Table 1: Patient characteristics ($n = 102$)

Age (years)	
Median	67
Range	34–89
Sex	
Female	52
Male	50
Size of tumour on HR-CT (mm)	
Median	18
Range	8–29
Histology	
Adenocarcinoma	91
Squamous cell	7
Adenosquamous	2
Large cell	1
Carcinoid	1
Serum CEA	
≤5.0 ng/ml	89
>5.0 ng/ml	13
Clinical stage	
IA	102 (T1a: 84, T1b: 18)
Pathologic stage	
IA	92
IB	8
IIA	1
IIIA	1

CEA, carcinoembryonic antigen.

Table 2: Location of burdened lung ($n = 102$)

Right upper lobe: 30
S^1: 8, $S^1 + S^2a$: 1, S^2: 9, $S^2b + S^3a$: 6, $S^2 + S^3a$: 1, S^3: 5
Right lower lobe: 26
S^6: 16, $S^6 + S^8$: 1, $S^7 + S^8$: 1, S^8: 3, $S^8 + S^9$: 2, $S^9 + S^{10}$: 1, S^7–S^{10}: 2
Left upper lobe: 22
$S^{1+2} + S^3$: 10, $S^{1+2}a$: 1, S^{1+2}: 3, $S^3 + S^{1+2}a$: 1, $S^3a + b + S^4 + S^5$: 1, $S^4 + S^5$: 6
Left Lower lobe: 24
S^6: 16, S^8: 2, S^9: 2, $S^9 + S^{10}$: 3, S^8–S^{10}: 1

a, posterior subsegment; b, anterior subsegment; S^1, apical; S^2, posterior; S^{1+2}, apicoposterior; S^3, anterior; S^4, superior; S^5, inferior; S^6, superior; S^7, medial basal; S^8, anterior basal; S^9, lateral basal; S^{10}, posterior basal.

Table 3: Surgical results ($n = 102$)

Operation time (min)	
Median	129
Range	60–275
Bleeding (ml)	
Median	50
Range	10–350
Utility access incision (mm)	
Median	50
Range	40–80
Complication	10
Prolonged air leak (>7 days)	4
Late alveolopleural fistula	3
Supraventricular arrhythmia	2
Interstitial pneumonia	1
Operative mortality	0
Mortality during follow-up	9
Cancer death	5
Other	4
Recurrence	12
Locoregional	
Dissemination	2
Mediastinum	2
Margin	1
Distant	
Lung	4
Brain	2
Meningitis	1

Follow-up of alive patients (month) median: 61 (range: 8–84).

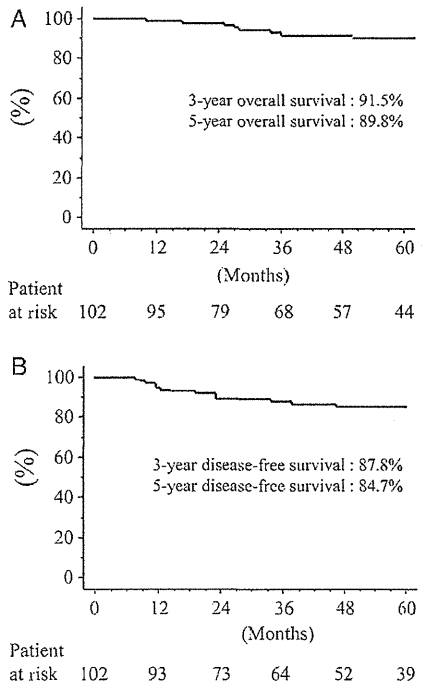

Figure 3: Postoperative Kaplan–Meier curves following radical hybrid VATS segmentectomy. (A) Overall survival; (B) disease-free survival.

conversion to thoracotomy requiring an incision of ≥8 cm in length. The perioperative complication rates were very low (9.8%, 10/102) and no operative mortality occurred. Since the selection of patients was not random in this series, which may have produced a bias in the selection of patients for specific procedures, we cannot describe the prognosis in detail even at a median follow-up of more than 5 years. The locoregional and distant recurrence rates were low at 4.9% (5/102) and 6.9% (7/102), respectively. The overall and disease-free 5-year survival rates of 89.8 and 84.7%, respectively, were comparable to previously published data [4, 13]. The outcomes of recent studies including ours indicate that VATS segmentectomy for recent smaller and more indolent tumours should be satisfactory from an oncological viewpoint [13, 14]. The ongoing clinical trial comparing the surgical results between lobectomy and sublobar resection conducted by the Cancer and Leukemia Group B (CALGB 140503) and by the Japan Clinical Oncology Group/West

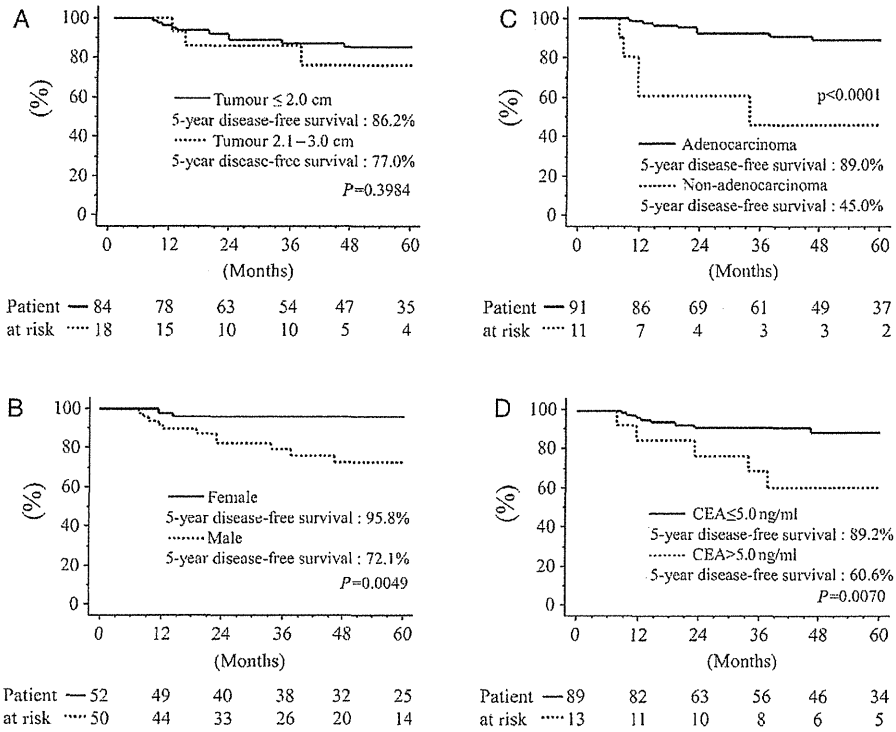

Figure 4: Disease-free survival following radical hybrid VATS segmentectomy, according to (A) tumour size, (B) gender, (C) histology and (D) serum CEA value.

Table 4: Multivariate analysis of factors predicting recurrence

Variable	Hazard ratio	95% confidence interval	*P*-value
Continuous factors			
Age	0.999	0.926–1.078	0.9880
Tumour size	1.069	0.887–1.288	0.4827
Skin incision	1.011	0.974–1.050	0.5623
Categorized factors			
Gender			
Male	Reference		
Female	0.240	0.047–1.216	0.0847
Side			
Right	Reference		
Left	1.028	0.269–3.919	0.9681
Lobe			
Upper	Reference		
Lower	0.599	0.151–2.387	0.4679
Histology			
AD	Reference		
Non-AD	6.775	1.488–30.839	0.0133
CEA			
≥5.0 ng/ml	Reference		
>5.0 ng/ml	1.452	0.330–6.383	0.6217

AD, adenocarcinoma; CEA, carcinoembryonic antigen.

Japan Oncology Group (JCOG0802/WJOG4607L) [15] will demonstrate the role of segmentectomy for patients with NSCLC 2 cm or smaller. The former randomly assigns patients to undergo segmentectomy or wedge resection and lobectomy, but the latter cannot permit wedge resection as a sublobar resection although surgeons in both trials can freely select the access technique by VATS or by thoracotomy.

The primary target of ideal function-saving surgery is to preserve the pulmonary parenchyma, and the secondary aim is to reduce trauma through selecting the appropriate surgical approach. As you know from accumulated experience, complete VATS approach can be used in simple lobectomy for lung cancer. However, we have applied a hybrid VATS approach to more technically demanding surgical procedures for treating lung cancer, such as bronchoplasty, angioplasty and atypical segmentectomy [9]. Segmentectomy allows optimal resection of small deep non-palpable lesions with suspected malignancy, which helps preserve lung function. Under these conditions, the hybrid VATS approach can provide safe surgical margins, although lobectomy even using a complete VATS approach must not be an option for any small undiagnosed tumour. The three-dimensional view provided by direct vision through hybrid VATS enables surgeons to understand the appropriate margins, thus minimizing the likelihood of missing diseased lung tissue or removing an excessive volume of healthy tissue. The advantages of treating lung cancer with VATS using only monitor visualization are arguable for the patient, although the surgeon seems to get a great sense of achievement. Whether or not direct vision is applied in VATS is in essence a peripheral matter. Thoracic surgeons need to balance the benefits and disadvantages of complete VATS, for example, to avoid lobectomy thoughtlessly performed for a tiny undiagnosed tumour. The most important aspects of cancer surgery are radicality and the preservation of functionality for the patients.

