according to the modified RECIST criteria for MPM proposed by Byrne and Nowak [4]. Time to progression was defined as the period from the start of treatment to the date of disease progression or death, whichever occurred first. Overall survival was defined as the time from the initial visit until death from any cause. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (version 3).

Patient characteristics are summarized in Table 1. The patients were men aged 65, 60, 53, or 69 years. Tumor histology was epithelial in three patients and biphasic in one patient. All patients received four cycles of treatment with cisplatin and pemetrexed as the first-line chemotherapy. Patient 1 achieved a partial response (Fig. 1) and patient 2 achieved a complete response, with no evidence of disease progression for 6.4 and 11.4 months, respectively. Patients 3 and 4 had stable disease for a duration of 7.8 and 5.0 months, respectively.

At the time of this analysis, all four patients were no longer undergoing retreatment with cisplatin and pemetrexed. Patients 1 and 2 showed a partial response to retreatment, with a time to progression of 5.0 and 8.2 months, respectively (Fig. 1, Table 1). Patients 3 and 4 manifested progressive disease, with a time to progression of only 1.0 and 1.4 months, respectively. With the exception of hyponatremia of grade 3 observed in one patient (patient 1), no toxicities of grade 3 or 4 were apparent during retreatment.

Discussion

We have presented four patients who were retreated with the same chemotherapy regimen on progression of their MPM after initial treatment with cisplatin plus pemetrexed and durable tumor control. Two of the four patients achieved an objective response after four cycles of retreatment with acceptable toxicity.

For many types of malignant neoplasm, the standard treatment options for disease progression after first-line chemotherapy are chemotherapeutic regimens that differ from the initial treatment. However, in the case of MPM, no drugs have been approved for second-line treatment. We elected to retreat the present patients after disease recurrence with the same regimen as that used for the initial chemotherapy, given that all four individuals manifested disease control (one a partial response, one a complete response, and two stable disease) after the first-line treatment. Retreatment of ovarian cancer patients with the combination of carboplatin and paclitaxel is established for individuals who show sensitive relapse, defined as disease that responds to first-line chemotherapy but which relapses more than 6 months after the last dose of the first-line treatment [5]. A previous report presented four patients with relapsed MPM who achieved long-lasting tumor control with the combination of platinum and pemetrexed for retreatment [6]. The time to progression after initial platinum–pemetrexed chemotherapy was unusually long in these patients, ranging from 23 to 73 months. In the present report, the time to progression after the initial chemotherapy was 6.4 or 11.4 months for the two patients who achieved a second response, times that are substantially shorter than those in the previous study [6]. Our findings suggest that patients who show a time to progression of 6 months or more after initial chemotherapy with cisplatin plus pemetrexed may show a response on retreatment. The histological subtype of the two patients who responded to the retreatment was epithelioid histology, consistent with

Table 1 Patient characteristics and response to first-line chemotherapy and retreatment

	Patient 1	Patient 2	Patient 3	Patient 4
Age, years	65	60	53	69
Sex	Male	Male	Male	Male
Histology	Epithelial	Epithelial	Epithelial	Biphasic
Dose of first-line chemotherapy[a]	P500 + C60	P500 + C75	P500 + C75	P500 + C75
No. of cycles of first-line chemotherapy	4	4	4	4
Time to progression (months) after first-line chemotherapy	6.4	11.4	7.8	5.0
Response to first-line chemotherapy)	Partial response	Complete response	Stable disease	Stable disease
Dose of retreatment[a]	P500 + C60	P500 + C75	P500 + C75	P500 + C60
No. of cycles of retreatment	4	4	1	1
Time to progression (months) after retreatment	5.0	8.2	1.0	1.4
Response to retreatment	Partial response	Partial response	Progression disease	Progression disease
Overall survival[b] (months)	19.0	26+	10.0	9.3

[a] Doses for pemetrexed (P) and cisplatin (C) are given in milligrams per square meter (mg/m^2)

[b] Overall survival was defined as the time from the initial visit until death from any cause

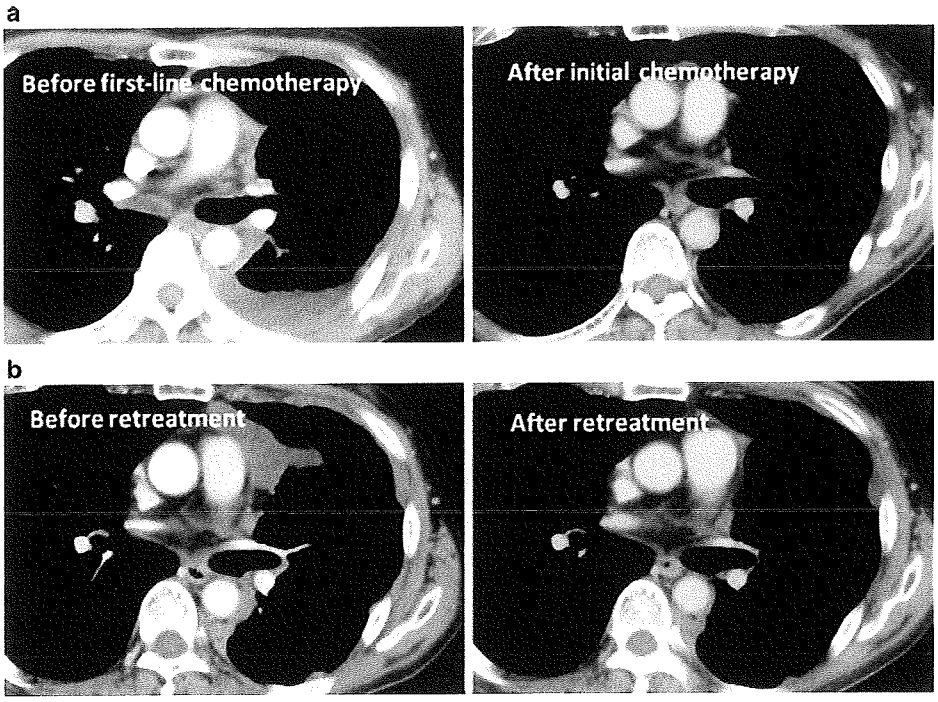

Fig. 1 Responses of patient 1 to first- and second-line chemotherapy with cisplatin plus pemetrexed. Computed tomography scans revealed that pleural nodules of patient 1 showed a partial response to first-line chemotherapy (**a**) and that a second response was obtained after retreatment with cisplatin plus pemetrexed (**b**)

the previous report [6]. These findings suggest that epithelioid histological subtype may also define a response on retreatment.

