Table 20. Characteristics of Commonly Used Antiarrhythmic Drugs During Pregnancy and Lactation

Drug	V-W classification*1	Pregnancy categories*2	Indications	Characteristics/ adverse effects	Teratogenicity	Breast feeding	Package insert*3	
							Pregnancy	Lactation
Quinidine	IA	C	Various arrhythmias	Thrombocytopenia	Absent	Probably compatible	2	1
Procainamide	IA	C	Various arrhythmias	Lupus-like syndrome	Absent	Probably compatible	2	1
Disopyramide	IA	C	Various arrhythmias	Uterine contraction	Absent	Probably compatible	2	1
Lidocaine	IB	B	VT	Bradycardia, CNS adverse effects	Absent	Probably compatible	2	
Mexiletine	IB	C	VT	Bradycardia, CNS adverse effects, low birth weight infants	Absent	Probably compatible	2	1
Phenytoin	IB	D	Digitalis intoxication	Fetal hydantoin syndrome, not covered for arrhythmias	Present	Compatible	2	
Flecainide	IC	C	VT, SVT	No in normal heart	Absent	Probably compatible	1	1
Propafenone	IC	C	VT, SVT	No in normal heart	Absent	Probably compatible	2	1
Atenolol	II	D	SVT, VT, AF	IUGR, hypoglycemia, bradycardia	Absent	Potential toxicity	2	1
Propranolol	II	C→D	SVT, VT, AF	IUGR, hypoglycemia, bradycardia	Absent	Potential toxicity	2	1
Metoprolol	II	C→D	SVT, VT, AF	IUGR, hypoglycemia, bradycardia	Absent	Potential toxicity	1	1
Amiodarone	III	D	VT	Thyroid disorder, bradycardia, IUGR	Absent	Contraindicated	2	1
Sotalol	III	B→D	VT, SVT	Bradycardia	Absent	Potential toxicity	2	1
Verapamil	IV	C	SVT, VT, AF	Hypotension, bradycardia	Absent	Probably compatible	1	1
Adenosine	NA	C	SVT	Nausea, facial flushing	Absent	Probably compatible	2	
Digoxin	NA	C	SVT, AF	Bradycardia, low birth weight infants	Absent	Compatible	2	

AF, atrial fibrillation; CNS, central nervous system; IUGR, intrauterine growth retardation; NA, not applicable; SVT, supraventricular tachycardia; VT, ventricular tachycardia.

*1Vaughan-Williams (V-W) classification of antiarrhythmic drugs. The above information is based on "Drugs in pregnancy and lactation, 8th edition (2008)".[40]

*2B→D/C→D: Pregnancy category B or C during the first trimester but pregnancy category D during the second and third trimesters.

*3Information on the use during pregnancy and lactation in the package insert (Blank columns represent no information in the source material).

1. Contraindication: This drug should not be administered to women who are or may be pregnant. Treatment should be discontinued without delay when pregnancy is detected. The drug should not be given to lactating women, and, when treatment is necessary, should be given after lactation is stopped.
2. Relative contraindication: The drug should be used when the benefits of use outweigh the risks. It is desirable that the treatment be avoided in women who are or may be pregnant. The safety in pregnant women has not been established.

[Precautions]

1) Indications and contraindications should be confirmed when considering the use during pregnancy.
2) When drugs contraindicated or not indicated for pregnant women in the package inserts, the physicians must fully explain the use of such drugs to the patients and their families and obtain informed consent.

to induce labor and patients with uterine inertia should be indicated for oxytocics.[109]

5. Delivery Methods

Vaginal delivery is generally recommended, although cesarean section is performed for selected cases (**Table 17**). Cesarean section is indicated for those with Marfan syndrome associated with an increase in ascending aortic diameter and those using artificial valves without hemostasis through switching from warfarin to heparin.[69] Cesarean section may be considered for other high-risk women. Epidural anesthesia is beneficial in reducing cardiac load by decreasing cardiac output, and in alleviating pain and anxiety of the patient.

6. Anesthesia for Delivery (Tables 18, 19)

Hemodynamics during delivery is significantly affected by the body position, delivery method, severity of labor pain, and depth of anesthesia. Epidural anesthesia is an excellent method to provide analgesic effect with limited effect on systemic hemodynamics.

Table 21. Characteristics of Drugs Commonly Used for the Treatment of Heart Failure During Pregnancy and Lactation

Drug	Class	Pregnancy categories*1	Characteristics/ adverse effects	Teratogenicity*1	Breast feeding	Package insert*2	
						Pregnancy	Lactation
Furosemide	Diuretic	C (D)	Decreased uteroplacental circulation, fetal dehydration	Absent	Probably compatible	2	1
Spironolactone	Diuretic	C (D)	Possible feminization	Absent	Probably compatible	2	1
Chlorothiazide	Diuretic	C (D)	Thrombocytopenia, hemolytic anemia	Absent	Compatible	2	1
Digoxin	Digitalis	C	Bradycardia, low birth weight infants	Absent	Compatible	2	
Nitroglycerin	Nitrate	B	Few reports	Absent	Probably compatible	2	1
Isosorbide dinitrate	Nitrate	C	Few reports	Absent	Probably compatible	2	1
Carvedilol	β-blocker	C→D	IUGR, bradycardia, hypoglycemia	Absent	Potential toxicity	1	1
Metoprolol	β-blocker	C→D	IUGR, bradycardia, hypoglycemia	Absent	Potential toxicity	1	1
Hydralazine	Peripheral vasodilator	C	Headache, neonatal thrombocytopenia	Absent	Probably compatible	2	1
Captopril*3	ACE inhibitor*3	C→D	Fetal renal dysplasia, renal failure, oligohydramnios	Present*3	Compatible	1	1
Enalapril*3	ACE inhibitor*3	C→D	Fetal renal dysplasia, renal failure, oligohydramnios	Present*3	Probably compatible	1	1
Candesartan*4 Losartan*4	Angiotensin receptor blocker*4	C→D	Fetal renal dysplasia, renal failure, oligohydramnios	Present*4	Probably compatible	1	1
Milrinone	PDE III inhibitor	C	Few reports	Absent	Probably compatible	2	1
Amrinone	PDE III inhibitor	C	Few reports	Absent	Probably compatible	1	1
Olprinone	PDE III inhibitor		Few reports			1	1
Carperitide	hANP		Few reports			2	1
Dopamine	Catecholamine	C	Few reports	Absent	Probably compatible	2	
Dobutamine	Catecholamine	B	Few reports	Absent	Probably compatible	2	
Isoproterenol	Catecholamine	C	Few reports	Absent	Probably compatible	2	

ACE, angiotensin converting enzyme; hANP, human atrial natriuretic peptide; IUGR, intrauterine growth retardation; PDE III, phosphodiesterase III.

Note) The above information is based on "Drugs in pregnancy and lactation, 8th edition (2008)"[40] (Blank columns represent no information in the source material).

*1C→D: Pregnancy category C during the first trimester but pregnancy category D during the second and third trimesters. C (D): Pregnancy category C for patients without gestational hypertension, and pregnancy category D for patients with gestational hypertension. Teratogenicity: Since ACE inhibitors have been reported to be teratogenic, strict caution should be needed for the use of these drugs even in the first trimester.

*2Information on the use during pregnancy and lactation in the package insert (Blank columns represent no information in the source material).

1. Contraindication: This drug should not be administered to women who are or may be pregnant. Treatment should be discontinued without delay when pregnancy is detected. The drug should not be given to lactating women, and, when treatment is necessary, should be given after lactation is stopped.
2. Relative contraindication: The drug should be used when the benefits of use outweigh the risks. It is desirable that the treatment be avoided in women who are or may be pregnant.

*3Since ACE inhibitors have been reported to be teratogenic, strict caution should be needed for the use of these drugs even in the first trimester.

*4Strict caution in terms of teratogenicity should be needed for the use of angiotensin receptor blockers, which exert their effects in a way similar to ACE inhibitors.

[Precautions]

1) Indications and contraindications should be confirmed when considering the use during pregnancy.
2) When drugs contraindicated or not indicated for pregnant women in the package inserts, the physicians must fully explain the use of such drugs to the patients and their families and obtain informed consent.

Table 22. Directions of Future Research on Pregnancy and Childbirth in Patients With Heart Disease

1. Counseling	Management of pregnancy and delivery, hereditary (risk of familial recurrence), maternal and fetal prognosis, support by family, and psychological approaches
2. Organization	Team-based practice, criteria for desirable hospitals, and cooperation with perinatal medical centers
3. Maternal management	Hemodynamics monitoring, management corresponding to types of heart disease, contraceptions, drug therapy, cardiac intervention (catheter intervention, cardiovascular surgery), and paternal management
4. Fetal management	Effects of maternal heart disease on the fetus, effects of drug therapy in the mother on the fetus, monitoring of fetal well-being, diagnosis of congenital anomalies of the fetus, and fetal treatment
5. Perinatal management	Perinatal monitoring, induction of delivery, anesthetic methods, delivery management, neonatal management (premature birth, low birth weight infants, and infants with congenital heart disease), excretion of drugs to the mother in the milk, effects of lactation on maternal heart disease, and caring for baby
6. Long-term management for child and mother	Assessment of maternal cardiac function, effects of pregnancy and delivery on the natural history of heart disease, growth and development of the children, and precautions for next pregnancy

V Types and Key Points of Treatment of the Mother

1. Antiarrhythmic Treatment (Table 20)[110–112]

2. Heart Failure Treatment (Table 21)[113–116]

3. Invasive Treatment

It has been reported that intervention using balloon catheters during pregnancy is effective for patients with pulmonary stenosis, aortic stenosis or mitral stenosis.[117,118] Cardiovascular surgery during pregnancy is required in rare cases.[2,71] The appropriateness of cardiovascular surgery during pregnancy should be determined according to the progression of lesions in aortic stenosis; the worsening of valvular regurgitation or heart failure due to diseases associated with valvular regurgitation; the severity of aortic dissection or giant aneurisms in aortic dilatation, or the status of vegetation or worsening of heart failure in infective endocarditis, among other conditions.[119] When surgery during pregnancy is unavoidable, those performed at 16 to 20 weeks of gestation or 24 to 28 weeks of gestation or thereafter are safer to the fetus than in other periods. When surgery may be waited to 28 to 30 weeks of gestation or thereafter, surgery after childbirth may be feasible.[117,120]

VI Directions of Future Research (Table 22)

It is expected that team management of high-risk pregnant women will advance, the number of women with heart disease who become pregnant and have children will increase, and that patient registration systems will be operated more efficiently. We hope that the directions for future research will be delineated more clearly and many of current problems will be solved by the time of the next revision of the present guidelines.

