flow cytometer and CXP software (Beckman Coulter, Inc., Fullerton, CA).

Western blotting

Western blotting was performed as described earlier (Eguchi et al., 2009). All antibodies used were from Cell Signaling Technology (Beverly, MA) except those against JNK and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

RNA interference (RNAi)

Small interfering RNA (siRNA) targeting JNK1 (MAPK8 Stealth Select RNAi HSS108548), JNK2 (MAPK9 Stealth Select RNAi HSS108550) and Stealth RNAi negative control were purchased from Invitrogen (Carlsbad, CA). Transient transfection of siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Stealth RNAi compounds were used at a concentration of 10 nM in transfections as described earlier (Eguchi et al., 2008). Briefly, 2×10^5 NCI-H2052 cells were incubated in 10 ml of RPMI-1640 medium in a 100 mm dish overnight. Lipofectamine RNAiMAX and siRNA were resuspended in 1 ml each of RPMI-1640 medium for 5 min at room temperature, combined and incubated for 15 min at room temperature to form complexes. Two milliliters of the mixture was added to the cell culture and incubated for 48 h. The cells were harvested by trypsinization and the 2×10^5 cells were seeded in a dish for As_2O_3 treatment as described above.

Statistical analysis

All data are presented as the mean ± standard error (SE) of three independent experiments. Comparisons between two groups were performed using Student's unpaired *t*-test ($^*P < 0.05$, $^{**}P < 0.005$).

Results

As_2O_3 induces apoptosis in human mesothelioma cells

Analysis of the effect of As_2O_3 on viability of human mesothelioma NCI-H2052 cells showed that As_2O_3 suppressed viability of the cells with IC_{50} values of 5 μM (Fig. 1A), which falls within the range of concentrations used clinically (1–10 μM) (Shen et al., 1997; Hayashi et al., 2002). We analyzed the ability of As_2O_3 to induce apoptosis by flow cytometry using double staining with Ax and PI. The results showed that As_2O_3 significantly increased the proportion of early apoptotic cells (Ax+/PI−) and late apoptotic and necrotic cells (Ax+/PI+) in NCI-H2052 cells (Fig. 1B).

We then examined whether caspase cascade is involved in As_2O_3-induced apoptosis in NCI-H2052 cells. As_2O_3 induced cleavage of caspase-3 at 24 h (Fig. 1C). zVAD(OMe)-fmk (zVAD), a broad spectrum caspase inhibitor, blocked cleavages of caspase-3 and PARP (Fig. 1C), and blocked significantly, but not entirely, an increase in the proportion of Ax (+) apoptotic cells (Fig. 1D). These results indicate that As_2O_3 mainly induced apoptosis through caspase cascade in NCI-H2052 cells.

As_2O_3 treatment activates both JNK1/2 and ERK1/2

We investigated signal transduction involved in As_2O_3-induced apoptosis (Fig. 2). We found that treatment with As_2O_3 in NCI-H2052 cells did not alter phosphorylation of both Akt and Src (Fig. 2A). However, ERK1/2 and JNK1/2, but not p38, were markedly phosphorylated at 24 h after As_2O_3 treatment (Fig. 2A). Time-course analysis showed that phosphorylation of JNK1/2 increased at 2 h after As_2O_3 treatment. While As_2O_3 induced ERK1/2 phosphorylation at 10 h, As_2O_3-induced caspase-3 cleavage began to increase at 16 h (Fig. 2B). zVAD, which suppressed caspase cascade (Fig. 1C), did not suppress phosphorylation of ERK1/2 and JNK1/2 (Fig. 2C).

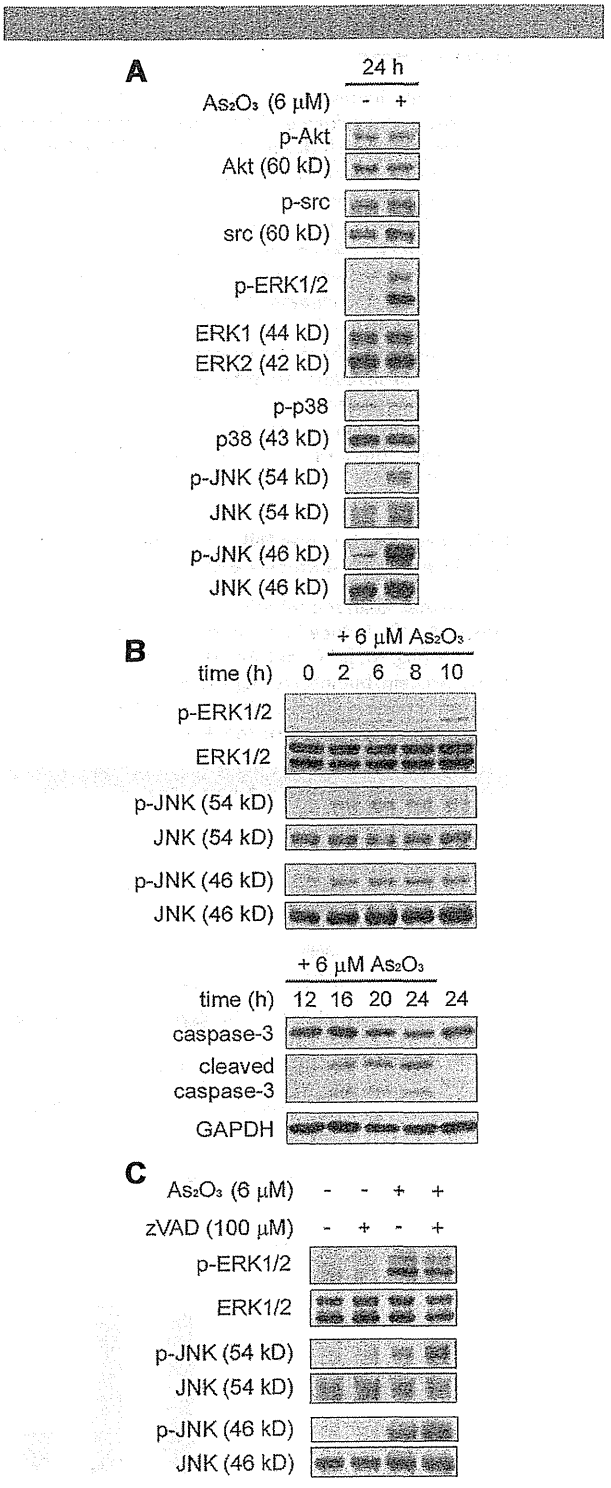

Fig. 2. As_2O_3 induces activation of ERK1/2 and JNK1/2, which is not suppressed by zVAD. A: As_2O_3 induces activation of ERK1/2 and JNK1/2. NCI-H2052 cells were treated with 6 μM As_2O_3 for 24 h. B: As_2O_3 induces activation of JNK1/2 and ERK1/2 before that of caspase-3. NCI-H2052 cells were treated with 6 μM As_2O_3 for 2–24 h. C: Inhibition of caspase activity fails to inhibit activation of both ERK1/2 and JNK1/2. Cell extracts were prepared from NCI-H2052 cells treated with 6 μM As_2O_3 alone or together with 100 μM zVAD for 24 h. All experiments were performed three times and the representative data are shown.

JNK1/2 regulate As_2O_3-induced apoptosis through caspase cascade

To elucidate involvement of JNK1/2 in As_2O_3-induced apoptosis, we performed RNAi experiments using siRNA that targeted JNK1 and JNK2 mRNAs in NCI-H2052 cells. JNK1 and JNK2 siRNAs successfully suppressed the expression of respective proteins (Fig. 3A). As described above, As_2O_3 first induced JNK1/2 phosphorylation and then caspase-3 cleavage (Fig. 2B), while zVAD did not suppress JNK1/2 phosphorylation (Fig. 2C). Here, JNK1 and JNK2 siRNA abrogated As_2O_3-induced caspase-3 cleavage (Fig. 3A), suggesting that JNK1/2 are located upstream of caspase-3. Knockdown of JNK1 or JNK2 with respective siRNA suppressed As_2O_3-induced apoptosis (Fig. 3B). These results, together with that of Figure 1D, showed that JNK1/2 regulate As_2O_3-induced apoptosis through caspase cascade. It should be noted that JNK1 siRNA exerted a greater suppressive effect on As_2O_3-induced apoptosis than JNK2 siRNA (Fig. 3C).

JNK1 protein, but not JNK2 protein or activated JNK1/2, regulates As_2O_3-induced ERK1/2 activation

We further analyzed whether JNK1 and JNK2 siRNA affects As_2O_3-induced phosphorylation of both JNK1/2 and ERK1/2 (Fig. 4). JNK1 siRNA had little effect on expression of JNK2 protein, but interestingly it blocked As_2O_3-induced JNK2 phosphorylation. Conversely, JNK2 siRNA had little effect on JNK1 expression but blocked As_2O_3-induced JNK1 phosphorylation. Unexpectedly, As_2O_3-induced ERK1/2 phosphorylation was markedly abrogated by JNK1 siRNA, but not by JNK2 siRNA. These findings suggested that As_2O_3-induced ERK1/2 phosphorylation requires expression of JNK1 protein, but not that of JNK2 protein or phosphorylated JNK1/2.

ERK1/2 regulate As_2O_3-induced apoptosis when JNK1/2 are inactivated

We determined the role of ERK in As_2O_3-induced apoptosis using PD98059, a specific MAPK/ERK kinase inhibitor (Fig. 5). PD98059 suppressed As_2O_3-induced ERK1/2 phosphorylation, but had no effect on As_2O_3-induced JNK1/2 phosphorylation (Fig. 5A). However, As_2O_3-induced caspase-3, -6, -7 and PARP cleavages were not suppressed by PD98059. In addition, given the fact that As_2O_3-induced phosphorylation of ERK1/2 was not suppressed by zVAD (Fig. 2C), it is unlikely that ERK1/2 regulate caspase cascade. Unexpectedly, treatment with JNK2 siRNA together with PD98059 suppressed As_2O_3-induced apoptosis more significantly than that with JNK2 siRNA alone (Fig. 5B). These results suggested that ERK1/2 is involved in As_2O_3-induced apoptosis in caspase-independent manner when JNK1/2 are dephosphorylated.

