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Novel clinical role of angiopoietin-1 in malignant pleural mesothelioma

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ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor associated with asbestos exposure with limited response to conventional therapy, so diagnosing MPM early is very important. We have previously reported that angiopoietin (Ang)-1 was correlated with bleomycin-induced pulmonary fibrosis. Here, we investigated the association of Ang-1 with the development of MPM which originate from mesenchymal cells similar to lung fibroblasts, and demonstrated that Ang-1 stimulated the cell growth and migration of MPM cells in *in vitro* studies. We also demonstrated that patients with MPM had significantly higher serum levels of Ang-1 in comparison to a population who had been exposed to asbestos but had not developed MPM. The patients with advanced stage MPM showed higher levels of Ang-1 than the early stage MPM patients and the Kaplan-Meier method revealed a significant correlation between serum Ang-1 levels and survival. We propose the possibility that Ang-1 plays an important role in MPM tumor growth and our data suggest that the serum concentration of Ang-1 could be useful as prognostic factor.

The number of words: 168

Key words: cytokines, pleural disease, serum marker

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure (1-3). Although recently, asbestos usage has 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 recently, the multi-targeted anti-folate pemetrexed has 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 beneficial than late intervention. So, diagnosing MPM at an early stage is very important (1). However, diagnosis can often be very difficult in histological studies. In the diagnosis of lung cancer, serum markers such as CEA, CYFRA, proGRP, and SCC provide supportive roles to confirm the diagnosis. Serum biomarkers for MPM such as mesothelin and osteopontin (7, 8) have been reported and used to assist the diagnosis of MPM. For the further improvement of specificity and sensitivity of diagnosis, research into the development of novel biological markers is urgently required.

We have previously reported that angiopoietin (Ang)-1 was correlated with bleomycin-induced pulmonary fibrosis in mice (9). In the report, we demonstrated that the Ang-1 mRNA level was increased in bleomycin-treated mouse lung tissues compared to that in control tissues. Moreover, we found that human lung fibroblasts and myofibroblasts produced Ang-1, which might be the underlying mechanism of pulmonary fibrosis. MPM involves the malignant transformation of mesothelial cells, which originate from mesenchymal cells similar to lung fibroblasts. Here, we investigated the effect of Ang-1 on the cell growth and/or migration of MPM cells in *in vitro* studies, and the serum levels of Ang-1 in the patients with MPM in comparison to a population that had been exposed to asbestos without developing MPM.

METHODS

Cell culture

The human malignant pleural mesothelioma (MPM) cell lines H28 (epithelioid), H2052 (sarcomatoid), H2452 (biphasic), and MSTO-211H (biphasic) and the human mesothelial cell line MeT-5A were obtained from the American Type Culture Collection (Rockville, MD). These cells were cultured in RPMI 1640 (Sigma Chemical Co., St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum.

Quantitative real-time RT-PCR

Total RNA was isolated using the RNeasy Mini kit (QIAGEN, Valencia, CA) and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed as previously described (10-12) using TaqMan Gene expression products for human Tie-2, Ang-1, and Ang-2. 18SrRNA served as an endogenous control (Applied Biosystems).

Measurement of Ang-1

Concentrations of Ang-1 and/or Ang-2 of cell culture supernatant and serum were measured by an enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Oxford, UK). The mean minimum detectable dose for Ang-1 of this ELISA Kit is 3.45 pg/ml.

Measurement of total and phosphorylated Tie-2

Cells were cultured for ten minutes with or without recombinant human Ang-1 (100 ng/ml) (R&D Systems), and cytoplasmic extracts were prepared by Nuclear Extract Kit (Active Motif, Carlbad, CA), then total and phospho-Tie-2 were detected by ELISA Kit (R&D Systems). We measured the total protein content in the cytoplasmic extracts (Protein Quantification Kit-Rapid, DOJINDO, Kumamoto, Japan) and the values of total and phosphorylated Tie-2were corrected as proportion to the total protein content.

Cell proliferation assay

The cell proliferation assay was performed as previously described (9, 10, 12). The MPM cell lines and MeT-5A cells were cultured in 96-well flat-bottomed culture plates for 2 days with or without recombinant human Ang-1 (1–100 ng/ml) (R&D Systems).