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CHEST

Original Research

PULMONARY VASCULAR DISEASE

Subpleural Perfusion as a Predictor for a Poor Surgical Outcome in Chronic Thromboembolic Pulmonary Hypertension

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Background: **Small vessel disease is a major determinant of poor outcome after pulmonary endarterectomy for chronic thromboembolic pulmonary hypertension (CTEPH). Out-of-proportion pulmonary vascular resistance (PVR) may indicate the presence of small vessel disease, but it is a very subjective evaluation. We investigated poor subpleural perfusion as a marker for small vessel disease and assessed its association with disease severity and surgical outcome of CTEPH.**

Methods: **We assessed the subpleural perfused area in the capillary phase of pulmonary angiography in 104 consecutive patients, including 45 who underwent surgery, and then divided the patients into either the well-perfused group (the subpleural space in at least one segment was well perfused [n = 75]) or the poorly perfused group (subpleural spaces were either unperfused or minimally perfused in all segments [n = 29]). We compared the pulmonary hemodynamics, degree of distal thrombi, and surgical outcome between these two groups.**

Results: **The poorly perfused group had significantly higher PVR (937 ± 350 dyne/s/cm^5 vs 754 ± 373 dyne/s/cm^5, $P = .02$) and more distal thrombi, resulting in fewer surgically treated patients (27.6% vs 49.3%, $P = .04$) compared with the well-perfused group. This group showed a higher surgical mortality (62.5% vs 2.7%) and higher postoperative PVR (656 ± 668 dyne/s/cm^5 vs 319 ± 223 dyne/s/cm^5, $P = .04$). Even in a multivariate analysis, poor subpleural perfusion was associated with surgical mortality.**

Conclusions: **Poor subpleural perfusion in the capillary phase of pulmonary angiography might be related to small vessel disease and a poor surgical outcome of CTEPH.**

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Abbreviations: CTEPH = chronic thromboembolic pulmonary hypertension; DSA = digital subtraction angiography; PAP = pulmonary arterial pressure; PE = pulmonary embolism; PVR = pulmonary vascular resistance

Chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by pulmonary hypertension caused by nonresolving thromboemboli of the pulmonary artery. The true incidence and prevalence of CTEPH are unknown. From 0.1% to 0.5% of patients who survive an episode of acute pulmonary embolism (PE) have been reported to develop CTEPH.[1] A prospective study that followed patients with acute PE showed that 3.8% developed CTEPH within 2 years.[2] However, up to 40% of the patients with CTEPH demonstrate no clinically apparent acute embolic

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episodes.[3] In addition, Galiè and Kim[4] suggested that acute PE might be an initiating event and that pulmonary hypertension may result from pulmonary vascular remodeling (small vessel disease).

Although pulmonary endarterectomy is an effective modality for the treatment of CTEPH, the most important cause of mortality is the inability to reduce pulmonary arterial pressure (PAP) due to small vessel disease. A high pulmonary vascular resistance (PVR) without parallel evidence of substantial proximal obstruction suggests significant distal vasculopathy and an unsuccessful postoperative outcome,[4,5] but it is difficult to evaluate the degree of pulmonary vascular remodeling in CTEPH from the central portions of pulmonary angiograms and the degree of PVR.

Patients with severe disease may show narrowing and a complete obstruction of pulmonary arteries due to thrombi as well as the elongation and pruning of pulmonary arteries in nonobstructed areas by thrombi on pulmonary angiograms. In these cases, subpleural spaces are either unperfused or minimally perfused in all segments in the capillary phase. In other cases, the subpleural space is well perfused in at least one segment in a nonobstructed area. We hypothesized that poor subpleural perfusion in all segments indicates a severe degree of small vessel disease, resulting in a poor outcome of surgery, and we retrospectively evaluated this hypothesis.

Materials and Methods

Design and Subjects

This was a retrospective single-center cohort study involving consecutive patients. Between July 2000 and December 2009, 110 patients were diagnosed with CTEPH at Chiba University Hospital. CTEPH was defined as mean PAP $\geq$ 25 mm Hg with a normal wedge pressure in patients who had dyspnea on exertion for $>$ 6 months. Additionally, lung perfusion scans were required to demonstrate segmental or larger defects concomitant with a normal ventilation scan. Helical CT scan angiography also was performed to confirm the diagnosis and to exclude large vessel arteritis and tumors. Finally, chronic thromboembolic findings were confirmed by pulmonary angiography.[6] Adequate selective angiography was performed in 104 of these 110 patients.

In the 104 cases, there were more female patients (n = 70) than male patients (n = 34). The age at catheterization ranged from 16 to 78 years (mean $\pm$ SD, 55.3 $\pm$ 13.0). Altogether, 43 patients (41.3%) had a history of DVT, 28 (26.9%) showed abnormalities in the screening for coagulopathy, and 23 (22.1%) had antiphospholipid antibodies. The mean PAP, cardiac index, and PVR were 44.6 $\pm$ 11.9 mm Hg, 2.59 $\pm$ 0.66 L/min/m^2, and 814 $\pm$ 400 dyne/s/cm^5, respectively. Pa_{O_2} while breathing room air was 58.3 $\pm$ 10.6 mm Hg. The 6-min walk distance while breathing appropriate oxygen was 344 $\pm$ 89 m. The number of patients in each World Health Organization functional class was as follows: class 1, n = 3; class 2, n = 29; class 3, n = 67; class 4, n = 5.

Pulmonary Endarterectomy Criteria

The selection criteria for pulmonary endarterectomy were slightly modified from those defined by Moser and colleagues.[7] Our criteria were (1) a mean PAP $>$ 30 mm Hg, resulting in a calculated PVR of $>$ 300 dyne/s/cm^5, even after oral anticoagulant therapy for $>$ 6 months; (2) World Health Organization functional class of $\geq$ 2; (3) thrombi defined as accessible to current surgical techniques (ie, presence at main, lobar, or segmental arteries); and (4) the absence of severe associated disease.[8] Forty-five patients underwent pulmonary endarterectomy. Fifty-nine patients were excluded from surgery because of mild disease (mean PAP $\leq$ 30 mm Hg) (n = 12), relatively peripheral-type thrombi (n = 42), and other associated conditions (age $>$ 70 years, n = 3; COPD, n = 1; thrombocytopenia, n = 1).

Pulmonary Angiography

Informed consent was obtained regarding the performance and risk of right-sided heart catheterization and digital subtraction angiography (DSA) and the respective exposure to radiation and contrast media. Serum creatinine level never exceeded 1.5 mg/dL. Pulmonary angiography was done in conjunction with right heart catheterization. For pulmonary DSA (Infinix; Toshiba Medical Systems Corporation), the right- and left-side pulmonary arteries were selectively catheterized using a 7F Berman catheter.

Arteriograms were acquired at 2 to 3 frames/s. Posteroanterior and lateral projections of each lung were obtained. The contrast bolus consisted of 18 to ~20 mL contrast material with iodine 300 mg/mL for each of the four series. The flow rate was 9 to ~10 mL/s. DSA images were digitally recorded and printed and then analyzed at a PACS workstation (DrABLE-EX; Fujitsu Limited).

We assessed the subpleural perfused area to be $\leq$ 1.5 cm (approximately one rib width) from the lateral pleura in the capillary phase of selective pulmonary angiography in the right- and left-side posterior and anterior views followed by lateral views of the dorsal area, and then divided the patients into either the well-perfused group (subpleural space in at least one segment was well perfused) (Fig 1) or the poorly perfused group (subpleural spaces were either unperfused or minimally perfused in all segments) (Fig 2). The analysis of pulmonary angiograms was done by two trained pulmonologists blinded to the patient's identity. To fully visualize subpleural perfusion, the level and contrast needed to be adjusted on the PACS workstation by each observer. The interobserver agreement between the two investigators also was confirmed by the McNemar test for the first 50 patients ($\kappa = 0.67$, $P < .0001$, n = 50). Final evaluations were achieved by consensus.

Assessment of the Extent of Central Thrombi and Intraoperative Classification

Using the Bergin method by CT scan angiography, the central arteries were defined as vessels proximal to the segmental branches and were divided into four portions. The central disease score was quantified by adding up the number of abnormal central portions in each patient up to a maximum score of 4.[9] Thromboembolic disease was visualized during surgery, and each patient was classified into one of the following four groups as reported by Thistlethwaite et al[10] (intraoperative classification): type 1, fresh thrombus in the main lobar pulmonary arteries; type 2, intimal thickening and fibrosis proximal to the segmental arteries; type 3, disease within the distal segmental arteries only; and type 4, distal arteriolar vasculopathy without visible thromboembolic disease.