Discussion

Malignant mesothelioma is an asbestos-related malignancy of serosal membranes characterized by rapid progression and

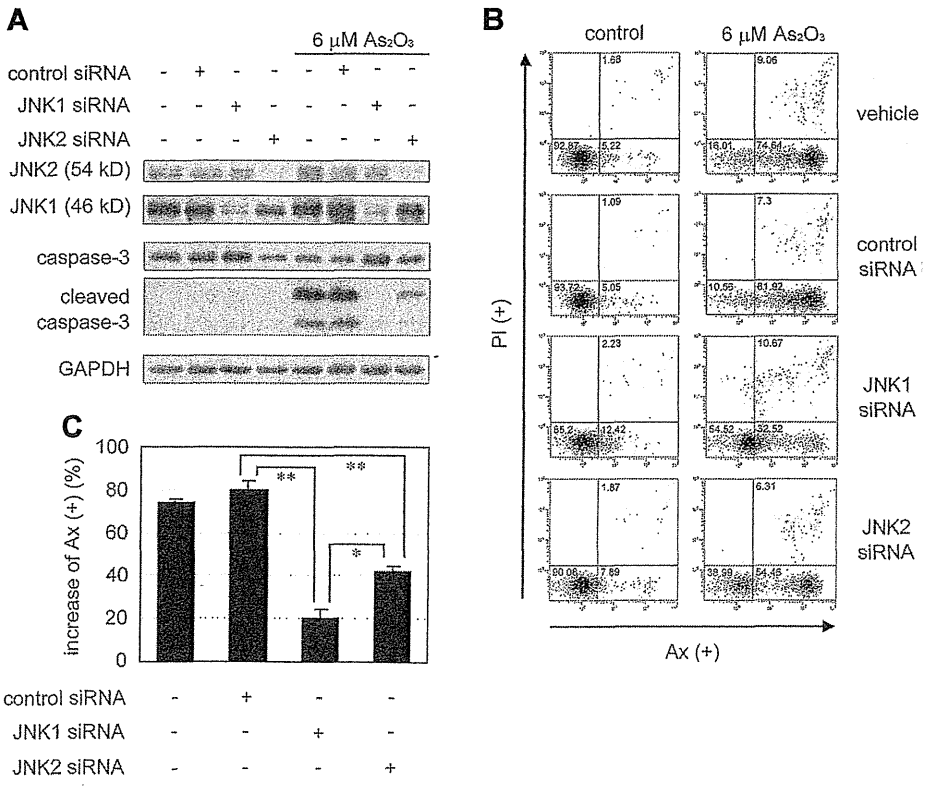

Fig. 3. As_2O_3-induced apoptosis through caspase cascade is regulated by JNK1/2. A: JNK1/2 regulate As_2O_3-induced caspase-3 activation. NCI-H2052 cells, transfected with 10 nM siRNA against JNK1 or JNK2 mRNA or control siRNA, were treated with 6 μM As_2O_3 for 24 h. B: Representative flow cytometric analysis of apoptosis treated with JNK1 and JNK2 siRNA. NCI-H2052 cells transfected with control, JNK1 or JNK2 siRNA were treated with 6 μM As_2O_3 for 72 h. C: As_2O_3-induced apoptosis is regulated by JNK1/2, particularly JNK1. Ax (+) apoptotic cells were determined using flow cytometry after staining Ax-FITC. The experiments in A and B were performed three times and the representative data are shown.

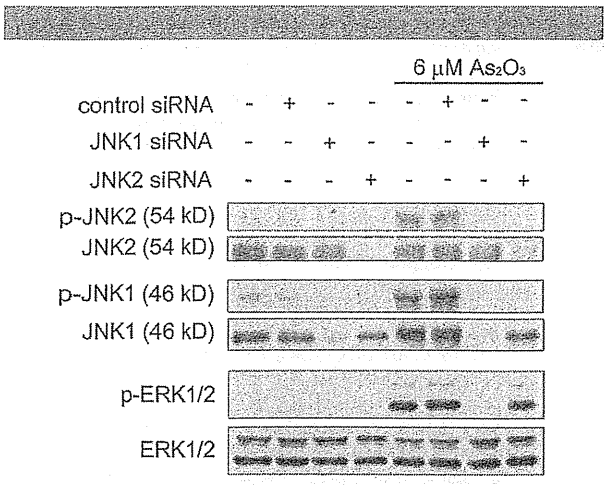

Fig. 4. JNK1 protein, but not JNK2 protein or activated JNK1/2, regulates As_2O_3-induced ERK1/2 activation. NCI-H2052 cells, transfected with 10 nM siRNA against JNK1 or JNK2 mRNA or control siRNA, were treated with 6 μM As_2O_3 for 24 h. Representative data of three independent experiments with similar results are shown.

diffused local growth (Robinson et al., 2005). Malignant mesothelioma is refractory to conventional chemotherapy, which is related to the resistance to induction of apoptosis by chemotherapeutic agents (Vogelzang, 2008). As_2O_3 is an old drug but has recently been explored for its ability to induce differentiation and apoptosis in leukemia cells (Antman, 2001), but the effect of As_2O_3 on mesothelioma cells has not been examined. In the present study, we examined apoptotic effect of As_2O_3 on human mesothelioma cells and analyzed its signal transduction pathway.

We found for the first time that As_2O_3 decreased cell viability and induced apoptosis in mesothelioma cells. In the As_2O_3-induced apoptosis, caspase-3 was activated, and zVAD suppressed the apoptosis significantly but not entirely (Fig. 1), indicating that As_2O_3-induced apoptosis is mainly regulated by the caspase cascade in mesothelioma cells.

Members of the MAPK families including ERK, JNK, and p38 are activated in response to stimuli by growth factors as well as extracellular stresses (Zhang and Liu, 2002). It has been shown that As_2O_3 activates p38 in prostate cancer cells (Maeda et al., 2001) and in leukemia cells (Iwama et al., 2001). In this study, we observed that JNK and ERK, but not p38, were activated in mesothelioma cells following As_2O_3 treatment (Fig. 2). In addition, we examined the effect of cisplatin on NCI-H2052 cells. Cisplatin-induced activation of JNK, ERK, and caspase-3 followed by apoptosis (Eguchi et al. unpublished observation). These results indicated that JNK and ERK signaling is involved in induction of apoptosis of NCI-H2052 cells.

JNKs are activated by cytokines or environmental stresses and induce both pro-apoptotic and pro-survival responses. JNK1 and JNK2 share some biological functions (Kuan et al., 1999), but they also have distinct or even opposing biological functions (Conze et al., 2002). Our initial experiments using JNK specific inhibitors, SP600125 and JNK inhibitor peptide-1 (both from Calbiochem, La Jolla, CA), showed inconsistent results between experiments. SP600125 has been reported to inhibit phosphatidylinositol 3-kinase non-specifically (Tanemura et al., 2009). Therefore, we performed knockdown of JNK 1 and JNK 2 separately using respective siRNAs. Our results indicated that JNK1 and JNK2 siRNAs were able to suppress caspase activation and JNK1 siRNA suppressed As_2O_3-induced apoptosis more significantly than JNK2 siRNA (Fig. 3). In addition, we found that JNK1 siRNA

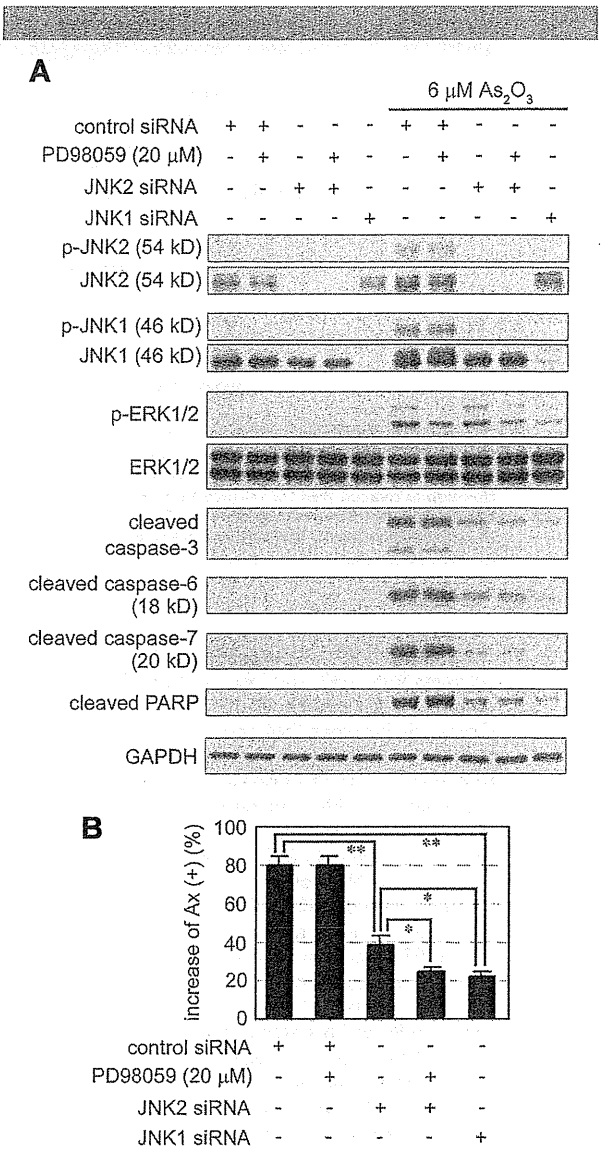

Fig. 5. As_2O_3-induced ERK1/2 activation is involved in caspase-independent apoptosis when JNK1/2 are inactivated. A: As_2O_3-induced caspase cascade is not suppressed by inhibition of ERK1/2 activation. NCI-H2052 cells transfected with control, JNK1 or JNK2 siRNA were treated with 6 μM As_2O_3 alone or together with 20 μM PD98059 for 24 h. Representative data of three independent experiments with similar results are shown. B: As_2O_3-induced apoptosis is regulated by ERK1/2 when JNK1/2 are inactivated. NCI-H2052 cells transfected with control, JNK1 or JNK2 siRNA were treated with 6 μM As_2O_3 alone or together with 20 μM PD98059 for 72 h, and analyzed for Ax(+) apoptotic cells by flow cytometry.

suppressed JNK2 phosphorylation and JNK2 siRNA suppressed JNK1 phosphorylation (Fig. 4), indicating that there is a cross-talk between JNK1 and JNK2. It has been reported that JNK2 phosphorylation is mediated by JNK1 in apoptosis of lung cancer cells, suggesting that JNK1 is located upstream of JNK2 (Oleinik et al., 2007). It has also been reported that JNK1, but not JNK2, is a major stress transduction pathway of extracellular stimuli including TNF-α and UV (Liu et al., 2004). Our results suggested that JNK1 and JNK2 are arranged in parallel in signal transduction, but able to carry cross-talk in As_2O_3-induced apopotosis in mesothelioma cells.

