2±6.1	4.3±4.7	6.9±7.8
mPAP, mmHg	47.9 ±13.2	48.8 ±13.9	46.2 ±11.7
mPCWP, mmHg	6.4±3.0	5.7±2.9	7.6±2.7*
CO, L/min	4.2±1.4	3.9±1.2	4.7±1.5*
CI, L/min per m^2	2.7±0.9	2.5±0.8	3.1±1.0*
PVR, dyne sec cm^{-5}	898.7±504.6	984.6±521.2	754.8±446.1*
mSAP, mmHg	89.3±14.9	90.5±13.4	87.2±17.2
Heart rate, beats/min	78.2±13.8	79.3±13.9	76.4±13.8
SvO_2, %	68.4±8.6	68.3±9.0	68.5±8.0
WHO functional class			
I, n (%)	1 (1.2%)	0 (0%)	1 (2.7%)
II, n (%)	32 (37.2%)	17 (32.7%)	15 (40.5%)
III, n (%)	47 (54.7%)	30 (57.7%)	17 (46.0%)
IV, n (%)	6 (7.0%)	5 (9.6%)	1 (2.7%)
Treatment			
Bosentan, n (%)	22 (21.4%)	8 (12.1%)	14 (37.8%)
Ambrisentan, n (%)	1 (1.0%)	0 (0.0%)	1 (2.7%)
Sildenafil, n (%)	20 (19.4%)	3 (4.5%)	17 (45.9%)
Tadalafil, n (%)	2 (1.9%)	0 (0.0%)	2 (5.4%)
Epoprostenol, n (%)	4 (3.9%)	3 (4.5%)	1 (2.7%)
Oral beraprost, n (%)	36 (35.0%)	20 (30.3%)	16 (43.2%)

*p<0.05; vs 1983-2004

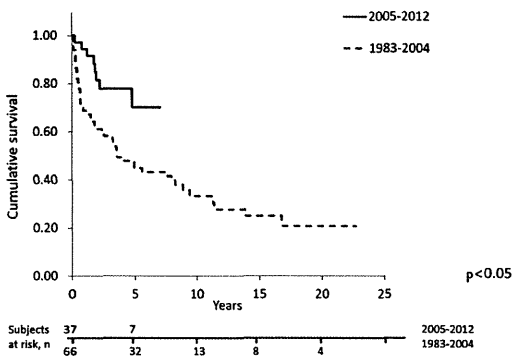

Figure 1. The Kaplan-Meier survival estimates for the PAH patients. The survival rate for patients treated between 2005 and 2012 (solid line) was 70.1% at 5-years compared with 44.8% for patients treated between 1983 and 2004 (dashed line; p<0.05 by the Cox-Mantel log-rank test).

41.5±61.3 months (range: 1 to 276). The mean pulmonary arterial pressure (PAP) and mean pulmonary vascular resistance (PVR) were 47.9 ±13.2 mmHg and 898.7±504.6 dyne. sec. cm^{-5}, respectively.

Survival

Patients diagnosed between 1983 and 2004 (n=66) vs. those diagnosed between 2005 and 2012 (n=37)

We divided the patients into two groups based on the timing of diagnosis (Table 1). We found that the patients diagnosed between 2005 and 2012 had a better survival rate than the patients diagnosed between 1983 and 2004 (5-year survival: 70.1% vs. 44.8%) (p<0.05) (Fig. 1). However, cardiac output (CO) and pulmonary vascular resistance (PVR) at baseline significantly differed between the two groups (Table 1), and these differences make it difficult to attribute the superior outcome to the introduction of ERAs and PDE5 inhibitors.

Outcomes of patients treated with ERAs and/or PDE5 inhibitors (n=36) and those treated with conventional therapy and/or oral beraprost (n=67)

To elucidate the absolute benefits of ERAs and/or PDE5 inhibitors on the survival of Japanese patients with PAH, an analysis was completed comparing the results of patients treated with ERAs and/or PDE5 inhibitors (n=36) and the

Table 2. Baseline Clinical Characteristics and Hemodynamics in the Patients with PAH

	The PAH patients, n=106		The idiopathic and heritable PAH patients, n=44		The associated PAH patients, n=59	
	ERA and/or PDE5 Inhibitors therapy, n=36	PAH therapies without ERA and PDE5 inhibitor, n=67	ERA and/or PDE5 Inhibitors therapy, n=16	PAH therapies without ERA and PDE5 inhibitor, n=28	ERA and/or PDE5 Inhibitors therapy, n=20	PAH therapies without ERA and PDE5 inhibitor, n=39
Female/male	33/3	53/14	13/3	19/9	20/0	34/5
Age(yrs)	47.3±14.4	46.7±15.5	44.8±14.0	41.7±15.2	49.2±14.7	50.2±14.9
Survivor, n (%)	28 (69.4%)	21 (29.9%)	14 (87.5%)	5 (17.9%)	14 (70.0%)	16 (41.0%)
Hemodynamics						
mRAP, mmHg	5.5±7.9	5.1±4.9	3.9±2.7	5.3±6.6	6.8±410.2	5.0±3.4
mPAP, mmHg	44.9 ±11.8	49.5 ±13.7	44.4 ±11.1	54.5±16.7*	45.3 ±12.6	46.2±10.1
mPCWP, mmHg	6.7±2.4	6.2±3.2	6.6±2.5	6.0±3.4	6.8±2.4	6.4±3.2
CO, L/min	4.4±1.1	4.1±1.5	4.4±0.9	3.7±1.4	4.4±1.3	4.4±1.6
CI, L/min per m^2	2.9±0.7	2.7±1.0	2.7±0.5	2.4±0.8	3.0±0.9	2.8±1.1
PVR, dyne $sec\ cm^{-5}$	771.4±440.3	971.4±527.6*	736.7±338.9	1166.7±609.7*	799.2±514.4	842.9±427.2
mSAP, mmHg	88.8±14.2	89.6±15.3	84.4±14.9	89.2±14.1	92.3±12.9	89.9±16.3
Heart rate, beats/min	75.9±12.7	79.6±14.3	73.1±13.3	83.0±14.3	78.1±12.1	77.2±14.0
SvO_2, %	68.1±7.7	68.5±9.1	67.5±6.1	66.1±9.5	68.7±8.9	70.1±8.7
WHO functional class						
I, n (%)	0 (0.0%)	1 (1.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.6%)
II, n (%)	15 (41.7%)	16 (23.9%)	6 (37.5%)	5 (17.9%)	9 (45.0%)	11 (28.2%)
III, n (%)	12(33.3%)	32(47.8%)	8 (50.0%)	14 (50.0%)	7 (35.0%)	18(46.2%)
IV, n (%)	1 (2.8%)	5 (7.5%)	0 (0.0%)	3 (10.7%)	1 (5.0%)	2 (5.1%)
Treatment						
Bosentan, n (%)	22 (61.1%)	0 (0.0%)	11 (68.8%)	0 (0.0%)	11 (55.0%)	0 (0.0%)
Ambrisentan, n (%)	1 (2.8%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Sildenafil, n (%)	18 (50.0%)	0 (0.0%)	7 (43.8%)	0 (0.0%)	11 (55.0%)	0 (0.0%)
Tadalafil, n (%)	2 (5.6%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	1 (5.0%)	0 (0.0%)
Epoprostenol, n (%)	3 (8.3%)	1 (1.5%)	3 (18.8%)	0 (0.0%)	0 (0.0%)	1 (2.6%)
Oral beraprost, n (%)	14 (38.9%)	22 (32.8%)	7 (43.8%)	8 (28.6%)	7 (35.0%)	14 (35.9%)

*p<0.05; vs ERA and/or PDE5 Inhibitors therapy

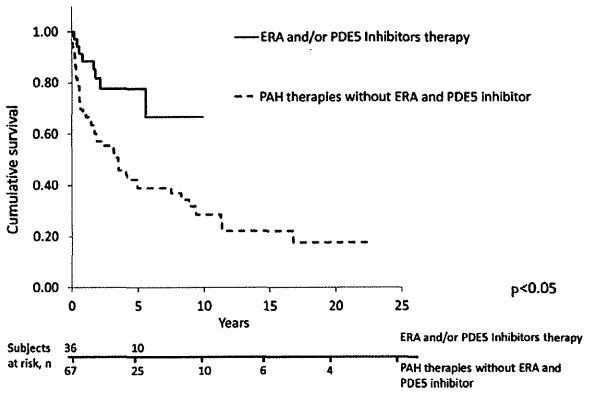

Figure 2. The Kaplan-Meier survival estimates for the PAH patients. The survival rates for patients treated with ERA and/or PDE5 inhibitor therapy (solid line) were 77.8% and 66.7% at five and eight years compared with 39.0% and 37.0% for patients treated with PAH therapies without ERA or PDE5 inhibitors (dashed line; p<0.05 by the Cox-Mantel log-rank test).

results of patients treated without ERAs and/or PDE5 inhibitors (n=67) (Table 2). A significant difference was observed between the two groups in the Kaplan-Meier survival curve (77.8% and 66.7% vs. 39.0% and 37.0%, respectively) (p< 0.05) (Fig. 2) and in PVR (Table 2). In particular, the patients with idiopathic and heritable PAH treated with ERAs and/or PDE5 inhibitors (n=16) showed significantly better survival outcomes than those not treated with these drugs (n=28) (Table 2) (5- and 8-year survival: 92.9% and 69.6% vs. 26.0% and 20.8%, respectively) (p<0.05) (Fig. 3). However, in the associated PAH patients (Table 2), no significant differences were observed between the groups (Fig. 4).