The key to successful high-quality procedures using the hybrid VATS approach is the upside-down backhand grip on long scissors for incisive dissection, and the long needle holder for bronchoplasty or angioplasty developed by Belsey (Frenchay Hospital; Bristol, UK) and Pearson (Toronto General Hospital; Toronto, ON, Canada) to facilitate deep manoeuvring. This skill

was advanced before the introduction of VATS and we consider it suitable for manoeuvring through a small access thoracotomy in this heyday of VATS. In general, segmentectomy is technically more demanding than lobectomy because it requires a thorough three-dimensional knowledge of all relevant bronchoarterial relationships and possible anomalies of the arterial branches. Sharp dissection with scissors that can accurately and rapidly expose the segmental hilar structures is important for radical hybrid VATS segmentectomy.

We concluded that hybrid VATS segmentectomy is a safe and useful option for patients with small N0 NSCLC who can tolerate lobectomy. The accurate identification of the intersegmental plane and dissection along an adequate cutting line by electrocautery prevents local failure and maintains lung function that are crucial during radical segmentectomy even through the VATS approach. Minimally invasive strategies will be applied to ever more challenging segmentectomies such as radical atypical resection of segments and/or subsegments, and thus become a standard approach for treating lung cancer.

Conflict of interest: none declared.

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Chapter 2

Effect of Asbestos on Anti-Tumor Immunity and Immunological Alteration in Patients with Malignant Mesothelioma

Yasumitsu Nishimura, Megumi Maeda, Naoko Kumagai-Takei, Hidenori Matsuzaki, Suni Lee, Kazuya Fukuoka, Takashi Nakano, Takumi Kishimoto and Takemi Otsuki

Additional information is available at the end of the chapter

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1. Introduction

It is well known that malignant mesothelioma is caused by exposure to asbestos, which comprises a group of naturally occurring fibrous minerals. However, the mechanism by which asbestos causes malignant mesothelioma remains unclear. Many researchers have examined the effect of exposure to asbestos on the body. To date, it has been confirmed that asbestos can cause various forms of damage to cells, including cellular toxicity and mutagenicity, as well as produce reactive oxygen species (ROS) (Mossman & Churg, 1998; Mossman et al., 1996; Sporn & Roggli, 2004). The levels of oxidized pyrimidine and alkylated bases correlate with the period of occupational exposure to asbestos (Dusinska et al., 2004), and the increase in mutation frequency of lung DNA is caused by instillation of asbestos through the trachea (Topinka et al., 2004). All of these factors are thought to generate the tumorigenic effect of asbestos on mesothelial cells. However, the development of malignant mesothelioma caused by exposure to asbestos shows the noteworthy characteristics of this condition, which differ from those induced by other toxic materials. Malignant mesothelioma develops under a relatively low or medium dose of exposure to asbestos. A high dose of exposure to asbestos causes the development of pneumoconiosis, i.e., asbestosis rather than mesothelioma. Thus, the development of mesothelioma caused by exposure to asbestos cannot be explained only by a general rule regarding a dose-response relationship of toxic materials. In addition, it takes a long period of about forty years to develop malignant mesothelioma after exposure to asbestos. These findings suggest the existence of other factors related to the development of malignant mesothelioma that are modified by exposure to asbestos in the body, and which differ from the well-known

tumorigenic effect of asbestos on mesothelial cells. One possible factor seems to be the effect of exposure to asbestos on anti-tumor immunity. In the body, the development of tumors is protected by anti-tumor immunity, composed of various kinds of cells including dendritic cells (DC), natural killer (NK) cells, helper T (Th) cells, cytotoxic T lymphocytes (CTLs), and so on. Exposure to asbestos might cause a suppressive effect on anti-tumor immunity in addition to the tumorigenic effect on mesothelial cells, and the combination of immune-suppressive and tumorigenic effects of asbestos might contribute to the development of malignant tumor (Fig. 1).

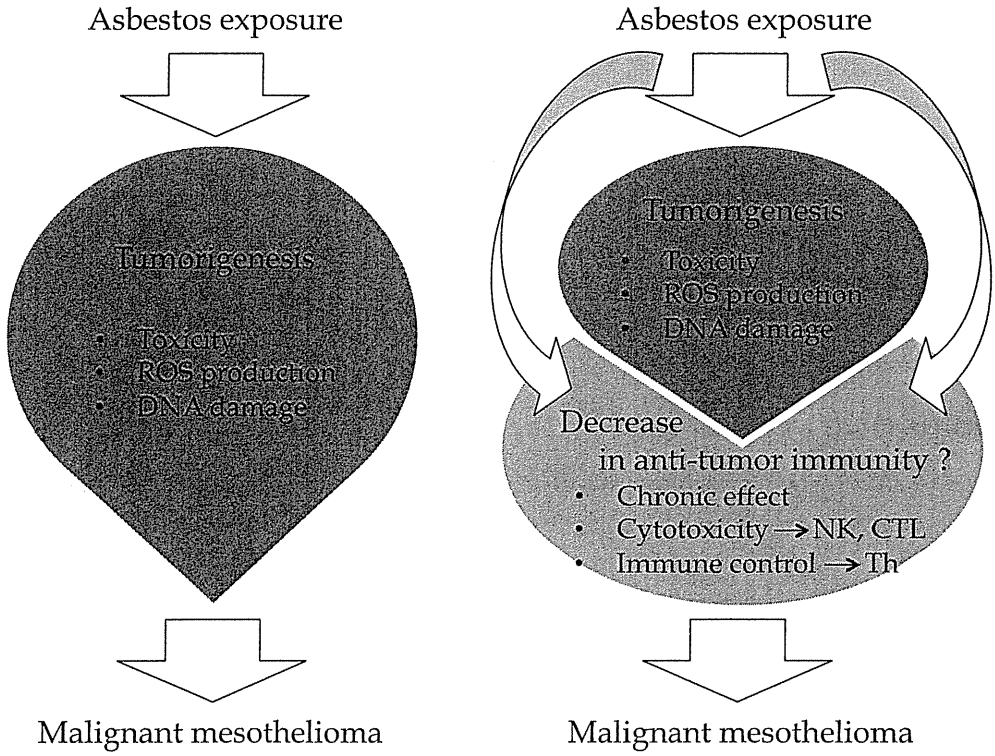

Figure 1. Hypothesis concerning the development of malignant mesothelioma caused by the immunological and tumorigenic effects of asbestos exposure. Many researchers have attempted to examine the tumorigenic effect of asbestos, thought to cause malignant mesothelioma (left). We propose the hypothesis that immune-suppressive effects, including the effect on NK- and T-cell functions, and the tumorigenic effects of asbestos exposure might contribute to the development of malignant mesothelioma (right).

The lung is not the only place where immune competent cells are able to meet asbestos fibers. Inhaled asbestos fibers reach the lung via the trachea, but they do not remain at that site. Those fibers translocate into the lung-draining lymph nodes over a long period. Dodson et al. examined the amount of asbestos in the lungs, lymph nodes, and plaque in a cohort of former shipyard workers, and reported that an analysis of asbestos in the lymph nodes confirmed accumulation in these sites (Dodson et al., 1991). In addition, asbestos fibers in the lymph nodes can translocate into the blood and may be observed in any tissue of the body, even in the brain where the accumulation of asbestos is low because of the blood-

brain barrier (Miserocchi et al., 2008). Thus, immune competent cells have many opportunities to encounter asbestos fibers in the body, and the primary place where these cells meet asbestos seems to be the lung-draining lymph nodes (Fig. 2).

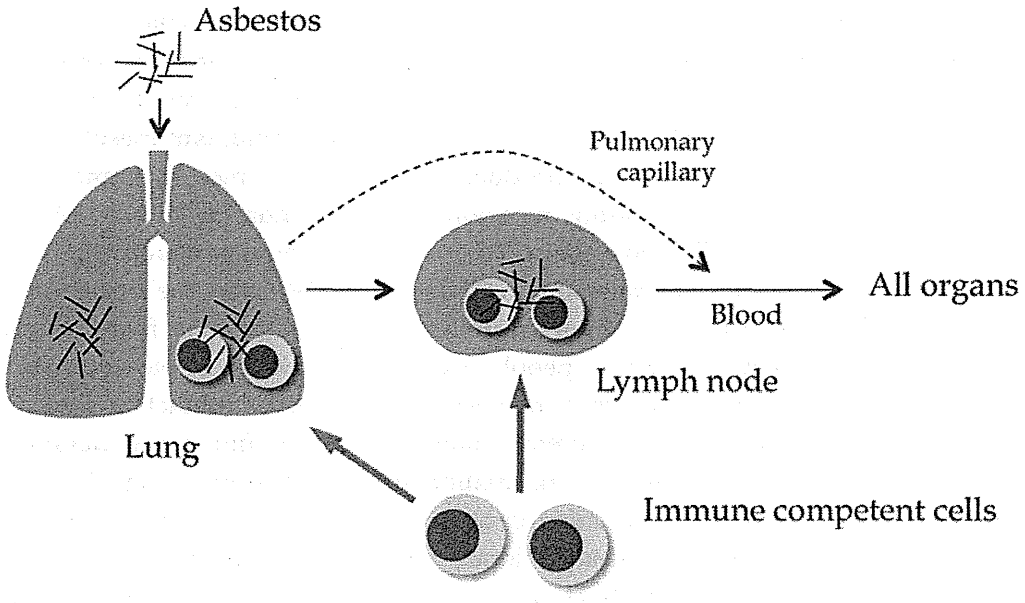

Figure 2. Places where immune competent cells encounter asbestos. Inhaled asbestos fibers reach the lung and translocate into the lung-draining lymph nodes. Therefore, the primary places where immune cells encounter asbestos seem to be the lung-draining lymph nodes.