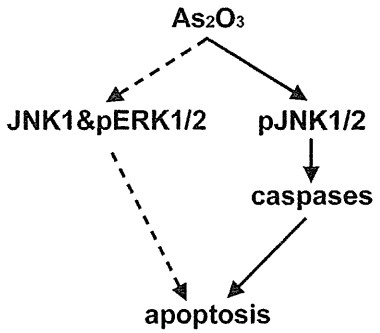

Fig. 6. Mechanistic scheme of As_2O_3-induced apoptosis in the NCI-H2052 human mesothelioma cell line. As_2O_3 induces apoptosis through activated JNK1/2 followed by caspase cascade. When JNK1 exists and JNK1/2 are inactivated, As_2O_3 induces apoptosis in caspase-independent manner through activated ERK1/2.

Liu et al. (1996) have reported that arsenite differentially activates JNK, p38, and ERK, and that JNK and p38 may share common upstream regulators distinct from those of ERK. Cross-talk between JNK and ERK has been described, in which the JNK pathway suppresses survival-promoting activity of the ERK pathway. In the present study, we observed that knockdown of JNK1 abrogated ERK1/2 phosphorylation (Fig. 4). Our results suggest that JNK1 cross-talked to ERK and that As_2O_3-induced ERK1/2 phosphorylation depends on existence of JNK1 protein, but not that of JNK2 protein and phosphorylated JNK1/2. These types of cross-talk between JNK and ERK have not been reported previously.

The ERK pathway mostly regulates cell proliferation, but it also mediates apoptosis (Lee et al., 2003). It has been reported that ERK1/2 are not in As_2O_3-induced apoptosis of breast cancer cells and hepatoma cells (Kang et al., 2003; Ye et al., 2005). Present results suggest involvement of ERK in As_2O_3-induced apoptosis, because JNK1 siRNA suppressed increases of not only phosphorylated JNK1/2 but also phosphorylated ERK1/2 in As_2O_3-induced apoptosis (Fig. 4). This suggests that As_2O_3-induced apoptosis is suppressed by inhibition of phosphorylation of both JNK1/2 and ERK1/2. To confirm the ERK involvement in As_2O_3-induced apoptosis, we conducted experiments using JNK2 siRNA, which suppressed As_2O_3-induced phosphorylation of JNK1/2 but not that of ERK1/2, together with PD98059. The results indicated that ERK1/2 are involved in As_2O_3-induced apoptosis of mesothelioma cells, when JNK1 exists and JNK1/2 are inactivated (Fig. 5). Furthermore, As_2O_3-induced apoptosis through ERK1/2 signaling was independent of caspase cascade. Thus, ERK1/2 constitute a part of signal transduction pathway of As_2O_3-induced apoptosis under cross-talk with JNK1. Further investigation is warranted for the mechanism of ERK1/2 engagement in As_2O_3-induced apoptosis.

In conclusion, As_2O_3 activates both JNK1 and JNK2, which in turn activate caspase-3, and JNK1-regulated ERK1/2, leading to apoptosis in human mesothelioma cells (Fig. 6). These results provide further understanding of As_2O_3-induced apoptosis of mesothelioma cells, which would be helpful for developing chemotheraputic agent against malignant tumor cells.

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Pleural effusion VEGF levels as a prognostic factor of malignant pleural mesothelioma

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KEYWORDS
Mesothelioma;
Tumor marker;
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Summary
Background: Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure. MPM has a limited response to conventional chemotherapy and radiotherapy so early diagnosis of MPM is very important. Vascular endothelial growth factor (VEGF), a potent mitogen for the vascular endothelium, is also known to be an autocrine growth factor for MPM. Here, we investigated the pleural effusion VEGF levels in patients with MPM and compared them to those of a population with a non-malignant pleuritis or lung cancer involving malignant pleural effusion.
Methods: The pleural effusion VEGF concentrations were measured in 46 MPM patients and 45 individuals with non-MPM individuals (25 individuals with non-malignant pleural effusions, and 20 individuals with lung cancer involving malignant pleural effusion).
Results: We demonstrated that patients with MPM had significantly higher pleural effusion VEGF levels than a population with non-malignant pleuritis or lung cancer involving malignant pleural effusion, and the patients with advanced stage MPM showed higher levels of VEGF than the early stage MPM patients. The difference in overall survival between the groups with pleural effusion VEGF levels lower and higher than the assumed cut-off of 2000 pg/ml was significant.
Conclusions: Our data suggest that the pleural effusion VEGF concentration could be useful as an aid for the diagnosis of MPM and as a prognostic factor.

Abbreviations: AUC, area under the ROC curve; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MPM, malignant pleural mesothelioma; PDGF, platelet derived growth factor; ROC, receiver operating characteristic; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure.[1–3] Although asbestos usage has recently decreased in Western countries and Japan, the incidence of MPM is expected to markedly increase over the next few decades because there is the long latency period (20–40 years) between asbestos exposure and tumor development.[4] MPM shows limited response to conventional chemotherapy and radiotherapy. Although the multi-targeted anti-folate pemetrexed has recently been approved as a first-line agent in combination with cisplatin for the treatment of MPM, overall survival remains very poor[5] with a median survival duration of 8–18 months.[6] In several centers, potentially curative surgery combined with some form of adjuvant therapy has been performed. Such early therapeutic intervention seems to be more beneficial than late intervention. Therefore, diagnosing MPM early is very important.[1] However, cytological diagnosis of pleural effusions can often be very difficult in MPM because MPM cells can not be easily distinguished from lung cancer cells and sometimes they can not be detected in pleural effusions.[1] Pleural effusion biomarkers for MPM such as hyaluronic acid and CYFRA 21-1 have been reported and used to assist the diagnosis of MPM.[7, 8] To further improve the specificity and sensitivity of diagnosis, research into the development of novel biological markers is urgently required.

Tumor growth and metastasis are associated with angiogenesis. We previously reported that vascular endothelial growth factor (VEGF), a potent mitogen for the vascular endothelium, is associated with bleomycin-induced pulmonary fibrosis in mice.[9] MPM is a malignant transformation of mesothelial cells, which originate from mesenchymal cells similar to lung fibroblasts. Moreover, we recently reported that patients with MPM had significantly higher serum levels of VEGF than a population with a history of asbestos exposure without developing MPM, which suggested its usefulness as a marker for MPM.[10] In this study, we evaluated the clinical role of pleural effusion VEGF levels in MPM and found that patients with MPM had significantly higher pleural effusion VEGF levels than a population with a non-malignant pleuritis or lung cancer involving malignant pleural effusion, which suggested its usefulness as a marker for MPM. Our results are consistent with previous reports demonstrating that VEGF is expressed in MPM, and moreover, that it acts as an autocrine growth factor for MPM.[11]

Materials and methods

Patients and pleural effusion samples

We studied the VEGF levels in pleural effusions collected from 91 individuals presenting at the Department of Respiratory Medicine of Hyogo College of Medicine Hospital from 2005 to 2009. The pleural effusions were obtained by thoracocentesis. All patients were diagnosed by pathologists, and it was confirmed that their clinical course matched their diagnosis. Forty-six individuals had malignant pleural mesothelioma involving a documented asbestos exposure history. These cases were diagnosed by pathologists skilled in the diagnosis of MPM using histopathological samples. All patients were classified using the staging system of the International Mesothelioma Interest Group (IMIG).[12] Patients with MPM were treated according to our therapeutic guideline: combination chemotherapy including multi-targeted anti-folate pemetrexed was performed for patients with PS 0–1 and age < 70, and for the remainders, best supportive care was chosen. Surgical treatment was not performed for the patients in the present study. Twenty-five individuals had non-malignant pleural effusions, and 20 individuals had lung cancer involving malignant pleural effusion. The study was approved by our ethics committee in accordance with the 1975 Declaration of Helsinki. Informed consent was obtained from all patients. Fresh pleural effusion samples were collected before treatment and centrifuged for 10 min at 2000 g, before the resultant supernatants were immediately frozen in liquid nitrogen and stored at −80° until use.

Measurement of VEGF

The VEGF concentrations of the pleural effusions were measured using an enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Oxford, UK) according to the manufacturers' instructions.

Statistical analysis

The nonparametric Mann–Whitney *U*-test was used to compare two groups of samples. Comparisons of data between various groups were performed with the nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test. In all tests, a *p*-value <0.05 was considered significant. In order to estimate the significance of the pleural effusion VEGF values, receiver operating characteristic (ROC) curves, the area under the ROC curve (AUC), and their 95% confidence intervals (95% CI) were calculated using standard techniques. To examine the cut-off values for the pleural effusion VEGF levels, we calculated the total sensitivity and specificity for each cut-off value and then chose the cut-off values that maximized each factor. Correlations between data were analyzed using Spearman's rank correlation test. Estimates of the probability of survival were calculated by the Kaplan–Meier method and compared using the log-rank test. In order to evaluate the prognostic significance of VEGF on the survival of patients with MPM, Cox's proportional hazards regression analysis was carried out as a multivariate analysis.

Results

VEGF pleural effusion levels in patients with MPM and individuals with non-MPM

We recruited a total of 91 subjects suffering from pleural effusion. Of them, 46 had confirmed MPM, 25 had no malignant pleural effusion, and 20 had lung cancer involving malignant pleural effusion. Their characteristics are shown in Table 1.

The ROC curves for the pleural effusion VEGF levels showed that the patients with MPM had an AUC of 0.8304 in

comparison to those with non-malignant pleural effusion (95% CI: 0.7528–0.9081). At the optimal cut-off value of 2000 pg/ml, the diagnostic sensitivity was 71.7%, and the specificity was 76.0% (Fig. 1A). The mean pleural effusion VEGF concentration of the patients with MPM was significantly higher (5303.6 ± 1711.0 pg/ml) than that of the patients with non-malignant pleural effusion and the patients with lung cancer involving malignant pleural effusion (1172.2 ± 1212.8 pg/ml, 2429.9 ± 2173.8 pg/ml) ($p = 0.0003$, $p = 0.0017$, respectively) (Fig. 1B). Moreover, scatter plots of the pleural effusion VEGF levels of the MPM patients showed statistically significant tendencies to increase as the stage increased (stage I: 1919.2 ± 1802.8 pg/ml, stage II: 3764.0 ± 2432.0 pg/ml, stage III: 3956.7 ± 2316.5 pg/ml, and stage IV: 4789.8 ± 2281.3 pg/ml) ($p = 0.025$, Fig. 1C). There were no statistically significant differences between the pleural effusion VEGF levels of the MPM histological groups (epithelioid: 4000.0 ± 2499.3 pg/ml, non-epithelioid: 4185.3 ± 2245.5 pg/ml) or sex (male: 4253.9 ± 2381.9 pg/ml, female: 3070.6 ± 2470.9 pg/ml), and there were no significant differences in the pleural effusion VEGF levels between the subjects with benign asbestos pleurisy and those with benign pleurisy without a history of asbestos exposure (1853.8 ± 1438.7 pg/ml, 1139.6 ± 1223.5 pg/ml, respectively).