A univariate Cox proportional hazard analysis showed that cardiac index (CI), mean pulmonary arterial pressure (mPAP) and the use of ERA and/or PDE5 inhibitor therapy were associated with cumulative survival. Moreover, CI and ERA and/or PDE5 therapy were the significant predictors of survival in the multivariate analysis. The use of ERA and/or PDE5 inhibitor therapy was an independent predictor for superior outcomes (Table 3).

Discussion

The data presented here show that the patients treated be-

Table 3. Univariate and Multivariate Cox Proportional Hazards Models for the Predictors of Survival

Factors	Univariate		Multivariate	
	HR (95% CI)	p	HR (95% CI)	p
CI (L/min per m^2)	0.56 (0.423-0.747)	0.0001	0.60 (0.458-0.810)	0.0008
mPAP (mmHg)	1.03 (1.005-1.048)	0.0160	1.02 (0.992-1.038)	0.1875
ERA and/or PDE5 Inhibitors therapy (vs. PAH therapies without ERA and PDE5 inhibitor)	0.32 (0.137-0.636)	0.0008	0.39 (0.168-0.796)	0.0084

ERAs: endothelin receptor antagonists, PDE5: phosphodiesterase type 5 (PDE5)

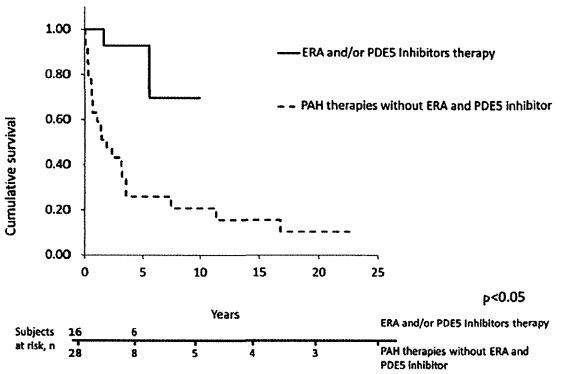

Figure 3. The Kaplan-Meier survival estimates for the idiopathic and heritable PAH patients. The survival rates for patients treated with ERA and/or PDE5 inhibitor therapy (solid line) were 92.9% and 69.6% at five and eight years compared with 26.0% and 20.8% for patients treated with PAH therapies without ERA or PDE5 inhibitors (dashed line; p<0.05 by the Cox-Mantel log-rank test).

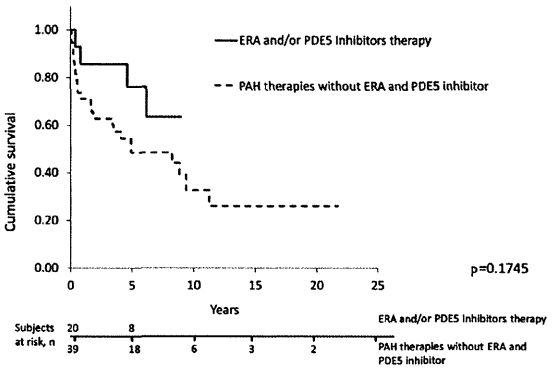

Figure 4. The Kaplan-Meier survival estimates for the associated PAH patients. The survival rates for patients treated with ERA and/or PDE5 inhibitor therapy (solid line) were 76.2% and 63.5% at five and eight years compared with 48.5% and 32.6% for patients treated with PAH therapies without ERA or PDE5 inhibitors (dashed line; p=0.1745 by the Cox-Mantel log-rank test).

tween 2005 and 2012 had a better survival rate ($p<0.05$) (Fig. 1); however, these patients had significantly less serious hemodynamic alterations (Table 1). The hemodynamic difference observed between the groups makes it difficult to attribute the superior outcome to the introduction of ERAs and PDE5 inhibitors (Fig. 1).

Although it is beyond the scope of this paper to argue delaying the diagnosis, the presence of less serious hemodynamic alterations in the patients diagnosed between 2005 and 2012 suggests that earlier detection of PAH in patients with mild/moderate hemodynamic changes was achieved more often in that group than in the group diagnosed between 1983 and 2004. Since signs and symptoms of PAH do not generally manifest until hemodynamic changes are advanced, there are significant delays in diagnosing this disease. Although primary pulmonary hypertension (PPH) registry data from 1987 show that, at that time, the time from symptom onset to diagnosis with catheterization was 2.3 years (12), there have not been any recent advances in the diagnostic processes. The registry to evaluate early and long-term PAH (REVEAL) study showed that the average time from onset to diagnosis is still more than two years (13). The advances observed in this study may be based on the development and approval of oral therapies and increased doctor recognition of PAH following the introduction of these drugs.

This single-center, uncontrolled study demonstrated that idiopathic and heritable PAH patients treated with ERAs and/or PDE5 inhibitors (n=13) have a higher survival rate than those treated with conventional therapy and/or oral beraprost (Fig. 3, Table 2). In line with previous reports (3), this result may support the concept that the use of these drugs provides benefits for the survival of Japanese patients with PAH. Nevertheless, the benefits of ERAs and/or PDE5 inhibitors on survival may be restricted to idiopathic and heritable PAH patients because no significant differences were observed between the groups in the associated PAH patients (Table 2, Fig. 4).

Because oral beraprost has a weak recommendation in the PAH evidence-based treatment algorithm (4) and has so far only been approved in Japan and Korea (10), this study was conducted without regard to oral beraprost therapy. However, this is a limitation of this study. It is impossible to deny that oral beraprost has a beneficial effect on the treatment of PAH. A randomized and properly controlled dose-response study of beraprost is currently underway (14).

There may be beneficial effects of high-dose oral beraprost on exercise capacity and hemodynamics in patients with PAH.

Epoprotenol was approved in Japan in 1999, and 23 patients treated after 1999 died in our center. In this study, only four patients (three between 1983 and 2004 and one between 2005 and 2012) were treated with epoprostenol and all survived. Although epoprotenol is strongly recommended by the WHO/NYHA class IV according to recent guidelines, we were unable to administer intravenous treatments in some of the 23 non-survivor cases because the patients were elderly (>70 years of age) (n=2), had comorbidities (n=5) or were unwilling (n=6) to undergo intravenous treatments. However, the early administration of epoprostenol therapy is suggested to improve survival in patients with a PVR >1,000 dynes.sec.cm^{-5}.

The data presented in this study were limited because this was an observational study from a single center and the PAH patients were not treated in a randomized manner according to hemodynamics and comorbidities, i.e., this study included patients treated with conventional therapy, which may have favorably biased the results. We realize the limitations of interpreting our results. We interpreted the results of this study as part of a hypothesis-generating analysis, which suggested that there are beneficial effects of treatment with ERAs and/or PDE5 inhibitors on overall survival in idiopathic and heritable PAH patients. This hypothesis will need to be further investigated in a large confirmatory long-term trial in the future.

This study has evolved over the 28-year time period of our practice. The results of six minute walk distance (6 MWD) tests and brain natriuretic peptide (BNP) tests were not obtained as consistently in the past as they have been in the most recent eight years. For this reason, in this observational study, we evaluated survival benefits only in PAH patients treated with PAH-specific therapy in comparison to patients treated with conventional therapy, instead of comparing the 6MWD and BNP results.

In conclusion, this study suggests that superior survival rates are observed in patients with idiopathic and heritable PAH after the introduction of ERAs and PDE5 inhibitors, and the use of these drugs provides a survival benefit in patients with idiopathic and heritable PAH.

Author's disclosure of potential Conflicts of Interest (COI).
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Expression of a murine homolog of apoptosis-inducing human IL-24/MDA-7 in murine tumors fails to induce apoptosis or produce anti-tumor effects

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ABSTRACT

Expression of human interleukin (IL)-24 in tumors achieved anti-tumor effects through apoptosis. IL-24 also induced secretion of proinflammatory cytokines, suggesting the role in immunity. We showed that murine IL-24 transcripts started from the second initiation codon and that expressed *mIL-24* in tumors failed to induce apoptosis. Proliferation of murine cells expressing mIL-24 was the same as that of the parent cells and inoculation of the mIL-24-expressing tumors into syngeneic mice did not produce anti-tumor effects. Secretory mIL-24 did not induce the expression of the *IL-6*, *TNF-α* or *IFN-γ* gene in spleen cells. Expression of mIL-24 receptor subunits, *IL-22R* and *IL-20R1*, was undetectable in spleen cells even though they were stimulated by anti-CD3, anti-CD40 antibody or concanavalin A. Transduction of murine tumors with adenoviruses expressing the human *IL-24* gene however suppressed the viability and decreased the tumor growth. These data suggest that mIL-24 is functionally irrelevant to the human counterpart.

1. Introduction

Human interleukin-24 (hIL-24), belonging to the IL-10 family, is expressed in lymphoid cells including monocytes, natural killer and T cells, and can induce interferon-γ (IFN-γ) and proinflammatory cytokines such as IL-6 and tumor necrosis factor-α (TNF-α) [1,2]. The cytokine was initially identified as a melanoma differentiation-associated gene 7 (MDA-7) and the expression was linked inversely with pathological progression of melanocytes to melanoma [3]. Subsequent studies demonstrated that forced expression of hIL-24 with adenoviruses vector (Ad/hIL-24) had a strong apoptosis-inducing activity in various types of human tumors but not in normal tissues, and produced anti-tumor effects through a number of non-immunological mechanisms including endoplasmic reticulum stress responses and anti-angiogenesis [3,4]. IL-24 thereby has at least two functions as a cytokine and a tumor suppressor, and Ad/hIL-24 have been investigated for its clinical feasibility [3]. Immunological activities of hIL-24 in the anti-tumor responses have not however been well characterized compared with its non-immunological properties although hIL-24 can increase several T helper 1 (Th1) type cytokines and CD8-positive cell numbers [2].