The study was approved by the ethics committee of Chiba University (approval number 826). Written informed consent was obtained from each patient before catheterization.

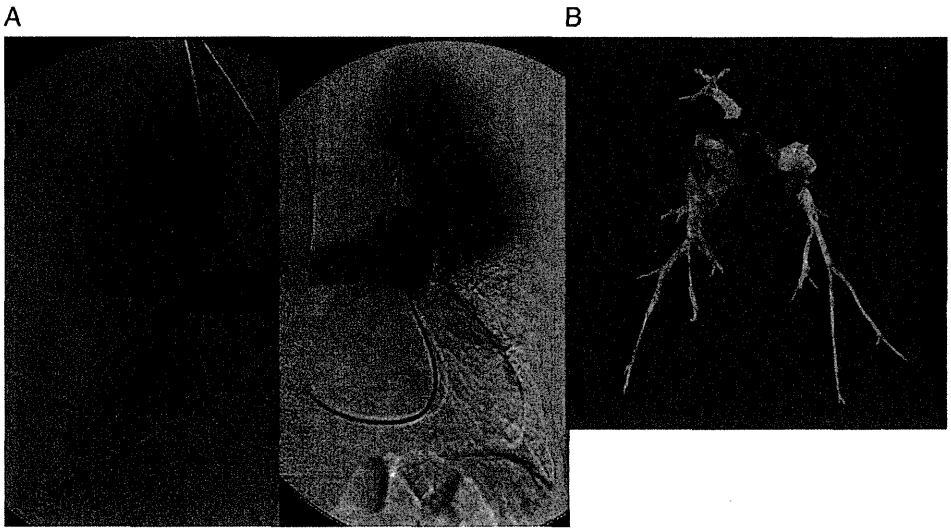

FIGURE 1. A, B, Capillary phase of pulmonary angiograms (A) and endarterectomized material (type 1) (B) in a well-perfused case (pulmonary arterial pressure, 73/23 [41] mm Hg; cardiac index, 2.1 L/min/m^2; PVR, 823 dyne/s/cm^5).

Statistical Analysis

The statistical analysis was performed using a commercially available software program (JMP 9 [Japanese version]; SAS Institute Inc). Comparisons of the well-perfused and poorly perfused groups in terms of the subpleural space were performed using the unpaired Student *t* test for continuous variables and by either the χ^2 test or the Wilcoxon test for categorical data, where appropriate. Risk factors for in-hospital death in the surgical group were identified using a univariate or multivariate logistic regression analyses. Correlations between postoperative PVR and preoperative parameters were analyzed by either univariate or multivariate linear regression analysis. $P < .05$ was considered to be significant.

RESULTS

Seventy-five patients comprised the well-perfused group, and 29 patients comprised the poorly perfused group. The poorly perfused group had a significantly higher mean PAP (49.5 ± 10.5 mm Hg vs 42.7 ± 11.9 mm Hg, $P = .008$) and PVR (969 ± 428 dyne/s/cm^5 vs 754 ± 367 dyne/s/cm^5, $P = .013$) and a lower central disease score compared with the well-perfused group, resulting in fewer patients meeting the operative criteria (27.6% vs 49.3%, $P = .04$) (Table 1). The overall hospital mortality after surgery

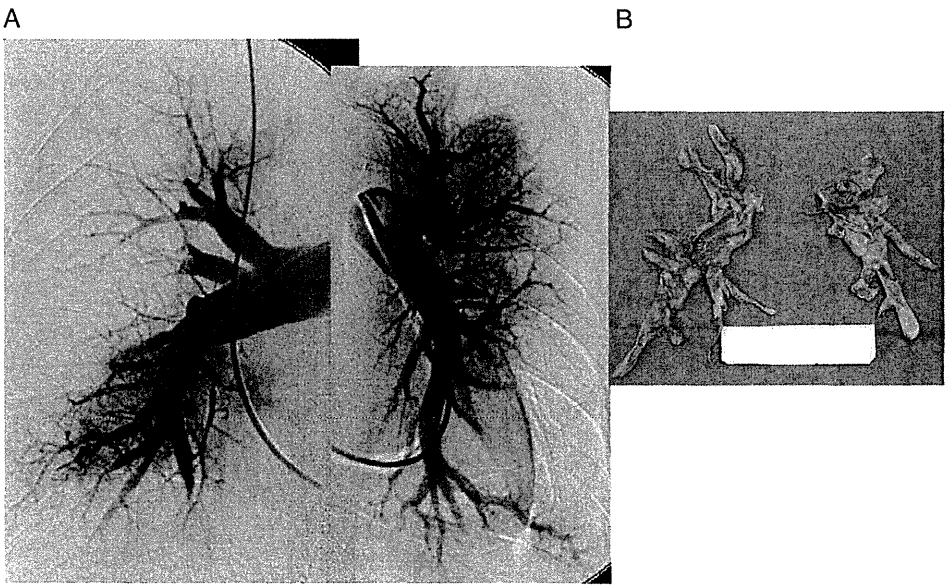

FIGURE 2. A, B, Capillary phase of pulmonary angiograms (A) and endarterectomized material (type 4) (B) in a poorly perfused case (pulmonary arterial pressure, 113/36 [62] mm Hg; cardiac index, 3.11 L/min/m^2; PVR, 1,414 dyne/s/cm^5). The picture of thrombotic material was provided with permission by Motomi Ando, MD, PhD.

Table 1—*Comparison of Clinical Characteristics Between the Well-Perfused and Poorly Perfused Groups*

Parameter	Well Perfused (n = 75)	Poorly Perfused (n = 29)	*P* Value
Age, y	56.9 ± 11.6	51.8 ± 11.6	.08
Female sex, No.	52	18	.48
Acute embolic episodes, %	50.7	44.8	.59
Underlying disease, %			
DVT	42.7	44.8	.84
Coagulopathy	30.7	17.2	.15
Anticardiolipin antibody	25.3	13.8	.19
Hemodynamics			
Mean right atrial pressure, mm Hg	5.0 ± 4.0	8.1 ± 5.1	.0015
Mean PAP, mm Hg	42.7 ± 11.9	49.5 ± 10.5	.008
Cardiac index, L/min/m^2	2.66 ± 0.69	2.41 ± 0.53	.08
PVR, dyne/s/cm^5	754 ± 367	969 ± 428	.013
Pa_{O_2}, mm Hg	58.6 ± 10.7	57.4 ± 10.5	.62
WHO functional class, No.			.009
I	3	0	
II	25	4	
III	46	21	
IV	1	4	
6-min walk distance, m	354 ± 87	317 ± 92	.07
Central disease score, No.			.027
0	18	16	
1	27	8	
2	18	4	
3	10	1	
4	2	0	
Surgical cases, %	49.3	27.6	.04

Data are presented as mean ± SD, unless otherwise indicated. PAP = pulmonary arterial pressure; PVR = pulmonary vascular resistance; WHO = World Health Organization.

was 13.3% (six of 45). Five patients died of residual pulmonary hypertension, whereas one patient in the poorly perfused group died of bleeding. The poorly perfused group had more patients with type 3 or 4 disease during surgery and showed a significantly higher hospital mortality (62.5% vs 2.7%, $P < 00001$) and higher postoperative PVR than the well-perfused group (Table 2). Although a univariate logistic regression analysis showed that a lower 6-min walk distance and poor subpleural perfusion to be associated with hospital death, a multiple regression analysis revealed that only poor subpleural perfusion was associated with hospital death (Table 3). The postoperative PVR was significantly correlated with the preoperative PVR, 6-min walk distance, World Health Organization functional class, and subpleural perfusion by univariate regression analysis. Multivariate regression analysis revealed poor subpleural perfusion to be an independent significant predictor for postoperative PVR (Table 4).

Discussion

Poor subpleural perfusion was found to be associated with surgical mortality as well as with a higher postoperative PVR. Even in a multivariate analysis, poor subpleural perfusion was associated with poor surgical outcome, although the poorly perfused group showed significantly more severe disease and more distal thrombi compared with the well-perfused group at baseline. To our knowledge, this report is the first to show poor subpleural perfusion to be associated with poor surgical outcome of patients with CTEPH.

Several issues still need to be considered when interpreting these results. First, we hypothesized that poor subpleural perfusion in all segments would reflect the degree of the severity of small vessel disease. As expected, the poorly perfused group showed a significantly lower central disease score, more cases of type 3 and 4 disease, and a higher PVR, suggesting a greater contribution of small vessel disease than that of the central thrombi to the severe pulmonary hemodynamic impairment in this group. In addition, poor subpleural perfusion was significantly associated with surgical mortality. Galiè and Kim[4] categorized the mechanism for small vessel disease seen in CTEPH into three processes as follows: (1) obstruction of small subsegmental elastic arteries, (2) classic pulmonary arteriopathy in small muscular arteries and arterioles distal to nonobstructed elastic pulmonary arteries, and (3) arteriopathy in small muscular arteries and arterioles distal to obstructed elastic pulmonary arteries. We excluded some cases with high PVR with respect to only subsegmental emboli or a few segmental emboli

Table 2—*Comparison of Surgical Outcome Between Well-Perfused and Poorly Perfused Groups*

Parameter	Well Perfused (n = 37)	Poorly Perfused (n = 8)	*P* Value
Preoperative mean PAP, mm Hg	46.7 ± 11.0	56.8 ± 6.2	.017
Preoperative PVR, dyne/s/cm^5	857 ± 342	1,184 ± 344	.019
Intraoperative classification			
Type, No.			< .0001
1	32	5	
2	5	0	
3	0	2	
4	0	1	
Postoperative mean PAP, mm Hg	24.8 ± 9.7	36.3 ± 18.9	.08
Postoperative PVR, dyne/s/cm^5	319 ± 223	656 ± 668	.04
Surgical mortality, No. (%)	1 (2.7)	5 (62.5)	< .0001

Data are presented as mean ± SD, unless otherwise indicated. See Table 1 for expansion of abbreviations.

from surgery. However, we had five patients with type 1 disease based on the intraoperative classification in the poorly perfused group, two of whom died of persistent pulmonary hypertension. Small vessel disease could have been present, even though the cases were classified as type 1 disease while also showing central thrombi.