Correlation of VEGF levels between pleural effusions and serum

We examined pleural effusion and serum VEGF levels in 16 MPM patients and showed that there was a significant correlation between them ($r = 0.51$, $p = 0.046$, Fig. 2). The numbers of MPM patients with higher VEGF levels in both their serum and pleural effusion; higher VEGF levels in their serum alone; higher VEGF levels in their pleural effusion alone; and levels lower than 460 pg/ml (serum cut-off level) and 2000 pg/ml (cut-off level for pleural effusion) in the serum and pleural effusion, respectively,[10] were 7, 5, 2, and 2, respectively. The two MPM patients with lower VEGF levels in both their serum and pleural effusion were classified as stage I.

Relationship between pleural effusion VEGF levels and overall survival

Among the 46 MPM patients, we were able to follow 28 patients closely for up to 600 days. To study the relationship between the pleural effusion VEGF levels and the patients' clinical courses, we separated the patients according to their pleural effusion VEGF levels at the time of the first measurement. The first group included patients with pleural effusion VEGF levels of lower than 2000 pg/ml (the cut-off value that we chose). In this group of 11 patients, the mean VEGF value was 1070.2 pg/ml (interquartile range: 634.3–1498.4). The other group included the remaining 17 patients with pleural effusion VEGF levels of higher than 2000 pg/ml, whose mean VEGF value of pleural effusions was 5101.1 pg/ml (interquartile range: 3184.9–6311.4). The difference in overall survival between the groups with lower and higher pleural effusion VEGF values than the assumed cut-off point of 2000 pg/ml was significant ($p = 0.041$, Fig. 3). Cox's regression analysis was performed on 28 MPM patients for whom data on age, sex, histology, stage, and the pleural effusion VEGF level were available. Independent

Table 1 Characteristics of the MPM patients and non-malignant subjects.

		Cases (%)	Total
MPM			
Age	69.17 ± 9.64		
Sex	Male/Female	38(82.6)/8(17.4)	46
Histology	Epithelioid	34(73.9)	
	Sarcomatoid	10(21.7)	
	Biphasic	2(4.4)	
Stage	I/II/III/IV	8(17.5)/6(13.0)/6(13.0)/26(56.5)	
No-malignancy			
Age	70.50 ± 13.02		
Sex	Male/Female	21(84.0)/4(16.0)	25
	Benign asbestos pleurisy	5(20.0)	
	Tuberculous(Tb) pleurisy	8(32.0)	
	Infectious (non-Tb) pleurisy	8(32.0)	
	Para-pneumonic	1(4.0)	
	Heart failure	1(4.0)	
	Hepatic failure	1(4.0)	
	Renal failure	1(4.0)	
Lung cancer			
Age	69.00 ± 8.84		
Sex	Male/Female	10(50.0)/10(50.0)	20
	Adenocarcinoma	17(85.0)	
	Squamous cell	2(10.0)	
	Small	1(5.0)	

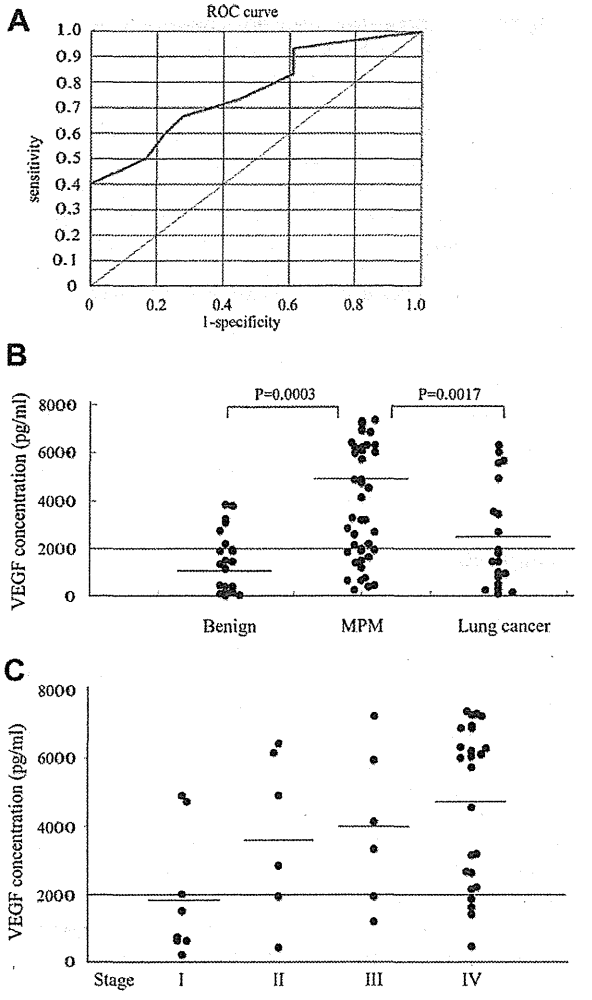

Figure 1 Pleural effusion VEGF levels in patients with MPM and non-MPM subjects. (A) An analysis that included 46 MPM and 25 non-malignant pleural effusion patients revealed an area under the curve (AUC) of 0.8304 (95%CI: 0.7528–0.9081). At the optimal cut-off value of 2000 pg/ml, the diagnostic sensitivity was 71.7%, and the specificity was 76.0%. (B) The pleural effusion VEGF levels of the patients with MPM versus those of the patients with non-malignant pleural effusion or lung cancer involving malignant pleural effusion were measured as described in Materials and Methods. (C) The pleural effusion VEGF levels in the MPM patients divided into 4 stages are shown. The nonparametric Mann–Whitney *U*-test (B) or the nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test (C) was used. *p*-values <0.05 were considered significant. The horizontal bars represent the mean of each group. The cut-off value is shown as a horizontal line.

statistically significant prognostic effects on survival were found for age (65 ≥ versus < 65years; HR, 15.6; 95% CI, 1.068–229.125; $p = 0.045$), sex (male versus female; HR, 0.380.0031; 95% CI, 0.000062–0.1555; $p = 0.0038$), histology (sarcomatoid versus epithelioid; HR, 8.663; 95% CI, 1.114–67.351; $p = 0.0039$), and pleural effusion VEGF level (2000 ≥ versus < 2000 pg/ml; HR, 961.23; 95% CI, 7.083–130446.4; $p = 0.0061$).

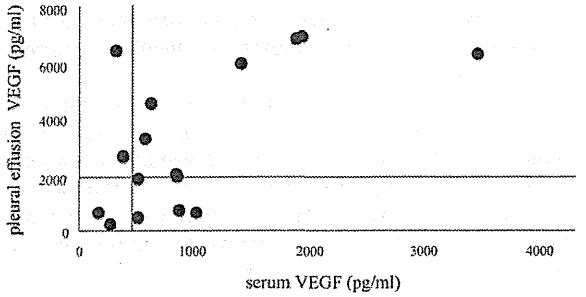

Figure 2 Correlation of VEGF levels between pleural effusions and sera. We examined the pleural effusion and serum VEGF levels in 16 MPM patients and showed that there was a significant correlation between the two ($r = 0.51$, $p = 0.046$). Correlations between data were analyzed using Spearman's rank correlation test.

Discussion

MPM is a malignant transformation caused by the exposure of mesothelial cells to asbestos, which shows a limited response to conventional chemotherapy and radiotherapy, and its prognosis is very poor. The lifetime risk of MPM is associated with occupational and/or environmental asbestos exposure history.[13] Due to the long latency period (typically longer than 30 years) between the first asbestos exposure and the onset of the disease, MPM remains a universally fatal disease of increasing incidence all over the world.[1, 2]

Although in advanced cases, resection of the tumor only prolongs survival by about 3 months, patients with stage IA disease survive for five or more years after total resection of the tumor.[14] Due to the difficulty of the differential diagnosis of MPM among individuals with pleural effusion by radiological, cytological, and/or histological examinations, efficient and practical pleural effusion biomarkers are required to aid the diagnosis of MPM. To date, there have been several reports concerning candidates for clinically useful markers for MPM.[14–17] Indeed, the level of mesothelin-related protein (SMRP), the soluble form of mesothelin, has been reported to be a useful pleural effusion marker in MPM.[18] Mesothelin is thought to play a role in cell-adhesion, cell-to-cell recognition, and signaling; however, its biological functions in MPM cells have not been

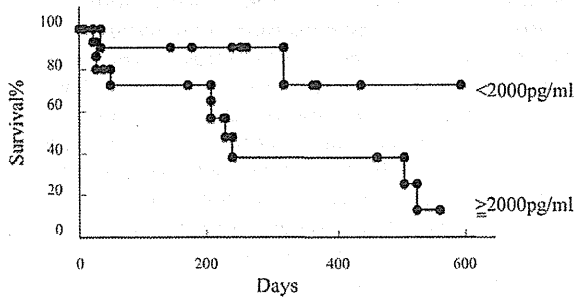

Figure 3 Survival of MPM subjects according to pleural effusions VEGF levels. Estimates of the probability of survival were calculated using the Kaplan–Meier method and compared using the log-rank test.

fully investigated and there was no significant difference between pleural effusion mesothelin levels and survival in MPM patients.[18]

Therefore, it is important to find biological markers with effects on MPM cells that are closely related to MPM progression. MPM cells are known to exhibit increased and/or dysregulated growth. Several factors, including transforming growth factor (TGF)-β_1, platelet derived growth factor (PDGF), TGF-α, and interleukin (IL)-8, have been reported to be associated with MPM cells.[19–24] Moreover, tumor development is closely related to angiogenesis. Malignant tumors require new blood vessel formation, and it has been reported that increased vascularity in MPM is associated with a poor prognosis.[2, 25] VEGF is known to be an important regulator of angiogenesis and plays critical roles in endothelial cell proliferation, vascular permeability, and angiogenesis in several inflammatory lesions.[26–28] We previously reported that VEGF is associated with bleomycin-induced pulmonary fibrosis in mice.[9] MPM is a malignant transformation of mesothelial cells, which originate from mesenchymal cells similar to lung fibroblasts, so it would not be surprising if VEGF was found to be associated with MPM progression. Moreover, we recently reported that patients with MPM had significantly higher serum levels of VEGF than a population with a history of asbestos exposure, which suggested its usefulness as a marker for MPM. In this study, we evaluated the clinical role of VEGF as a pleural effusion biomarker in MPM and found that patients with MPM had significantly higher pleural effusion VEGF levels than a population with non-malignant pleural effusion or lung cancer involving malignant pleural effusion.