Murine FISP (IL-4-induced secreted protein), which is about 70% identical to hIL-24 at the amino acid level, is considered to be a homolog of hIL-24 [1,5] (Supplementary Fig. 1). The initial study showed that murine IL-24 (mIL-24) was induced by IL-4 in Th2 type cells [5]. The biological and immunological functions however remained uncharacterized in contrast to hIL-24-mediated biological activities. There were used to be two types of mIL-24 cDNA reported in the database of the National Center for Biotechnology Information, AF333251 (originally reported as FISP cDNA) and the previous version of NM_053095 (the sequences are currently revised to be identical to AF333251). Predicted amino acid sequences of both cDNAs are identical in the coding region but different in the signal sequence. There are two forms of IL-24 receptors (IL-24R) in human, IL-22R/IL-20R2 and IL-20R1/IL-20R2 complexes, which mediate hIL-24 signals [1,6] but the functional murine IL-24R structures have not yet been analyzed. Moreover,

Abbreviations: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MDA-7, differentiation-associated gene 7; Ad, adenoviruses; Th1, T helper 1; IL-24R, interleukin-24 receptors; RT-PCR, reverse transcription-polymerase chain reaction; MOI, multiplicity of infection; GFP, green fluorescence protein; Con A, concanavalin A; PMA, phorbol myristate acetate.

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mIL-24-mediated anti-tumor effects from immunological standpoints have been scarcely investigated.

Expression of cytokine genes in tumors can augment cell-mediated immunity and consequently produce possible anti-tumor effects when they are inoculated in mice [7]. The forced expression in tumors activates dendritic cells and augments the activities of natural killer cells and cytotoxic T cells. The animal experiments with such cytokine-producing cells thereby are a method to examine whether the cytokine can produce immunological responses in *in vivo* settings. In this study, we examined whether mIL-24 could have an apoptosis-inducing activity on murine tumors and achieve anti-tumor effects in syngeneic mice.

2. Materials and methods

2.1. Cells and mice

BALB/c and C57/BL6 mice (6-week-old females) were purchased from Japan SLC (Shizuoka, Japan). Both ecotropic ψ2 and amphotropic PA317 packaging cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

2.2. Isolation of mIL-24 cDNA

mIL-24 cDNA was prepared by a reverse transcription–polymerase chain reaction (RT–PCR) with mRNA extracted from anti-CD3 antibody-stimulated spleen cells of BALB/c mice. Synthesized first-strand cDNAs were amplified with two primers, 5′-CCGGAATTCCACAGATGAGTTGGGGACTAC-3′ (as a 5′ primer) and 5′-GTACTCGAGTCAGAGATGGTAGAATTTCTG-3′ (as a 3′ primer). Amplification was performed according to the manufacturer's recommendation (Perkin-Elmer Cetus, Norwalk, CT) and it consisted of 30 cycles under the following conditions: 30 s at 94 °C for denaturation, 30 s at 60 °C for primer annealing and 30 s at 72 °C for primer extension. The sequence of the product was confirmed to be identical to the published sequence [5].

2.3. Western blot analysis

COS-7 cells were transfected with pcDNA3.1myc-His vector (Invitrogen, Carlsbad, CA) bearing mIL-24 cDNA, the vector or pcDNA3.1 vector bearing human *c-myc* gene with lipofectin reagent (Invitrogen), and cultured for two days. Equal amounts of culture supernatants and cell lysate of the COS-7 cells were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the protein was transferred to a nylon membrane filter. The filter was reacted with monoclonal anti-human c-Myc antibody (Santa Cruz Biotech, Santa Cruz, CA) and then horseradish peroxidase-conjugated anti-mouse IgG_1 antibody. The membrane was developed with the ECL system (GE Healthcare, Buckinghamshire, UK).

2.4. Transduction of tumor cells

The retrovirus LXSN DNA vector with mIL-24 cDNA was transfected into ψ2 cells. After drug selection with G418 (Invitrogen), the G418-resistant cell supernatants were incubated with PA317 cells in the presence of polybrene (Aldrich, Milwaukee, WI). Among the G418-resistant PA317 cells, a clone that produced the largest amount of mIL-24 mRNA (PA317/mIL-24) was selected and the culture supernatants were used for infecting murine cells. G418-resistant murine cells were cloned and used for experiments. As a control, G418-resistant PA317 cells that were retrovirally transduced with LXSN bearing the *β-galactosidase* gene (PA317/LacZ) were also used.

2.5. Flow cytometry

Cells were infected with PA317/mIL-24 or PA317/LacZ cells-derived culture supernatants at a multiplicity of infection (MOI) of 10 and were cultured for 48 h. They were stained with fluorescein-isothiocyanate-conjugated annexin-V (Bender MedSystems, Vienna, Austria) and propidium iodide (PI), and were analyzed by FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). For detecting Ad-mediated infectivity, cells were incubated with Ad bearing the *green fluorescence protein* gene (Ad/GFP) at MOI = 300 for 24 h and were examined for the GFP-positive cell populations.

2.6. Northern blot analysis

RNA (20 μg) from parent and transduced cells was subjected to electrophoresis on a formaldehyde-agarose gel and transferred to a nylon filter. The filter was hybridized with [α-^{32}P]dCTP-labeled mIL-24 cDNA in a QuickHyb solution (Stratagene, La Jolla, CA). The filter was hybridized with mouse elongation factor-1α (EF-1α) as a control. Spleen cells were stimulated with plate-bound anti-CD3 (1 μg/ml, PharMingen, San Diego, CA), anti-CD40 antibody (1 μg/ml, PharMingen), or concanavalin A (Con A, 5 μg/ml, Sigma, St. Louis, MO). The filter blotted with RNA from the spleen cells and liver was hybridized with mIL-22R, mIL-20R1 or mIL-10R2 cDNA.

2.7. Ad-mediated inhibition of viability

Cells were seeded into 96-well plates and treated with Ad/hIL-24 (Introgen, Huston, TX) or Ad expressing *β-galactosidase* gene (Ad/LacZ). The growth suppressive activity was measured with a cell-counting WST kit (Dojindo, Kumamoto, Japan). The amount of formazan produced was determined with the absorbance at 450 nm.

2.8. Animal study

B16 cells untransduced or transduced with Ad/hIL-24 or Ad/LacZ, or B16 cells retrovirally transduced with mIL-24 were subcutaneously inoculated into syngeneic C57/BL6 mice. Colon 26 cells untransduced or retrovirally transduced with mIL-24 were injected subcutaneously or intraperitoneally into syngeneic BALB/c mice. Tumor volume was calculated according to the formula [1/2 × length × width^2]. Mice were assessed for survival and analysis was conducted by the Kaplan–Meier test. Statistical analysis was performed using the log-rank test.

2.9. RT–PCR for detecting cytokines

First-strand cDNA was synthesized with RNA from spleen cells that were inoculated with Ad/hIL-24 or Ad/LacZ, or were incubated with 10 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin or with supernatants from either COS-7 or COS-7 cells transfected with pcDNA3.1myc-His DNA expressing *mIL-24* gene for 6 h. Amplification of an equal amount of respective cDNA was performed for 25 cycles for *β-actin*, 30 cycles for the *IL-6* and 33 cycles for *IFN-γ* and *TNF-α* genes with the following primers and conditions: for *IL-6* gene expression, forward (5′-ACTGATGCTGGTG ACAAC-3′) and reverse (5′-TCCACAAACTGATATGCT-3′) primers, and 45 s at 94 °C for denaturation/60 s at 50 °C for primer annealing/2 min at 72 °C for primer extension: for *IFN-γ*, forward (5′-TGCGGCCTAGCTCTGAGACAATG-3′) and reverse (5′-TGAATGCTTG GCGCTGGACCTG-3′) primers, and 30 s at 94 °C/30 s at 60 °C/1 min at 72 °C; for *TNF-α*, forward (5′-TCTCAGCCTCTTCTCATTCC-3′) and reverse (5′-TACTTGGGCAGATTGACCTC-3′) primers, and 5 s at 95 °C/10 s at 56 °C/1 min at 72 °C; for *β-actin*, forward

(5′-ATGGATGACGATATCGCT-3′) and reverse (5′-ATGAGGTAGTCTGTCAGGT-3′) primers, and 5 s at 95 °C/10 s at 54 °C/70 s at 72 °C.