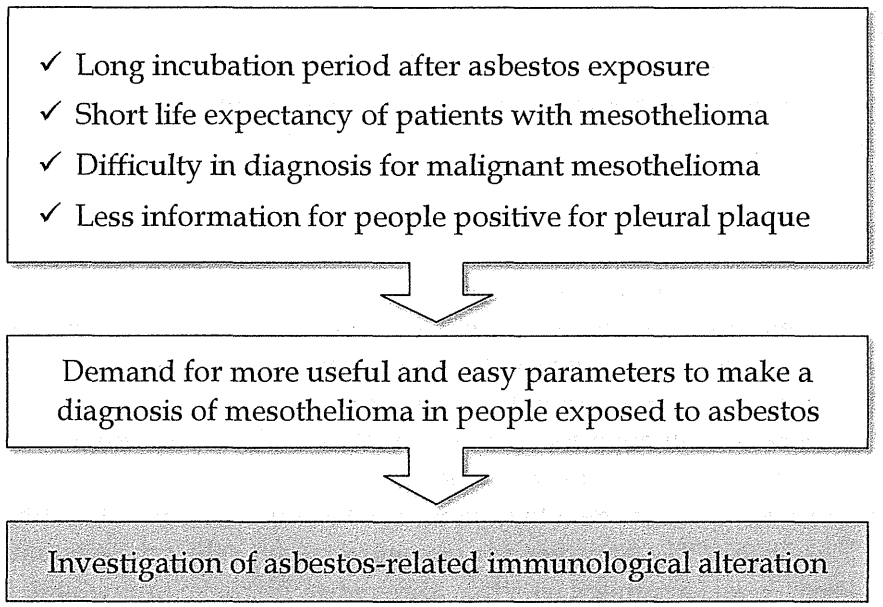

Figure 3. Present problems in malignant mesothelioma and the demand for a new parameter useful in making a diagnosis of malignant mesothelioma. Due to the problems shown in the top box, more useful and easy parameters to detect malignant mesothelioma in people exposed to asbestos are needed, to which our investigation of asbestos-related immunological alteration might contribute.

The present diagnosis for malignant mesothelioma is based on X-ray and CT image analyses, as well as pathohistological analysis. However, diagnosis using these procedures is sometimes accompanied with a risk of radiation exposure or invasiveness, and results are not regarded equally among doctors because they have learned these analyses separately and it is not easy to master all of these methods. In addition, although it takes a long period to develop malignant mesothelioma after exposure to asbestos, the mean life expectancy of patients with malignant mesothelioma is short, and people exposed to asbestos need a safer analysis that can be used frequently in a year in order to detect malignant mesothelioma as early as possible. Recently, the use of products derived from mesothelioma cells for diagnosis has been proposed, including megakaryocyte potentiating factor (MPF) and mesothelin (Creaney et al., 2007; Onda et al., 2006). However, those products might not be observed in the blood that early because they will appear exactly after the development of mesothelioma, and it may take some time for those products to transfer from the pleural cavity into the blood stream. Many people exposed to asbestos worry about the development of malignant mesothelioma; however, there is little predictive information regarding the onset of mesothelioma. Therefore, there is a need to find a new parameter or method useful for the early diagnosis of malignant mesothelioma (Fig. 3). If some characteristic alteration of immune function is caused by exposure to asbestos that is also found in patients with malignant mesothelioma, which can be measured by checking lymphocytes or other cells in peripheral blood, the analysis for that alteration might contribute to the early detection of malignant mesothelioma.

On the basis of these ideas, we started to investigate the immunological effect of exposure to asbestos and immunological alteration in patients with malignant mesothelioma. In this chapter, we show the results obtained from these studies concerning the effect of asbestos on anti-tumor immunity, focusing on NK and Th cells, and discuss the immune-suppressive effect of asbestos and the possible application of our results for the early diagnosis of malignant mesothelioma.

2. NK cells

The role in cytotoxicity against target cells in anti-tumor immunity is played by two populations of cells, natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). NK cells have a cytotoxicity for targets which they are ready to kill without prior stimulation, whereas this readiness is absent from the killing activity of CTLs and is induced by antigen stimulation. Therefore, although the cytotoxicity of NK cells has no antigen specificity, it is thought to contribute widely to the early deletion of unhealthy cells such as virus-infected cells or transformed cells. On the other hand, it takes time to induce the differentiation of naïve $CD8^+$ T cells to CTLs, but they can recognize target cells precisely and injure them effectively. Imai et al. examined whether differences between individuals in regard to natural immunological host defense, i.e., NK cytotoxicity, can predict the future development of cancer. They reported that medium and high cytotoxic activity of peripheral blood lymphocytes is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk, and these findings suggest a role for natural immunological host

defense mechanisms against cancer (Imai et al., 2000). Therefore, we examined the effect of exposure to asbestos on cytotoxicity of NK cells and alteration in cytotoxicity of NK cells in patients with malignant mesothelioma.

2.1. Mechanism of cytotoxicity in NK cells

The mechanism of cytotoxicity in NK cells and CTLs can be separated into two parts. The role of one part is to recognize target cells, which is followed by transduction of the stimulation signal into the cytosol, while the other part acts to kill target cells. In the killing mechanism, both NK cells and CTLs use the common molecules perforin and granzymes. Perforin- and granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to eliminate virus-infected or transformed cells (Trapani & Smyth, 2002). Perforin and granzymes are produced and accumulate in the cytotoxic granules of NK cells. Once NK cells are optimally stimulated, perforin and granzymes are released into the gap of the immune synapse by degranulation and act on target cells to induce apoptosis. Perforin is the protein that can disrupt the cellular membrane and create a pore in the membrane of the target cell. Granzyme is a family of structurally related serine proteases, which enters target cells through the pore made by perforin, and induces apoptosis of the target cells. The second pathway to kill targets is Fas-mediated apoptosis, induced by ligation of the Fas ligand (FasL) expressed on NK cells or CTLs with Fas on target cells. In addition to these two pathways, tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) is also known to control the growth and metastasis of tumors (Smyth et al., 2001; Takeda et al., 2001). These killing mechanisms are followed by recognition of target cells by NK cells. In contrast to T cell, which utilizes the T cell receptor (TCR) to recognize targets, NK cells utilize various kinds of receptors for target recognition. These receptors could be of either type: inhibitory or activating. In the next section, we explain the significance of the expression levels of NK cell receptors for cytolytic activity.

2.2. NK cell receptors

NK cells do not have clonal diversity like T cells, which include many repertoires, rearrangements, and somatic mutations of TCRs. However, NK cells can recognize various target cells using various kinds of receptors expressed on the cell surface of NK cells (Moretta, L. & Moretta, A., 2004; Yokoyama & Plougastel, 2003). Some NK cell receptors, the ligands of which are human leukocyte antigen (HLA) molecules, genetically differing among individuals and recognized by T cells with T cell receptor (TCR) to find abnormal cells, have the role of transducing an inhibitory signal. Those inhibitory receptors include a KIR family of receptors and heterodimer of NKG2A and CD94. The inhibitory signals derived from those receptors contribute to prohibition of cytotoxicity against normal self cells. In contrast, several other receptors transduce an activation signal after ligation with their respective ligands to induce cytotoxicity against abnormal target cells (Fig. 4). NKG2D is the best characterized activating receptor expressed on NK cells. NKG2D is a receptor belonging to the same group as NKG2A, NKG2 family, characterized by a lectin-like domain, but can transduce an activation signal unlike NKG2A. The signaling lymphocytic

activation molecule (SLAM) family is another group of activating receptors expressed on NK cells. A representative receptor of the SLAM family is 2B4, which induces cytotoxicity by stimulation with the natural ligand, CD48, or the antibody to 2B4 (Endt et al., 2007; Garni-Wagner et al., 1993; Valiante & Trinchieri, 1993). Moreover, natural cytotoxicity receptors (NCRs) make a family of receptors that includes NKp46, NKp44 and NKp30, which play a major role in the NK-mediated killing of most tumor cells (Moretta, A. et al., 2001; Sivori et al., 1999). These activating receptors transduce the stimulation signal leading to the phosphorylation of c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinase (ERK), which cause polarization of the microtubule organizing center (MTOC) and cytotoxic granules followed by release of perforin and granzymes, producing degranulation (Chen et al., 2006; Chen et al., 2007). Thus, the various kinds of receptors expressed on NK cells control induction of cytotoxicity for target cells, and alteration in expression of these receptors is thought to affect the strength of the stimulation signal to induce cytotoxicity of NK cells. In addition, if NK cells exposed to asbestos show some characteristic alteration in expression of NK cell receptors, this alteration might be used as a possible marker of asbestos exposure-related immune alteration. Therefore, we planned to study the effect of asbestos exposure on NK cells, focusing on the expression level of NK cell receptors, as well as investigate the cytotoxicity of NK cells.