Our cohort included two patients who developed progressive disease with retreatment. These two patients did not develop an objective response to initial chemotherapy with cisplatin–pemetrexed, instead manifesting stable disease, whereas the two patients who achieved a response to retreatment showed a partial or complete response to first-line treatment. This observation suggests that failure to respond to initial chemotherapy may be a negative predictive factor for the effectiveness of retreatment.

The overall survival of the two patients who achieved a second response was 19 and more than 26 months, respectively, suggesting that successful retreatment with cisplatin plus pemetrexed can prolong survival time. Our observations thus suggest that retreatment with cisplatin plus pemetrexed may yield clinical benefits in patients who show a partial or complete response of long duration (>6 months) to the initial combination chemotherapy. Further studies are warranted to evaluate the efficacy of such second-line treatment and to clarify the criteria for selection of patients likely to respond to retreatment with cisplatin plus pemetrexed.

Conflict of interest statement I. Okamoto and K. Nakagawa received honoraria from Boehringer Ingelheim. The other authors have no conflict of interest.

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Efficacy and Safety of Pemetrexed in Combination with Cisplatin for Malignant Pleural Mesothelioma: A Phase I/II Study in Japanese Patients

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Background: Pemetrexed in combination with cisplatin (Pem/Cis) is used globally for the treatment of malignant pleural mesothelioma (MPM). This Phase I/II study was conducted to determine the recommended dose (RD) (Phase I) of Pem/Cis, and evaluate the efficacy and safety (Phase II) in Japanese MPM patients.
Methods: Key eligibility criteria were histologic diagnosis of MPM incurable by surgery, no prior chemotherapy, and a performance status 0–1. Under full vitamin supplementation, pemetrexed was intravenously administered on Day 1 of a 21-day cycle, followed by cisplatin. A cohort of six patients, starting from pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2 (Level 1), were studied in the dose-escalation Phase I (Step 1). The RD determined in Step 1 was carried forward into Phase II (Step 2). Planned number of patients treated with Pem/Cis was 18–38.
Results: In Step 1, 13 patients were enrolled: seven in Level 1 and six in Level −1 (pemetrexed 500 mg/m^2, cisplatin 60 mg/m^2). Two of six evaluable patients had dose-limiting toxicities (pneumonitis and neutropenia) in Level 1, establishing Level 1 as the RD. In Step 2, 12 patients were enrolled, for a total of 19 patients treated at the RD. Seven patients achieved a partial response among these patients, for a response rate of 36.8% (95% confidence interval: 16.3–61.6); overall survival was 7.3 months. One drug-related death occurred due to worsening of a pre-existing pneumonia. Common grade 3/4 toxicities were neutropenia and decreased-hemoglobin.
Conclusion: The Pem/Cis combination provides promising activity and an acceptable safety profile for chemonaive Japanese MPM patients with the same recommend dosage and schedule used in rest of the world.

Key words: cisplatin – mesothelioma – pemetrexed – phase I/II

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a tumor derived from the mesothelium covering the surface of pleural membranes or from undifferentiated mesenchymal cells in connective tissue under the membranes. MPM is a locally invasive and aggressive tumor with a poor prognosis and a median survival time (MST) of ≈9–16 months (1).

MPM is known to be linked to asbestos exposure, and the incidence of this tumor is expected to increase in the next 10–20 years according to an estimation of asbestos consumption in

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the world (9). Recently, the prevalence of MPM in Japan was widely recognized after uncovering the high incidence of MPM and MPM-related deaths in ex-workers of asbestos factories and in residents of the surrounding areas who may have been subject to non-occupational exposure to asbestos fibers.

Surgical resection offers local control of the tumor but its effect on survival remains unclear. In addition, application of radiation therapy is limited because of the diffuse extension of tumor spread. Regimens applied to lung cancer such as platinum-containing chemotherapy have been used for MPM in Japan; however, the efficacy outcomes of these therapies are not satisfactory. Therefore, effective systemic chemotherapy for MPM is clearly needed.

Pemetrexed is a novel antifolate (12) that inhibits three enzymes in folate metabolism: thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyltransferase (11). Because of the multi-targeted profile of this compound, broad and preferable anti-tumor activity is expected. Pemetrexed has shown clinical activity in various tumors including mesotheliomas (6). A pivotal multicenter, randomized Phase III study of pemetrexed (500 mg/m^2) in combination with cisplatin (75 mg/m^2) versus cisplatin alone (cisplatin 75 mg/m^2) in patients with MPM who had no prior chemotherapy was conducted in 20 countries (not including Japan) (16). A total of 448 patients were randomized and treated in this study (226 treated by pemetrexed/cisplatin (Pem/Cis) and 222 treated by cisplatin). MST in the Pem/Cis arm was 12.1 months compared with 9.3 months in the cisplatin arm ($P = 0.020$, two-sided log rank test). This was the first confirmation of significant prolongation of survival for patients with MPM. On the basis of this evidence, the combination of pemetrexed and cisplatin was approved for the treatment of MPM in the USA in 2004. Since then, the combination therapy has been approved in more than 80 countries and regions for the treatment of MPM, and recognized as a standard care for MPM (8).

In 2005, we initiated a Phase I/II study of Pem/Cis therapy in Japanese patients with MPM who had no prior chemotherapy. The primary objectives of this study were to determine the clinically recommended dose (RD) of Pem/Cis therapy in the Phase I portion of the study (Step 1), and to examine tumor response of the combination therapy in the Phase II portion (Step 2). The secondary objectives included time-to-event efficacy outcomes [the duration of response, progression free survival (PFS), and overall survival time], 1-year survival rate, quality of life (QOL) assessments, pulmonary function tests and safety.