3. Results

3.1. Secretion of mIL-24

We initially tried to clone full-length mIL-24 cDNA based on the AF333251 cDNA sequences with RT-PCR, but failed to obtain it with mRNA from anti-CD3 antibody-stimulated spleen cells and with different 5′ primers designed between 1 and 39 of the AAK52470 (amino acid sequences based on AF333251) (Fig. 1A). We then used a downstream 5′ primer and cloned cDNA corresponding to the previous version of NM_053095 cDNA sequences which encoded the previous version of NP_444325 amino acid sequences (the sequences are currently revised to be identical to AAK52470). The AAK52470 contained the complete NP_444325 sequences and possessed extra 39 amino acids in the 5′ region (Fig. 1A). The NM_053095 sequences showed that the previous NP_444325 started from the second initiation codon of the AF333251. Both amino acid sequences consequently encoded the same mature secretory protein with different putative signal peptides which had the same 3′ side sequences (Fig. 1A). The Kyte-Doolittle hydropathy plot suggested that the sequences of NP_444325 amino acids at 1–19 has less possibility of a putative signal sequence compared with those of AAK52470 at 1–65 (Fig. 1B). We nevertheless examined possible production of mIL-24 from COS-7 cells transfected with the previous NM_053095 cDNA (Fig. 1C). Western blot analyses showed two bands from the culture supernatants, which migrated at 28 kDa and 24 kDa of molecular masses, and a single 24 kDa-band from the cell lysate. A previous study using AF333251 cDNA demonstrated the same-sized two bands in culture supernatants [5]. None of signals was detected from COS-7 cells transfected with vector DNA alone. These data indicated that the previous NM_053095 cDNA also encoded mIL-24. A band with the higher molecular mass, in comparison with the secretory form, was detected in the supernatants despite of a cleavage at the signal peptide, but secretory hIL-24 had been also demonstrated to have a higher molecular mass than the non-secretory uncleaved form [2]. Calculated molecular masses of the secreted and the uncleaved cytoplasmic form were 18.6 and 20.8 kDa, respectively and the differential molecular sizes between the estimation and experimental data could be due to possible post-translational modifications [1].

We also compared sequence homology between mIL-24 and hIL-24 and confirmed that they were homologous each other at 69.5% at the amino acid level (Supplementary Fig. 1).

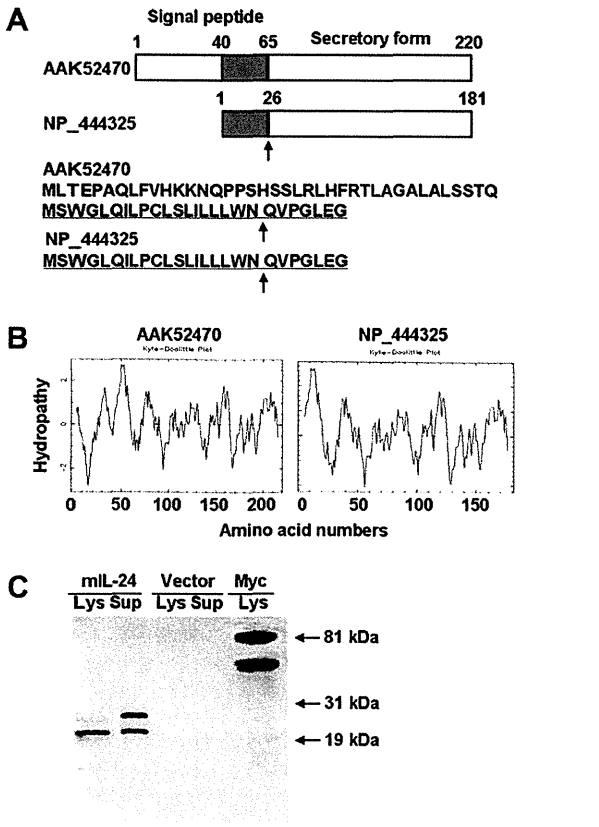

Fig. 1. Structure of mIL-24 and mIL-24 expression. (A) Diagrams of mIL-24 based on AAK52470 and the previous version of the NP_444325 sequences, both of which are encoded by AF333251 and the previous NM_053095 cDNA sequences, respectively. The 3′ portions (shaded) of the putative signal peptides (40–65 or 1–26) and the mature secretory forms (66–220 or 27–181) are identical. Predicated signal sequences are also shown below and the shared sequences are underlined. The arrows indicate a possible cleavage site. (B) The Kyte-Doolittle hydropathy blot of the AAK52470 and the NP_444325 sequences. Y axis numbers indicated hydrophilicity. (C) Production of mIL-24 in COS-7 cells. Culture supernatants (Sup) and cell lysate (Lys) of COS-7 cells transfected with pcDNA3.1myc-His DNA/mIL-24 (mIL-24) or pcDNA3.1myc-His DNA (Vector), and cell lysate of COS-7 cells transfected with pcDNA/c-myc DNA (Myc) were examined with western blot analysis using anti-c-Myc antibody. Molecular sizes are also shown.

3.2. Growth of mIL-24-expressing cells

We examined whether expression of mIL-24 induced apoptosis in murine tumors, colon tumors Colon 26 cells and Lewis lung carcinoma-derived A11 cells, and in fibroblasts, NIH3T3 cells. They were retrovirally transduced with the *mIL-24* gene under the condition that 100% of the cells were infected, and then stained with PI and annexin V 48 h after the transduction (Fig. 2). All the cells infected were not morphologically different from uninfected cells and apoptotic cell fractions were not different among *mIL-24*- or *LacZ*-transduced, and uninfected cells, irrespective of the cell types. We established G418-resistant Colon 26, A11 and NIH3T3 cells that stably expressed mIL-24 (Fig. 3A). Proliferation *in vitro* of the mIL-24-expressing cells was not different from that of respective parent or the *LacZ* gene-transduced cells (Fig. 3B). These data suggest that mIL-24 did not induce cell death in murine cells or suppressed the proliferation. We could not demonstrate secretion of mIL-24 from the G418-resistant cells since anti-mIL-24 antibody is currently unavailable and anti-hIL-24 antibody (provided by Introgen) did not cross-react with mIL-24 (data not shown).

3.3. Ad/hIL-24-mediated decreased viability and growth of murine tumor cells

We examined sensitivity of murine tumors to Ad/hIL-24 to know possible effects of hIL-24 in murine cells. We firstly tested growth inhibitory activities of Ad/hIL-24 on human tumors and confirmed that Ad/hIL-24 but not Ad/LacZ suppressed the viability of human pancreatic carcinoma cells (Supplementary Fig. 2). Transduction with Ad/hIL-24 decreased viability of A11 and murine melanoma B16 cells but not Colon 26 cells (Fig. 4A). We also examined the infectivity of Ad to these cells and found that B16 and A11 cells were infected well with Ad/GFP but Colon 26 cells were resistant to Ad infection (Fig. 4B). These data showed that Ad/hIL-24 could suppress the viability of murine cells and that the susceptibility to Ad/hIL-24 was influenced by Ad infectivity. Ad used in the present study were type 5 Ad and the expression levels of the Ad cellular receptor, the coxsackie and adenovirus receptor, were different among the cells tested (data not shown).

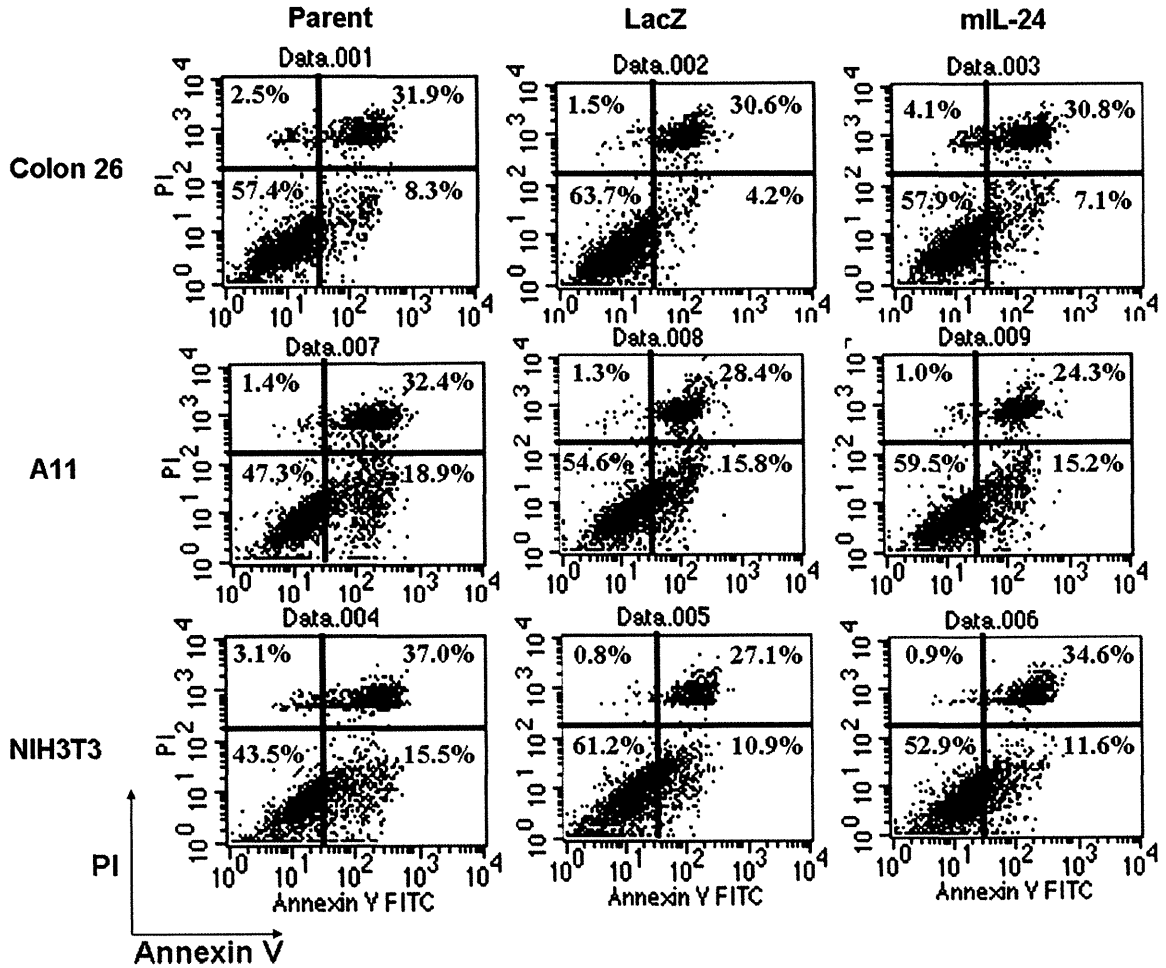

Fig. 2. Representative flow cytometry data of murine cells transduced with *mIL-24* gene. Colon 26, A11 and NIH3T3 cells that were retrovirally transduced with culture supernatants of PA317/mIL-24 (mIL-24) or PA317/LacZ (LacZ) cells and the respective parent (Parent) cells were stained with PI and annexin V. One of three independent experiments and percentages of respective fractions are shown.