Second, there have been several reports showing a higher PVR to be a significant prognostic factor for surgery.[3-5,8-12] In the present surgical series, the preoperative PVR in the surgical death cases was slightly higher than that in survivors (1,115 ± 260 dyne/s/cm^5 vs 884 ± 369 dyne/s/cm^5, $P = .15$), but it did not reach significance. Excluding the patients with a high PVR without substantial proximal obstruction may result in a smaller contribution of the PVR to the surgical outcome in our series.

Third, predicting the postoperative PVR is important because residual pulmonary hypertension correlates with surgical risk as well as with a poor quality of life at follow-up,[13,14] although a recent article reported that patients with residual pulmonary hypertension still do extremely well in the long term after surgery.[15] The presence of poor subpleural perfusion significantly correlated with postoperative PVR according to a multiple regression analysis. Thistlethwaite et al[10] reported that type 3 or type 4 diseases correlated with a higher postoperative PVR. Although the poorly perfused group included more patients with type 3 or 4 disease, the preoperative angiographic marker could be more helpful than intraoperative classification for determining the surgical indications and predicting postoperative PVR.

Fourth, we excluded 42 patients with relatively peripheral-type disease from surgery because of peripheral thrombi and out-of-proportion PVR without substantial proximal obstruction. A number of good surgical candidates may have been excluded from surgery in this series because out-of-proportion PVR is a subjective evaluation. Angiographic evaluation of the subpleural perfusion in addition to the out-of-proportion PVR may be needed to assess small vessel disease and improve patient selection for surgery.

Fifth, the use of CT scan pulmonary angiograms recently has been substituted for invasive pulmonary angiograms in the diagnosis of CTEPH. However, the present data indicate that pulmonary angiograms still play an important role in the evaluation of small vessel disease. Therefore, pulmonary angiogram remains an important tool in the preoperative evaluation of patients with CTEPH despite recent improvements in CT scan pulmonary angiograms.

Sixth, the guidelines show that the surgical mortality of 13.3% in the present series is higher than that of the current best CTEPH centers (4%-7%),[16] although it had decreased to 10% over the past 5 years. This higher mortality may be associated with inexperience in our center (fewer than five cases per year) rather than with the poor subpleural perfusion by angiographic evaluation.

Table 3—*Preoperative Parameters Associated With Surgical Death by Univariate and Multivariate Logistic Regression Analyses*

	Univariate Analysis		Multivariate Analysis	
Preoperative Assessment	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Mean PAP	1.075 (0.99-1.185)	.10	...	...
PVR	1.007 (0.999-1.004)	.147	...	...
6-min walk distance	0.985 (0.968-0.997)	.031	0.985 (0.959-1.003)	.12
Subpleural perfusion (well vs poorly)	0.017 (0.0007-0.143)	.001	0.0197 (0.00049-0.248)	.0019

See Table 1 legend for expansion of abbreviations.

Table 4—*Correlation Between Preoperative Parameters and Postoperative PVR by Univariate and Multivariate Regression Analyses*

Preoperative Assessment	Univariate Analysis		Multivariate Analysis	
	Partial Regression Coefficient	*P* Value	Partial Regression Coefficient	*P* Value
Mean PAP	1.85	.07	...	...
PVR	2.59	.014	1.28	.21
6-min walk distance	−2.18	.037	−0.73	.35
WHO functional class	2.40	.02	1.69	.10
Central disease score	−1.64	.11		
Subpleural perfusion (well = 1, poorly = 0)	−2.86	.007	−2.39	.024

See Table 1 legend for expansion of abbreviations.

A finally limitation of the present study is related to it being retrospective and based on findings at a single institution. The results need to be confirmed prospectively in a large series from multiple institutions.

In conclusion, poor subpleural perfusion in the capillary phase of pulmonary angiography might be a useful new marker for small vessel disease. It may also be associated with a poor surgical outcome of CTEPH.

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Author contributions: Dr Tanabe had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Dr Tanabe: contributed to the design of the study, data analysis and interpretation, and writing and review of the entire manuscript.
Dr Sugiura: contributed to the data analysis and critical review of the manuscript.
Dr Jujo: contributed to the data analysis and critical review of the manuscript.
Dr Sakao: contributed to the data interpretation and critical review of the manuscript.
Dr Kasahara: contributed to the data interpretation and critical review of the manuscript.
Mr Kato: contributed to the imaging analysis, writing of the methods, and critical review of the manuscript.
Dr Masuda: contributed to performing the surgery, data interpretation, and critical review of the manuscript.
Dr Tatsumi: contributed to the data interpretation and critical review of the manuscript.
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ORIGINAL ARTICLE

Upregulated p53 expression activates apoptotic pathways in wild-type *p53*-bearing mesothelioma and enhances cytotoxicity of cisplatin and pemetrexed

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The majority of malignant mesothelioma possesses the wild-type *p53* gene with a homologous deletion of the INK4A/ARF locus containing the $p14^{ARF}$ and the $p16^{INK4A}$ genes. We examined whether forced expression of p53 inhibited growth of mesothelioma cells and produced anti-tumor effects by a combination of cisplatin (CDDP) or pemetrexed (PEM), the first-line drugs for mesothelioma treatments. Transduction of mesothelioma cells with adenoviruses bearing the *p53* gene (Ad-p53) induced phosphorylation of p53, upregulated Mdm2 and p21 expression levels and decreased phosphorylation of pRb. The transduction generated cleavage of caspase-8 and -3, but not caspase-9. Cell cycle analysis showed increased G0/G1- or G2/M-phase populations and subsequently sub-G1 fractions, depending on cell types and Ad-p53 doses. Transduction with Ad-p53 suppressed viability of mesothelioma cells and augmented the growth inhibition by CDDP or PEM mostly in a synergistic manner. Intrapleural injection of Ad-p53 and systemic administration of CDDP produced anti-tumor effects in an orthotopic animal model. These data collectively suggest that Ad-p53 is a possible agent for mesothelioma in combination with the first-line chemotherapeutics.
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Keywords: malignant mesothelioma; adenovirus; p53; chemotherapy

Introduction

Malignant mesothelioma, most of which are linked with asbestos exposure, remains intractable despite recent treatment modalities.[1] The latent period is long and no preventive procedure is currently available. The patient numbers will continuously increase in the next decades in many industrialized countries as well as in newly developing countries.[2] Extrapleural pneumonectomy is a therapeutic option applicable to an early-staged case, but the recurrence is common even after the radical surgery. Mesothelioma is resistant to radiotherapy, which is mainly used for a palliative purpose. Chemotherapy is therefore a primary treatment in most of the cases and a combination of cisplatin (CDDP) and pemetrexed (PEM) is currently the first-line chemotherapy.[3] Nevertheless, a mean survival rate with the CDDP plus PEM regimen is 12.1 months[3] and the prognosis remains poor. A novel therapeutic strategy is thereby required to improve the prognosis and quality of the patients.

Genetic characterization of mesothelioma cells and the clinical specimens has shown that the majority is defective in the INK4A/ARF locus containing the $p14^{ARF}$ and the $p16^{INK4A}$ genes, but possesses the wild-type *p53* gene.[4] Deletion of p16 increases cyclin-dependent kinase 4/6 activities and subsequently phosphorylates pRb, which induces cell cycle progression. In contrast, p14 deficiency augments Mdm2 activities and consequently downregulates p53 expression, which renders mesothelioma cells resistant to apoptotic pathways. Reintroduction of the defective genes can be a possible treatment modality by activating the suppressor gene functions. Forced expression of p16 or p14 with adenovirus vectors (Ad-p16,

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Ad-p14) in fact inhibited proliferation of mesothelioma cells through dephosphorylation of pRb and restoration of the p53 pathways.[5–8] Expressed p14 however did not fully activate the p53-mediated signal transduction,[9] and Ad-p14-mediated anti-tumor effects required intact p53 downstream pathways.[6] Reactivation of p53 by Ad-p53 can therefore be a more direct way to reconstitute all the p53 signal pathways and can dephosphorylate pRb through p53-induced upregulation of p21. On the other hand, the Ad-p53-mediated anti-tumor effects are difficult to be achieved in wild-type *p53*-bearing tumors compared with those with *p53* mutations.[10] None of the studies in fact has demonstrated Ad-p53-mediated anti-tumor effects in mesothelioma cells, except a short report that showed the suppressed tumor growth using one mesothelioma cell line, but did not investigate the inhibitory mechanism.[11] It remains unknown as to how overexpressed p53 induces growth inhibition to *p14/p16*-defective mesothelioma cells and whether Ad-p53 can produce anti-tumor effects in an orthotopic animal model. Moreover, combinatory effects of Ad-p53 and anticancer agents, in particular CDDP or PEM, have not been investigated with mesothelioma cells. In this study, we examined a possible Ad-p53-mediated cytotoxicity in five kinds of *p14/p16*-defective and *p53*-wild-type mesothelioma cells and demonstrated that upregulated p53 itself induced the p53 phosphorylation, activated p53 downstream pathways and produced combinatory anti-tumor effect with the anticancer agents.