Cell Migration Assay

In vitro migration assays were performed using the CytoSelect 24-well Cell Migration Assay (8μm, Colorimetric Format) (CELL BIOLABS, Huissen, Netherlands), according to the manufacturer's instructions as previously described (12). Briefly, MPM cell lines and MeT-5A cells were suspended at a density of 1 x 10^6 cells/ml in RPMI1640 and placed in the upper half of a Boyden chamber. The lower half of the Boyden chamber was filled with RPIM1640 containing 100 ng/ml recombinant human Ang-1 or

RPMI1640 alone. The cells were incubated for 16 hours, and non-migratory cells which stayed in the upper chamber were removed. The migratory cells were stained with Cell Stain Solution, and extracted by Extraction Solution, then measured using plate reader at the OD560 nm.

Animals

Six-week-old C.B-17/Icr-*scid* Jcl (*scid* /*scid*) (SCID) female mice were purchased from Clea Japan (Tokyo, Japan) and maintained in our specific pathogen-free animal facility. All animals were kept according to the Animal Protection Guidelines of Hyogo College of Medicine. All protocols for animal use and euthanasia were reviewed and approved by the Institute of Laboratory Animals, Graduate School of Medicine, Hyogo College of Medicine, Japan.

Ectopic (subcutaneous) xenograft model

To produce subcutaneous (s.c.) tumors, a single-cell suspension of 10^7 MSTO-211H cells was implanted s.c. into the back of SCID mice.

Immunohistochemistry

Mice were sacrificed at day 28 after the implantation, and the tumors were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin and sectioned. Sections were

immunostained by using rabbit anti-mouse Ang-1 polyclonal antibody (Fitzgerald, Concord, MA) followed by anti- rabbit polyclonal antibody (Dako Cytomation, Glostrup, Denmark) and, peroxidase activity was envisioned with diaminobenzidine kit (Dako Cytomation).

Patients

We studied the Ang-1 levels in the serum of 102 patients admitted for diagnosis to the Department of Respiratory Medicine of Hyogo College of Medicine Hospital from 2000 to 2007. All of the patients had a documented asbestos exposure history. Sixty-two individuals had malignant pleural mesothelioma, which was diagnosed using histopathological samples by pathologists skilled in the diagnosis of MPM. Forty individuals had benign asbestos-related diseases (asbestosis or pleural plaques) or were healthy despite their asbestos-exposure. All patients were classified by the staging system of the International Mesothelioma Interest Group (IMIG) (13). Samples from 11 patients with lung cancer with malignant pleuritis and 5 healthy volunteers were also studied. The study was approved by our ethics committee in accordance with the 1975 Declaration of Helsinki. Informed consent was obtained from all patients. Samples were immediately frozen in liquid nitrogen and stored at -80 degrees until use.

Statistical analysis

For the in vitro study, the results are given as the mean ± SD of three experiments performed in triplicate. Statistical analysis was performed using the Bonferroni/Dunn multiple comparisons test. The nonparametric Mann-Whitney U-test was used to compare two groups of serum samples. Comparisons of data between various groups of serum samples 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 value of serum Ang-1, receiver operating characteristic (ROC) curves, areas under the ROC curves (AUC), and their 95% confidence intervals (95% CI) were calculated using standard techniques. To examine the cut-off values of serum levels, we calculated the total sensitivity and specificity for each cut-off value and then chose the cut-off 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. In order to evaluate the Ang-1 prognostic significance on survival of patients with MPM, Cox's proportional hazards regression analysis (backward) was carried out as multivariate analysis.

RESULTS

Evaluation of Ang-1, Ang-2, and Tie-2 expression

We evaluated mRNA expression of Ang-1, Ang-2, and Tie-2 by real time RT-PCR analysis. As shown in Table 1, Ang-1 mRNA was expressed in H2052 and MSTO-211H cells. However, H28, H2452, and MeT-5A showed no Ang-1 mRNA expression. On the other hand, Tie-2 mRNA was expressed in H2452, MSTO-211H, and MeT-5A cells. No MPM or MeT-5A cells expressed Ang-2. The protein levels of Ang-1 and Ang-2 of the cells were analyzed by ELISA. H2052 and MSTO-211H cells were demonstrated to produce Ang-1 (2384.4±372.6 and 140.9±22.1 pg/ml, respectively), while H28, H2452, and MeT-5A showed no Ang-1 production. On the other hand, none of them produced Ang-2 (data not shown). We also examined total and phosphorylated Tie-2, and found that in MSTO-211H cells, phosphorylated Tie-2 was increased (1.2±0.036-fold increase, p=0.026), whereas total Tie-2 was not affected by the addition of Ang-1 (1.0±0.0028-fold increase). However, neither total nor phosphorylated Tie-2 changed by the addition of Ang-1 in H2452 (1.0±0.052-fold increase, 1.0±0.016-fold increase, respectively) and MeT-5A (1.0±0.0060-fold increase, 1.1±0.052-fold increase, p-o.o60, respectively) cells. Tie-2 was not detected in H28 and H2052 cells.