PATIENTS AND METHODS

Patient selection

Chemonaive patients with histological diagnosis of MPM, regardless of clinical stage and who were not candidates for curative surgery, were assessed for eligibility. Eligible patients needed to be 20–74 years old with a life expectancy $\geq$12 weeks and an Eastern Cooperative Oncology Group performance status (PS) 0 or 1. Patients were also required to have adequate organ functions: bone marrow reserve [platelets $\geq 100 \times 10^3/mm^3$, hemoglobin $\geq$9.0 g/dl, and absolute neutrophil count (ANC) $\geq 2.0 \times 10^3/mm^3$], hepatic function [bilirubin $\leq$1.5 $\times$ upper limit of normal (ULN), aspartate/alanine transaminase (AST/ALT) $\leq$2.5 $\times$ ULN, and serum albumin $\geq$2.5 g/dl], renal function (serum creatinine $\leq$ULN, and calculated creatinine clearance $\geq$45 ml/min using the Cockcroft and Gault formula), lung function (functional oxygen saturation [SpO_2] $\geq$92%) and normal electrocardiogram.

Patients were excluded from this study for active infection, symptomatic brain metastasis, a wide-spread diffuse shadow in the lung caused by interstitial pneumonitis diagnosed by chest X-ray, pregnancy, serious concomitant systemic disorders incompatible with the study, clinically significant effusions, Common Terminology Criteria for Adverse Events (CTCAEs) v3 grade $\geq$2 peripheral neuropathy, the inability to discontinue aspirin and other non-steroidal anti-inflammatory agents or the inability or unwillingness to take folate and vitamin B_{12} during the study.

This study was conducted in compliance with the guidelines of good clinical practice and the Declaration of Helsinki, and it was approved by the local institutional review boards. All patients gave written informed consent before study entry. The Efficacy and Safety Evaluation Committee (ESEC), an independent body, was consulted if any efficacy and safety issues arose in the study.

Study Design

This was a Phase I/II, multicenter, single-arm, open-label study, performed in two steps. The RD level established in Step 1 was carried forward in Step 2. Patients enrolled in Step 1 at the RD level could continue in Step 2 unless otherwise indicated. The planned number of patients in total of Steps 1 and 2 treated with Pem/Cis was 18–38 for examination of efficacy and safety profile. In Step 1, six patients were to be enrolled in each dose level. The lower number of the planned number of patients, 18, was set as the minimum number of patients needed to confirm that the response rate of the study drugs was significantly larger than the threshold rate of 10% at one-sided significant level 0.05 with $\geq$80% power.

Study Treatment

Pemetrexed was intravenously administered as a 10-min infusion on Day 1 of a 21-day cycle, followed by cisplatin administration intravenously as a 2-h infusion 30 min after pemetrexed administration. Patients were instructed to take a daily 1 g multivitamin containing 500 μg of folate beginning 1 week prior to Day 1 of Cycle 1 until study discontinuation. Vitamin B_{12} (1000 μg) was intramuscularly injected, starting 1 week prior to Day 1 of Cycle 1 and repeated every 9 weeks until study discontinuation. Patients remained on study unless they were discontinued, for instance, due to disease progression and unacceptable adverse events.

Determination of RD for Step 2

In Step 1 (Phase I), four escalating dose levels were planned: pemetrexed at 500 (Level 1), 700 (Level 2), 900 (Level 3) and 1000 mg/m^2 (Level 4) with cisplatin held at 75 mg/m^2. In addition, a lower dose level (Level −1) was planned at pemetrexed 500 mg/m^2 and a lower dose of cisplatin 60 mg/m^2 for a failure case of dose-escalation in Level 1. In the dose-escalation procedure, the starting dose of pemetrexed was set to be 500 mg/m^2 which is ca. 40% of the maximum tolerated dose (MTD) of pemetrexed monotherapy with folic acid and vitamin B_{12} supplementation determined in a Japanese Phase I study; the MTD and RD of pemetrexed were determined to be 1200 and 1000 mg/m^2, respectively (7). The percentage of the starting dose to the MTD was based on a guideline for Phase I/II study on anticancer drugs (10). For escalation of pemetrexed dose, a modified Fibonacci dose-escalation method was used (2). Dose level reduction or escalation depended on the incidence of dose-limiting toxicity (DLT) at a given dose level (Fig. 1). If two of six patients at Levels 1, 2 or 3 developed DLT, that dose level was considered the RD for Step 2 (Phase II) of the study, and then Step 2 was initiated. This was also the case for Level −1 or 4 if 0–2 patients developed DLT. If three or more patients developed DLT at a given dose level (except dose Level −1), the next lower dose level was considered the RD level for Step 2. If three or more patients had DLT at Level −1, a decision was made as to whether the study should be continued.

A DLT was defined as a toxicity occurring in Cycle 1 meeting one of the following criteria: any grade ≥3 non-hematologic toxicity (except nausea, vomiting, anorexia and fatigue), grade ≥2 peripheral neuropathy or hearing loss/impairment, grade ≥3 febrile neutropenia (<1000/mm^3 with ≥38.5°C), grade 4 leukopenia (<1000/mm^3) or neutropenia (<500/mm^3) lasting ≥3 days, thrombocytopenia (<25000/mm^3), or thrombocytopenia requiring platelet transfusion. A failure to start the second cycle by Day 29 due to toxicity was also considered a DLT. All toxicities were assessed according to CTCAE.

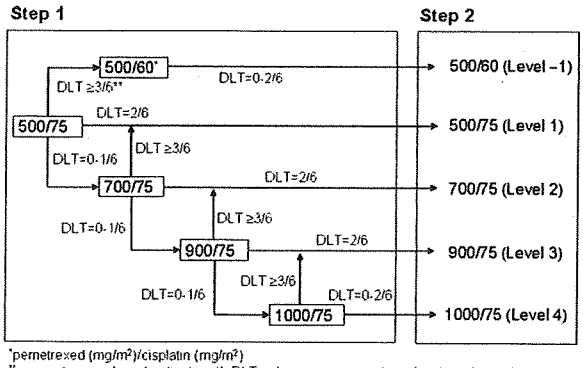

Figure 1. Scheme of dose-escalation Steps 1 and 2. DLT, dose-limiting toxicity.