Paracrine and autocrine mechanisms have been reported for several cytokines in malignant tumors. We recently reported the autocrine and/or paracrine mechanism of TGF-β_1 in MPM.[19] VEGF has also been reported to be an autocrine growth factor of MPM.[11, 29] On the other hand, Li et al.[30] reported that treatment with anti-VEGF neutralizing antibody suppressed MPM progression in a mouse model, mainly by preventing angiogenesis, especially during the formation of pleural effusion. These reports suggested that VEGF plays an important role in the tumor growth of MPM. Strizzi et al.[11] reported that higher VEGF levels were found in the pleural effusions of MPM patients than in those of patients with non-malignant pleural diseases; however, there was no significant correlation between VEGF levels and MPM patient survival. In the present study, we demonstrated that patients with MPM had significantly higher pleural effusion VEGF levels than a population with non-malignant pleuritis or lung cancer involving malignant pleural effusion, and moreover, there was a significant correlation between VEGF levels and MPM patient survival. The discrepancy between our observations and those of the previous study with regard to the correlation between VEGF pleural effusion levels and MPM patient survival may be explained as follows: first, they examined 12 MPM patients; whereas, we examined more patients; second, their MPM patients were classified into IA ($n = 1$), IB ($n = 5$), II ($n = 5$), and III stages ($n = 1$); whereas, our patients included more advanced stages.

Here, we examined the pleural effusion and serum VEGF levels in 16 MPM patients and demonstrated that there was a significant correlation between them. In these patients, 14 patients showed higher VEGF levels in their serum and/or pleural effusion. On the other hand, 12 and 9 patients had higher VEGF levels in their serum and pleural effusion, respectively. Only 2 stage I patients demonstrated lower VEGF levels in both their serum and pleural effusion. So the measurement of VEGF levels in both serum and pleural effusions seems to be an efficient way of diagnosing MPM.

The prognostic significance of VEGF in MPM has been estimated previously.[31] However, the previous study demonstrated a significant correlation between the VEGF staining of resected specimens and short survival. In the present study, we demonstrated that pleural effusion VEGF levels gradually increased according to the progression of the disease, and the Kaplan–Meier method revealed a significant correlation between pleural effusion VEGF levels and survival, which suggested its usefulness as a marker for estimating prognosis.

Conclusion

In summary, we demonstrated that patients with MPM had significantly higher pleural effusion VEGF levels than a population with non-malignant pleuritis involving benign asbestos pleurisy or lung cancer involving malignant pleural effusion, suggesting VEGF to be a useful marker for MPM. The patients with advanced stage MPM showed higher levels of VEGF than the patients with early stage MPM, and the Kaplan–Meier method revealed a significant correlation between pleural effusion VEGF levels and patient survival. Moreover, Cox's regression analysis demonstrated that the pleural effusion VEGF level had an independent statistically significant prognostic effect on survival, which suggested its usefulness as a marker for estimating prognosis.

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Contributors: Tabata C, Tabata R and Nakano T designed research. Tabata C and Hirayama N performed research. Tabata C, Hirayama N, Maeda R, Yasumitsu A, Yamada S, Kuribayashi K and Fukuoka K collected data. Tabata C and Tabata R analyzed and interpreted data. Tabata C performed statistical analysis. Tabata C and Tabata R wrote the manuscript.

Conflicts of interest

We declare that no conflicts of interest exist.

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Is Serum Thioredoxin-1 a Useful Clinical Marker for Malignant Pleural Mesothelioma?

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Abstract

Malignant pleural mesothelioma (MPM), an asbestos-related aggressive malignant tumor of mesothelial origin, shows limited response to therapy and overall survival remains very poor. Reactive oxygen species play an important role in asbestos toxicity. Here, we found that the patients with MPM had significantly higher serum levels of thioredoxin-1 (TRX) than control population. The patients with advanced-stage MPM showed higher levels of TRX than those with early-stage MPM. The difference in overall survival between the groups with lower and higher serum TRX levels was significant. Our data suggest that serum TRX concentration could be a useful clinical marker for MPM. *Antioxid. Redox Signal.* 15, 685–689.

Introduction

MALIGNANT PLEURAL MESOTHELIOMA (MPM) is an aggressive malignant tumor of mesothelial origin, which shows limited response to conventional chemotherapy and radiotherapy (20, 21, 30). Although the multitarget antifolate pemetrexed was recently approved as a first-line agent in combination with cisplatin for the treatment of MPM, the overall survival of MPM patients remains very poor (29), with a median survival duration of 8–18 months (14). In several centers, potentially curative surgery combined with some form of adjuvant therapy has been performed. Such early therapeutic intervention seems to be more beneficial than late intervention. Therefore, diagnosing MPM at an early stage is very important (21). To detect MPM in its early stages, we have screened outpatients with a history of asbestos exposure but no symptoms for several years. However, diagnosis by radiological and/or histological examinations can be often very difficult. Therefore, efficient and practical serum biomarkers are required to aid the diagnosis of MPM.

In the diagnosis of lung cancer, serum markers such as CEA, CYFRA, proGRP, and SCC play supportive roles to confirm the diagnosis. There have been several reports about candidates for clinically useful markers for MPM. Indeed, some of them have been reported to be useful serum markers for MPM, such as mesothelin and osteopontin (16, 19); however, little is known about their biological functions or effects on MPM cells. For further improvement of the specificity and sensitivity of diagnosis, research into the development of novel biological markers is urgently required.

Innovation

MPM is an aggressive malignant tumor of mesothelial origin and shows limited response to conventional chemotherapy and radiotherapy. Therefore, diagnosing MPM early is very important. ROS play an important role in asbestos toxicity, which is associated with the pathogenesis of MPM development. TRX is a small redox-active protein that demonstrates antioxidative activity associated with tumor growth. Here, we investigated the serum levels of TRX in 57 MPM patients and compared them with those of a population that had been exposed to asbestos without developing MPM.

We demonstrated that the patients with MPM had significantly higher serum levels of TRX than the population who had been exposed to asbestos but had not developed MPM. Also, we demonstrated that the patients with advanced-stage MPM showed higher levels of TRX than those with early-stage MPM. As the clinical stage of MPM is not related to the presence or absence of pleural effusion, and the early distinction of MPM patients from those with benign asbestos-related diseases is necessary, we propose that measuring serum TRX levels is an easy and useful method for the clinical management for MPM. Moreover, the difference in overall survival between the groups with serum TRX levels that were lower and higher than the assumed cutoff of 60 ng/mL was significant. It is suggested that TRX would be a useful serum prognostic factor for MPM. This is the first report about the relationship between serum TRX and MPM.

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It is well known that MPM is associated with asbestos exposure (20, 21, 30). The lifetime risk of MPM is closely related to occupational and/or environmental asbestos exposure history (17). Although asbestos usage has recently decreased in Western countries and Japan, the incidence of MPM is expected to markedly increase over the next few decades because there is the long latency period (20–40 years) between asbestos exposure and tumor development (24).

A previous report demonstrated that reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion are important mediators of malignancies of the human lung and asbestos toxicity (8), which is associated with the pathogenesis of MPM development. In oxidative stress conditions, thioredoxin-1 (TRX), a small redox-active protein that possesses antioxidative activity and acts as a redox-regulating multifunctional protein, reduces the levels of ROS as part of the antioxidant defense (5).

In this study, we evaluated the clinical role of serum TRX in MPM and found that the patients with MPM had significantly higher serum levels of TRX than a population with a history of asbestos exposure, which suggested its usefulness as a marker for MPM.

Results and Discussion

Possible important roles of TRX in MPM

TRX was first identified from extracts of *Escherichia coli* B as a hydrogen donor that transfers hydrogen from NADPH to ribonucleotide reductase (5). TRX is a 12-kDa multifunctional protein containing a redox-active site (Cys-Gly-Pro-Cys), which undergoes NADPH-dependent reduction by TRX reductase, and reduced oxidized cysteine protein groups. TRX is ubiquitously expressed in various organisms. Human TRX has been cloned independently by two groups: it was first cloned as an IL-2 receptor α chain (CD25)–induced factor in HTLV-1–infected T-cell lines and designated as adult T-cell leukemia–derived factor (27), and another group cloned it as an IL-1–like growth factor produced by Epstein-Barr virus–transformed cells (2).

TRX is ubiquitously expressed in normal tissues and cells, and its expression can also be induced by a variety of physiochemical stresses, such as viral infection, mitogens, ultraviolet irradiation, hydrogen peroxide, and oxidative stress (12). TRX is mainly localized in the cytoplasm, and its secretion mechanism remains unclear. As human TRX was first cloned as a secreted adult T-cell leukemia–derived factor, its expression is markedly enhanced in HTLV-I–infected T cells (27). The plasma TRX level is elevated in certain human diseases including HIV infection and hepatitis C virus infection (13, 26).

Recent reports have shown that TRX affects a number of cellular responses, including cell growth, inflammation, and apoptosis, and that the redox regulation mediated by TRX is correlated with the pathogenesis of several oxidative stress–associated diseases (6, 28). On the other hand, it is well known that both oxidative stress and hypoxia are common features of tumors. Growing tumor masses need vascular networks. Although angiogenesis occurs in malignant tumors, it does not occur in an efficient manner, leading to switching between hypoxia and re-oxygenation conditions because of the irregular flow of oxygen, which causes oxidative stress (3). As cancer cells are often under high oxidative or hypoxic stress, they express high levels of antioxidant proteins, including TRX. For example, TRX expression is increased in several primary cancers, including lung, cervix, pancreatic, colorectal, breast, hepatocellular, and gastric carcinomas (9, 15, 25).