Effect of Ang-1 on the proliferation of MPM cells and MeT-5A cells

To clarify the involvement of Ang-1 in the development of MPM tumor growth, we studied the effect of Ang-1 on the proliferation of MPM cells, and the human mesothelial cell line MeT-5A, which expresses Tie-2 alone. As shown in Fig. 1A and 1B, the addition of Ang-1 stimulated MSTO-211H cell growth which express both Ang-1 and Tie-2, and H2452 which express only Tie-2, in a dose-dependent manner and reached a plateau at the concentration of 100 ng/ ml (1.3±0.045-fold increase [$p< 0.0001$] and 1.1±0.014-fold increase [$p< 0.0001$], respectively). The proliferation of H2052, which produced Ang-1 without Tie-2 expression, and H28 with neither Ang-1 production nor Tie-2 expression was not affected by the addition of Ang-1 (1.0±0.019-fold increase, 1.0±0.020-fold increase, respectively). On the other hand, Ang-1 had no effect on the proliferation of human mesothelial cell line MeT-5A although it showed Tie-2 expression (1.0±0.049-fold increase, Fig. 1C).

Effect of Ang-1 on the migration of MPM cells

It is well known that cell migration plays an important role in tumor cell invasion, especially in the spread of MPM tumors. We therefore performed an *in vitro* migration assay to study the effect of Ang-1 on MPM progression and revealed that in both MSTO-211H which express both Ang-1 and Tie-2, and H2452 which express only Tie-2, cell migration was induced (1.4 fold increase, $p=0.002$ and 1.3 fold increase, $p=0.002$,

respectively) by Ang-1 (Fig. 2A, 2B). The migration of H2052, which produced Ang-1 without Tie-2 expression, and H28 with neither Ang-1 production nor Tie-2 expression was not induced by the addition of Ang-1 (data not shown). Ang-1 had no effect on the migration of MeT-5A mesothelial cells, although it showed Tie-2 expression (Fig. 2C).

Immunohistochemical findings

We previously reported that only MSTO-211H cells could grow on the back of SCID mice when a single cell suspension of 10^7 H28, H2052, H2452, and MSTO-211H cells with a viability of >95% was implanted subcutaneously into the back of SCID mice (12). To examine the physiopatholigical roles of Ang-1 *in vivo*, tumor tissue from the ectopic xenograft model implanted with MSTO-211H cells was available for Ang-1 staining. Ang-1 was stained in cytoplasm of the tumor cells (Fig. 2E).

Serum levels of Ang-1 in patients with MPM, benign asbestos-related diseases (asbestosis or pleural plaques), and healthy individuals with a history of asbestos-exposure

We recruited a total of 102 subjects with a history of asbestos exposure. Of them, 62 had confirmed MPM, 25 had pleural plaque and/or asbestosis, and 15 had no asbestos-related lesions despite their exposure to asbestos; i.e., were healthy. Their characteristics are shown in Table 2.

ROC curves for serum Ang-1 levels showed that patients with MPM had an AUC of 0.7974 in comparison to those with benign asbestos-related diseases (asbestosis or pleural plaques) and those who were healthy despite their asbestos-exposure (95% CI, 0.7261-0.8687). At a cut-off value of 34.5 ng/ml, the diagnostic sensitivity was 71.0%, and the specificity was 77.5% (Fig. 3A). Positive predictive value (PPV) was 83.0 %, and negative predictive value (NPV) was 63.3 %. The level of Ang-1 in patients with MPM was significantly higher (44.4 ± 16.1 ng/ml) in comparison to those with benign asbestos-related diseases (asbestosis or pleural plaques) and those who were healthy (28.5 ± 10.7 ng/ml) ($p<0.0001$). On the other hand, serum levels of Ang-1 from the patients with lung cancer with malignant pleuritis (n=11) and found the lower Ang-1 levels (31.9 ± 9.1 ng/ml, $p=0.0088$) than MPM patients (Fig. 3B). Moreover, the scatter plots of the serum Ang-1 levels in MPM showed tendencies to increase as the stage went up (stage I: 35.7 ± 4.7 ng/ml, stage II: 36.1 ± 11.4 ng/ml, stage III: 40.2 ± 13.7 ng/ml, and stage IV: 49.3 ± 17.2 ng/ml) ($p=0.049$ by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney U-test, Fig. 3C). The differences in Ang-1 levels between the different MPM histological stages were not statistically significant (data not shown). There were no significant differences in Ang-1 levels among the subjects with benign

asbestos-related diseases (asbestosis or pleural plaques) and those that were healthy despite having a history of asbestos-exposure (data not shown).