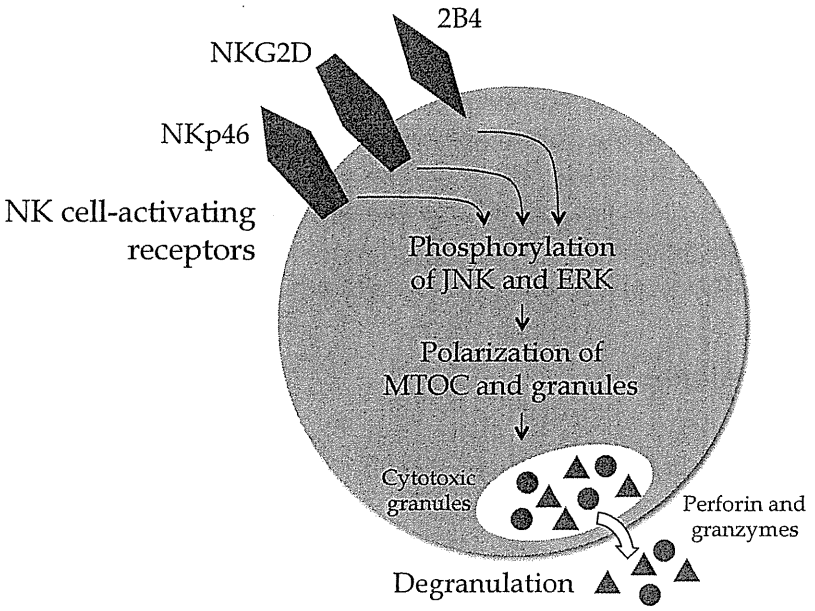

Figure 4. NK cell-activating receptors and the machinery of degranulation caused by stimulation with these receptors. The ligation of NK cell-activating receptors with their respective ligands induce phosphorylation of JNK and ERK, leading to polarization of the microtubule organizing center (MTOC) and granules, where perforin and granzymes are released and induce apoptosis of target cells.

2.3. Cytotoxicity of human NK cell line cultured with asbestos for a long period

We initially started by making the sub-line of an NK cell line by culturing cells exposed to asbestos for a long period. The human NK cell line of YT-A1 was kindly provided by Dr.

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Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemistry of cytokeratins 7, 8, 17, 18, and 19, and GLUT-1 aids differentiation of desmoplastic malignant mesothelioma from fibrous pleuritis

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Summary. It is difficult to distinguish desmoplastic malignant mesothelioma (DMM) from fibrous pleuritis (FP). We investigated the utility of immunohistochemistry as a way of differentiating between DMM and FP. We examined 11 DMMs and 46 FPs with the aid of antibodies against 18 cytokeratin (CK) subtypes, calponin, caldesmon, desmin, and GLUT-1. The best sensitivity and specificity cut-off values in the receiver operating characteristic curves (ROC) for CKs 7, 8, 17, 18, and 19, and GLUT-1 were each above 60%. When cases with either DMM or FP were partitioned by the staining score associated with the best sensitivity and specificity cut-off values in ROC, the incidence of a positive expression for CKs 7, 8, 17, 18, and 19, and GLUT-1 was significantly higher in DMM than in FP. In conclusion, immunohistochemistry for CKs 7, 8, 17, 18, and 19, and GLUT-1 may be useful, alongside histological characteristics, for separating DMM from FP.

Key words: Immunohistochemistry, Desmoplastic malignant mesothelioma, Fibrous pleurisy, Cytokeratins, GLUT-1

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Introduction

Malignant mesothelioma is a relatively rare tumor that originates from the serosal membrane of the pleura, peritoneum, pericardium, or tunica vaginalis. The latent period between asbestos exposure and onset of mesothelioma is reported to be between 15 and 60 years (Bianchi et al., 1997; McElvenny et al., 2005). In Japan, the Ministry of Health, Labor, and Welfare has disclosed that the number of deaths due to mesothelioma increased gradually from 500 in 1995 to 1156 in 2009 (Ministry of Health, Labour and Welfare 2010). On the above basis, mesothelioma cases are expected to continue to increase in Japan, and to peak in about the year 2025 because large amounts of asbestos were used in Japan between 1960 and 1975.

In the 2004 WHO classification, malignant mesotheliomas were essentially classified as epithelioid, biphasic, sarcomatoid, or desmoplastic. Although the desmoplastic type had been classified as a subtype of sarcomatoid mesothelioma until 2004, it is now considered a new entity because it is characterized by a shorter survival than either epithelioid or sarcomatoid mesothelioma (Churg et al., 2004). To qualify for a diagnosis of desmoplastic mesothelioma (DMM), the paucicellular collagen-rich tissue must occupy at least 50% of a tissue specimen. In addition to the above findings, a diagnosis of DMM requires a storiform pattern or the "patternless pattern" of Stout (Stout,

1965), plus one or more of the following four findings: invasion of chest wall or lung, bland necrosis, frankly sarcomatoid areas, and distant metastases. However, it is difficult to distinguish the histological features of DMM from those of fibrous pleuritis (FP) because the inflammation and hyperplasia of connective tissue cause a change in the form of the epithelioid cell so that it shows cytological atypia.

In recent years, a number of immunohistochemical markers -- including antibodies to cytokeratin (CK) 5/6, calretinin, Wilm's tumour-1 (WT-1), and thrombomodulin -- have become available for the diagnosis of mesothelioma. These markers have proven very useful for differentiating epithelioid mesothelioma from lung adenocarcinoma (Cury et al., 2000; Oates and Edwards 2000; Carella et al., 2001; Ordóñez 2003; Suster and Moran 2006; Addis and Roche 2009; Husain et al., 2009). In contrast, the frequency and degree of expression of these markers in DMM has not been characterized, and the effectiveness of immunohistochemistry for differentiating DMM from FP is unclear. To address these issues, we used antibodies against 18 cytokeratins, calponin, caldesmon, desmin, and GLUT-1 to examine a series of 11 DMMs and 46 FPs.

Materials and methods

We obtained surgically resected or autopsie specimens from patients with DMM (11 specimens) o FP (46 specimens). The DMM specimens were collecte in 7 different hospitals across Japan. The DMM patient were 9 men and 2 women (mean age 68.3 years; rang 57 to 83). Among them, seven patients had had previou occupational or environmental exposure to asbestos. Te patients had pleural effusion with a high hyaluronic aci concentration, and 2 cases had positive cytology. Seve patients (excluding the 4 autopsy cases) died after a operation, such as panpleuropneumonectomy pleurectomy or pleural biopsy. The four autopsy case died at 1 to 8 months after the onset of symptoms.

Macroscopically, pleural thickening and adhesio were seen in DMM. Microscopically, DMM wa characterized by the presence, in at least 50% of th tumor, of dense collagenized tissue separated by atypica tumor cells arranged in a storiform or "patternless' pattern. These areas included micronodular proliferatio and foci of bland necrosis. For the definitive diagnosis o mesothelioma, we performed immunohistochemistr using 8 antibodies. Tumor cells were positive fo

Table 1. Antibodies used in this study.

Antibody/ antigen	Type*/clone	Source	Dilution	Pretreatment
CK† 1	G	Acris Antibodies GmbH, Herford, Germany	1/25	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 2	M/BM5091	Acris Antibodies GmbH, Herford, Germany	1/100	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
CK 3	M/AE5	Enzo Life Sciences, Plymouth Meeting, PA	1/1,000	boil in 0.05M citrate-0.002M EDTA buffer, pH6.0, for 60 min
CK 4	M/EP1599	Novus Biologicals, Littleton, CO	1/100	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 5	M/XM26	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/300	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 6	M/LHK6B	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/20	boil in 0.01M Tris buffer, pH 10.0, for 60 min
CK 7	M/OV-TL 12/30	Nichirei Biosciences Inc, Tokyo, Japan	1/5	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
CK 8	M/DE-K	DAKO, Glostrup, Denmark	1/2	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 9	M/Ks9.70+Ks9.216	Progen Biotechnik GmbH, Heidelberg, Germany	1/10	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 10	M/LHP1	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/400	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
CK 12	R	TransGenic Inc, Hyogo, Japan	1/500	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 13	M/KS-1A3	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/500	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 14	M/LL002	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 15	M/LHK15	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/300	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 16	M/LL025	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 17	M/E3	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	autoclave in 0.05M citrate-0.002M EDTA buffer, pH6.0, for 20 min
CK 18	M/DC-10	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 19	M/b170	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/1000	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
Calponin	M/ CALP	DAKO, Glostrup, Denmark	1/400	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
Caldesmon	M/ h-CD	DAKO, Glostrup, Denmark	1/200	boil in 0.05M citrate-0.002M EDTA buffer, pH6.0, for 60 min
Desmin	M/ D33	Nichirei Biosciences Inc, Tokyo, Japan	ready to use	No pretreatment
GLUT-1	R	Immuno-Biological Laboratories Co. Ltd, Fujioka, Japan	1/50	No pretreatment

*: G, guinea pig: M, mouse: R, rabbit; †: cytokeratin.

calretinin and AE1/AE3 in all cases, D2-40 in 9 cases, and WT-1 in 7 cases, but negative for CEA, BerEP4, MOC31, and TTF-1 in all cases. Since the first four antibodies yield positive reactions in mesothelioma, while the last four yield positive reactions in pulmonary adenocarcinoma, we diagnosed DMM in all 11 cases.