Materials and methods

Cells and mice

Human mesothelioma cells, NCI-H2452, NCI-H2052, NCI-H226, NCI-H28 and MSTO-211H, were obtained from ATCC (Manassas, VA). All of them are defective of the *p14* and *p16* genes, but possess the wild-type *p53* gene,[6] which were also confirmed by our sequencing data. BALB/c *nu/nu* mice (6-week-old females) were purchased from Japan SLC (Hamamatsu, Japan).

Ad preparation

Replication-incompetent type 5 Ad expressing wild-type *p53* (Ad-p53) or β-*galactosidase* gene (Ad-LacZ), in which the cytomegalovirus promoter activated the transgene, were prepared with an Adeno-X expression vector system (Takara, Shiga, Japan) and were purified with Adeno-X virus purification kit (BD Biosciences, Palo Alto, CA). The number of virus particles (vp) per ml was estimated with the formula (absorbance at 260 nm of purified Ad in the presence of 0.1% sodium dodecyl sulfate $\times 1.1 \times 10^{12}$).

Cell cycle analysis

Ad-infected cells were fixed in ice-cold 70% ethanol, incubated with RNase (50 μg ml^{-1}) and stained with propidium iodide (PI) (50 μg ml^{-1}). The staining profiles were analyzed with the FACScan and CellQuest software (BD Biosciences, San Jose, CA).

Viability test in vitro

Cells (1×10^3 per well) were seeded in 96-well plates and were infected with Ad at different doses. In a combinatory treatment, cells were treated with various concentrations of CDDP or PEM and then infected with Ad-p53 or Ad-LacZ. Cell viability was determined with a cell-counting WST kit (Wako, Osaka, Japan) on day 5 and the relative viability was calculated based on the absorbance without any treatments. IC_{50} (half maximal inhibitory concentration) was estimated with a program related to nonlinear least squares in FORTRAN77 Version 3.5 developed by Dr Yamaoka (Kyoto University, Kyoto, Japan; http://www.pharm.kyoto-u.ac.jp/byoyaku/Kinetics/program/manual.htm). Combinatory effects of Ad and an anticancer agent were assessed by calculating combination index (CI) values with the CalcuSyn software (Biosoft, Cambridge, UK). $CI < 1$, $CI = 1$ and $CI > 1$ indicate synergistic, additive and antagonistic actions, respectively.

Western blot analysis

Cells were infected with Ad-LacZ or Ad-p53, and the cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to a nylon filter and was hybridized with antibodies against p53 (Lab Vision, Fremont, CA), phosphorylated p53 at serine (Ser) residues 15 or at Ser 46, Fas, TRAIL receptor (DR5) (Cell Signaling, Danvers, MA), Mdm2 (Santa Cruz Biotech, Santa Cruz, CA), p21 (Santa Cruz Biotech), p27 (BD Biosciences), pRb, phosphorylated pRb at Ser 795, Bid, caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, caspase-9, cleaved caspase-9 (Cell Signaling) or actin (Sigma-Aldrich, St Louis, MO). The membranes were developed with the ECL system (GE Healthcare, Buckinghamshire, UK).

Animal experiments

MSTO-211H cells (1×10^6) were injected into the intrapleural cavity of BALB/c *nu/nu* mice. The mice were treated with intrapleural injection of Ad alone or with a combination of intraperitoneal injection of CDDP on day 3, and the tumor weights were measured on day 28. Phosphate buffered-saline and Ad-LacZ were used as a control. The animal experiments were approved by the animal experiment and welfare committee at Chiba University and were performed according to the guideline on animal experiments.

Results

Phosphorylated p53 and activated caspases in CDDP-treated mesothelioma cells

We examined whether CDDP, a DNA-damaging agent, augmented p53 expression in *p14/p16*-defective and *p53*-wild-type MSTO-211H cells (Figure 1). The treatment upregulated p53 expression and induced the phosphorylation at Ser 15 and 46, both of which are markers for p53-mediated cell cycle arrest and apoptosis. Moreover, cleavage of caspase-8 and -9 together with the decreased pro-caspase forms, which were involved in the extrinsic

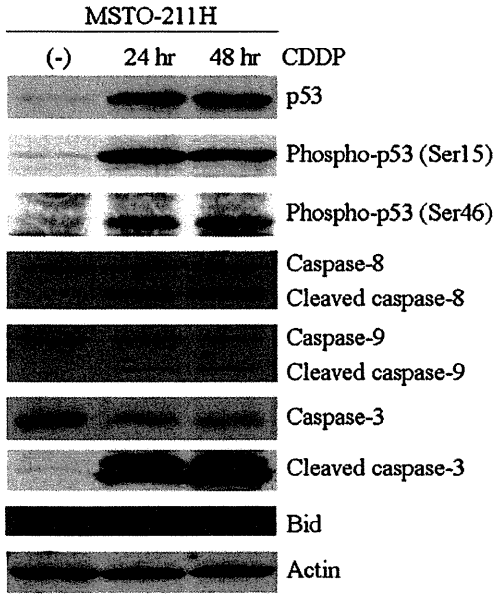

Figure 1 Activation of the p53-mediated pathways in mesothelioma cells. MSTO-211H cells were treated with cisplatin (CDDP) (20 μM) for 24 and 48 h and the extracted protein were subjected to western blot analyses as indicated. Untreated cells were used as a control.

death receptor- and the intrinsic mitochondria-linked apoptosis, respectively, were detected in the CDDP-treated cells. We noted that Bid expression was down-regulated, but truncated Bid, which contributes to the linkage between the death receptor- and the mitochondria-mediated apoptosis, was not induced (data not shown). Caspase-3, an enzyme mapped in a common apoptotic pathway after caspase-8 and -9 activations, was also cleaved. These data collectively suggest that *p14/p16*-defective mesothelioma maintains p53 downstream functions and that death receptor-mediated and mitochondrial-mediated pathways were independently activated upon CDDP treatments.

Decreased viability of mesothelioma treated with Ad-p53

We examined whether transduction of *p53* could suppress viability of five kinds of human mesothelioma cells with *p14/p16*-defective and *p53*-wild-type genes. Relative viabilities of mesothelioma cells were examined with different doses of Ad-p53 or Ad-LacZ as a control (Figure 2). Ad-p53 but not Ad-LacZ treatment decreased viability of all the mesothelioma cells with a dose-dependent manner, except NCI-H2052 cells. The IC_{50} values suggested that NCI-H28 cells were the most sensitive to Ad-p53 and the others, NCI-H226, NCI-H2452 and MSTO-211H cells, became susceptible when infected with increased virus numbers. Insensitivity of NCI-H2052 cells to Ad-p53 could be partly due to the low expression level of the major Ad receptor, coxsackie adenovirus receptor (Supplementary Figure 1). Ad infectivity to NCI-H2025 cells was in fact poor compared with that to other cells (Supplementary Figure 2), but the differential susceptibility was also attributable to other factors than the Ad infectivity, such as the vulnerability to Ad-induced cell death. In addition, greater amounts of Ad-p53 overcame the insensitivity and induced the growth suppression in NCI-H2052 cells (data not shown).

Cell cycle changes induced by Ad-p53

We examined cell cycle changes induced by Ad-p53 or Ad-LacZ at two different doses (1×10^4 and 3×10^4 vp per cell) (Table 1). Transduction of NCI-H2452 cells with Ad-p53 increased G0/G1-phase populations with reduced S-phase fractions at 24 h and the infection at 3×10^4 vp per cell increased sub-G1 populations at 48 h. The cell cycle changes, G0/G1 arrest followed by increased sub-G1 populations, were more significant in MSTO-211H cells than NCI-H2452 cells, although the IC_{50} value of MSTO-211H cells was greater than that of NCI-H2452 cells. Ad-p53-infected NCI-H28 cells showed increased G2/M-phase populations with decreased S-phase populations at 24 h and augmented sub-G1 populations thereafter. Sub-G1 fractions increased significantly in NCI-H28 cells compared with NCI-H2452 and MSTO-211H cells, showing their high susceptibility to Ad-p53-mediated apoptosis. Interestingly, Ad-p53-infected NCI-H28 cells did not show G0/G1 arrest in contrast to NCI-H2452 and MSTO-211H cells. Moreover, G2/M fractions increased in NCI-H28 cells with Ad-p53 treatment at 24 h and with Ad-LacZ treatment at 48 h, suggesting that increased G2/M fractions can be at least partly due to Ad infections. These cell cycle changes caused by Ad-p53 were thus subjected to cell types, and were dependent on Ad doses and treated periods as well.