Relationship between Ang-1 levels and overall survival

To study the relationship between serum Ang-1 levels and the patients' clinical courses, we separated the patients based on their serum Ang-1 levels at the time of the first measurement. The first group included patients with serum Ang-1 levels lower than 34.5 ng/ml, the cut-off value that we used. In this group of 13 patients, the mean serum Ang-1 value was 29.2±4.7 ng/ml. The other group included the remaining 37 patients with serum Ang-1 levels higher than34.5 ng/ml, whose mean serum Ang-1 value was 52.5±14.2 ng/ml. The difference in overall survival between the groups with lower and higher serum Ang-1 values than the assumed cut-off point of 34.5 ng/ml was significant (p=0.029, Fig. 4).

Cox's regression analysis was done on 50 MPM patients for whom data on age, sex, histology, stage, performance status, treatments (pemetrexed-based chemotherapy or surgery), and serum Ang-1 level were available. An independent statistically significant prognostic effect on the survival of age (65≥ versus < 65years; HR, 2.63; 95% CI, 1.04-6.68; p=0.042), pemetrexed-based chemotherapy (with versus without; HR, 0.38;

95% CI, 0.15-0.941; p=0.035), and serum Ang-1 level (34.5 ≥ versus < 34.5 ng/ml; HR, 3.43; 95% CI, 1.08-10.87; p=0.036) was found.

DISCUSSION

MPM is a malignant transformation caused by the exposure of mesothelial cells to asbestos, which has 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 (14). Due to the long latency period (typically longer than 30 years) between first asbestos exposure and the onset of the disease, the diagnosis of MPM remains difficult with 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 (13) IA disease survive for five or more years after total resection of the tumor (8). We have screened outpatients with a history of asbestos exposure but no symptoms for several years in order to detect MPM in its early stages. Due to the difficulty of the early diagnosis of MPM by radiological and/or histological examinations, efficient and practical serum biomarkers are required to aid the diagnosis of MPM. To date, there have been several reports about candidates for clinically useful markers for MPM (7, 8, 15, 16, 17). Indeed some of them have reported to be useful serum markers of MPM such as mesothelin and osteopontin (7, 8, 15), however, little is known about their biological functions or effects on MPM cells.

Angiopoietin-1(Ang-1) and Ang-2 are counteracting ligands for the endothelial specific receptor, tyrosine kinase Tie-2, and important regulators of blood vessel growth, maturation, and function. Ang-1 promotes angiogenesis, induces vascular maturation, and decreases vascular permeability. On the contrary, Ang-2 has the ability to destabilize blood vessels, enhance vascular leaking, and antagonizes Ang-1 (18-20).

We previously reported that Ang-1 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 we investigated the effects of Ang-1 on mesothelial cells.

First, we demonstrated the production of Ang-1 in 2 of 4 MPM cells, and expression of mRNA of its receptor, Tie-2, in 2 of them. The human mesothelial cell line Met-5A, which was used as a control, expressed Tie-2 mRNA, but not Ang-1 production. MSTO-211H cells expressed mRNA of both Ang-1 and its receptor. To clarify the involvement of Ang-1 in the development of MPM tumor growth, we studied the effect of Ang-1 on the proliferation of MPM cells and demonstrated that the addition of Ang-1 stimulated MPM cell growth in MSTO-211H and H2452 cells with Tie-2 expression in a dose-dependent manner. On the other hand, Ang-1 had no effect on the proliferation of MPM cell lines H2052 and H28, without Tie-2 expression, suggesting the functional

importance of Tie-2 in Ang-1-induced proliferation. Ang-1 had also no effect on the proliferation of MeT-5A, suggesting the absence functional expression of Tie-2 in normal mesothelial cells, although it might become functional when the cells are transformed. The present data suggest the proliferation of MPM cells is increased via the Ang-1 autocrine and/or paracrine mechanisms of MPM cells and Ang-1-producing surrounding cells, such as fibroblasts (9) and pericytes (19).