Immunohistochemistry

We used the polymer-peroxidase method (EnVision+/HRP; Dako Cytomation, Denmark) on deparaffinized sections of DMM and FP. All antibodies were incubated overnight at 4 degree Celsius. The monoclonal and polyclonal antibodies used are listed in Table 1, together with the antigen-retrieval conditions.

For the analysis of immunoreactivity, the extent of moderate-to-strong staining was scored as: 0, indicating negative reaction of tumor cells; 1, ≤10% of tumor area stained; 2, 11 to 25% stained; 3, 26 to 50% stained; 4, 51 to 75% stained; or 5, ≥76% stained. For each antibody, a

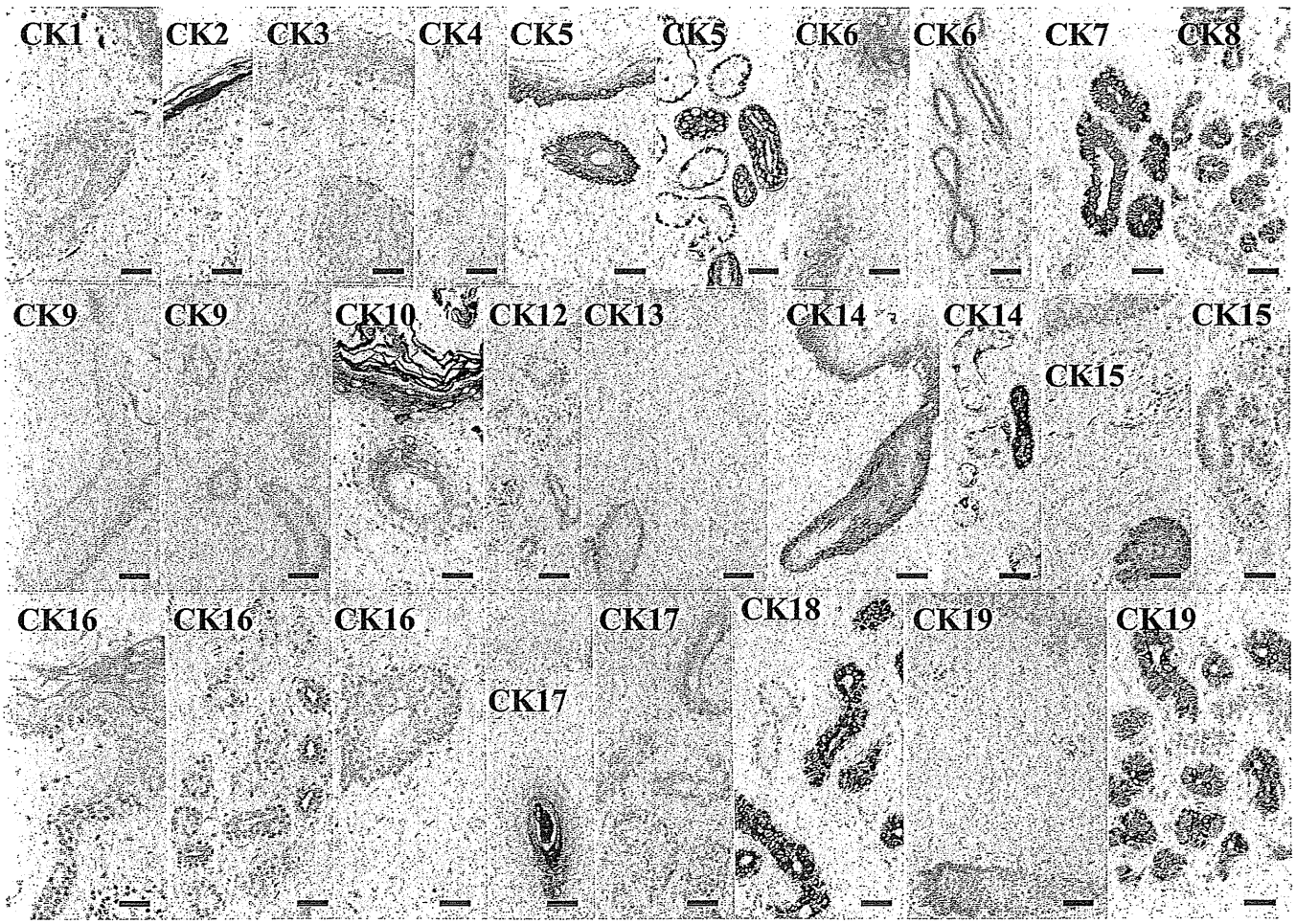

Fig. 1. Immunohistochemistry of cytokeratins within normal skin. CK1 was revealed in squamous, granular, and cornified layers of the epidermis, and inner root hair sheath, CK2 in granular and cornified layers of the epidermis, CK3 in squamous, granular, and cornified layers of the epidermis, and inner root hair sheath, CK4 in inner root hair sheath, CK5 in basal, squamous, and granular layers of the epidermis, outer root hair sheath, inner and outer layers of the eccrine ducts, and myoepithelial cells of the secretory glands, CK6 in squamous, granular, and cornified layers of the epidermis, inner root hair sheath, and inner layer of the eccrine ducts, CK7 in inner layer of the eccrine ducts, and secretory and myoepithelial cells of the secretory glands, CK8 in secretory and myoepithelial cells of the secretory glands, CK9 in basal and cornified layers of the epidermis, outer root hair sheath, outer layers of the eccrine ducts, and myoepithelial cells of the secretory glands, CK10 in granular and cornified layers of the epidermis, and inner root hair sheath, CK12 in inner layer of the eccrine ducts, and secretory and myoepithelial cells of the secretory glands, CK13 in outer root hair sheath, CK14 in basal and squamous layers of the epidermis, outer root hair sheath, inner and outer layers of the eccrine ducts, and myoepithelial cells of the secretory glands, CK15 in outer root hair sheath, and secretory and myoepithelial cells of the secretory glands, CK16 in cornified layer of the epidermis, inner root hair sheath, and inner layers of the eccrine ducts, CK17 in inner root hair sheath, inner layer of the eccrine ducts, and myoepithelial cells of the secretory glands, CK18 in secretory and myoepithelial cells of the secretory glands, and CK19 in outer root hair sheath, inner layer of the eccrine ducts, and secretory and myoepithelial cells of the secretory glands. Bars: 50 μm.

Table 2. Cytokeratin (CK) expression in the skin.

	CK 1	CK 2	CK 3	CK 4	CK 5	CK 6	CK 7	CK 8	CK 9	CK 10	CK 12	CK 13	CK 14	CK 15	CK 16	CK 17	CK 18	CK 19
Epidermis																		
Basal layer	-	-	-	-	+	-	-	-	+	-	-	±	+	-	-	-	-	-
Squamous layer	+	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-
Granular layer	+	+	+	-	±	+	-	-	-	+	-	-	-	-	-	-	-	-
Cornified layer	+	+	+	-	-	+	-	-	+	+	+	-	-	-	±	-	-	-
Hair sheath																		
Inner root sheath	+	-	±	±	-	+	-	-	-	±	+	-	-	-	+	+	-	-
Outer root sheath	-	-	-	-	+	-	-	-	+	-	-	+	+	+	-	-	-	+
Eccrine duct																		
Inner layer	-	-	-	-	+	+	±	-	-	-	+	-	+	-	+	±	-	+
Outer layer	-	-	-	-	±	-	-	-	+	-	-	-	±	-	-	-	-	-
Secretory gland																		
Secretory cells	-	-	-	-	-	-	+	+	-	-	±	-	-	±	-	-	+	+
Myoepithelial cells	-	-	-	-	+	-	±	+	±	-	±	-	+	±	-	+	+	+