3.4. Anti-tumor effects of IL-24 in vivo

We subcutaneously inoculated parent Colon 26 and B16 cells or the mIL-24-expressing cells (Colon 26/mIL-24, B16/mIL-24) into respective syngeneic mice to examine whether anti-tumor effects were produced by the expressed mIL-24. Growth of Colon 26/mIL-24 and B16/mIL-24 tumors was not different from that of respective parent tumors (Fig. 5A). Survival of the mice that received Colon 26/mIL-24 cells intraperitoneally or injected with B16/mIL-24 cells subcutaneously was not different from that of respective parent cells-injected mice (Table 1). We also examined mIL-24-mediated anti-tumor effects in a different assay to examine possible bystander effects. Syngeneic mice were inoculated with Colon 26 cells mixed equally with either allogenic PA317/mIL-24 or PA317/LacZ cells. The growth was not different among parent Colon 26- and the mixed cells-injected groups (data not shown).

We then investigated possible anti-tumor effects by Ad/hIL-24 *in vivo* (Fig. 5B). B16 cells transduced with Ad/hIL-24 or Ad/LacZ were inoculated into syngeneic mice and we detected growth retardation of B16 tumors infected with Ad/hIL-24 but not Ad/LacZ. These data together with the viability data *in vitro* (Fig. 4A) indicated that Ad/hIL-24 produced anti-tumor effects on B16 cells by suppressing the viability.

3.5. Inability to induce cytokines and IL-24R expression in activated spleen cells

We examined whether mIL-24 induced the expression of proinflammatory cytokines and IFN-γ. Spleen cells were incubated with the supernatants of either COS-7 or COS-7 cells transfected with the *mIL-24* gene (Fig. 6A). Naïve spleen cells increased the *IL-6*, *TNF-α* or *IFN-γ* expression when stimulated with PMA plus ionomycin but the supernatants of mIL-24-transfected COS-7 cells did not up-regulate any of the gene expressions in contrast to previous reports on hIL-24 acting on human peripheral blood cells [1,2]. Our data also showed that stimulation with the supernatants of mIL-24-transfected COS-7 cells did not influence the expression levels of *IL-17*, *IL-21* or *IL-28* gene (data not shown). We then examined the expression of the IL-24R complexes in spleen cells stimulated with anti-CD3 or anti-CD40 antibody, or Con A (Fig. 6B). Spleen cells constitutively expressed the *mIL-10R2* gene but not the *mIL-22R* or *mIL-20R1* gene. Moreover, the *mIL-22R* and *mIL-20R1* genes, both of which are components of the IL-24R in human, were not activated in spleen cells even after the stimulations. These data therefore suggest that functional IL-24R complexes were not expressed in murine lymphoid cells. We also examined Colon 26, B16, A11 and NIH3T3 cells with RT–PCR and found that they were negative for the *mIL-22R* and *mIL-20R1* genes (data not shown).

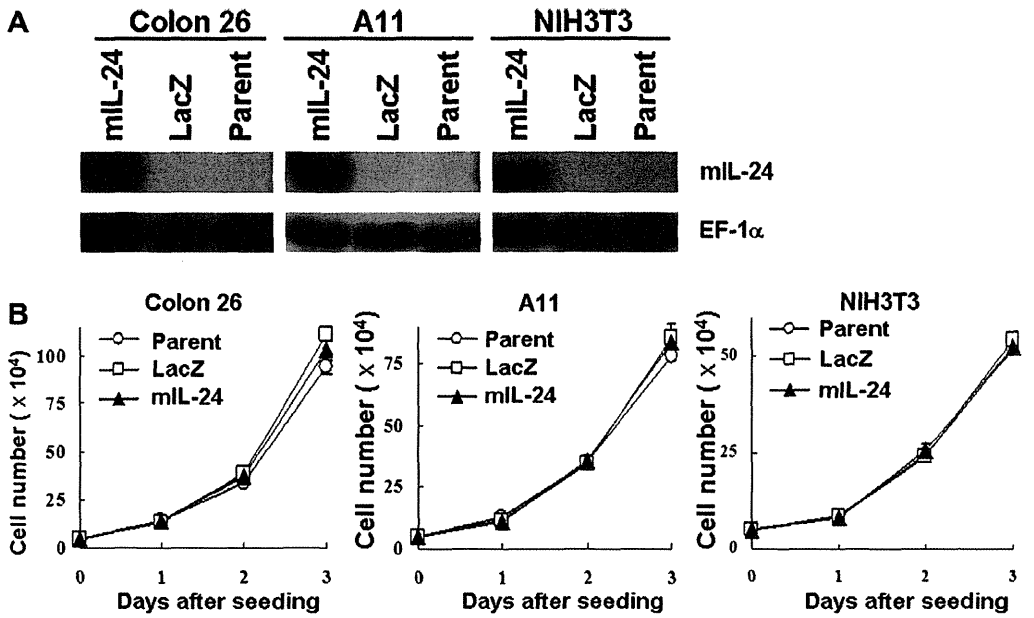

Fig. 3. Growth of mIL-24-expressing cells. (A) Northern blot analysis of the *mIL-24* gene expression in parent and G418-resisitant cells transduced with either *mIL-24* or *LacZ* gene. The same filter was rehybridized with EF-1α probe. (B) Proliferation of parent (open cycle) and transduced cells with either *mIL-24* (closed) or *LacZ* (open square) gene. SE bars are also shown.

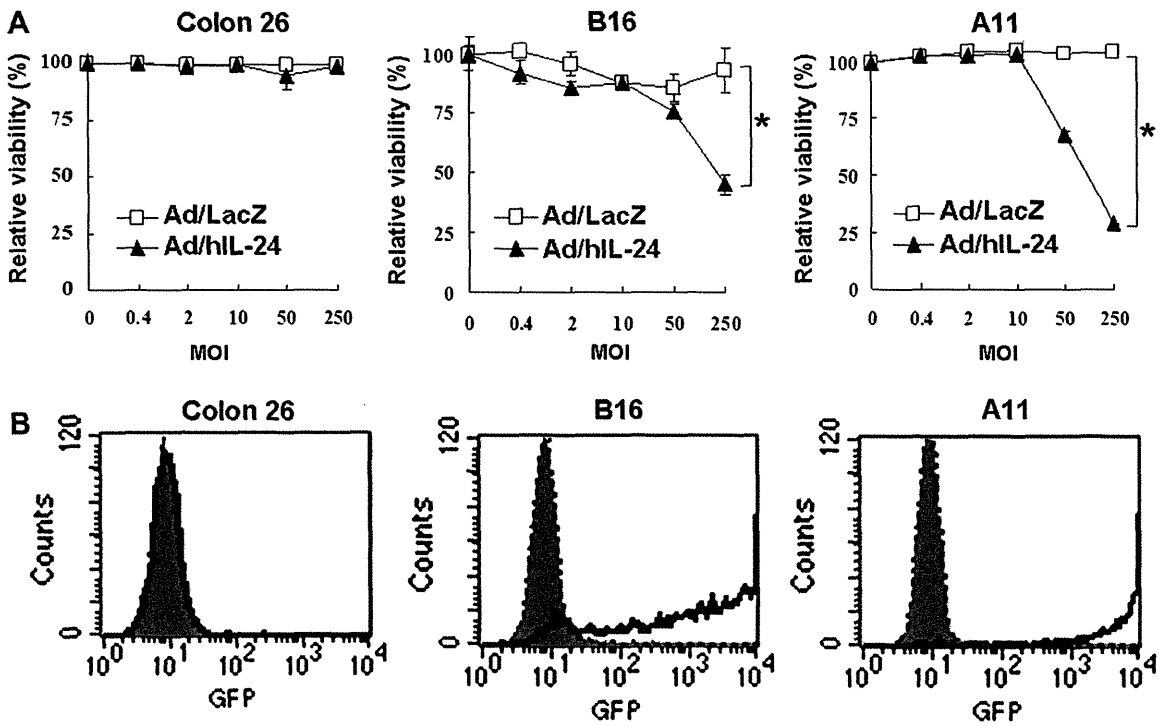

Fig. 4. Ad/hIL-24-mediated suppression of cell viability of murine tumor cells (A) Cells were infected with Ad/hIL-24 or Ad/LacZ at different MOIs and the cell viability without Ad treatment was calculated as 100%. SE bars are also shown. *$P < 0.05$ (B) Staining profiles of uninfected (shaded with dotted line) and Ad/GFP-infected cells (bold line) at 300 MOI.