Serum levels of TRX in patients with MPM as a diagnostic marker

The human lung is more exposed to ROS caused by cigarette smoke and chemical pollutants than any other organ (10). Here, we investigated the serum levels of TRX in patients with MPM and compared them with those of a population that had been exposed to asbestos without developing MPM. We recruited a total of 91 subjects with a history of asbestos exposure. Of them, 57 had confirmed MPM, 19 had pleural plaques and/or asbestosis, and 15 had no asbestos-related lesions despite being exposed to asbestos, i.e., were healthy. Their characteristics are shown in Table 1.

The receiver operating characteristic (ROC) curves for serum TRX levels showed that the patients with MPM had an area under the ROC curves of 0.8178, compared with those with benign asbestos-related diseases (asbestosis or pleural plaques) and those who were healthy despite asbestos exposure (95% confidence intervals [95% CI]: 0.7482–0.8873). At the optimal cutoff value of 60 ng/mL, the diagnostic sensitivity was 71.9% and the specificity was 85.0% (Fig. 1A).

The serum TRX concentration of the patients with MPM was significantly higher (119.1 ± 125.2 ng/mL) than that of the patients with benign asbestos-related diseases (asbestosis or pleural plaques) and the healthy individuals (40.1 ± 20.4 ng/mL) ($p < 0.0001$; Fig. 1B). Serum TRX level of healthy volunteers ($n = 5$; age: 32.0 ± 8.0; male/female: 3/2) who had never been exposed to asbestos was 4.8 ± 2.7 ng/mL. The difference in serum TRX levels between the population with a history of asbestos exposure and the healthy volunteers was not statistically significant, suggesting that not only asbestos exposure

TABLE 1. CHARACTERISTICS OF THE MALIGNANT PLEURAL MESOTHELIOMA PATIENTS AND THE NONMALIGNANT PLEURAL MESOTHELIOMA SUBJECTS WITH A HISTORY OF ASBESTOS EXPOSURE

	No. of cases (%)	*Total*
MPM[a]		57
Age, 66.04 ± 9.26		
Sex, male/female	44 (77.2)/13 (22.8)	
Histology		
Epithelioid	39 (68.4)	
Sarcomatoid	9 (15.8)	
Biphasic	6 (10.5)	
Desmoplastic	2 (5.3)	
Stage I/II/III/IV	4 (7.0)/6 (10.5)/11 (19.3)/36 (63.2)	
Non-MPM[a]		
Age, 69.18 ± 8.71		34
Sex, male/female	30 (88.2)/4 (11.8)	
CT findings		
Plaque	15 (44.1)	
Asbestosis	1 (3.0)	
Plaque and asbestosis	3 (8.8)	
None	15 (44.1)	

[a]All individuals were exposed to asbestos.
MPM, malignant pleural mesothelioma.

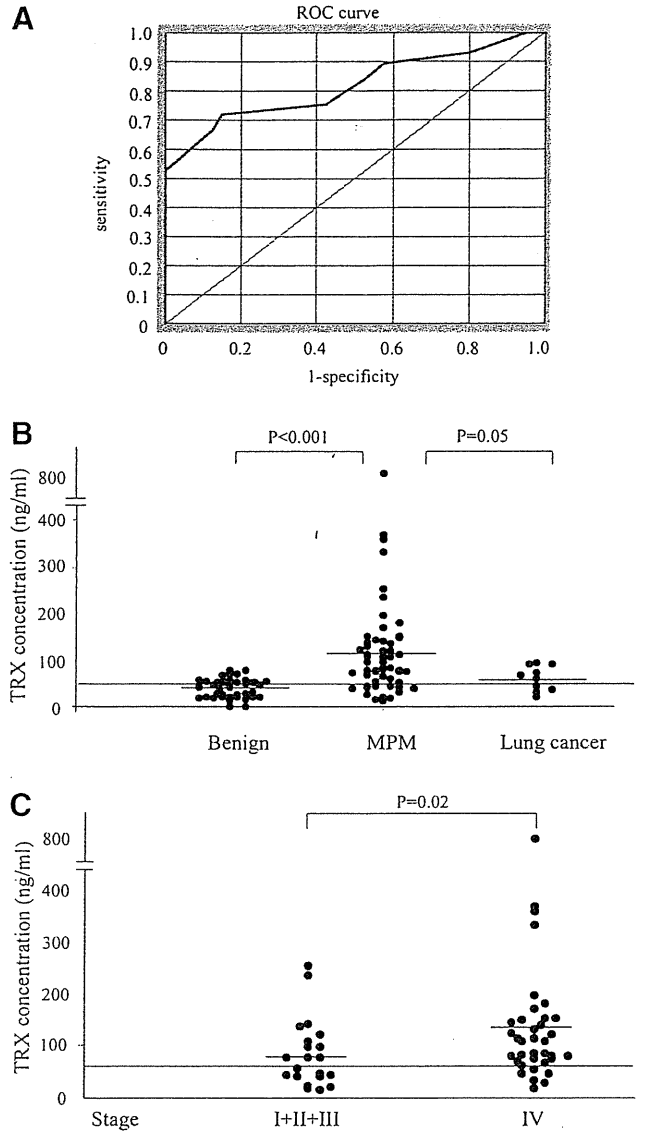

FIG. 1. Serum thioredoxin-1 (TRX) levels in patients with malignant pleural mesothelioma (MPM) and non-MPM subjects. (A) Sensitivity and specificity of serum TRX for distinguishing patients with MPM from non-MPM subjects (receiver operating characteristic [ROC] curve). An analysis that included 57 MPM patients and 34 non-MPM subjects with a history of asbestos exposure revealed an area under the ROC curve of 0.8178 (95% confidence interval: 0.7482–0.8873). At a cutoff value of 60 ng/mL, the diagnostic sensitivity was 71.9% and the specificity was 85.0%. **(B)** Serum TRX levels in non-MPM subjects, MPM patients, and lung cancer patients with malignant pleuritis were measured as described in the Materials and Methods section. **(C)** Serum TRX levels of MPM patients divided into four groups according to disease stage. The nonparametric Mann–Whitney *U* test was used. A *p*-value of <0.05 was considered significant. Horizontal bars represent the mean value of each group. The cutoff value is shown as a horizontal line.

but also MPM development is required for the elevation of serum TRX levels.

The positive predictive value of high TRX value for MPM was 89.1%, and the negative predictive value was 64.4%. Although the diagnostic sensitivity was not high (71.9%), its positive predictive value was fairly high (89.1%), suggesting that high serum TRX levels are supportive of a diagnosis of MPM. Interestingly, we found that the serum TRX levels of patients with lung cancer involving malignant pleuritis ($n=10$) were significantly lower (61.3 ± 27.0 ng/mL; $p=0.05$) than those of the MPM patients (Fig. 1B). The differences in serum TRX levels between the MPM histological groups (epithelioid: 111.0 ± 90.4 ng/mL; sarcomatoid: 104.6 ± 62.8 ng/mL; biphasic: 73.8 ± 19.8 ng/mL; and desmoplastic: 119.4 ± 32.9 ng/mL) were not statistically significant. There were no significant differences in TRX levels among the subjects with benign asbestos-related diseases (asbestosis or pleural plaques) and those who were healthy despite having a history of asbestos exposure (40.1 ± 18.7 and 40.0 ± 23.1 ng/mL).

It has been reported that TRX mRNA is strongly expressed in MPM cells (18), and TRX protein was immunohistochemically detected in MPM tissues (7). Although its precise cellular mechanism has not been fully investigated, these reports indicated that a correlation exists between the TRX system and MPM development. Here, we evaluated the clinical role of TRX as a serum biomarker for MPM and found that patients with MPM had significantly higher serum levels of TRX than control population, suggesting its useful roles as a diagnosing and prognostic marker for MPM.

Serum levels of TRX in patients with MPM as a prognostic marker

An elevated level of plasma TRX was reported in AIDS patients with poor prognoses and was found to be inversely correlated with the intracellular glutathione level. Also, the serum TRX levels of patients with HCV infection increased with the progression of liver fibrosis (26). Therefore, the plasma or serum level of TRX is a good marker of the oxidative stress caused by viral infection.

On the other hand, the overexpression of TRX in cancer cells seems to be associated with the promotion of cancer cell growth, a worse prognosis, and the development of resistance to anticancer agents. These data suggest a role for TRX as a target of cancer therapy or a tumor marker (1).

Here, we demonstrated that the serum TRX levels of the MPM patients gradually increased according to the progression of the disease. The patients with stage IV MPM had significantly higher levels of TRX (139.9 ± 146.1 ng/mL) than the patients with stage I–III disease (82.7 ± 68.0 ng/mL) (stage I: 67.1 ± 25.6 ng/mL; stage II: 110.5 ± 73.0 ng/mL; stage III: 74.5 ± 72.8 ng/mL) ($p=0.024$; Fig. 1C).

To study the relationship between serum TRX levels and the patients' clinical courses, we separated the patients based on their serum TRX levels at the time of the first measurement. Among the 57 MPM patients, we were able to closely follow-up 56 patients for 1400 days. The first group included patients with serum TRX levels lower than 60 ng/mL, the cutoff value that we used. In this group of 16 patients, the mean serum TRX value was 34.2 ng/mL (interquartile range: 21.2–43.6). The other group included the remaining 40 patients with serum TRX levels higher than 60 ng/mL, whose mean serum TRX value was 160.0 ng/mL (interquartile range: 79.6–151.4). The difference in overall survival between the two groups was significant ($p=0.030$; Fig. 2), which suggested the usefulness of serum TRX value as a marker for estimating prognosis. On the other hand, Kahlos *et al.* (7) reported that there was no

significant association between TRX expression in the tissues of MPM patients and survival. Although they assessed the levels of TRX expression using the staining intensities of resected specimens, we consider that serum TRX levels are more closely associated with the general condition of the patient.

Moreover, Cox's regression analysis was performed on 56 MPM patients for whom data on age, sex, histology, stage, and serum TRX level were available, and an independent statistically significant prognostic effect of stage on survival (IV *versus* I–III; HR, 3.15; 95% CI: 1.23–8.04; $p=0.017$) was found.

Several studies have demonstrated that overexpression of TRX enhances cancer cell growth (4). In addition, high levels of TRX expression have been shown to correlate with highly invasive and metastatic tumor activity (11). Further, high levels of TRX and other antioxidant proteins have also been shown to be correlated with resistance to various chemotherapeutic agents such as cisplatin (23). From these observations, TRX seems to play an active role in both cancer growth and cancer progression through the inhibition of apoptosis, the stimulation of metastatic and invasive activity, and chemotherapy resistance. The present data suggests that TRX also plays an important role in tumor progression of MPM and could be a useful marker for prognosis of the patients with MPM.