Cell migration plays an important role in tumor cell invasion, and we previously demonstrated the important role of MPM cell migration in tumor growth (9). We therefore performed an *in vitro* migration assay to study the effect of Ang-1 on MPM spreading and revealed that in MSTO-211H and H2452 cells, cell migration was induced by Ang-1. The migration of H2052 or H28, without Tie-2 expression, was not induced by the addition of Ang-1, suggesting the importance of Tie-2 in also Ang-1-induced migration. On the other hand, Ang-1 had no effect on the migration of MeT-5A mesothelial cells, suggesting the limited effect of Ang-1 on MPM cells. From these findings, one possible mechanism is that MPM cell spread is triggered by the Ang-1 produced by the MPM cells themselves and/or Ang-1-producing surrounding cells.

Moreover, it is well known that malignant tumors require new blood vessel formation, and it has been reported that increased vascularity in MPM is associated with a

poor prognosis (2, 23). Ang-1 is also an important regulator of blood vessel growth, maturation, and function. Ang-1 promotes angiogenesis, induces vascular maturation, and decreases vascular permeability (18-20).

Although the precise cellular mechanism has not been fully investigated, we propose the possibility that Ang-1 plays an important role in MPM tumor growth through stimulation of both proliferation and cell migration. Of course, as the main function of Ang-1 is the promotion of angiogenesis, we consider that in addition to its effects on the proliferation and migration of MPM cells, increased angiogenesis might be one of the mechanisms involved in Ang-1 induced growth of MPM tumors (21).

Next, we evaluated the clinical role of Ang-1 as a serum biomarker for MPM. In this study, we found that patients with MPM had significantly higher serum levels of Ang-1 in comparison to a population with a history of asbestos exposure, or healthy volunteers (n=5, age: 32.0 ± 8.0, male/female: 3/2) never exposed to asbestos (33.7 ± 4.3 ng/ml). Serum Ang-1 levels between a population with a history of asbestos exposure and healthy volunteers never exposed to asbestos were not statistically significant. Although the diagnostic sensitivity of Ang-1 for MPM from the ROC curve was not high (71.0%), PPV was fairly well (83.0 %), suggesting that the high serum Ang-1 levels might be supportive for the diagnosis of MPM. We also measured serum levels of Ang-1 from the

patients with lung cancer with malignant pleuritis and found the lower Ang-1 levels than MPM patients.

Moreover, the levels gradually increased according to the disease progression, although only stage IV MPM had potentially significant higher values of Ang 1, and the Kaplan-Meier method revealed a significant correlation between serum Ang-1 levels and survival. Furthermore, serum Ang-1 level was revealed to be a statistically significant prognostic effect on the survival by Cox's regression analysis, which suggested its usefulness as a marker to estimate prognosis. Since 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 the Ang-1 levels in serum seems an easy and useful tool of clinical management for MPM.

In summary, we demonstrated that Ang -1 stimulated the cell growth and migration of MPM cells in *in vitro* studies. We also demonstrated that patients with MPM had significantly higher serum levels of Ang-1 in comparison to a population with a history of asbestos exposure that did not develop MPM, and the patients with advanced stage MPM showed higher levels of Ang-1 compared to patients with early stage MPM. It

is suggested that Ang-1 would be a novel useful serum prognostic factor. This is the first report about the relationship between Ang-1 and MPM.

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FIGURE LEGENDS

FIGURE 1. Effect of Ang-1 on the proliferation of MPM and mesothelial cells

MSTO-211H (A), H2452 (B) MPM cells and the human mesothelial cell line MeT-5A (C) were cultured in 96-well flat-bottomed culture plates for 48 hours in serum-free medium with or without various concentrations (1, 10, and 100 ng/ml) of Ang-1 for 48 hours, and cell proliferation was assayed as described in Materials and Methods. The results are indicated as the mean ± SD of three separate experiments performed in triplicate. Statistical analysis was performed using the Bonferroni/Dunn multiple comparisons test.