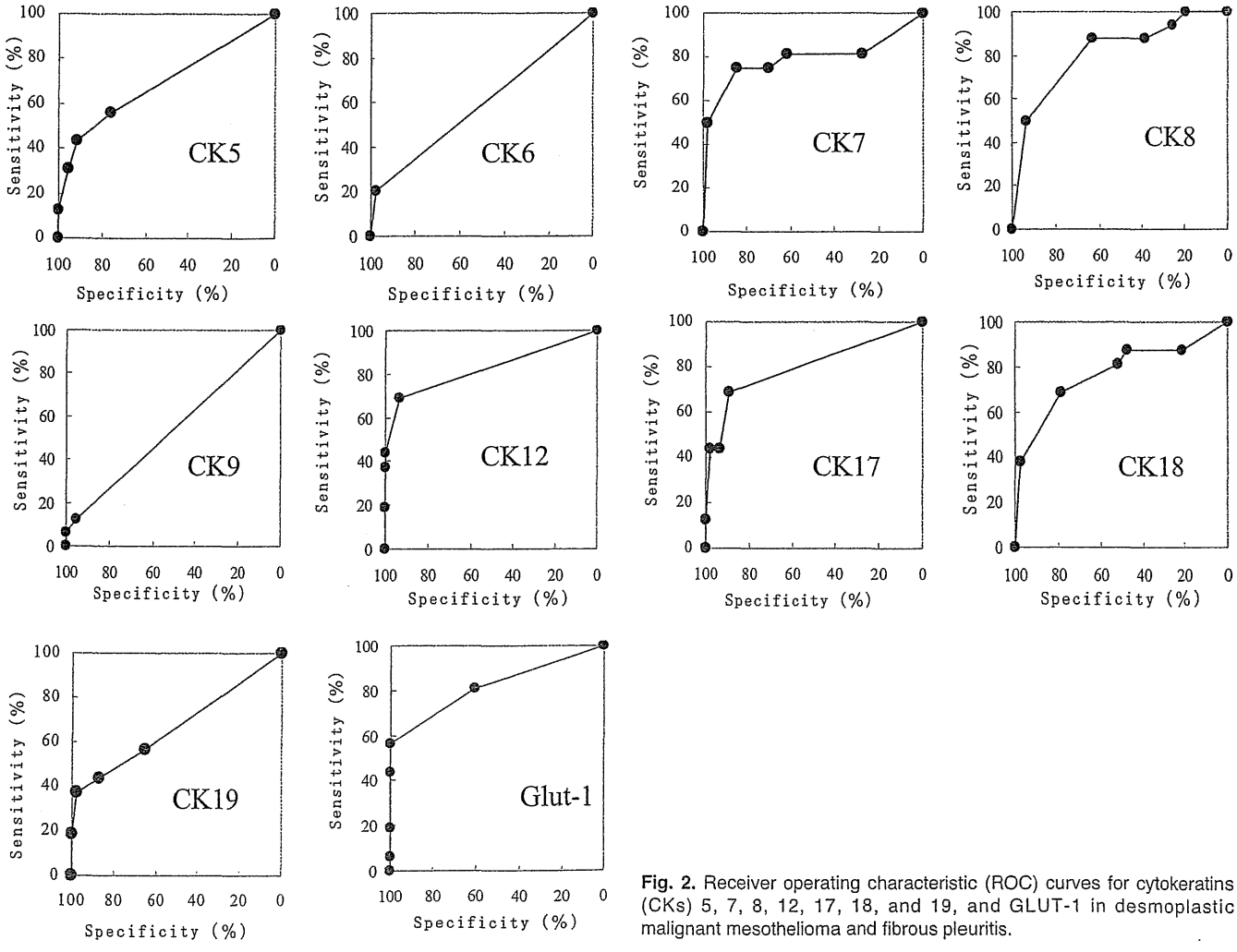

Fig. 2. Receiver operating characteristic (ROC) curves for cytokeratins (CKs) 5, 7, 8, 12, 17, 18, and 19, and GLUT-1 in desmoplastic malignant mesothelioma and fibrous pleuritis.

receiver operating characteristic (ROC) curve was employed to identify the best cut-off values for sensitivity and specificity (13). Then, tumors with a staining score equal to or above that associated with the best sensitivity and specificity were graded as positive.

Data analysis

Statistical analysis of the difference in incidence between two groups was performed using the Chi-square analysis. A p value of less than 0.05 was considered significant.

Results

By immunohistochemistry, each CK was revealed in the cytoplasm of positive cells in the epidermis, dermis, eccrine ducts, and/or secretory glands within normal skin (Table 2, Fig. 1). Calponin, caldesmon, and desmin were detected in the cytoplasm of both vascular smooth muscle cells and bronchial surface epithelial cells within the normal lung. GLUT-1 was found in the membrane of red blood cells within blood vessels and in the membrane and cytoplasm of bronchial surface epithelial cells, each within the normal lung. Expressions of CKs 5, 7, 8, 9, 12, 17, 18, and 19, calponin, caldesmon, and desmin were confined to the cytoplasm of DMM tumor cells and of reactive spindle cells in FP, while GLUT-1 expression was detected in the membrane and cytoplasm of DMM tumor cells and reactive spindle cells in FP (Fig. 2). However, the staining intensity of their proteins sometimes varied within a given case. No ROC curve could be obtained for CKs 1, 2, 3, 4, 6, 9, 10, 13, 14, 15, or 16, or for calponin, caldesmon, or desmin.

Among CKs 5, 7, 8, 12, 17, 18, and 19, and GLUT-1, the best sensitivity and specificity cut-off values in the ROC curves were above 60% for each of CKs 7, 8, 17, 18, and 19, and GLUT-1 (Table 3, Fig. 3). The incidence of a positive expression for CK5, CK12, or CK17 was significantly higher in DMM than in FP (tumors were graded as positive if 1% or more of their cells showed staining) (Table 4; p=0.046, p=0.001, or p<0.0001, respectively). The incidence of a positive expression for CK7 or CK18 was significantly higher in DMM than in FP (tumors were graded as positive if 51% or more of their cells showed staining) (p<0.0001 or p=0.0001, respectively). The incidence of a positive expression for CK18 was significantly higher in DMM than in FP (tumors were graded as positive if 76% or more of their cells showed staining) (p=0.0001). Further, the incidence of a positive expression for GLUT-1 was significantly higher in DMM than in FP (tumors were graded as positive if 11% or more of their cells showed staining) (p<0.0001). Given the best sensitivity and specificity cut-off values in the ROC curves, and the statistical analysis of the difference in incidence between the two groups, CKs 7, 8, 17, 18, and 19, and GLUT-1 were identified as potentially useful markers for diagnosis between DMM and FP.

Table 3. Sensitivity and specificity data for cytokeratins (CKs) and GLUT-1.

	CK5	CK7	CK8	CK12	CK17	CK18	CK19	GLUT-1
Sensitivity*	55	82	64	55	91	82	64	73
Specificity*	76	85	94	94	89	78	87	100

*: CK5, CK12, CK17: positive if staining score >1, CK19, GLUT-1: positive if staining score ≥2, CK7, CK18: positive if staining score >4, CK8: positive if staining score >5

Discussion

Although several proteins -- such as CK5/6, calretinin, WT-1, thrombomodulin, and mesothelin -- are useful markers for distinguishing epithelioid mesothelioma from pulmonary adenocarcinoma (Cury et al., 2000; Oates and Edwards 2000; Carella et al., 2001; Ordóñez 2003), no immunohistochemical marker for

Table 4. Expressions of cytokeratins (CKs) and GLUT-1 in desmoplastic malignant mesothelioma (DMM) and fibrous pleuritis (FP).

	DMM	FP	p value*
CK5			
>1%	6	11	0.046
0%	5	35	
CK7			
>51%	9	7	<0.0001
<50%	2	39	
CK8			
>76%	7	3	<0.0001
<75%	4	43	
CK12			
>1%	6	3	0.001
0%	5	43	
CK17			
>1%	10	5	<0.0001
0%	1	41	
CK18			
>51%	9	10	0.0001
<50%	2	36	
CK19			
>11%	7	6	0.0047
<10%	4	40	
GLUT-1			
>11%	8	0	<0.0001
<10%	3	46	

*: Statistical analysis of the difference in incidence between two groups was performed using the Chi-square analysis.

differential diagnosis between DMM and FP has been reported. In the present study, in which we examined 18 CKs, calponin, caldesmon, desmin, and GLUT-1 in 11 DMMs and 46 FPs, we observed that the best sensitivity and specificity cut-off values in the ROC curves were above 60% for each of CKs 7, 8, 17, 18, and 19, and GLUT-1, and that for each of these, the incidence of positive expression was significantly higher in DMM than in FP. On that basis, immunohistochemistry for CK 7, 8, 17, 18, and 19, and GLUT-1 may provide useful markers for separating DMM from FP, alongside such histological characteristics as cellular atypia, storiform