Materials and Methods

Patients and serum samples

We studied the TRX levels in sera collected from 91 individuals who presented at the Department of Respiratory Medicine of Hyogo College of Medicine Hospital from 2005 to 2007. All of the individuals had a documented asbestos exposure history. Fifty-seven individuals had MPM, which was diagnosed using histopathological samples by pathologists skilled in the diagnosis of MPM. All patients were classified according to the staging system of the International Mesothelioma Interest Group (22). Thirty-four individuals had benign asbestos-related diseases (asbestosis or pleural plaques) or were healthy despite their previous asbestos exposure. The study was approved by our ethics committee in accordance with the 1975 Declaration of Helsinki. Informed consent was obtained from all patients. Serum samples were collected before treatment, immediately frozen in liquid nitrogen, and stored at −80°C until use.

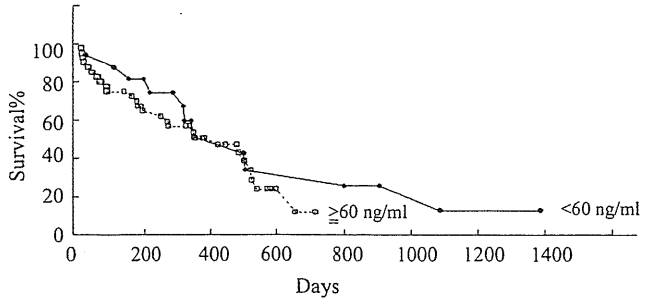

FIG. 2. Survival of MPM subjects according to serum TRX levels. Estimates of the probability of survival were calculated using the Kaplan–Meier method and compared using the log-rank test.

Measurement of TRX

Serum TRX concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Redox BioScience, Kyoto, Japan) according to the manufacturer's instructions.

Statistical analysis

The nonparametric Mann–Whitney *U* test was used to compare two groups of serum samples. In all tests, a *p*-value of <0.05 was considered significant. To estimate the significance of serum TRX values, ROC curves, area under the ROC curves, and their 95% CI were calculated using standard techniques. To obtain appropriate serum level cutoff values, we calculated the total sensitivity and specificity for each cutoff value and then chose the cutoff values that maximized each factor. Estimates of the probability of survival were calculated by the Kaplan–Meier method and compared using the log-rank test. To evaluate the prognostic significance of TRX with regard to the survival of patients with MPM, Cox's proportional hazards regression analysis was carried out.

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Author Contributions

C. Tabata, R. Tabata, and T. Nakano designed the research. C. Tabata and R. Maeda performed the research. C. Tabata, R. Eguchi, and Y. Fujimori collected data. C. Tabata and R. Tabata analyzed and interpreted the data. C. Tabata performed statistical analysis. C. Tabata and R. Tabata wrote the manuscript.

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Abbreviations Used

CI = confidence interval
ELISA = enzyme-linked immunosorbent assay
MPM = malignant pleural mesothelioma
ROC = receiver operating characteristic
ROS = reactive oxygen species
TRX = thioredoxin-1

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Clinical significance of pleural effusion mesothelin in malignant pleural mesothelioma

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Abstract

Background: Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure. MPM has a limited response to conventional chemotherapy and radiotherapy, so early diagnosis of MPM is very important. This study investigated the pleural effusion mesothelin levels in patients with MPM and compared them to those of a population with a non-malignant pleuritis or lung cancer involving malignant pleural effusion.
Methods: The pleural effusion mesothelin concentrations were measured in 45 MPM patients and 53 non-MPM individuals (24 individuals with non-malignant pleural effusions and 29 individuals with lung cancer involving malignant pleural effusion).
Results: This study demonstrated that patients with MPM had significantly higher pleural effusion mesothelin levels than a population with non-malignant pleuritis or lung cancer involving malignant pleural effusion. The difference in overall survival between the groups with pleural effusion mesothelin levels lower and higher than the assumed cut-off of 10 nM was significant.
Conclusions: The data suggest that the pleural effusion mesothelin concentration could be useful as an aid for the diagnosis of MPM.

Keywords: mesothelin; mesothelioma; tumor marker.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure (1–4). The lifetime risk of MPM is associated with occupational and/or environmental asbestos exposure history (5). Although asbestos usage has recently decreased in Western countries and Japan, MPM remains a universally fatal disease of increasing incidence all over the world (1, 2), because of the long latency period (typically longer than 30 years) between the first asbestos exposure and the onset of the disease. MPM shows limited response to conventional chemotherapy and radiotherapy. Although the multi-targeted anti-folate pemetrexed has recently been approved as a first-line agent in combination with cisplatin for the treatment of MPM, overall survival remains very poor (6), with a median survival duration of 8–18 months (7). In several centers, potentially curative surgery combined with some form of adjuvant therapy has been performed (1). Although such early therapeutic intervention seems to be more beneficial than late intervention, no single or combined intervention has been conformed to prolong survival after early diagnosis. Currently, however, early detection is important, because significant progress in systemic biologic therapy of MPM has been made, which may change as effective treatments become available (8). However, cytological diagnosis of pleural effusions can often be very difficult in MPM because MPM cells cannot be easily distinguished from lung cancer cells and sometimes they cannot be detected in pleural effusions (1). To further improve the specificity and sensitivity of diagnosis, research into the development of novel biological markers is urgently required.

Mesothelin is a cell surface glycoprotein which is overexpressed in malignant mesothelioma and other cancers (9). Some reports showed that serum mesothelin (soluble mesothelin-related protein) is a useful marker for MPM (10, 11). On the other hand, the clinical usefulness of pleural fluid mesothelin has not been fully defined. In this study, we evaluated the clinical role of pleural effusion mesothelin levels in Japanese MPM patients and found that patients with MPM had significantly higher pleural effusion mesothelin levels than a population with a non-malignant pleuritis or lung cancer involving malignant pleural effusion, which suggested its usefulness as a diagnostic marker for MPM.

Materials and methods

Patients and pleural effusion samples

We studied the mesothelin levels in pleural effusions collected from 98 individuals presenting at the Department of Respiratory Medicine of Hyogo College of Medicine Hospital from 1999 to 2007. The pleural effusions were obtained by thoracocentesis. All patients were diagnosed by pathologists and it was confirmed that their clin-

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ical course matched their diagnosis. Forty-five individuals had malignant pleural mesothelioma involving a documented asbestos exposure history. These cases were diagnosed by pathologists skilled in the diagnosis of MPM using histopathological samples. All patients were classified using the staging system of the International Mesothelioma Interest Group (IMIG) (12). Patients with MPM were treated according to our therapeutic guideline: combination chemotherapy, such as cisplatin was performed for patients with PS 0–1 and age <70 years and, for the remainders, best supportive care was chosen. Surgical treatment radiation therapy was performed for some patients in the present study. Twenty-four individuals had non-malignant pleural effusions and 29 individuals had lung cancer involving malignant pleural effusion. The study was approved by our Ethics Committee in accordance with the 1975 Declaration of Helsinki. Informed consent was obtained from all patients. Fresh pleural effusion samples were collected before treatment and centrifuged for 10 min at 2000 *g*, before the resultant supernatants were immediately frozen in liquid nitrogen and stored at –80°C until use.

Measurement of mesothelin

The mesothelin concentrations of the pleural effusions were measured using an enzyme-linked immunosorbent assay (ELISA) Kit (Mesomark™; Fujirebio Diagnostics Inc., Malvern, PA, USA) according to the manufacturers' instructions.

Statistical analysis

The non-parametric Mann-Whitney U-test was used to compare two groups of samples. Comparisons of data between various groups were performed with the Bonferroni/Dunn multiple comparisons test. In all tests, a p-value <0.05 was considered significant. In order to estimate the significance of the pleural effusion mesothelin values, receiver operating characteristic (ROC) curves, the area under the ROC curve (AUC) and their 95% confidence intervals (95% CI) were calculated using standard techniques. To examine the cut-off values for the pleural effusion mesothelin levels, we calculated the total sensitivity and specificity for each cut-off value and then chose the cut-off value that maximizes the sum of sensitivity plus 1-specificity. Estimates of the probability of survival were calculated by the Kaplan-Meier method and compared using the log-rank test. In order to evaluate the prognostic significance of mesothelin on the survival of patients with MPM, Cox's proportional hazards regression analysis was carried out as a multivariate analysis.

Results

Mesothelin pleural effusion levels in patients with MPM and individuals with non-MPM

We recruited a total of 98 subjects suffering from pleural effusion. Of them, 45 had confirmed MPM, 24 had no malignant pleural effusion and 29 had lung cancer involving malignant pleural effusion. Their characteristics are shown in Table 1.

The ROC curves for the pleural effusion mesothelin levels showed that the patients with MPM had an AUC of 0.863 in comparison to those with non-malignant pleural effusion (95% CI: 0.792–0.935). At the optimal cut-off value of 10 nM, the diagnostic sensitivity was 80.0% and the specificity was 83.3% (Figure 1A). The mean pleural effusion mesothelin concentration of the patients with MPM was significantly higher (52.3 ± 57.6 nM) than that of the patients with non-malignant pleural effusion (6.4 ± 4.6 nM) and the patients with lung cancer involving malignant pleural effusion (5.7 ± 4.5 nM) ($p<0.001$, $p<0.001$, respectively) (Figure 1B). There were no statistically significant differences between the pleural effusion mesothelin levels of the MPM

Table 1 Characteristics of the MPM patients and non-malignant subjects.