Activation of p53-mediated pathways

We further examined molecular events of Ad-p53-mediated effects in MSTO-211H and NCI-H28 cells with western blot analyses (Figure 3a). Transduction with Ad-p53 increased p53 levels and the phosphorylation was readily detected at Ser 15 and then at Ser 46 residues. Ad-LacZ infection did not upregulate endogenous p53 expression or induce the phosphorylation. Expression levels of Mdm2, 90-kDa full-length molecules and 60-kDa cleaved moieties that represented a p53-bound form[12] increased by transduction with Ad-p53, but not with Ad-LacZ. Anti-pRb antibody detected a change of pRb migration from the high to the low molecular weight accompanied by the Ad-p53 transduction, which corresponds to a shift from the phosphorylated to the dephosphorylated state. The decreased phosphorylation was confirmed by anti-phosphorylated pRb antibody, which detected the reduced intensity. Expression levels of p21 increased with Ad-p53 transduction in both cells, but p27 expressions altered differently. The p27 expression was upregulated in Ad-53-infected MSTO-211H cells, but the level in NCI-H28 cells remain unchanged or even decreased at 36 h after the Ad-p53 infection. These data demonstrated that Ad-p53 infection activated p53-mediated pathways, but subsequent induction of cyclin-

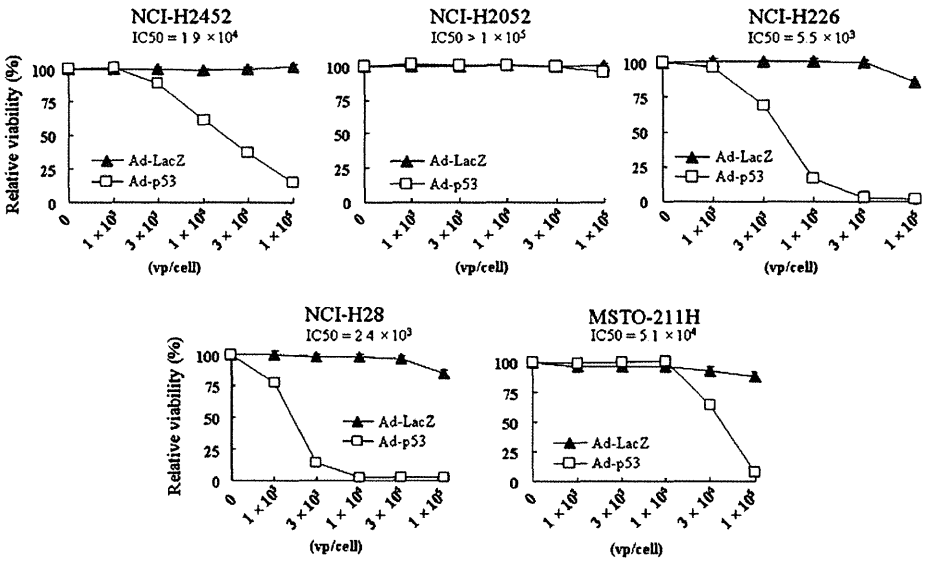

Figure 2 Adenoviruses bearing the *p53* gene (Ad-p53)-mediated suppression of cell viability. The relative viability of mesothelioma cells that were treated with various dose of Ad-p53 or Ad-LacZ was examined with the WST assay. IC_{50} (half maximal inhibitory concentration) values for Ad-p53-mediated suppression were shown as viral particles (vp) per cell. Standard errors (s.e.) was too small to be shown ($n=3$).

dependent kinase inhibitors, p21 and p27, was not coordinated.

We then examined activation of caspases in Ad-p53-infected MSTO-211H and NCI-H28 cells (Figure 3b). Transduction with Ad-p53 but not Ad-LacZ induced cleavage of caspase-3 and -8, whereas Ad-p53 transduction did not stimulate caspase-9 cleavage. Bid expression levels were basically unchanged and truncated Bid was not detected in both cells (data not shown). Expressions of Bax in MSTO-211H cells remained unchanged and those in NCI-H28 cells were barely detected (data not shown). We also examined expression levels of the molecules in upstream pathways of the extrinsic apoptosis system (Figure 3c). Expression levels of Fas were augmented in both cells and those of TRAIL receptors (DR5) were also upregulated in NCI-H28 cells. FADD expressions were however almost unchanged (data not shown). These data collectively suggest that Ad-p53 transduction activated the extrinsic death receptor-mediated pathways through caspase-8 and that the intrinsic mitochondrial-mediated apoptosis was less likely to be involved.

Combination of Ad-p53 and anticancer agents

We examined possible combinatory anti-tumor effects of Ad-p53 and CDDP or PEM with the WST assay (Figure 4). Mesothelioma cells were treated with different concentrations of CDDP or PEM and then were transduced with Ad-p53. Transduction with Ad-p53 enhanced CDDP-induced anti-tumor effects and the CI values showed that the Ad-p53 and CDDP were synergistic in the inhibitory activity in most of the cells tested, except NCI-H2452 cells, which showed minimal synergism between 0.3 and 0.6 at fraction affected (Figures 4a and b). Combination of Ad-p53 and PEM also produced synergistic effects except in NCI-H28 cells, which showed slight inhibitory effects at a lower fraction affected range (Figures 4c and d). Notably, NCI-H2052 cells, which were resistant to Ad-p53-mediated growth inhibitory effects (Figure 2), came to be more sensitive to CDDP and to PEM when treated with Ad-p53 than with Ad-LacZ. We also examined cell cycle changes induced by the combination of Ad-p53 and PEM in NCI-H2052 and MSTO-211H cells (Table 2). Ad-p53 transduction alone induced minimal cell cycle changes at the virus doses used. PEM treatments increased S-phase fractions in NCI-H2052 cells and both S-phase and sub-G1 populations in MSTO-211H cells as PEM is a DNA synthesis inhibitor. Concomitant Ad-p53 treatments increased sub-G1 populations in PEM-treated cells, whereas Ad-LacZ did not influence the cell cycles. These data showed that Ad-p53 transduction enhanced cytotoxicity of the anticancer agents.

In vivo *anti-tumor effects by Ad-p53 with CDDP*

We investigated the anti-tumor effects of Ad-p53 in an orthotopic animal model, in which nude mice were injected with MSTO-211H cells in the pleural cavity and received intrapleural administration of Ad-p53 or Ad-LacZ as a control on day 3. Tumor weights on day 28 showed that Ad-p53 produced anti-tumor effects in a dose-dependent manner, whereas Ad-LacZ administration did not influence the weight (Figure 5a). We also examined combinatory effects with Ad-p53 and CDDP, both of which were administered on day 3 (Figure 5b). Administration of Ad-p53 and CDDP, respectively, inhibited the tumor growth and the combination decreased the tumor weight greater than Ad-p53 or CDDP treatment alone. A combinatory use of Ad-p53 and PEM was not examined as an optimal PEM dose to

Table 1 Cell cycle distribution by Ad-p53 or Ad-LacZ treatment

Cells	*Treatment*[a] *(vp per cell)*	*Time (h)*	*Cell cycle distribution (%)*[b]			
			Sub-G1	*G0/G1*	*S*	*G2/M*
NCI-H2452	(−)	24	1.3 ± 0.1	61.4 ± 0.8	13.9 ± 0.4	23.6 ± 0.4
	Ad-LacZ (1×10^4)	24	2.0 ± 0.1	62.2 ± 0.6	13.2 ± 0.2	23.0 ± 0.3
	Ad-LacZ (3×10^4)	24	1.7 ± 0.1	57.9 ± 0.7	15.2 ± 0.2	25.6 ± 0.7
	Ad-p53 (1×10^4)	24	1.7 ± 0.1	70.8 ± 0.5[c]	4.4 ± 0.1	23.3 ± 0.4
	Ad-p53 (3×10^4)	24	1.8 ± 0.1	70.9 ± 0.7[c]	3.9 ± 0.1	23.5 ± 0.7
	(−)	48	1.8 ± 0.2	67.6 ± 0.8	10.0 ± 0.3	20.7 ± 0.6
	Ad-LacZ (1×10^4)	48	1.7 ± 0.1	59.8 ± 0.5	12.8 ± 0.4	25.6 ± 0.4
	Ad-LacZ (3×10^4)	48	1.8 ± 0.1	57.4 ± 0.5	12.8 ± 0.3	28.3 ± 0.5
	Ad-p53 (1×10^4)	48	2.2 ± 0.1	74.7 ± 0.3[c]	3.4 ± 0.1	19.8 ± 0.3
	Ad-p53 (3×10^4)	48	5.5 ± 0.2[c]	69.8 ± 0.5[c]	3.9 ± 0.1	21.1 ± 0.6
NCI-H28	(−)	24	0.3 ± 0.1	71.7 ± 0.7	10.7 ± 0.4	17.5 ± 0.5
	Ad-LacZ (1×10^4)	24	0.4 ± 0.1	69.4 ± 0.4	10.9 ± 0.3	19.6 ± 0.3
	Ad-LacZ (3×10^4)	24	0.4 ± 0.1	72.2 ± 0.2	8.5 ± 0.2	19.3 ± 0.1
	Ad-p53 (1×10^4)	24	0.9 ± 0.1	72.1 ± 0.4	1.1 ± 0.2	26.0 ± 0.4[c]
	Ad-p53 (3×10^4)	24	1.9 ± 0.1[c]	61.8 ± 0.4	4.4 ± 0.2	32.1 ± 0.4[c]
	(−)	48	0.2 ± 0.1	71.6 ± 0.4	9.1 ± 0.3	19.4 ± 0.5
	Ad-LacZ (1×10^4)	48	0.2 ± 0.1	68.1 ± 0.4	6.7 ± 0.3	25.2 ± 0.5[d]
	Ad-LacZ (3×10^4)	48	0.3 ± 0.1	63.8 ± 0.8	4.3 ± 0.1	31.8 ± 0.9[d]
	Ad-p53 (1×10^4)	48	20.5 ± 0.4[c]	56.6 ± 0.5	4.3 ± 0.1	18.7 ± 0.2
	Ad-p53 (3×10^4)	48	49.2 ± 0.8[c]	36.9 ± 0.3	3.0 ± 0.1	11.2 ± 0.1
MSTO-211H	Ad-LacZ (1×10^4)	24	3.6 ± 0.3	64.7 ± 0.4	13.7 ± 0.1	18.3 ± 0.3
	Ad-LacZ (3×10^4)	24	3.4 ± 0.1	64.0 ± 0.5	13.9 ± 0.2	19.0 ± 0.2
	Ad-p53 (1×10^4)	24	3.7 ± 0.2	84.7 ± 0.2[c]	3.9 ± 0.1	7.7 ± 0.1
	Ad-p53 (3×10^4)	24	7.9 ± 0.2[c]	84.5 ± 0.2[c]	1.8 ± 0.1	5.9 ± 0.2
	Ad-LacZ (1×10^4)	48	1.7 ± 0.1	90.8 ± 0.1	2.4 ± 0.2	5.1 ± 0.1
	Ad-LacZ (3×10^4)	48	1.7 ± 0.2	87.9 ± 0.2	3.1 ± 0.2	7.3 ± 0.2
	Ad-p53 (1×10^4)	48	5.5 ± 0.3[c]	81.3 ± 0.2	4.5 ± 0.2	8.8 ± 0.1
	Ad-p53 (3×10^4)	48	23.9 ± 0.3[c]	70.0 ± 0.6	1.9 ± 0.2	4.4 ± 0.2