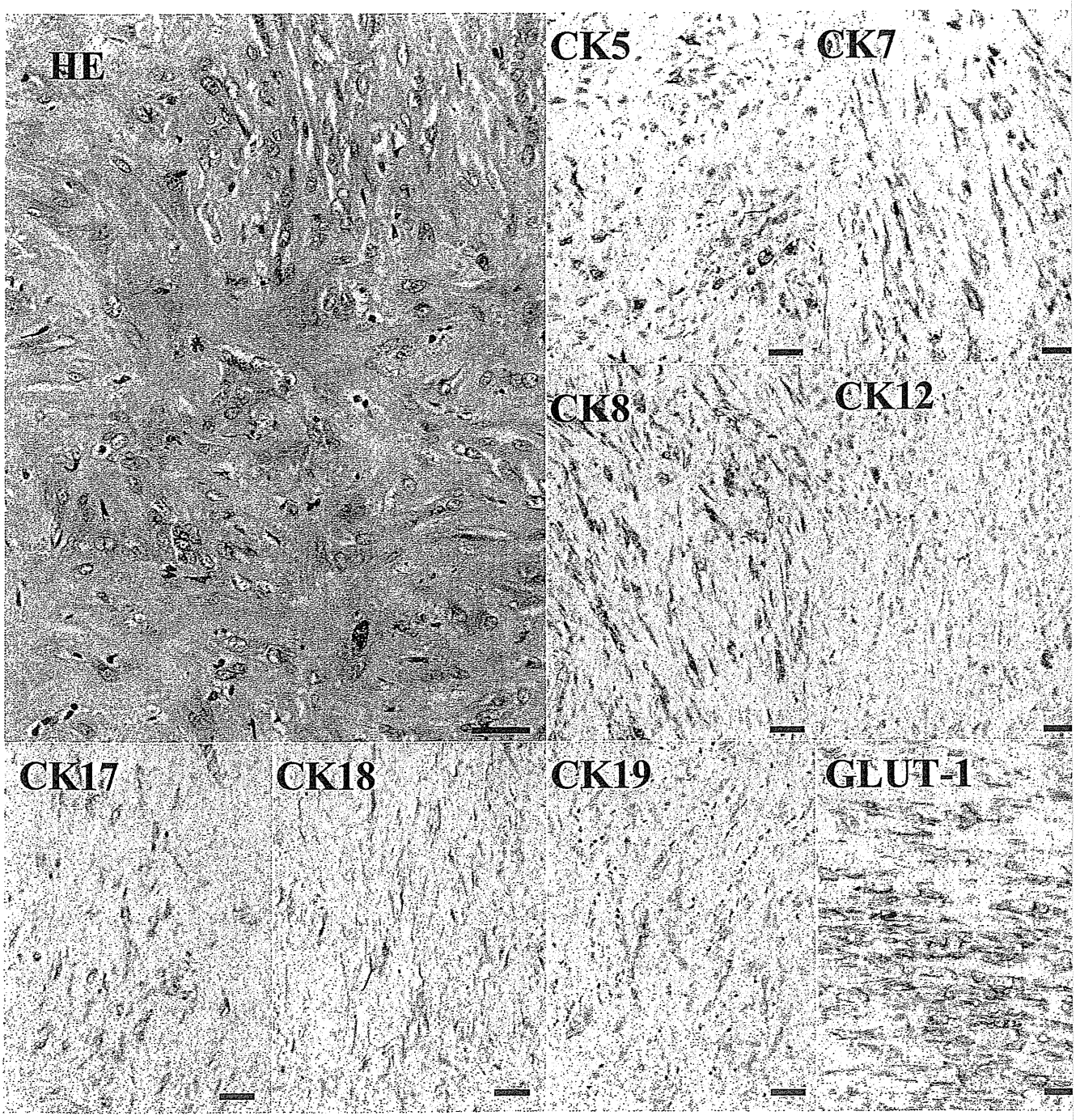

Fig. 3. Hematoxylin-eosin (HE) staining and immunohistochemistry of cytokeratins (CKs) 5, 7, 8, 12, 17, 18, and 19, and GLUT-1 in desmoplastic malignant mesothelioma (DMM). Bars: 50 μm.

pattern and/or bland necrosis.

It was previously reported that immunohistochemical staining for CK5/6, calretinin, WT-1, and mesothelin was useful in the diagnosis of sarcomatoid mesothelioma, as well as of epithelioid mesothelioma (Cury et al., 2000; Oates and Edwards 2000; Carella et al., 2001; Lucas et al., 2003; Ordóñez 2003; Suster and Moran 2006; Addis and Roche 2009; Husain et al., 2009). In epithelioid mesothelioma, CK5/6, calretinin, WT-1, and mesothelin have been found to be positive in 55-100%, 42-100%, 43-95%, and 100% of cases, respectively (Ordóñez 2003; Suster and Moran 2006). As far as we can tell, however, these positive expressions are lower in incidence in sarcomatous mesothelioma than in epithelioid mesothelioma, since CK5/6, calretinin, and WT-1 were detected in 0-29%, 39-100%, and 0-50%, respectively, in sarcomatoid mesothelioma (Suster and Moran 2006). However, there is little reported immunohistochemistry for DMMs (Lucas et al., 2003), although in routine practice pathologists frequently have need to differentiate DMM from FP.

It is well known that CKs are a family of intermediate filaments involved in epithelial differentiation, and several CKs are useful tools for differential diagnosis in surgical pathology (Quinlan et al., 1985; Moll et al., 2008; Klebe et al., 2010). So far, at least 20 distinct CKs have been identified. In mesothelioma, Bolen et al (1986), who examined 9 epithelial, 5 sarcomatoid, and 2 desmoplastic malignant mesotheliomas, using antibodies of both low molecular weight CKs (2 antibodies of 44 and 54 kDa, and 46, 52, and 54 kDa) and high molecular weight CKs (one antibody of 57 and 66 kDa), demonstrated that epithelial mesotheliomas were all positive for low and high molecular weight CKs, while sarcomatoid and desmoplastic mesotheliomas were all positive for low molecular weight CKs, but negative for high molecular weight CKs, except for one sarcomatous mesothelioma. In the present study, we investigated 18 CKs for their utility in differentiating DMM from FP. In the ROC curves for low molecular weight CKs (less than 55kDa), such as CKs 7, 8, 17, 18, and 19, sensitivity and specificity tended to be above 60%, while for high molecular weight CKs (equal or more than 55kDa), such as CKs 1, 2, 3, 4, 5, 6, 9, 10, 11, and 12, one or both of them tended to be below 60%. When tumors were graded as positive if 1% or more of their cells showed staining for CK17, if 11% or more of their cells showed staining for CK19, if 51% or more of their cells showed staining for CK7 or CK18, or if 76% or more of their cells showed staining for CK8 (criteria based on the sensitivity and specificity data in their ROC curves), we demonstrated that positive expressions of CKs 7, 8, 17, 18, and 19 were significantly more frequent in DMMs than in FPs. To judge from that finding, immunostaining for CKs 7, 8, 17, 18, and 19 may not only help to identify the presence of invasion into adipose tissue or invasion into the underlying lung in DMM, but also aid the diagnosis of DMM.

In the present study, we demonstrated that GLUT-1 immunohistochemistry was useful for separating DMM from FP. It is generally accepted that GLUT-1 is one of 14 members of the mammalian facilitative glucose transporter (GLUT) family of passive carriers that function as an energy-independent system for the transport of glucose down a concentration gradient (Olson and Pessin, 2005). Although GLUT-1 is not detectable in a large proportion of cells within normal tissues or benign lesions, it is expressed in various cancers (Macheda et al., 2005). In recent studies, GLUT-1 expression has been found to be useful for distinguishing malignant mesothelioma from reactive mesothelial hyperplasia (Kato et al., 2007; Acurio et al., 2008). In fact: (a) Kato et al. (2007) found that its immunoreactivity was negative in 40 reactive mesothelial cases, but positive in all of 40 malignant mesotheliomas, and (b) Acurio et al. (2008) found that 40 benign mesothelial tissues (20 normal, 20 reactive cases) were negative, while 34 of 45 malignant mesotheliomas were positive (unpublished observations). On the basis of the present data and those from the above two studies, GLUT-1 may be a useful marker for separating DMM from FP, as well as for separating malignant mesothelioma from FP or reactive mesothelial hyperplasia.

In conclusion, immunohistochemistry for CKs 7, 8, 17, 18, and 19, and GLUT-1 may be useful for differentiating DMM from FP, alongside their characteristic histological features. Even so, accurately diagnosing DMM in routine practice will require careful consideration of all the available findings by the pathologists.

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Naftopidil Induces Apoptosis in Malignant Mesothelioma Cell Lines Independently of α_1-Adrenoceptor Blocking

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Abstract. *Background: Naftopidil, an α_1-adrenoceptor blocker, has been clinically used for the treatment of benign prostate hyperplasia and hypertension. Emerging evidence has shown that naftopidil exhibits an antitumor effect on a variety of cancer types including prostate cancer. The aim of the present study was to investigate naftopidil-induced apoptosis in human malignant mesothelioma cells and to shed light on the underlying mechanism. Materials and Methods: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) staining, western blotting, and enzymatic assay of caspase-3, -8, and -9 activities were carried out on human malignant mesothelioma cell lines NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells. To knock-down α_{1D}-adrenoceptor, siRNA to silence human α_{1D}-adrenoceptor-targeted gene was constructed and transfected into cells. Results: Naftopidil induced apoptosis in all the investigated malignant mesothelioma cells, and a similar effect was obtained with prazosin, another α_1-adrenoceptor blocker. α_1-Adrenoceptor is linked to $G_{q/11}$ protein involving activation of protein kinase C (PKC). Naftopidil-induced reduction in cell viability was inhibited by GF109203X, while prazosin-induced in cell viability was less affected. Knocking-down α_{1D}-adrenoceptor promoted malignant mesothelioma cell proliferation. Both naftopidil and prazosin activated caspase-3 and -8 in all the investigated malignant mesothelioma cells. Conclusion: Naftopidil, as well as prazosin, has the potential to induce apoptosis in malignant mesothelioma cells by activating caspase-8 and the effector caspase-3, regardless of α_1-adrenoceptor blocking.*

Adrenaline/noradrenaline play a pivotal role in the autonomic nervous system by activating adrenoceptors, *e.g.*, regulation of cardiac motility and blood pressure. Adrenoceptors are classified into three groups: α_1-, α_2-, and β-receptors (1-3). α_1-Adrenoceptor is linked to $G_{q/11}$ protein involving phospholipase C activation, and further divided into α_{1A}-, α_{1B}-, and α_{1D}-subtypes. α_2-Adrenoceptor is linked to G_i protein involving adenylate cyclase inhibition, and further divided into α_{2A}-, α_{2B}-, and α_{2C}-subtypes. β-Adrenoceptor is linked to the G_s protein involving adenylate cyclase activation, and further divided into β_1-, β_2-, β_3-, and β_4-subtypes.