Treatment Assessments

Anti-tumor Activity

Disease staging was assessed according to International Mesothelioma Interesting Group Tumor Node Metastasis (IMIG TNM) staging criteria (13). Within 28 days before the first treatment and approximately every 4 weeks after the first treatment, computer tomography or X-ray imaging of each lesion was performed. Tumor response was assessed using the modified Southwest Oncology Group (SWOG) criteria. Unidimensionally measurable lesions were defined as *Measurable disease*, and assessed objectively by the sum of the greatest diameters of them. Bidimensionally measurable lesions defined in the standard SWOG criteria (5) were assessed in the similar way. Best overall response selected from total overall response assessments was determined according to assessment of the Extramural Case Judgment Committee (E-CJC). Duration of response was measured as from the date of the first objective assessment of complete response (CR) or partial response (PR) until the date of the first assessment of progression of disease (PD). PFS was measured as from the registration date of Cycle 1 treatment until the first date of PD or death from any cause. Overall survival time was measured as from the registration date of Cycle 1 treatment until the date of death from any cause or until the last follow-up date in survival surveillance period.

QOL Assessments and Pulmonary Function Tests

QOL surveillance was employed using the following questionnaires: QOL questionnaire for cancer patients treated with anticancer drugs (QOL-ACD), and functional assessment of cancer therapy for lung cancer (FACT-L). These questionnaires were used on Day 1 of Cycles 1 and 2, and on 3 months after Day 1 of Cycle 1. QOL-ACD consists of four subscales (activity, physical condition, psychological condition and social relationships) and a total QOL scale (face scale) (4). The lung cancer subscale (LCS) score of FACT-L was used (3). As pulmonary function tests, forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1) and vital capacity (VC) were measured using a spirometer in the sitting position. All tests followed the Japanese Respiratory Function Test guidelines (14).

Safety

Adverse events were recorded throughout the study and after the last drug administration until signs of recovery were evident. Adverse events were evaluated according to treatment-emergent adverse events (TEAEs) definitions, and coded using the Medical Dictionary for Regulatory Activities (MedDRA v9.0). The severity (grade) of an adverse event was assessed according to CTCAE v3.

Statistical Analysis

The evaluation period of efficacy and safety in this study was defined as from the beginning of the study treatment to 5 months after the last patient began study treatment. For the

evaluations of overall survival time and 1-year survival rate, survival surveillance period was defined as from the beginning of the study treatment to 1 year after the last patient began study treatment. Patients who received the study drugs and complied with all inclusion/exclusion criteria were included in full analysis set (FAS). Patients who were treated with the RD level in Step 1 or 2 among FAS were included in efficacy analysis set for efficacy evaluation. Patients who received the study drugs at least once were included in safety analysis set for safety evaluation.

Assessment results of the best overall response by the E-CJC were used for efficacy analysis. Statistical tests based on binominal distribution were done to confirm that the response rate of the study drugs was significantly larger than the threshold rate of 10% at one-sided significant level 0.05. The threshold rate 10% was set on the basis of historical data on the response rate of cisplatin alone arm reported in other studies (15,16).

RESULTS

Patient Characteristics

From 2005 to 2006, a total of 25 Japanese patients with MPM were enrolled in Steps 1 and 2 at seven centers in Japan. All patients met the eligibility criteria and received study treatment; all were included in FAS. One patient was still receiving the study drug at the time of the efficacy and safety evaluations in this report.

Patient characteristics are summarized in Table 1. The majority of patients were male (22 patients, 88.0%). The median age was 61 years (range: 50–74 years). Most patients had a PS of 1 (18 patients, 72.0%) and clinical stage IV (21 patients, 84.0%). The predominant histologic subtype was epithelial in 64% of patients. Two demographic characteristics showed differences among dose levels. There were more patients with PS 0 in Level −1 (50.0%) than in Level 1 (21.1%). All six (100%) patients in Level −1 had the epithelial subtype versus 10 (52.6%) patients in Level 1.

Dose-Escalation, Dose-Limiting Toxicity and RD

One patient in Level 1 of Step 1 died on Day 14 of Cycle 1 due to exacerbation of pneumonia, respiratory failure (hypoxia) and disseminated intravascular coagulation (DIC). The ESEC evaluated the case of the early death. Since the patient had had the shadow of the lung detected by radiographic image prior to receiving study treatment, it was unlikely that the administration of pemetrexed was the primary cause of the pneumonia. The autopsy of this patient showed that interstitial changes in the lung were mild and the pathological diagnosis was an organizing pneumonia. The result of the autopsy was compatible with the clinical course and suggested that the direct cause of the death was not the drug-induced interstitial pneumonia but the exacerbation of infectious pneumonia, worsened by the study treatment. The case, therefore, was considered not appropriate for the DLT evaluation.

Table 1. Patient characteristics

	Step 1 Level −1 ($n = 6$)	Level 1 ($n = 19$)	All treated ($n = 25$)
Gender			
Male	5	17	22
Female	1	2	3
Age			
Mean	61	61	61
SD	3.9	6.3	5.8
Med	61	59	61
Weight(kg)			
Mean	62.8	58.1	59.2
SD	8.51	11.19	10.65
Performance status prior to Cycle 1			
0	3	4	7
1	3	15	18
Histological subtype			
Epithelioid mesothelioma	6	10	16
Sarcomatoid mesothelioma	0	5	5
Biphasic mesothelioma	0	4	4
Other	0	0	0
Asbestos exposure			
Had no exposure	2	3	5
Had exposure	4	16	20
Stage of disease			
Ia	0	0	0
Ib	0	1	1
II	0	1	1
III	1	1	2
IV	5	16	21

Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2
Level −1: pemetrexed 500 mg/m^2 + cisplatin 60 mg/m^2
SD, standard deviation.

One patient was added in this dose level to assess the safety profile additionally. Among the six patients in Level 1 excluding the case inappropriate for the DLT evaluation, two patients showed DLTs: drug-induced pneumonitis in one patient and dose delay of Cycle 2 initiation due to decreased neutrophil count in the other. According to the protocol definition, Level 1 was determined to be an RD for the next phase (Fig. 1).