We further presumed a possible cross-reactivity of hIL-24 in a murine system and examined whether hIL-24 influenced production of proinflammatory cytokines and IFN-γ. Expressions of *IL-6*, *TNF-α* and *IFN-γ* genes were tested with spleens of mice that were inoculated with B16 cells or those transduced with Ad/hIL-24 or Ad/LacZ (Fig. 6C). All of the mice except one mice injected with B16 cells expressed the *IL-6* gene and all the mice expressed the *TNF-α* gene, whereas expression levels of the *IFN-γ* gene were different among the mice without any preference. The RT–PCR data of the tumor-inoculated mice collectively indicated that hIL-24 did not up-regulated the transcription of the target genes in mice.

4. Discussion

In the present study, we showed that expression of mIL-24 in murine tumors failed to produce anti-tumor effects. Expressed

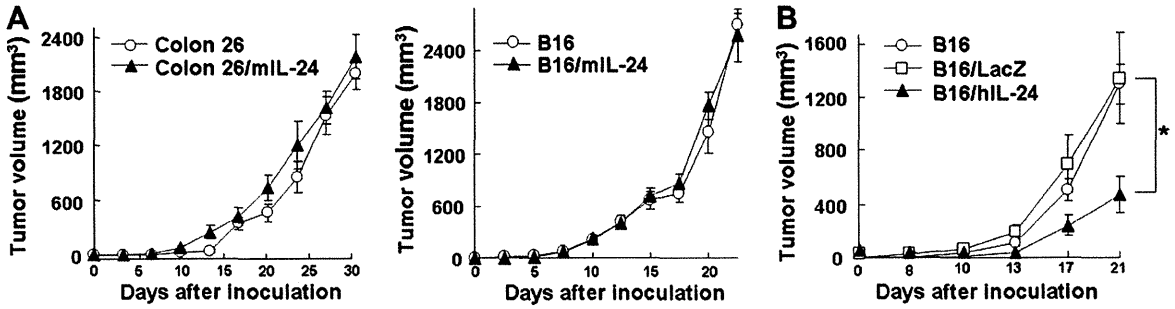

Fig. 5. Anti-tumor effects of mIL-24 and hIL-24 *in vivo*. (A) Subcutaneous tumor growth of parent (Colon 26 and B16) or transduced (Colon 26/mIL-24 and B16/mIL-24) cells. Colon 26 (1×10^6) and B16 (5×10^5) cells were inoculated into syngeneic BALB/c and C57/BL6 mice ($n = 7$), respectively. SE bars are also shown. (B) Growth of B16 tumors untransduced or transduced with Ad/hIL-24 or Ad/LacZ (MOI=250). Parent B16 (B16), Ad/LacZ-infected (B16/LacZ) or Ad/hIL-24-infected B16 (B16/hIL-24) cells (3×10^5) were injected subcutaneously into syngeneic C57/BL6 mice ($n = 6$). The average tumor volumes with SE bars are shown. $^*P < 0.05$.

Table 1
Survival of the mice inoculated with mIL-24-expressing tumors.

Cells inoculated[a]	Number of mice tested	Survival days (average ± SE)
Colon 26	6	14, 15, 18, 19, 20, 25 (18.5 ± 1.6)[b]
Colon 26/mIL-24	6	12, 15, 16, 19, 20, 21 (17.2 ± 1.4)[b]
B16	7	16, 19, 22, 22, 22, 26, 55 (26 ± 5.0)[c]
B16/mIL-24	7	22, 22, 23, 26, 27, 31, 34 (26.4 ± 1.8)[c]

[a] Colon 26 or Colon 26/mIL-24 cells (5×10^5) were injected into the intraperitoneal cavity and B16 or B16/mIL-24 cells (5×10^5) were injected subcutaneously.
[b] $\chi^2 = 0.157$, d.f. = 1, $P = 0.691$.
[c] $\chi^2 = 0.339$, d.f. = 1, $P = 0.560$.

mIL-24 did not induce apoptosis or suppress cell proliferation in both murine tumors and fibroblasts. Moreover, growth of the mIL-24-expressing tumors was not retarded and survival of mice bearing the mIL-24-transduced tumors was not prolonged in *in vivo* settings. In contrast, the murine tumor cells were susceptible to Ad/hIL-24, which was evidenced by growth inhibition *in vitro* and retarded growth of the transduced tumors *in vivo*. Previous studies showed that interaction of hIL-24/hIL-24R complexes was necessary to achieve anti-tumor effects in a certain human tumors but the interaction was not always required, which indicated that the expression within cytoplasm could produce biological effects [8,9]. Differential mechanisms as to how hIL-24 produces anti-tumor activities are thus partly associated with the secretory or the non-secretory form, and possible mitochondrial dysfunction and/or endoplasmic reticulum stress might be involved in the receptor-independent mechanisms [9]. Murine tumors used in the present study were negative for mIL-22R or mIL-20R1 mRNA. Functional mIL-24R complexes, currently not well described, consequently cannot be formed in the tumors if the mice receptor configuration is the same as the human counterpart. Suppressed viability of the mIL-24R-negative tumor cells with Ad/hIL-24 thereby suggested that hIL-24-induced inhibitory action was due to non-receptor-mediated actions even though hIL-24 can bind to murine IL-24R complexes, and indicated that the IL-24-induced intracellular signaling pathways were shared between the two species. Growth retardation of Ad/hIL-24-infected B16 tumors *in vivo* was directly attributable to the growth inhibitory effects since spleens of the mice bearing Ad/hIL-24-transduced tumors did not augment production of proinflammatory cytokines or IFN-γ, both of which could contribute to cell-mediated immune responses against tumors and were typical target molecules that hIL-24 induced.

The present study also demonstrated that activated spleen cells were negative for the *mIL-22R* and *mIL-20R1* expression even after the activation. Spleen cells may not respond to mIL-24 although functional mIL-24R structures have not yet been reported. The *hIL-22R* and *hIL-20R1* genes were not expressed even in lymphoid tissues and expression levels of the *hIL-20R2* gene in peripheral blood mononuclear cells were quite low [10,11]. Although hIL-24 was secreted from Con A-stimulated mononuclear cells and hIL-24 induced proinflammatory or pro-Th1 cytokine productions [2,6], immunocompetent cells are not the major target of IL-24 both in human and mouse because of their defective receptor expressions. In contrast, liver can be a potential target of IL-24 as well as other IL-10 family cytokines [12]. Recent studies demonstrated that hIL-24 activated neutrophils to secrete IL-12 and IFN-γ [13] and that hIL-24 also inhibited plasma cell differentiation in human [14]. Nevertheless, these reports did not directly provide evidences that hIL-24 produced anti-tumor effects through acquired immune systems.

Previous studies demonstrated anti-tumor effects of hIL-24 and mIL-24 in mice models [15,16]. Chen et al. showed that intramuscular electroporation of mIL-24 plasmid DNA inhibited growth of murine hepatoma in mice through anti-angiogenesis [15], which was the first report to show that mIL-24 produced anti-tumor activities but did not analyze any immunological responses. They showed that the tumor cells were positive for putative mIL-24R complexes but did not investigate possible tumor cell death. No other studies were reported regarding mIL-24-mediated anti-tumor effects irrespective of the mechanisms. In contrast, Miyahara et al. showed that vaccination of syngeneic mice with Ad/hIL-24-transduced murine fibrosarcoma inhibited growth of parent tumors subsequently inoculated and increased IFN-γ and proinflammatory cytokine production, and CD8-positive cell numbers in the vaccinated spleen [16]. These data did not necessary indicate that hIL-24 stimulated host immune systems since the transduction-induced dying fibrosarcoma cells themselves could activate host immune response and Ad vector might work as an adjuvant. These previous studies in fact did not directly demonstrated immunity-mediated anti-tumor effects by expressed mIL-24 or hIL-24 and the present study rather indicated that mIL-24 was irrelevant to anti-tumor immune responses.