FIGURE1

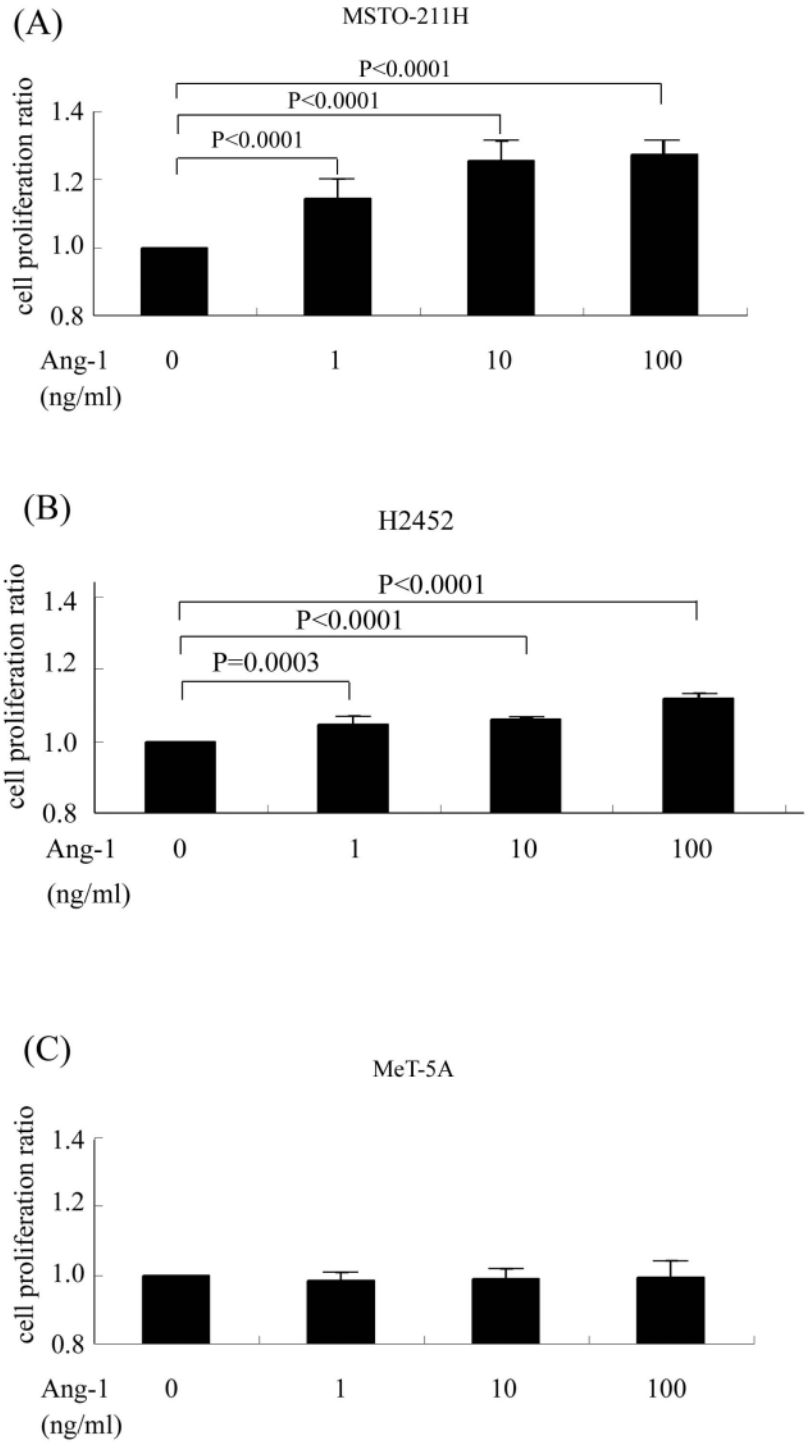

(A)
MSTO-211H
P<0.0001
P<0.0001
P<0.0001
cell proliferation ratio
1.4
1.2
1.0
0.8
Ang-1 (ng/ml)
0
1
10
100
(B)
H2452
P<0.0001
P<0.0001
P=0.0003
cell proliferation ratio
1.4
1.2
1.0
0.8
Ang-1 (ng/ml)
0
1
10
100
(C)
MeT-5A
cell proliferation ratio
1.4
1.2
1.0
0.8
Ang-1 (ng/ml)
0
1
10
100

FIGURE 2. Effect of Ang-1 on the migration of MPM and mesothelial cells

MSTO-211H (A), H2452 (B) MPM cells and the human mesothelial cell line MeT-5A (C) were cultured overnight in the presence or absence of 100 ng/ml of Ang-1, and a cell migration assay was performed as described in Materials and Methods. The results are indicated as the mean ± SD of three separate experiments performed in triplicate. Statistical analysis was performed using the Bonferroni/Dunn multiple comparisons test.

The tumors from SCID mice injected subcutaneously MSTO-211H cells were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin and sectioned. Sections were immunostained by using control (D) or rabbit anti-mouse Ang-1 (E) polyclonal antibody as described in Methods (original magnification x400).