The ESEC, however, recommended examining the treatment at Level −1 (pemetrexed 500 mg/m^2 and cisplatin 60 mg/m^2) exploratively to accumulate more safety information. Accordingly, six patients were enrolled and treated at Level −1, and no DLTs were observed in this dose level.

Evaluating the data of these two levels together, the ESEC agreed to continue Step 2 carefully with the dose of Level 1. The sponsor decided to carry forward into Step 2 with

an RD of Level 1 (pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2). In Step 2, 12 patients were treated at Level 1.

Efficacy

Nineteen patients (7 in Step 1 and 12 in Step 2) in Level 1 were included in the efficacy analysis set and of 19 patients, seven patients had PR, five patients had stable disease (SD), six patients had PD and one patient was classified as not evaluated. An overall response rate (ORR) was 36.8% [95% confidence interval (CI): 16.3%–61.6%]. The 95% one-sided confidence lower limit was 18.8%, exceeding the threshold level of 10%. The six patients in Level −1 had PR; thus, the ORR for all 25 patients treated with the study drug reached 52.0% (13 total PR, 95% CI: 31.3%–72.2%).

The secondary efficacy variables were time-to-event outcomes (the duration of response, PFS and overall survival time), 1-year survival rate, QOL and pulmonary function test. The median duration of response was 5.2 months (95% CI: 4.3–7.3 months) for the seven responders in the efficacy analysis set (Table 2). The median duration of response for the six responders at Level −1 was again 5.2 months. For the efficacy analysis set, median PFS was 4.7 months (95% CI: 1.3–6.5 months) and MST was 7.3 months (95% CI: 4.6–14.2 months, Fig. 2) with 1-year survival rate of 36.8% (95% CI: 15.2%–58.5%). Median PFS for the six patients at Level −1 was 10.1 months. MST at Level −1 could not be calculated by Kaplan–Meier method. The 1-year survival rate of Level −1 (66.7%) was beyond 50%.

The QOL-ACD and FACT-L measures were used for QOL evaluation. There were no major changes from prior to Cycle 1 to 3 months after Cycle 1 treatment in the mean scores for the activity and physical condition subscales of QOL-ACD (Table 3); however, mean scores from prior to Cycle 1 to 3 months after Cycle 1 treatment for the psychological condition and social relationships subscales numerically increased. The mean LCS score of FACT-L did not change substantially from prior to Cycle 1 to 3 months after Cycle 1 treatment (data not shown). These score changes indicate that QOL of the patients was maintained without worsening from baseline. Pulmonary function was also maintained with no worsening from baseline observed in the pulmonary function tests (FEV_1, FVC and VC) in the efficacy analysis set (data not shown).

Safety

Of 25 patients of the safety analysis set, three died during the study period: one (Level 1, Step 1) from exacerbation of pneumonia as a pre-existing complication, respiratory failure, and DIC, as described earlier, and the other two (Step 2) due to study disease. Two patients experienced non-fatal serious adverse events (fever and aspiration pneumonia, respectively). A causal relationship between fever and the study drugs could not be ruled out, but the aspiration pneumonia was not considered related to study drugs. Adverse events leading to discontinuation from study treatment were observed in six patients: one patient at Level 1 and three patients at Level −1 in Step 1 and in two patients in Step 2. Adverse event leading to discontinuation in two or more patients was increased blood creatinine (two patients).

Table 2. Summary of time-to-event outcomes and 1-year survival rates

	Step 1 Level −1 ($n = 6$)	Level 1 ($n = 19$)	All treated ($n = 25$)
Duration of response (months)			
Responders	6	7	13
Med	5.2	5.2	5.2
(95% CI)	3.1 – *	4.3–7.3	4.3–7.3
Range	2.7–9.6	2.0–7.3	2.0–9.6
Censored (%)	50	14.3	30.8
Progression free survival (months)			
Med	10.1	4.7	4.8
(95% CI)	4.3 – *	1.3–6.5	2.5–7.1
Range	3.3–12.1	0.5–9.6	0.5–12.1
Censored (%)	50	10.5	20
Overall survival (months)			
Med	NA	7.3	9.2
(95% CI)	11.1 – *	4.6–14.2	5.8–14.4
Range	8.6–19.3	0.5–21.5	0.5–21.5
Censored (%)	66.7	21.1	32
1-year survival rate (%)			
	66.7	36.8	44.0
(95% CI)	28.9–100.0	15.2–58.5	24.5–63.5

*Not calculated. NA, not assessed.
Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2.
Level −1: pemetrexed 500 mg/m^2 + cisplatin 60 mg/m^2.
CI, confidence interval.

Grade 3 or more laboratory TEAEs were observed in 16 patients: four patients at Level 1 and five patients at Level −1 in Step 1 and in seven patients in Step 2. Laboratory TEAEs observed in at least half of the 25 patients were decreased-hemoglobin, decreased red blood cell count, decreased neutrophil count, decreased white blood cell count, decreased lymphocyte count, increased blood urea and decreased body weight (Table 4). Grade 3 or more non-laboratory TEAEs were observed in eight patients: three patients at Level 1 and one patient at Level −1 in Step 1 and in four patients in Step 2. Non-laboratory TEAEs observed in at least half of the 25 patients were nausea, anorexia, vomiting and malaise. No major differences between Levels 1 and −1 (Step 1) in the incidence of TEAEs were noted.

For the 19 patients at Level 1, laboratory TEAEs of grade 3 or higher, possibly related to drug, and observed in at least two patients were decreased neutrophil count (seven patients, 36.8%), decreased hemoglobin (six patients, 31.6%), decreased white blood cell count (five patients, 26.3%), decreased lymphocyte count (five patients, 26.3%),