Intriguingly, α_1-adrenoceptor antagonists such as prazosin, doxazosin and terazosin have the potential to inhibit cell growth by arresting the cell cycle or induce apoptosis of malignant and non-malignant cells (4-20). In explanation of the apoptotic action of α_1-adrenoceptor antagonists, a variety of pathways have been proposed, such as mitochondria-mediated activation of caspase-3/-9 and c-Jun N-terminal kinase (JNK)1/2; recruitment of Fas-associated death domain (FADD) and the ensuing activation of caspase-8; transforming growth factor-β1 (TGF-β1) activation followed by IκBα induction; or antagonistic effect of BCL-2. α_1-Adrenoceptor antagonists, alternatively, exhibit anti-angiogenic effects, resulting in suppression of cell growth in human prostate cancer and human bladder cancer (21-25).

Naftopidil, an antagonist for α_1-adrenoceptor, with higher selectivity for α_{1A}- and α_{1D}-receptors, has been developed as a drug for treatment of benign prostate hyperplasia and hypertension (26). Naftopidil is still capable of inhibiting prostate cancer cell growth by arresting cells at the G_1 phase of cell cycling (27, 28). Moreover, a study showed that an

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asymptomatic meningioma markedly regressed two years after oral intake of naftopidil (29). Naftopidil might be available for the treatment of various malignant tumors. Malignant pleural mesothelioma, a highly aggressive neoplasm, has been increasing in incidence and is strongly associated with asbestos exposure (30). No efficient therapy or drug for malignant mesothelioma has been yet established, and therefore, patients with malignant mesothelioma cannot escape death.

The present study investigated the antitumor action of naftopidil on human malignant mesothelioma cell lines. We showed that naftopidil induces apoptosis of malignant mesothelioma cells by a mechanism independent of α_1-adrenoceptor blocking.

Materials and Methods

Cell culture. Human malignant pleural mesothelioma cell lines such as NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.003% L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell viability assay. Cell viability was evaluated by the method of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described (31).

Terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) staining. TUNEL staining was performed to detect *in situ* DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37°C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510; Carl Zeiss Co., Ltd., Oberkochen, Germany).

Construction and transfection of siRNA. siRNA to silence human α_{1D}-adrenoceptor-targeted gene (α_{1D}R siRNA) and negative control siRNA (NC siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The α_{1DR} siRNA or the NC siRNA was reverse-transfected into cells using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Western blotting. Western blotting was carried out on cells using an antibody to α_{1D}-adrenoceptor (Santa Cruz Biotechnology) and to β-actin (Sigma, St. Louis, MO, USA) by the method previously described (31).

Enzymatic assay of caspase-3, -8, and -9 activities. Caspase activity was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) by the method described previously (31). Briefly, cells were harvested before and after treatment with naftopidil or prazosin, and then centrifuged at 1,200 rpm for 5 min at 4°C. The cell pellet was incubated on ice in cell lysis buffer for 10 min, and reacted with the fluorescently-labeled tetrapeptide at 37°C for 2 h. The fluorescence was measured at an excitation of wavelength of 380 nm and an emission wavelength of 460 nm with a fluorometer (Fluorescence Spectrometer F-4500; Hitachi High-Tec, Tokyo, Japan).

Statistical analysis. Statistical analysis was carried out using unpaired *t*-test.

Results

Naftopidil induces apoptosis of malignant mesothelioma cells. For all the malignant mesothelioma cell lines examined here, naftopidil reduced cell viability in a concentration (1-100 μM)- and treatment time (24-48 h)-dependent manner, the extent reaching almost 0% of basal levels at 100 μM (Figure 1A-D). Likewise, prazosin, another α_1-adrenoceptor antagonist, reduced cell viability in a fashion mimicking the effect of naftopidil (Figure 1E-H).

In the TUNEL staining, naftopidil significantly increased the number of TUNEL-positive cells as compared with that for the untreated control in all the malignant mesothelioma cell lines (Figure 2A-D). A similar effect was obtained with prazosin (Figure 2A-D). These results indicate that α_1-adrenoceptor blockers, such as naftopidil and prazosin, are capable of inducing apoptosis of malignant mesothelioma cells.

Naftopidil induces apoptosis of malignant mesothelioma cells, regardless of α_{1D}-adrenoceptor blocking. α_1-Adrenoceptor is linked to $G_{q/11}$ protein involving phospholipase C activation, to hydrolyze phosphatidylinositol into inositol 1,4,5-trisphosphate (IP_3) and diacyl-glycerol allowing protein kinase C (PKC) activation. We postulated that naftopidil and prazosin induce apoptosis of malignant mesothelioma cells by inhibiting PKC as a result of α_1-adrenoceptor blocking. If this was true, then PKC inhibitors should enhance the cell death-inducing effect of naftopidil and prazosin. Unexpectedly, the effect of naftopidil on malignant mesothelioma cell death was attenuated by GF109203X, an inhibitor of PKC, for all the cell types examined here (Figure 3A-D). Naftopidil, thus, appears to induce apoptosis of malignant mesothelioma cells by a mechanism independent of α_1-adrenoceptor blocking and PKC inhibition.

GF109203X, in contrast, enhanced the effect of prazosin on cell viability, only for NCI-H28 cells (Figure 3E) or inhibited it for NCI-H2052 and NCI-H2452 cells (Figure 3F,G), but did not alter the effect for MSTO-211H cells (Figure 3H). It is even less likely that prazosin induces apoptosis of malignant mesothelioma cells by inhibiting α_1-adrenoceptor bearing PKC activation.

To obtain further evidence for naftopidil- or prazosin-induced apoptosis independent of α_1-adrenoceptor blocking,

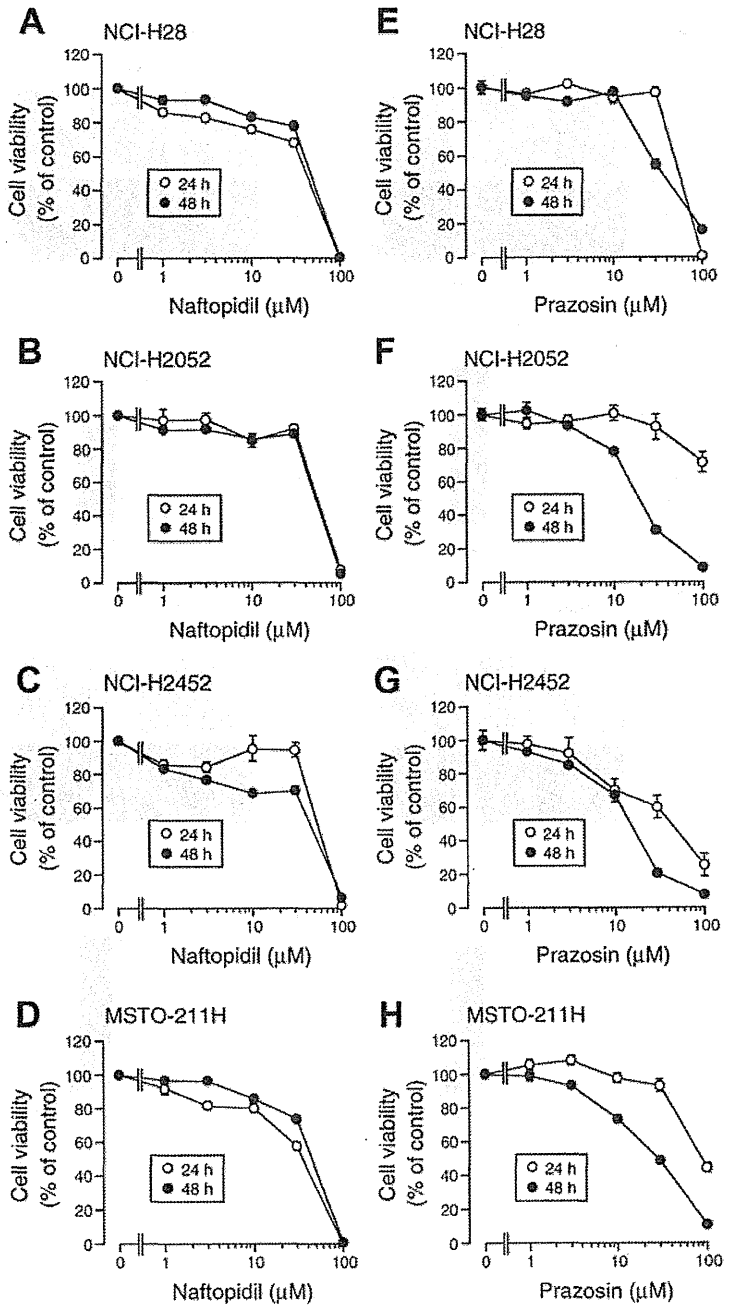

Figure 1. *The effects of naftopidil and prazosin on cell viability. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out on NCI-H28 (A, E), NCI-H2052 (B, F), NCI-H2452 (C, G), and MSTO-211H cells (D, H) untreated and treated with naftopidil or prazosin, at concentrations, as indicated for 24-48 h. In the graphs, each point represents the mean (±SEM) percentage of control cell viability (MTT intensities of cells untreated with naftopidil or prazosin) (n=4 independent experiments).*

an α_{1D}R siRNA was constructed and transfected into malignant mesothelioma cells. Expression of α_{1D}-adrenoceptor protein for cells transfected with α_{1D}R siRNA significantly decreased as compared with the expression for cells transfected with the NC siRNA (Figure 4A-D), confirming α_{1D}-adrenoceptor knock-down. Cell viability for all the malignant mesothelioma cell lines was not reduced by knocking-down α_{1D}-adrenoceptor, but conversely, it was