		Cases (%)	Total
MPM			
Age	66.16 ± 10.05		
Sex	Male/female	31 (68.9)/14 (31.1)	45
Histology	Epithelioid	37 (82.2)	
	Sarcomatoid	5 (11.1)	
	Biphasic	3 (6.7)	
Stage	I/II/III/IV	8 (17.8)/6 (13.3)/10 (22.2)/21 (46.7)	
No-malignancy			
Age	70.79 ± 10.66		
Sex	Male/female	19 (79.2)/5 (20.8)	24
	Benign asbestos pleurisy	2 (20.0)	
	Tuberculous (Tb) pleurisy	7 (32.0)	
	Infectious (non-Tb) pleurisy	8 (32.0)	
	Empyema	2 (4.0)	
	Hepatic failure	2 (4.0)	
	Renal failure	3 (4.0)	
Lung cancer			
Age	69.00 ± 9.95		
Sex	Male/female	26 (89.7)/3 (10.3)	29
	Adenocarcinoma	18 (62.1)	
	Squamous cell	6 (20.7)	
	Small	5 (17.2)	

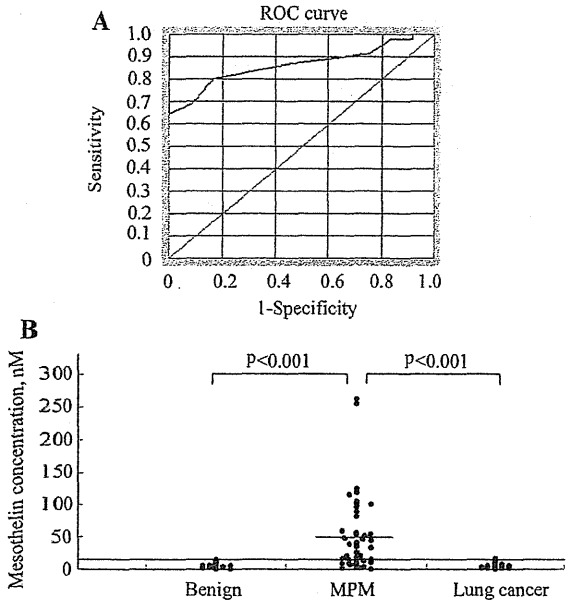

Figure 1 Pleural effusion mesothelin levels in patients with MPM and non-MPM subjects.
(A) An analysis that included 45 MPM and 24 non-malignant pleural effusion patients revealed an area under the curve (AUC) of 0.863 (95% CI: 0.792–0.935). At the optimal cut-off value of 10 nM, the diagnostic sensitivity was 80.0% and the specificity was 83.3%. (B) The pleural effusion mesothelin levels of the patients with MPM vs. those of the patients with non-malignant pleural effusion or lung cancer involving malignant pleural effusion were measured as described in Materials and methods.

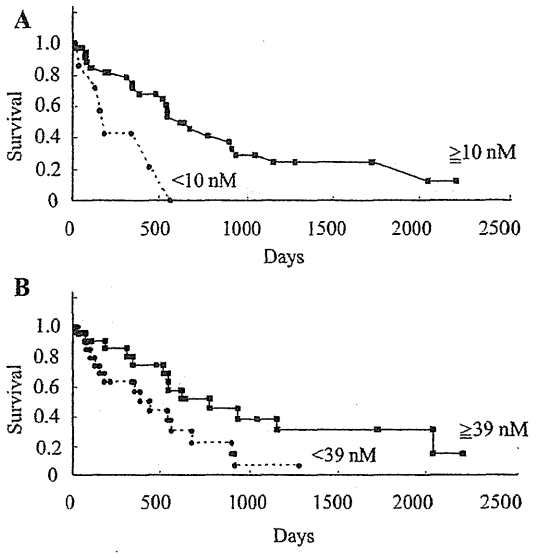

Figure 2 Survival of MPM subjects according to pleural effusions mesothelin levels.
Estimates of the probability of survival were calculated using the Kaplan-Meier method and compared using the log-rank test between the groups with higher and lower pleural effusion mesothelin levels than cut-off value (10 nM, A) or median value (39 nM, B).

histological groups (epithelioid: 56.2±61.8 nM, non-epithelioid: 33.9±27.9 nM), stages (stage I: 48.0±41.3 nM, stage II: 73.8±96.2 nM, stage III: 63.5±41.4 nM, stage IV: 42.4±57.5 nM) or sex (male: 45.9±53.3 nM, female: 66.4±66.1 nM) and there were no significant differences in the pleural effusion mesothelin levels between the subjects with benign asbestos pleurisy and those with benign pleurisy without a history of asbestos exposure (6.4±4.2 nM, 6.4±4.7 nM, respectively).

Relationship between pleural effusion mesothelin levels and overall survival

We were able to follow 45 MPM patients closely for 16–2200 days (mean 537 days). To study the relationship between the pleural effusion mesothelin levels and the patients' clinical courses, we separated the patients according to their pleural effusion mesothelin levels at the time of diagnosis. The first group included patients with pleural effusion mesothelin levels of lower than 10 nM (the cut-off value that we chose). In this group of nine patients, the mean mesothelin value was 4.76 nM (interquartile range: 1.80–7.25 nM). The other group included the remaining 36 patients with pleural effusion mesothelin levels of higher than 10 nM, whose mean mesothelin value of pleural effusions was 64.13 nM (interquartile range: 25.90–89.60 nM). The difference in overall survival between the groups with lower and higher pleural effusion mesothelin values than the assumed cut-off point of 10 nM was significant ($p=0.014$, Figure 2A). Next we examined the survival data for patients with pleural effusion mesothelin levels above the median level of the all 45 MPM patients (39.05 nM). The overall survival of the groups with lower pleural effusion mesothelin levels showed the tendency of poor prognosis than that with higher mesothelin levels ($p=0.053$, Figure 2B).

Cox's regression analysis was performed on 45 MPM patients for whom data on age (≥65 vs. <65 years), sex (male vs. female), histology (non-epithelioid vs. epithelioid), stages (I and II vs. III and IV), performance status (0 and 1 vs. 2, 3 and 4) and the pleural effusion mesothelin level (≥10 nM vs. <10 nM) were available. Independent statistically significant prognostic effects on survival were found for age (≥65 vs. <65 years; HR, 5.72; 95% CI, 1.54–21.23; $p=0.009$) and histology (non-epithelioid vs. epithelioid; HR, 5.47; 95% CI, 1.27–23.15; $p=0.022$).

Discussion

MPM is a malignant transformation caused by the exposure of mesothelial cells to asbestos, which shows a limited response to conventional chemotherapy and radiotherapy and its prognosis is very poor. It has been reported that patients with stage IA disease survive for 5 or more years after total resection of the tumor, whereas, in advanced cases, resection of the tumor only prolongs survival by approximately 3 months (13). However, there are no randomized trials demonstrating a clear survival benefit to surgery (14, 15). Due to the difficulty of the differential diagnosis of

MPM among individuals with pleural effusion by radiological, cytological and/or histological examinations, efficient and practical pleural effusion biomarkers are required to aid the diagnosis of MPM. Pleural effusion biomarkers for MPM, such as hyaluronic acid and CYFRA 21-1 have been reported and used to assist the diagnosis of MPM (16, 17).

Serum soluble mesothelin-related peptide (SMRP) has been demonstrated as a useful biological marker for MPM (18). Subsequently, some reports showed that serum SMRP is a useful marker for diagnosis of MPM (10, 11) or can be useful as a marker of treatment response and prognosis (14). However, its benefit in screening for MPM in asbestos-exposed individuals was hampered in a prospective study because of a low specificity and high number of false-positive values (19). Mesothelin is thought to play a role in cell-adhesion, cell-to-cell recognition and signaling; however, its biological functions in MPM cells have not been fully investigated and the clinical usefulness of pleural fluid mesothelin has not been fully defined, although pleural fluid mesothelin level has been demonstrated to provide additional diagnostic value relative to cytologic examination (20).

In this study, we evaluated the clinical role of mesothelin as a pleural effusion biomarker in MPM. We demonstrated that patients with MPM had significantly higher pleural effusion mesothelin levels than a population with non-malignant pleural effusion or lung cancer involving malignant pleural effusion, which was compatible with the previous reports (21, 22). It has been reported (20) that the pleural effusion mesothelin levels are reproducible and not affected by inflammatory pleural processes, also suggesting the usefulness of pleural effusion mesothelin as diagnostic value. It has been reported that there had been no significant difference between pleural effusion mesothelin levels and survival in MPM patients (22). On the other hand, in the present study, the patients with lower levels of pleural effusion mesothelin had a tendency of poor prognosis than those with higher mesothelin levels. The differences between our results and theirs might be explicable by the following data: first, the present study contained five (11.1%) patients with sarcomatoid type, known to have a worse prognosis, whereas their study contained nine (17.3%) sarcomatoid MPM patients; second, in the present study no statistically significant differences between the pleural effusion mesothelin levels of the epithelioid MPM and non-epithelioid MPM (56.2 ± 61.8 nM and 33.9 ± 27.9 nM, respectively) were observed, whereas sarcomatoid MPM patients in their study had significantly lower pleural effusion mesothelin levels. Also, there was no correlation of the pleural effusion mesothelin levels with MPM stages in our patients, which is compatible with the previous report demonstrating no significant differences in serum mesothelin levels between the individual stages (23). No statistically significant differences between the pleural effusion mesothelin levels of the MPM histological groups or clinical stages suggest that the pleural effusion mesothelin levels themselves could provide prognostic information.

Several factors, including transforming growth factor (TGF)-β_1, platelet derived growth factor (PDGF), TGF-α and interleukin (IL)-8, have been reported to be associated with MPM cells (24–29) via paracrine and/or autocrine mechanisms. Moreover, tumor development is closely related to angiogenesis. Malignant tumors require new blood vessel formation and it has been reported that increased vascularity in MPM is associated with a poor prognosis (2, 30). Vascular endothelial growth factor (VEGF) has been reported to be an autocrine growth factor of MPM (31, 32). Previously, we have demonstrated the usefulness of VEGF as a marker for both diagnosis and estimating prognosis of MPM (33, 34). Moreover, we also demonstrated the autocrine/paracrine mechanism of angiopoietin-1 in MPM cells in vitro and its clinical usefulness in both diagnosis and estimating prognosis in MPM patients (35). On the contrary, the biological effects of mesothelin on MPM cells are not fully investigated. So, it is unclear why the patients with lower levels of pleural effusion mesothelin had a tendency of poorer prognosis than those with higher mesothelin levels in the present study. Although one possible explanation is that anti-tumor immune responses are increased through over-expression of mesothelin (36) in patients with higher serum mesothelin levels, we consider that the prognostic impact of pleural effusion mesothelin levels needs further evaluation.

Conclusions

In summary, we demonstrated that patients with MPM had significantly higher pleural effusion mesothelin levels than a population with non-malignant pleuritis involving benign asbestos pleurisy or lung cancer involving malignant pleural effusion, suggesting mesothelin to be a useful diagnosing marker for MPM. Kaplan-Meier method revealed the tendency for the poor prognosis of the MPM patients with lower pleural effusion mesothelin levels, which needs further examination.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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