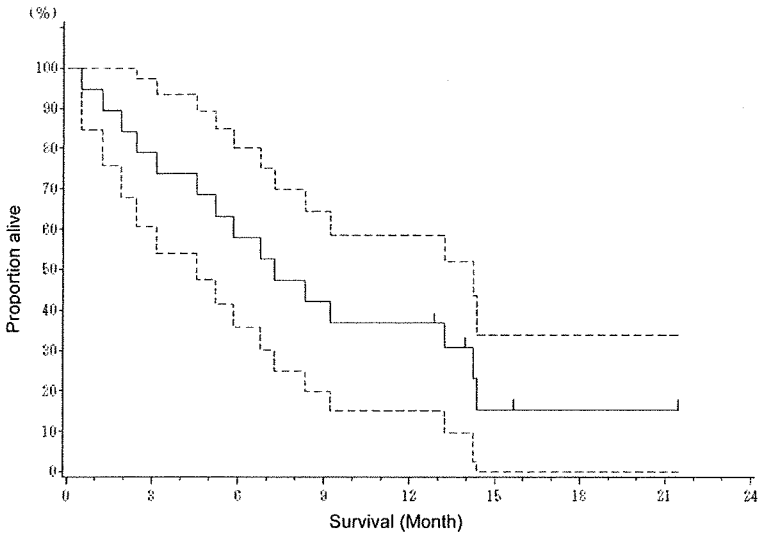

Figure 2. Kaplan–Meier plot of overall survival in the efficacy analysis set. Solid lines, overall survival; dotted lines, high and low limits of 95% confidence interval.

decreased platelet count (two patients, 10.5%) and decreased blood potassium (two patients, 10.5%). Non-laboratory adverse drug reactions of grade 3 or higher observed in at least two patients were vomiting (three patients, 15.8%), anorexia (three patients, 15.8%), nausea (two patients, 10.5%) and malaise (two patients, 10.5%). Adverse drug reactions of grade 3 or higher for the six patients in Level −1 were decreased neutrophil count (three patients), decreased-hemoglobin (two patients), decreased lymphocyte count (two patients) and decreased red blood cell count (one patient).

DISCUSSION

This Phase I/II study reports the first experience of the combination of pemetrexed and cisplatin therapy in Japanese patients. The RD of Pem/Cis combination therapy was established at pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2, with pemetrexed administration on Day 1 of each 21-day cycle followed by cisplatin, which is the same regimen used in worldwide for patients with MPM (16).

Of the 19 patients evaluable for efficacy at the RD level, there were PRs in seven patients, for an ORR of 36.8% (95% CI: 16.3%–61.6%). A pivotal Phase III study of the same regimen as that applied of the present study, yielded a response rate of 41.3% (95% CI: 34.8%–48.1%) in 225 patients (16). The response rates from both studies are comparable despite of the large difference in sample size.

The response rate of all the 25 treated patients was higher than the response rate for the 19 patients treated at the RD (52.0% versus 36.8%). This is due to the fact that all the six patients in Level −1 had PR. The excellent outcome observed in Level −1 may be attributed to differences between those patients who received the RD and those patients in Level −1 in the histological subtype of mesothelioma. All six patients in Level −1 had an epithelial subtype, which is known as a favorable prognostic factor, while only about half of the 19 patients at the RD had this subtype. In addition, the PS of the patients in Level −1 was better than the patients at RD.

A secondary efficacy endpoint MST showed 7.3 months in this study, shorter than that of the Pem/Cis arm in the Phase III study (12.1 months) (16). Although it would be difficult to compare MST of this study derived from a small sample size with the large Phase III study ($n = 226$), the discrepancy of survival between the two studies could be ascribed for the demographic characteristics of patients in both. There are less patients who had good prognostic factors in this study than in the Pem/Cis arm of the Phase III study: epithelial subtype: 52.6% versus 68.1%, a good PS: 21.1% (PS = 0) versus 51.8% (Karnofsky PS = 90/100) and clinical stage I/II: 8.0% versus 22.6% (16).

In this study, the most common adverse events (>50% of patients) were decreased-hemoglobin, erythropenia, neutropenia, leukopenia and lymphopenia for laboratory parameters, and nausea, anorexia, and vomiting for non-laboratory parameters. These hematologic and gastrointestinal events were similarly observed in the Pem/Cis arm of the pivotal Phase III study (16). No grade 3/4 febrile neutropenia toxicity which is a potentially life-threatening event was reported in our study. One death by pneumonitis was observed in this study; however, the patient was considered to have a pre-existing condition before initial treatment with study therapy. Adverse events observed in this study were predictable from safety profile observed in overseas trials and market experiences of pemetrexed and cisplatin combination therapy.

Table 3. Summary of QOL questionnaire for cancer patients treated with anticancer drugs (Level 1, $n = 19$)

Subscale	Measurement Point	*n*	Mean	SD	Min	Med	Max
Activity							
	Prior to Cycle1	19	62.9	25.35	20.0	60.0	100.0
	Prior to Cycle2	15	61.8	32.27	5.0	70.0	100.0
	Prior to Cycle3	14	69.6	21.79	20.0	75.0	95.0
	Cycle1 + 3M	11	60.5	32.13	5.0	70.0	100.0
Physical							
	Prior to Cycle1	19	64.7	22.33	15.0	70.0	100.0
	Prior to Cycle2	15	64.3	18.11	20.0	65.0	95.0
	Prior to Cycle3	14	66.2	18.33	30.0	70.0	85.0
	Cycle1 + 3M	11	61.4	21.46	35.0	60.0	95.0
Psychological							
	Prior to Cycle1	19	53.2	20.62	12.5	56.3	81.3
	Prior to Cycle2	15	59.6	24.87	12.5	62.5	100.0
	Prior to Cycle3	14	58.0	17.41	31.3	56.3	87.5
	Cycle1 + 3M	11	61.4	18.07	37.5	68.8	87.5
Social							
	Prior to Cycle1	19	32.9	21.56	5.0	25.0	75.0
	Prior to Cycle2	15	33.7	19.13	0.0	25.0	70.0
	Prior to Cycle3	14	43.6	19.94	10.0	42.5	85.0
	Cycle1 + 3M	11	36.4	22.59	10.0	30.0	85.0
Face scale							
	Prior to Cycle1	19	50.0	23.57	0.0	50.0	100.0
	Prior to Cycle2	14	55.4	24.37	0.0	50.0	100.0
	Prior to Cycle3	14	64.3	23.44	25.0	50.0	100.0
	Cycle1 + 3M	11	63.6	20.50	25.0	75.0	100.0

Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2. M, months.
QOL, quality of life.

CONCLUSION

The RDs for the Pem/Cis combination are pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2, which is the same regimen used in worldwide for patients with MPM. The combination shows promising efficacy with an acceptable safety profile in Japanese patients with MPM.