Abbreviations: Ad-LacZ, replication-incompetent type 5 adenoviruses expressing the β-*galactosidase* gene; Ad-p53, replication-incompetent type 5 adenoviruses expressing the wild-type *p53 gene*; h, hours; vp, virus particles.
[a]Cells were infected with or without Ad (1×10^4 or 3×10^4 vp per cell) and cultured for the indicated time.
[b]Cell cycle profiles were analyzed with flow cytometry and data are shown in mean percentages with s.e's ($n=3$).
[c]$P<0.05$, comparing between Ad-p53-infected cells and corresponding untreated or Ad/LacZ-infected cells.
[d]$P<0.05$, comparing between Ad-LacZ-infected cells and corresponding untreated or Ad-p53-infected cells.

produce anti-tumor effects in mice was difficult to be determined.

Discussion

We examined Ad-p53-mediated anti-tumor effects in five kinds of mesothelioma cells and firstly demonstrated to our knowledge combinatory effects of Ad-p53 and CDDP or PEM in *p53*-wild-type mesothelioma. We showed activation of p53 pathways with CDDP treatments and demonstrated that p53-mediated apoptosis was maintained in *p14/p16*-defective mesothelioma cells, which were evidenced by upregulated p53 expression, the phosphorylation at Ser 15 and 46 residues and cleavage of caspases. Ad-p53 likewise induced p53 expression and the phosphorylation at Ser 15 and thereafter at Ser 46, both of which are linked with cell cycle arrest and apoptosis, respectively.[13,14] It is however currently unknown about a precise mechanism of phosphorylation of p53 induced under no apparent DNA damage as Ad infection itself, as demonstrated with Ad-LacZ, did not increase endogenous p53 expression or the phosphorylation. Overexpressed p53 was not always associated with the phosphorylation,[9] but increased p53 expression can augment activities of p53-phosphorylating kinases and consequently result in p53 activation.

Upon p53 induction and phosphorylation, Mdm2 and p21 expression, both of which are induced by p53-mediated transcriptional activation, were upregulated. Dephosphorylation of pRb can be induced by the upregulated p21 through inhibiting cyclin-dependent kinase activities. Mdm2 can be overexpressed in *p14*-defective cells because p14 suppresses the expression, but previous studies as well as this study showed no such enhancement of Mdm2 expression in *p14/p16*-defective mesothelioma cells.[15] Full-length 90 kDa Mdm2 molecules were cleaved into 60 kDa molecules, which was induced by the binding to p53, and the cleavage level was linked with p53 induction. These data collectively demonstrated that Ad-p53 activated the downstream pathways and blocked pRb-mediated cell cycle progression, suggesting that Ad-p53

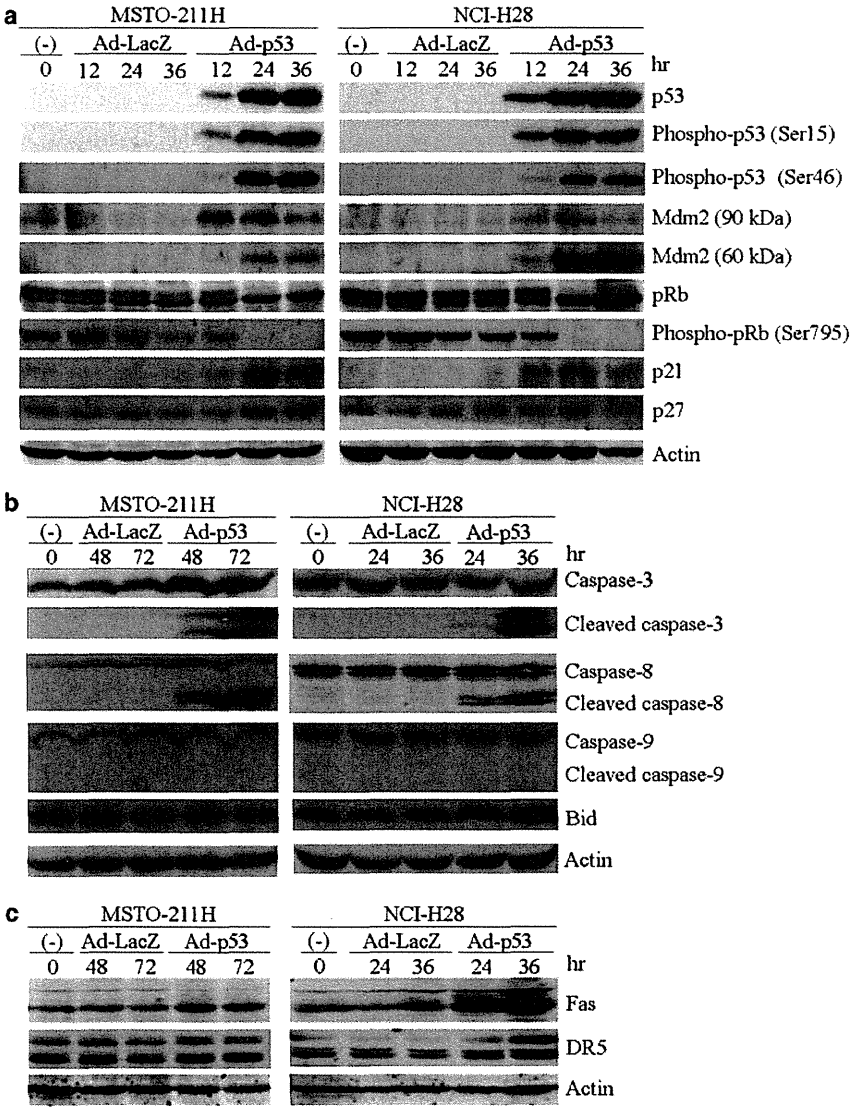

Figure 3 Adenoviruses bearing the *p53* gene (Ad-p53)-induced activation of p53 downstream pathways. MSTO-211H and NCI-H28 cells were infected with Ad-p53 or Ad-LacZ (7×10^3 viral particles (vp) per cell) and were cultured for the indicated time. Expression levels of p53- (**a**), caspase- (**b**) linked protein and (**c**) death receptors were analyzed with western blot analyses. Actin is used as a loading control.

restored loss of tumor suppressor functions in *p14/p16*-defective mesothelioma. Expression of p27 in NCI-H28 cells, in contrast to that in MSTO-211H cells, did not coordinately increase responding to p53 activation as found in p21 expression. The dissociated expressional changes between the two cyclin-dependent kinase inhibitors might be linked with differential cell cycle alterations, increased G0/G1-phase population in MSTO-211H cells and no G0/G1 arrest in NCI-H28 cells (discussed later).

Apoptosis induction was accompanied by Ad-p53 transduction. Increased cleaved caspase-8, but not caspase-9, suggested activation of the extrinsic receptor-mediated apoptosis pathways without involvement of the mitochondria-mediated pathways. Moreover, expressions of Bax remained unchanged and truncated Bid, which is involved in a cross-talk between the extrinsic and the intrinsic apoptosis pathways, was not detected. We however detected upregulated death receptors, Fas and TRAIL receptor molecules, as reported previously.[16] These biochemical data suggested that Ad-p53 activated primarily the extrinsic pathway in contrast to CDDP treatments that activated the both pathways. Shinoura *et al.*[17] demonstrated enhanced apoptosis by a combination of Ad-p53 and forced expressions of Apaf-1 and caspase-9, suggesting less involvement of the intrinsic pathways by Ad-p53.