FIGURE2

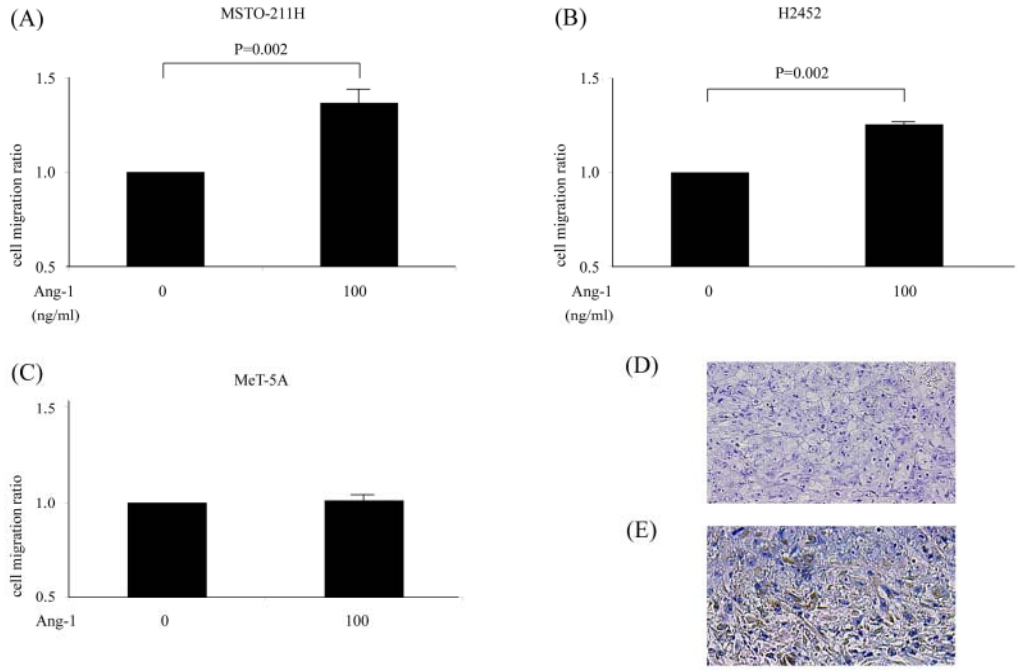


FIGURE 3. Serum Ang-1 levels in patients with MPM and non-MPM subjects

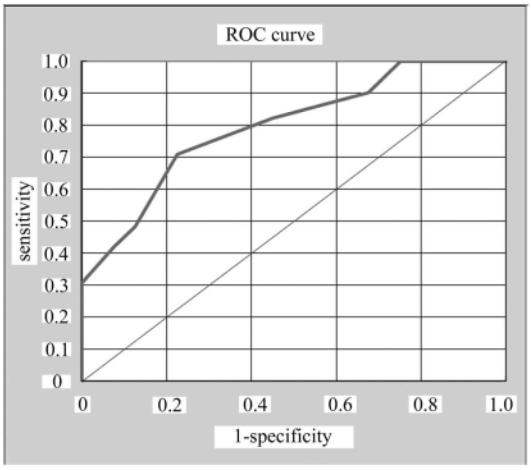
(A) Sensitivity and specificity of serum Ang-1 for distinguishing patients with MPM from non-MPM subjects (ROC curve). An analysis that included 62 MPM patients and 40 non-MPM subjects with a history of asbestos exposure revealed an AUC of 0.7974 (95% CI, 0.7261-0.8687). At a cut-off value of 34.5 ng/ml, the diagnostic sensitivity was 71.0%, and the specificity was 77.5%.

(B) Serum levels of Ang-1 in non-MPM subjects, MPM patients, and lung cancer with malignant pleuritis were measured as described in Materials and Methods.

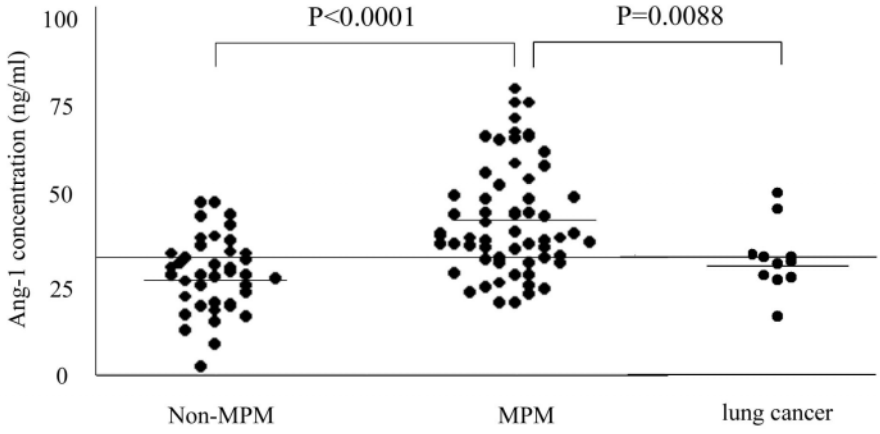
(C) Serum levels of Ang-1 in MPM patients divided into 4 stages are shown. The nonparametric Mann-Whitney U-test (B) or the nonparametric Kruskal-Wallis test followed by the Mann-Whitney U-test (C) was used. p-values < 0.05 were considered significant. The horizontal bars represent the mean value of each group. The cut-off value is shown as a horizontal line.