On January 2007, Pem/Cis combination therapy was approved and launched for the treatment of patients with MPM in Japan. Intensive post-marketing surveillance in patients with MPM is ongoing.

Funding

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Conflict of interest statement

S.A. and Y.N. are employed by the sponsor, Eli Lilly Japan K.K.; N.S. and M.F. are paid consultants to the sponsor.

Table 4. Summary of treatment-emergent adverse events (TEAEs) reported >25% patients

System organ class preferred term	Step 1 Level −1 ($n = 6$)	Level 1 ($n = 19$)	All treated ($n = 25$)
Patients with ≥1 TEAEs	6	19	25
Laboratory			
Hemoglobin decreased	6	18	24
Red blood cell count decreased	6	16	22
Neutrophil count decreased	5	16	21
White blood cell count decreased	5	15	20
Lymphocyte count decreased	5	12	17
Blood urea increased	5	11	16
Weight decreased	3	12	15
Blood albumin decreased	2	10	12
Platelet count decreased	4	8	12
Protein total decreased	3	9	12
Blood creatinine increased	4	7	11
Neutrophil count increased	2	8	10
White blood cell count increased	2	8	10
Blood sodium decreased	2	7	9
Alanine aminotransferase increased	1	7	8
Protein urine present	1	7	8
Aspartate aminotransferase increased	1	6	7
Blood magnesium decreased	2	5	7
Blood potassium decreased	0	7	7
Non-laboratory			
Nausea	6	18	24
Anorexia	6	16	22
Vomiting	3	15	18
Malaise	5	10	15
Constipation	3	9	12
Hiccups	3	5	8
Rash	2	6	8
Diarrhoea	1	6	7
Oedema	2	5	7
Pyrexia	2	5	7
Dysgeusia	3	4	7
Headache	1	6	7

Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2
Level −1: pemetrexed 500 mg/m^2 + cisplatin 60 mg/m^2
MedDRA Ver 9.0.

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Osteopontin Modulates Malignant Pleural Mesothelioma Cell Functions *In Vitro*

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Abstract. *Background: Although serum osteopontin (OPN) concentration is elevated in patients with malignant pleural mesothelioma (MPM), the role of OPN in the pathogenesis and development of MPM remains unknown. Materials and Methods: To determine the roles of OPN in MPM, immunohistochemical staining was performed to investigate the concentration of OPN in the pleural tumor of patients with mesothelioma; cell adhesion, proliferation and migration assays of H28 cells, an MPM cell line, were also carried out in vitro. Results: H28 cells cultured on OPN-coated plates revealed enhanced adhesion, proliferation, migration, cell survival and phosphorylated focal adhesion kinase activities. As expected, these enhancements were markedly suppressed with the addition of anti-αvβ3 antibody or arginine-glycine-aspartic acid serine (RGDS) peptide to the medium. Conclusion: OPN is speculated to play an important role in the enhancement of adhesion, proliferation and migration activities of H28 cells, presumably by interacting with the αvβ3 integrin.*

Malignant pleural mesothelioma (MPM) is a highly invasive tumor and resistant to conventional treatment modalities including chemotherapy, surgery and radiation (1, 2). In spite of recent advancements and developments in chemotherapy, the prognosis of patients with advanced MPM still remains poor: median survival for the epithelial type of mesothelioma is approximately 10 to 17 months, and 4 to 7 months for the sarcomatoid type (3). This dismal outcome of patients with mesothelioma may be attributable to the fact that its pathogenesis has not yet been elucidated. The progression of MPM is characterized by local tumor invasion, which consists of a multi-step process: migration, adhesion and proliferation. Interestingly, distant metastasis is very rare. Local invasion of MPM is dependent on interactions with the extracellular matrix (ECM) proteins that regulate tumor cell survival, invasion, angiogenesis and metastasis.

One of the ECM proteins, osteopontin (OPN), is a phosphoprotein that binds to the arginine-glycine-aspartic acid (RGD) complex in the central region of αv integrin, such as αvβ1, αvβ3, and αvβ5, and exerts cell adhesion, migration and cell growth activities (4-11). OPN is a tumor associated, secreted phosphoprotein that has been implicated in progression and metastasis of various types of cancer (9, 11, 12). In fact, breast cancer cells, in which OPN was knocked down with siRNA, revealed significantly lower invasion, proliferation and migration activities in Boyden chamber assays (13). Furthermore, it has, been reported that OPN influences myeloma cell survival by increasing proliferation and inhibiting apoptosis (14-16). Recently, it has been reported that measurement of serum OPN concentrations of patients exposed to asbestos and suspected of MPM are useful for early diagnosis of MPM (17, 18). However, there has been no sequential examination to confirm these results and its specificity. Moreover, the role of OPN in the pathogenesis of MPM has not yet been clarified.

Therefore, the aim of this study was to determine whether the interaction of an MPM cell line with OPN regulated mesothelioma cell functions.

Materials and Methods

Cell culture. Human mesothelioma cell lines, H28 (sarcomatoid), H2452 (epithelial), MSTO-211H (biphasic) and normal mesothelial cell, Met5A, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in RPMI-1640 (Kohjin Bio, Japan) containing 10% (v/v) fetal calf

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Key Words: Osteopontin, malignant pleural mesothelioma, αvβ3 integrin.

serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 atmosphere. For *in vitro* cell proliferation experiments, cells were grown in SITA (RPMI-1640 supplemented with 30 nM selenium, 5 μg/ml insulin, 10 μg/ml transferrin and 0.25% (w/v) bovine serum albumin (BSA). Cells were routinely tested for Mycoplasma contamination with MycoAlert Mycoplasma Detection Kit (Cambrex, Rockland, ME, USA), and were confirmed not to be contaminated.

Reagents. Anti-human monoclonal antibodies, including integrin αv (13C2), β3 (PM6/13) and αvβ3 (LM609), were purchased from Chemicon International (Temecula, CA, USA). Recombinant human OPN was purchased from R&D (Minneapolis, MN, USA). Poly-L-lysine solution (PLL) and hyaluronic acid (HA) were purchased from Sigma (St Louis, MO, USA).