Cell cycle analyses showed differential effects of Ad-p53 to respective mesothelioma cells. Ad-p53 induced G0/G1

arrest in NCI-H2452 cells and a small cell population was subjected to sub-G1 fractions. The similar effects were observed in MSTO-211H cells, but increase of sub-G1 fractions was significant. In contrast, NCI-H28 cells showed increased G2/M populations at 24 h and sub-G1-phase fractions thereafter. Ad infection itself increased G2/M populations in NCI-H28 cells as found in Ad-LacZ-treated cells at 48 h. The NCI-H28 cell cycle profiles thus suggested that p53 accelerated the Ad-mediated G2/M arrest. It remains unclear as to the mechanism how Ad infection itself increased G2/M phase and whether expressed p53 differentially arrested cell cycle progression at either the G0/G1 or G2/M phase. The present biochemical data supported p53-induced cell cycle arrest at the G0/G1 phase. Phosphorylated p53 at Ser 15 followed by at Ser 46 matched to the sequential change of cell cycle arrest followed by apoptosis. Increased p21 and p27 expressions and dephosphorylated pRb in MSTO-211H cells favored G0/G1 arrest, but unchanged or decreased p27 expression in NCI-H28 cells

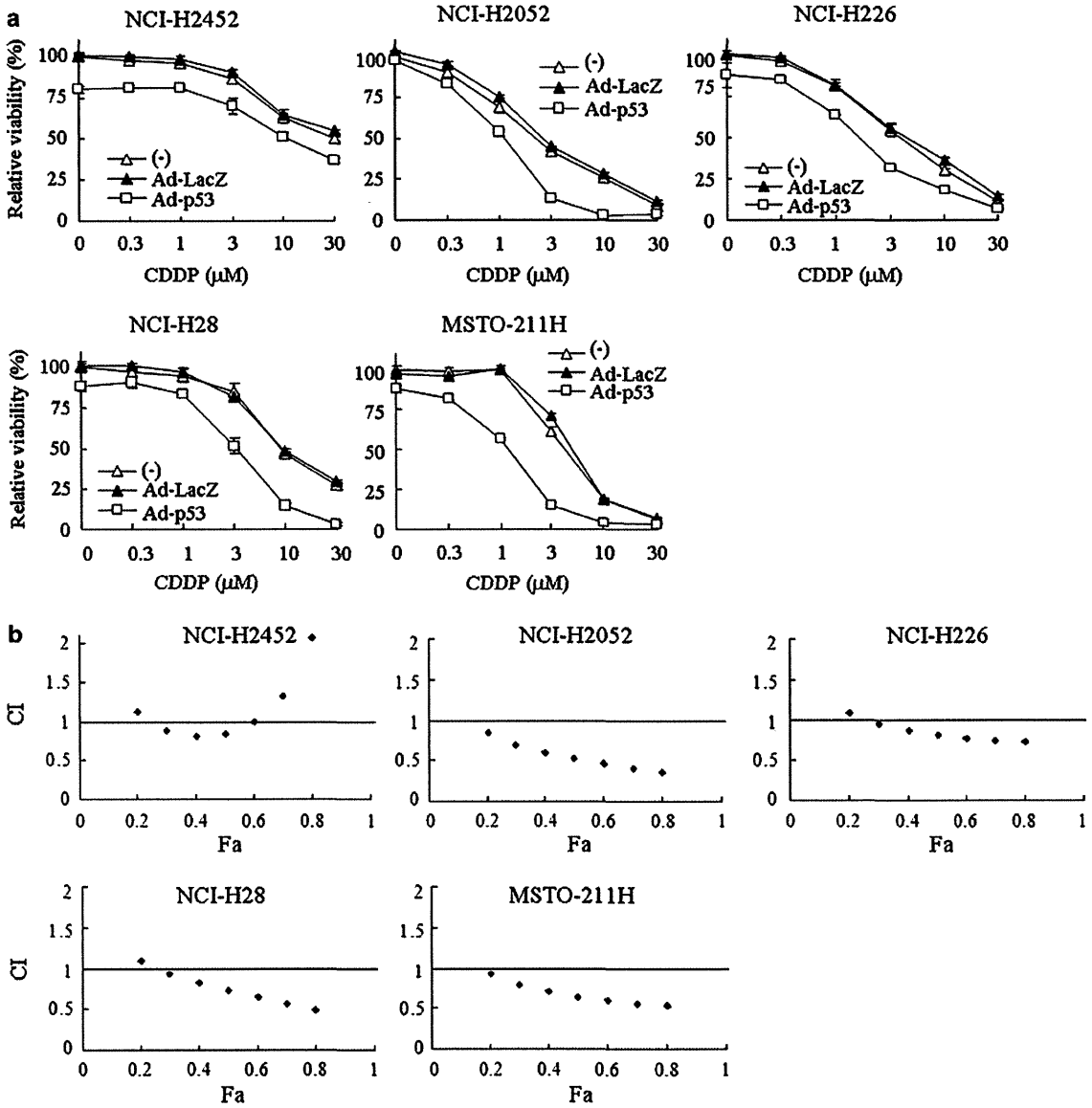

Figure 4 Combinatory effects of Adenoviruses bearing the *p53* gene (Ad)-p53 and anticancer agents. Cells were treated with cisplatin (CDDP) (**a**) or pemetrexed (PEM) (**c**) for 24 h, and then infected with Ad-p53 or Ad-LacZ (NCI-H2452: 1×10^4 viral particles (vp) per cell; NCI-H2052: 1×10^5 vp per cell; NCI-H226: 3×10^3 vp per cell; NCI-H28: 1×10^3 vp per cell; MSTO-211H: 3×10^4 vp per cell) and were cultured for 4 days after the Ad infection. Relative viability of cells was examined with the WST kit and s.e. bars are shown ($n=3$). Combination index (CI) of Ad-p53 and CDDP (**b**) or PEM (**d**) in respective fraction affected (Fa) values were shown.

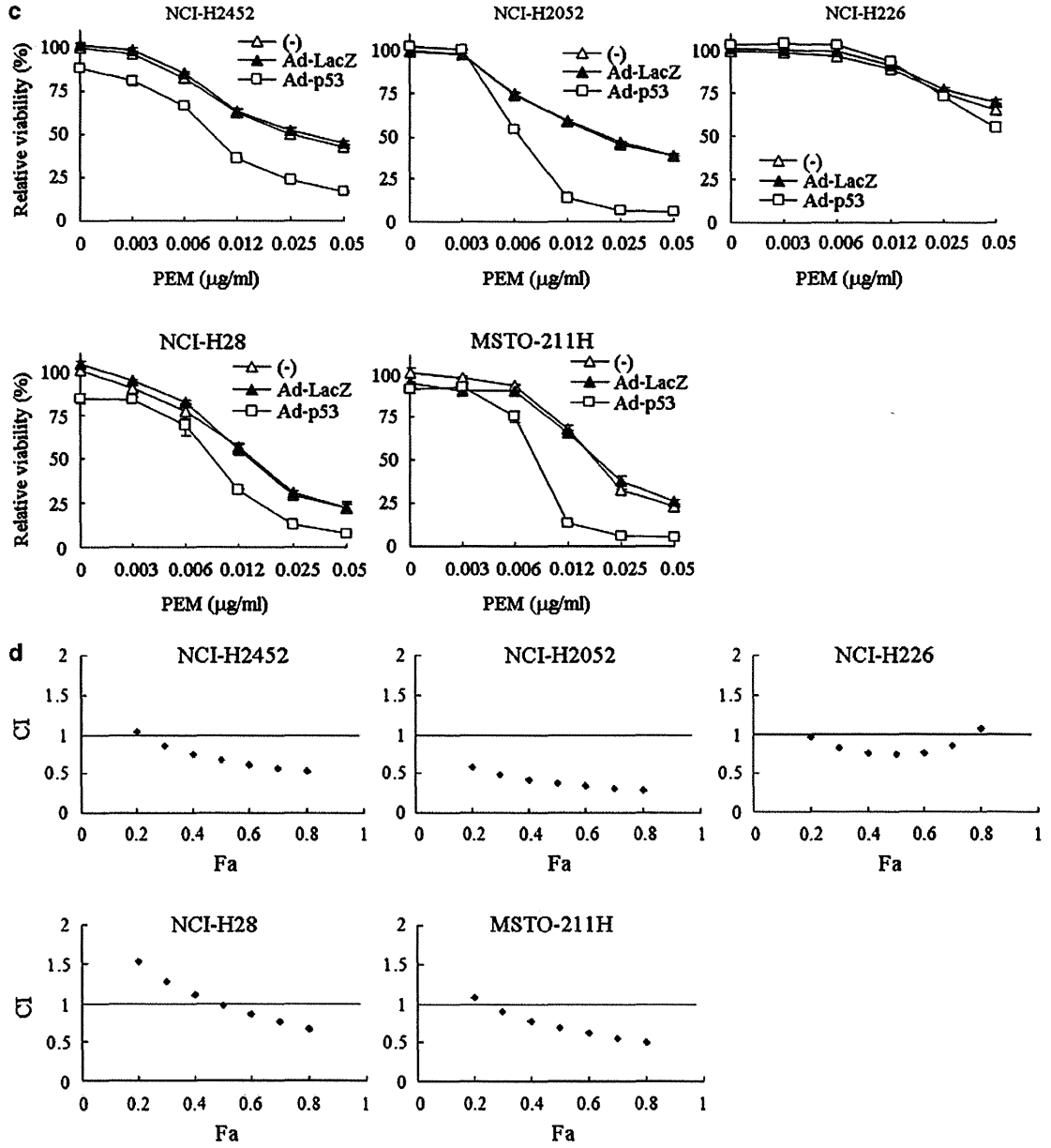

Figure 4 Continued.

may counteract cell cycle arrest at the G0/G1 phase. Cell viability data tested with the WST assay were not completely consistent with cell cycle analysis data in terms of Ad-p53 susceptibility. Cell cycle distributions showed that NCI-H2452 cells were resistant to Ad-p53-mediated apoptosis and MSTO-211H cells were sensitive, whereas the cell viability data indicated that NCI-H2452 cells were more susceptible than MSTO-211H cells. The differential results could be due to distinctive assays systems. Cell cycle analyses showed relative distributions of each cell cycle population, but the WST assay measured a metabolic activity of the whole cell populations. The p53 family members such as p73 and p63 may also differentially influence susceptibility to apoptosis, although expression levels of the p53 family protein were not well studied in mesothelioma cells.

Anti-tumor effects mediated by Ad-p53 need to be compared with those by Ad-p14 or Ad-p16 for the therapeutic efficacy. These Ad-mediated effects can be linked with the functions of respective genes re-expressed in mesothelioma. Ad-p14 induced upregulation of p53 expression and subsequently G0/G1 arrest and apoptosis.[6] Ad-p14 transduction however did not augment CDDP-induced apoptosis or cleaved caspase-3 levels,