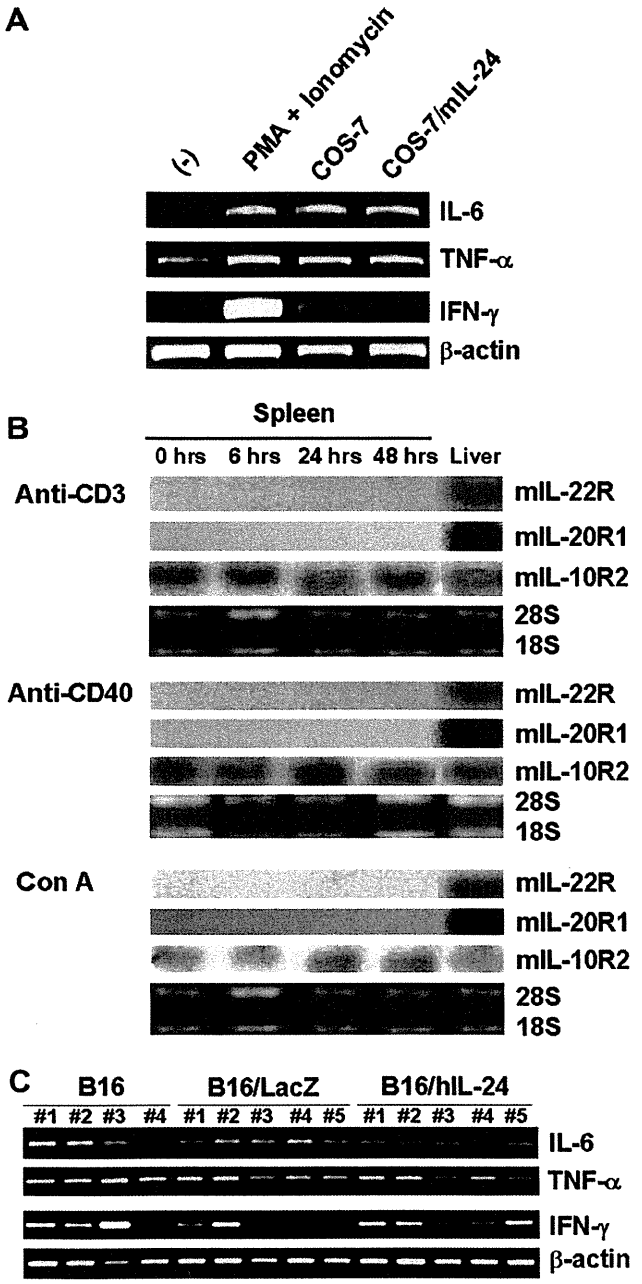

Fig. 6. Induction of proinflammatory cytokines and *IFN-γ* and IL-24R expressions in activated spleen cells. (A) RT-PCR analyses of the *IL-6*, *TNF-α* or *IFN-γ* gene expression in spleen cells stimulated with PMA and ionomycin, or with the culture supernatants of COS-7 or COS-7 cells transfected with the pcDNA3.1myc-His DNA/mIL-24 (COS-7/mIL-24). The supernatants used were 5 times more in volume than those used in Fig. 1C. (B) Northern blot analysis of *mIL-22R*, *mIL-20R1* or *mIL-10R2* gene expression in spleen cells stimulated with anti-CD3 or anti-CD40 antibody, or Con A for 6–48 h and in liver as a control. Ethidium bromide-stained ribosomal RNA is also shown as a control. (C) RT-PCR analyses in spleen cells from mice injected with B16 cells untransduced or transduced with Ad/hIL-24 or Ad/LacZ (the same mice shown in Fig. 5B). The numbers indicated the mice tested.

Biological and immunological functions of mIL-24 have been scarcely investigated and the present data indicated that mIL-24 did not induce cell death. Moreover, mIL-24 might not play a major role in anti-tumor immune responses. These data suggest that mIL-24 rather belongs to a different gene family with distinct functions from hIL-24 and is not the human homolog. Another possible explanation is that IL-24 is functionally diversified in evolutional processes and the biological activities thereby do not overlap each other despite the conserved genetic sequences. Recombinant hIL-24 can induce apoptosis of human tumors and inhibit angiogenesis in *in vivo* experiments [17], suggesting that Ad-mediated production is not a mechanism for the IL-24-mediated anti-tumor activity.

In summary, we showed that murine tumor cells expressing mIL-24 were not subjected to apoptosis or did not produce anti-tumor effects. Transduction with Ad/hIL-24 however suppressed viability and growth of the murine tumors transduced. Anti-tumor effects by IL-24 are evidenced in human IL-24 only and the murine homolog can be functionally distinct from the human counterpart. A deficient expression of mIL-24R complexes in spleen of immunocompetent mice raises a possibility that mIL-24 is not directly involved in immune responses but further investigation is required to explore a possible other biological significance of mIL-24.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2012.02.010.

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Antiproliferative action of metformin in human lung cancer cell lines

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Abstract. The oral antidiabetic agent metformin has anticancer properties, probably via adenosine monophosphate-activated protein kinase activation. In the present study, growth inhibition was assessed by a clonogenic and by a cell survival assay, apoptosis induction was assessed by Hoechst staining and caspase activities and cell cycle alteration after exposure to metformin, and the interaction of metformin with cisplatin *in vitro* were elucidated in four human lung cancer cell lines representing squamous, adeno-, large cell and small cell carcinoma. Clonogenicity and cell proliferation were inhibited by metformin in all the cell lines examined. This inhibitory effect was not specific to cancer cells because it was also observed in a non-transformed human mesothelial cell line and in mouse fibroblast cell lines. Inhibition of clonogenicity was observed only when the cells were exposed to metformin for a long period, (10 days) and the surviving fraction, obtained after inhibiting proliferation by increasing the dose, reached a plateau at approximately 0.1-0.3, indicating the cytostatic characteristics of metformin. Metformin induced significant apoptosis only in the small cell carcinoma cell line. A tendency of cell cycle accumulation at the G0/G1 phase was observed in all four cell lines. Cisplatin, in a dose-dependent manner, severely antagonized the growth inhibitory effect of metformin, and even reversed the effect in three cell lines but not in the adenocarcinoma cell line. The present data obtained using various histological types of human lung cancer cell lines *in vitro* illustrate the cytostatic nature of metformin and its cytoprotective properties against cisplatin.

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Key words: metformin, lung cancer, cisplatin, apoptosis, cell line, cell cycle

Introduction

Metformin is an oral biguanide agent used worldwide to treat non-insulin-dependent diabetes mellitus. The initial reports related to the anticancer effects of metformin were epidemiological studies demonstrating a lower incidence of the occurrence and death of cancer in patients with diabetes mellitus treated with metformin compared to those treated with other antidiabetic agents (1,2). Consequently, these reports have triggered several clinical observational studies. Jiralerspong *et al* reported a significantly increased pathologically complete response rate in induction chemotherapy for breast cancer in diabetes patients receiving metformin compared to those not receiving metformin (3). Mazzone *et al* reported that diabetes patients with lung cancer who were previously treated with metformin or thiazolidinediones had a lower incidence of metastatic disease at the time of diagnosis and a reduced risk of death compared to those who did not receive the same treatment (4). Thereafter, the antiproliferative action of metformin was confirmed via *in vivo* and *in vitro* experiments in various cancer cell lines including breast (5-8), prostate (9), pancreas (10), and ovarian cancer (11-13) as well as lung adenocarcinoma (8).

Metformin is considered to exert anticancer effects via inhibition of insulin and the mammalian target of rapamycin (mTOR) pathways. Since insulin is a growth-promoting hormone with a mitogenic effect (14), metformin could indirectly inhibit tumor growth by ameliorating hyperinsulinemia, which is frequently observed in patients with non-insulin-dependent diabetes mellitus (15). In fact, Algire *et al* reported that metformin inhibited mouse lung tumor growth under specific conditions in which the animals were bred on a high-calorie diet (16). Although this would explain the effect of the agent *in vivo*, its *in vitro* effects (5-12) cannot be explained by this anti-insulin action. It has been reported that metformin inhibits complex-I of the respiratory chain in mitochondria, leading to increased AMP expression and liver kinase B1 (LKB1)-mediated activation of AMP kinase, finally inhibiting the mTOR downstream (15). Despite these findings, the precise mechanisms of the metformin-induced effects are not fully understood. In particular, controversy remains about

whether metformin is apoptotic (6, 10) or just cytostatic (5, 9) and whether it kills cancer cells synergistically with cytotoxic agents including cisplatin (11,13), paclitaxel (8), and doxorubicin (17), or if it is antagonistic to cisplatin (18,19).

In the present study, the cytotoxic effects of metformin were elucidated in various types of human lung cancer cell lines including squamous, adeno-, large, and small cell carcinoma, together with non-transformed cell lines. The drug-drug interaction between metformin and cisplatin was also investigated.

Materials and methods

Reagents. Metformin (1,1-dimethylbiguanide hydrochloride, #D150959-5G; Sigma-Aldrich Co., St. Louis, MO, USA) was diluted in distilled water. Cisplatin solution at a concentration of 0.5 mg/ml (pH 2.5–5.5) was purchased from Nippon Kayaku Co. (Tokyo, Japan).

Cells and cell culture. The human lung cancer cell lines RERF-LC-AI (#RCB0444) and A549 (#RCB0098) were purchased from the Riken Cell Bank (Tsukuba, Japan), while IA-5 (#RCB0548) and WA-hT (#RCB2279) (20) were established and maintained in our laboratory. The established mouse fibroblast cell line Balb/3T3 clone A31 (A31, #RCB0005) was purchased from the Riken Cell Bank. A nontumorigenic human mesothelial cell line derived from pleural effusion and immortalized by the pRSV-T plasmid Met5A (#CRL-9444) was purchased from the American Type Culture Collection (Manassas, VA, USA). The histological cell line types included RERF-LC-AI, squamous cell carcinoma; A549, adenocarcinoma; IA-5, large cell carcinoma; and WA-hT, small cell carcinoma. They were cultured as a monolayer in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ ml streptomycin in a 37˚C humidified atmosphere containing 5% CO_2.

Clonogenic assay. For a clonogenic assay involving a 10-day exposure to metformin, subconfluent cultured cells were trypsinized to obtain cell suspensions. Subsequently, a varied number of cells, such that the resulting colony number per plate would be approximately 20-50, were immediately replated onto 6-cm culture dishes in triplicate, cultured for 24 h in the complete medium until administration of various concentrations of metformin, and further cultured for 10 days without changing the medium. In the clonogenic assay involving 1- and 24-h exposure to the agents, subconfluent cultured cells were treated with various concentrations of agents for 1 or 24 h and then trypsinized, washed twice with the agent-free complete medium, replated on culture dishes as in the 10-day exposure method, and further cultured for 10 days. In each case, the obtained colonies were counted under a dissecting microscope after a 1% crystal violet staining.