FIGURE3

(A)


ROC curve
sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1.0
1-specificity

(B)


P<0.0001
P=0.0088
Ang-1 concentration (ng/ml)
100
75
50
25
0
Non-MPM
MPM
lung cancer

(C)

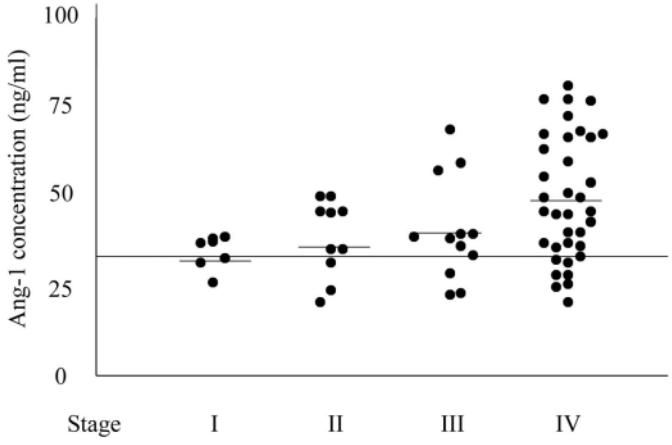

Ang-1 concentration (ng/ml)
100
75
50
25
0
Stage
I
II
III
IV

FIGURE 4. Survival of MPM subjects according to serum Ang-1 levels

Estimates of the probability of survival were calculated using the Kaplan-Meier method and compared using the log-rank test.

FIGURE4

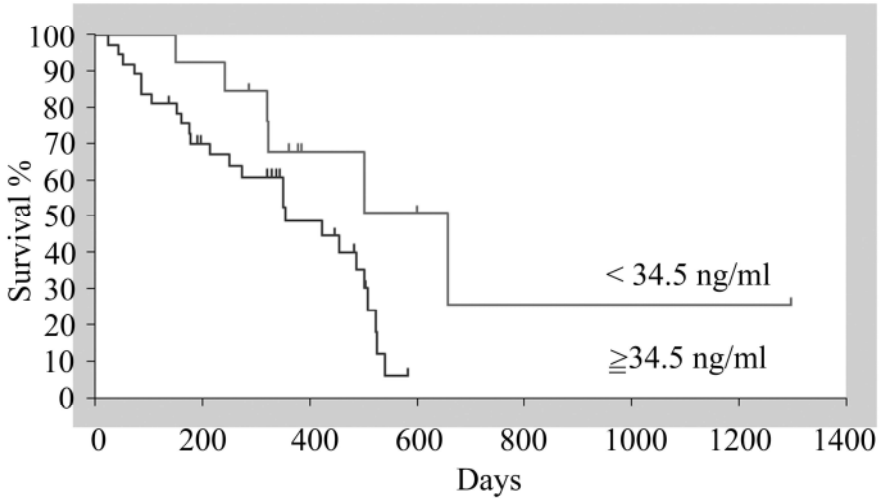


Table 1. Expression of Ang-1, Ang-2, and Tie-2 on MPM cells and MeT-5A

	H28	H2052	H2452	MSTO-211H	MeT-5A
Ang-1	–	+	–	+	–
Ang-2	–	–	–	–	–
Tie-2	–	–	+	+	+

Table 2. Characteristics of MPM patients and non-MPM subjects with Asbestos exposure

MPM*		cases	Total
Age	65.45±9.18		
Sex	Male / Female	46 / 16	62
Histology	Epithelioid	44	
	Sarcomatoid	8	
	Biphasic	5	
	Desmoplastic	5	
Stage	I / II / III / IV	6 / 7 / 13 / 36	
Non-MPM*			
Age	69.05±8.65		
Sex	Male / Female	33 / 7	40
CT findings	Plaque	20	
	Asbestosis	3	
	Plaque and asbestosis	2	
	None	15	

*All of the individuals were exposed to asbestos.