Cell survival assay. In the cell survival assay, cells were plated onto 6-cm culture dishes in triplicate at a cell concentration of $1x10^5$/plate in complete medium. The cells were cultured for 24 h, and metformin or cisplatin at various concentrations were added to the medium and cultured for an additional 4 days. Viable cells negatively stained with 0.4% trypan blue were then counted. In the cell survival assays using combined treatment with metformin and cisplatin, doses of cisplatin that reduced the surviving cells to 50% (IC_{50}) and 10% (IC_{90}) with single-agent administration in each cell line were admixed with various concentrations of metformin, with other methods being similar to the methods of the single-agent experiments.

Apoptosis assay. Apoptosis was evaluated using morphological and enzymatic assays, that is, with Hoechst staining and by assessing caspase 3, 8 and 9 activities. For Hoechst staining, trypsinized cells together with floating cells were harvested, fixed with 1% glutaraldehyde, and stained with 1 mM bisbenzimide H 33248 fluorochrome trihydrochloride (Hoechst 33248; Ana Spec, Inc., Fremont, CA, USA). The cells were examined under fluorescence microscopy. Aggregating cells and cells with fragmented chromatin were considered apoptotic. More than 500 cells were evaluated, and the apoptotic cell ratio was recorded in each experiment. The activities of caspases 3, 8 and 9 were evaluated with the synthetic substrates DEVD-, IETD-, and LEHD-pNA, respectively, with the colorimetric assay kits APOPCYTO (Medical & Biological Laboratories Co., Nagoya, Japan) by monitoring the absorbance at a wavelength of 405 nm to measure p-nitroanilide (pNA) cleaved from synthetic substrates with cell extracts.

Cell cycle analysis. Cell cycle distribution was determined by the propidium iodide single-color method using a flow cytometer (FACSCanto II; BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. In brief, cells were trypsinized, fixed with 70% ethanol, washed with PBS(-), and treated with PI/RNase Staining buffer (BD Biosciences) at a concentration of $2x10^6$ cells/ml. The data were analyzed using the BD FACSDiva software (BD Biosciences).

Results

Colony formation and cell proliferation. Metformin exerted inhibitory effects on the clonogenicity of the 4 human lung cancer cell lines as well as that of non-transformed human mesothelial cell line and a mouse fibroblast cell line in a dose-dependent fashion when they were exposed to metformin for 10 days. On the other hand, a 1-h exposure to metformin did not show any significant inhibitory effect on the clonogenicity of any cell line, whereas a 24-h exposure showed slight suppression of clonogenicity in the A549, IA-5, and Met5A lines (Fig. 1). According to the cell survival assay, inhibition of cell proliferation was observed in the 4 human lung cancer cell lines when they were exposed to metformin for 4 days (Fig. 2). Cell proliferation inhibitory effects on the 4 cell lines exposed to cisplatin for 4 days are shown in Fig. 3.

Apoptosis. Apoptosis was assessed by Hoechst staining and by determining the activities of caspases 3, 8 and 9. The effects of metformin at IC_{30} and IC_{70} were examined in each cell line. Experiments with cisplatin at IC_{70} were conducted for comparison with metformin, and those with cisplatin at a higher dose were conducted for assay control. Apoptosis assessed by Hoechst staining failed to show significant apoptosis in all lung cancer cell lines except WA-hT, which had a significantly higher ratio of apoptosis compared to the non-treated cells

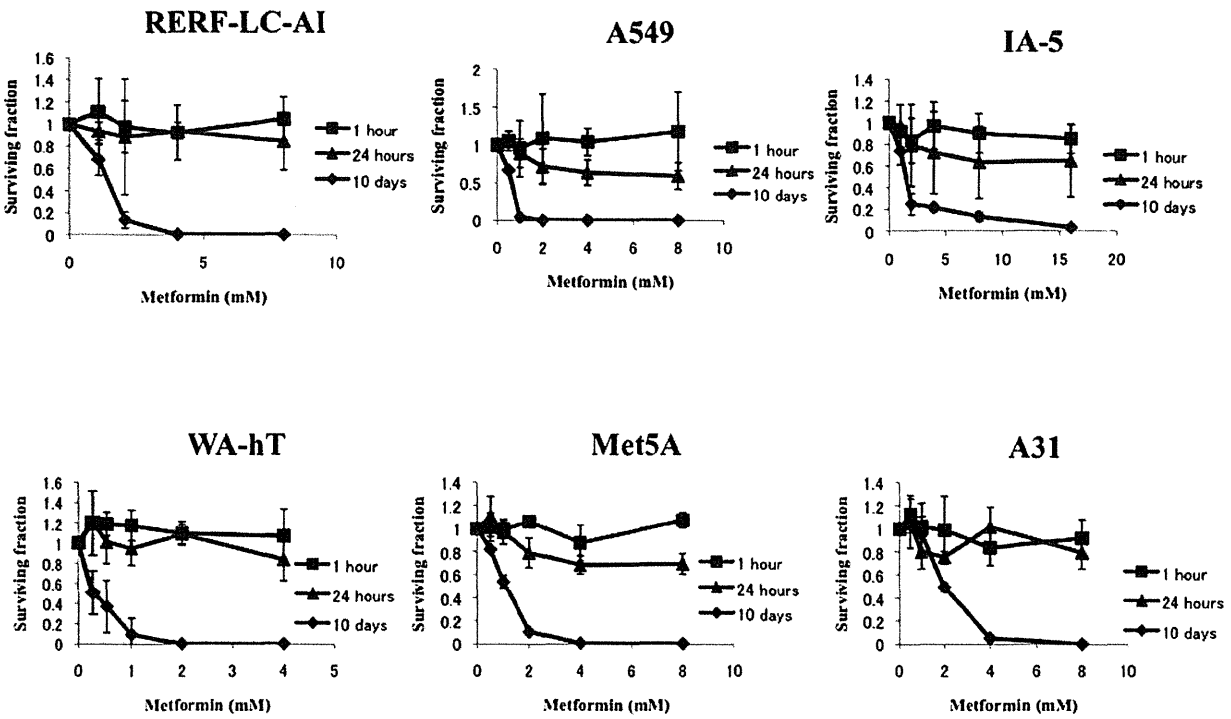

Figure 1. Survival curves drawn based on the results of the clonogenic assay. The cells were exposed to various concentrations of metformin for 1 or 24 h before being trypsinized and plated for colony formation in complete medium. Otherwise, the cells were trypsinized and plated for colony formation by further culture for 10 days in metformin-containing complete medium. RERF-LC-AI, A549, IA-5, and WA-hT represent squamous, adeno-, large cell, and small cell lung carcinoma cell lines, respectively. Met5A and A31 represent non-transformed human mesothelial and mouse fibroblast cell lines, respectively. Each experiment was conducted in triplicate and repeated 3 times. The mean value of each triplicate represents the value of each experiment, while the mean ± SD of the 3 experimental results were calculated. The dot and bar represent mean and SD at each point.

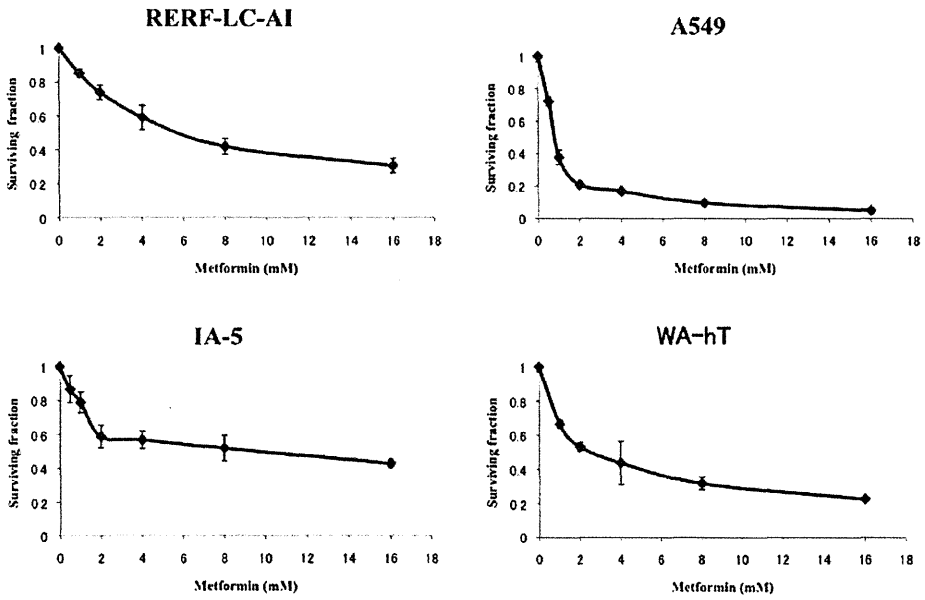

Figure 2. Survival curves drawn based on the results of the cell survival assay. The cells were plated at a concentration of 10^5 cells/plate with complete medium containing metformin at various concentrations, and were further cultured for 4 days with metformin until the surviving cells were counted. Each experiment was conducted in triplicate and repeated 3 times. The mean value of each triplicate represents the value of each experiment, and the mean ± SD of the 3 experimental results were calculated. The dot and bar represent mean and SD at each point.

(Fig. 4A). Apoptosis assessed by determining the activities of caspases 3, 8 and 9 revealed results similar to that of Hoechst staining (Fig. 4B).

Cell cycle distribution. In cell cycle analysis, the effects of metformin at IC_{30} and IC_{70} were compared to those of cisplatin at IC_{70} and at a higher concentration in each cell line. Although