To evaluate cell viability, the Cell Counting Kit-8 with WST-8 (2-(2-methoxy-4 nitrophenyl)-3-(4-nitrophenyl) 5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) was used (Dojindo, Kumamoto, Japan). The anti-focal adhesion kinase (FAK) polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The anti-phosphotyrosine py-69 antibody was purchased from BD Transduction Laboratories (Tokyo, Japan).

Immunohistochemical staining. The expression of OPN in the lungs of patients with mesothelioma was assessed with immuno-histochemical staining using OPN epitope-specific rabbit antibody (Spring-BioScience, Fremont CA, USA). Immunohistochemical analyses were performed as described elsewhere (19). Paraffin-embedded tumor specimens from 6 patients (epithelial type in three, desmoplastic type in two, sarcomatoid type in one) with MPM were obtained by surgical resection at Juntendo University Hospital. Briefly, sections were treated by autoclaving for 15 min at 120°C in 10 mM citrate buffer, pH 6.0, to retrieve the antigen. The sections were then incubated overnight with OPN epitope-specific rabbit antibody diluted to 1:50 at 4°C. Specific binding was detected through avidin-biotin peroxidase complex formation with a biotin-conjugated goat anti-rabbit immunoglobulin (Ig) G (Vectastatin ABC kit; Vector, Burlingame, CA, USA) and diaminobendizine (Sigma) as substrate. Staining was absent when isotype-matched immunoglobulin was used as the control. The protocol was approved by the Committee for Medical Ethics of Juntendo University, School of Medicine, and informed consent was obtained from all participants enrolled in this study.

RNA Isolation, cDNA synthesis, primers, and reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of OPN mRNA was assessed with RT-PCR. Total RNA was isolated from cultured cell lines with TRIzol reagent (Invitrogen, San Diego, CA). The primers for RT-PCR were generated by Invitrogen: OPN sense primer (5'-GTGATTTGCTTTTGCCTCCTA-3'), OPN anti-sense primer (5'-TCCTTACTTTTGGGGTCTACA-3'), β-actin sense primer (5'-GGCGGCAACACCATGTACCCT-3'), β-actin anti-sense primer (AGGGGCCGGACTCGTCATACT). RT-PCR was conducted using a Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, NJ, USA) according to the manufacturer's instructions.

Flow cytometric analysis. The adherent cells were detached from plates with 0.05% EDTA in phosphorylate-buffered saline (PBS), washed with PBS, and then incubated with anti-human αv integrin antibody (CD51), anti-human integrin β3 antibody (CD61) or αvβ3 antibody (LM609) in 1% FCS/PBS at 4°C for 30 min. After washing, the cells were incubated with fluorescein-labeled anti-mouse IgG (Chemicon). Cells were washed twice with PBS, then propidium iodide (PI) (Sigma) was added to a final concentration of 10 μg/ml to exclude dead cells. Flow cytometric analysis was performed with a FACScan™ (Becton-Dickinson Co., Mountain View, CA, USA).

Adhesion assay. The following procedures were performed as described elsewhere (20). Briefly, 96-well flat-bottom plates (Corning Incorporated, NY, USA) were coated with recombinant human OPN (0.1 μg/ml, 1 μg/ml, or 5 μg/ml), PLL (0.001%), BSA (10 mg/ml) or HA (2 mg/ml) in PBS overnight at 4°C. For some experiments, H28 suspensions were pretreated with anti-human αvβ3 antibody (10 μg/ml) or Gly-Arg-Gly-Asp-Ser (GRGDS) peptide (100 μM; Sigma) for 1 h at 37°C.

Immunoprecipitation-Western blotting analysis for FAK. Polystyrene dishes (Corning) were coated with OPN (0.1 1, or 5 μg/ml), HA (2 mg/ml), PLL (0.001%), or BSA (10 mg/ml) in PBS and incubated overnight at 4°C. The dishes were then washed three times with PBS and blocked with RPMI/SITA at 37°C for 1 h. H28 cells were harvested after 5 min incubation in 0.05% trypsin-EDTA solution and washed twice with PBS containing 0.5 mg/ml soybean trypsin inhibitor. Cells were resuspended in RPMI/SITA and 3×10^6 cells were added to the coated dishes and incubated at 37°C for 60 min in the absence or presence of anti-human αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM). The cells were then homogenized in lysis buffer (1% Triton® X-100 in PBS, 1.5 mM $MgCl_2$, 1 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Nuclei were removed with centrifugation and the lysate was precleared with protein G-magnetic beads (BioLabs, Ipswich, MA, USA). Cell lysates were then incubated overnight with protein G-magnetic beads conjugated with anti-FAK antibody at 4°C. The beads were washed three times and boiled in 1 volume of 2 × SDS sample buffer. Immunoprecipitates were analyzed with sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electroblotted at 4°C. After blocking with Tween-TBS containing 1% BSA, the filters were washed in Tween-TBS containing 1 M Tris-HCl and 0.1% Tween-20. Filters were incubated with anti-FAK polyclonal antibody or anti-phosphotyrosine py-69 antibody for 1 h at room temperature, respectively. Filters were then incubated with horseradish peroxidase-linked anti-rabbit antibody (Amersham Biosciences, Buckinghamshire, UK) for anti-FAK polyclonal antibody or anti-mouse antibody (Amersham Biosciences) for anti-phosphotyrosine py-69 antibody and specific proteins were detected with an enhanced chemiluminescence system (Amersham Bioscience).

In vitro cell proliferation assay. Ninety six-well microtiter plates coated with OPN (0.1 μg/ml, 1 μg/ml, 5 μg/ml), PLL (0.001%), BSA (10 mg/ml) or HA (2 mg/ml) were incubated overnight at 4°C. Two thousands cells were added to the coated plates in triplicate, and allowed to grow at 37°C with 5% CO_2 for 3 days. For some experiments, H28 suspensions were co-incubated with anti-human αvβ3antibody (10 μg/ml) or GRGDS peptide (100 μM) for 3days at 37°C. At the indicated time, cells were harvested from plates with 0.05% EDTA in PBS, suspended in SITA medium in single suspension and counted. The cell number was assessed with the Cell Counting Kit-8™ (Dojindo) according to the manufacturer's instruction.

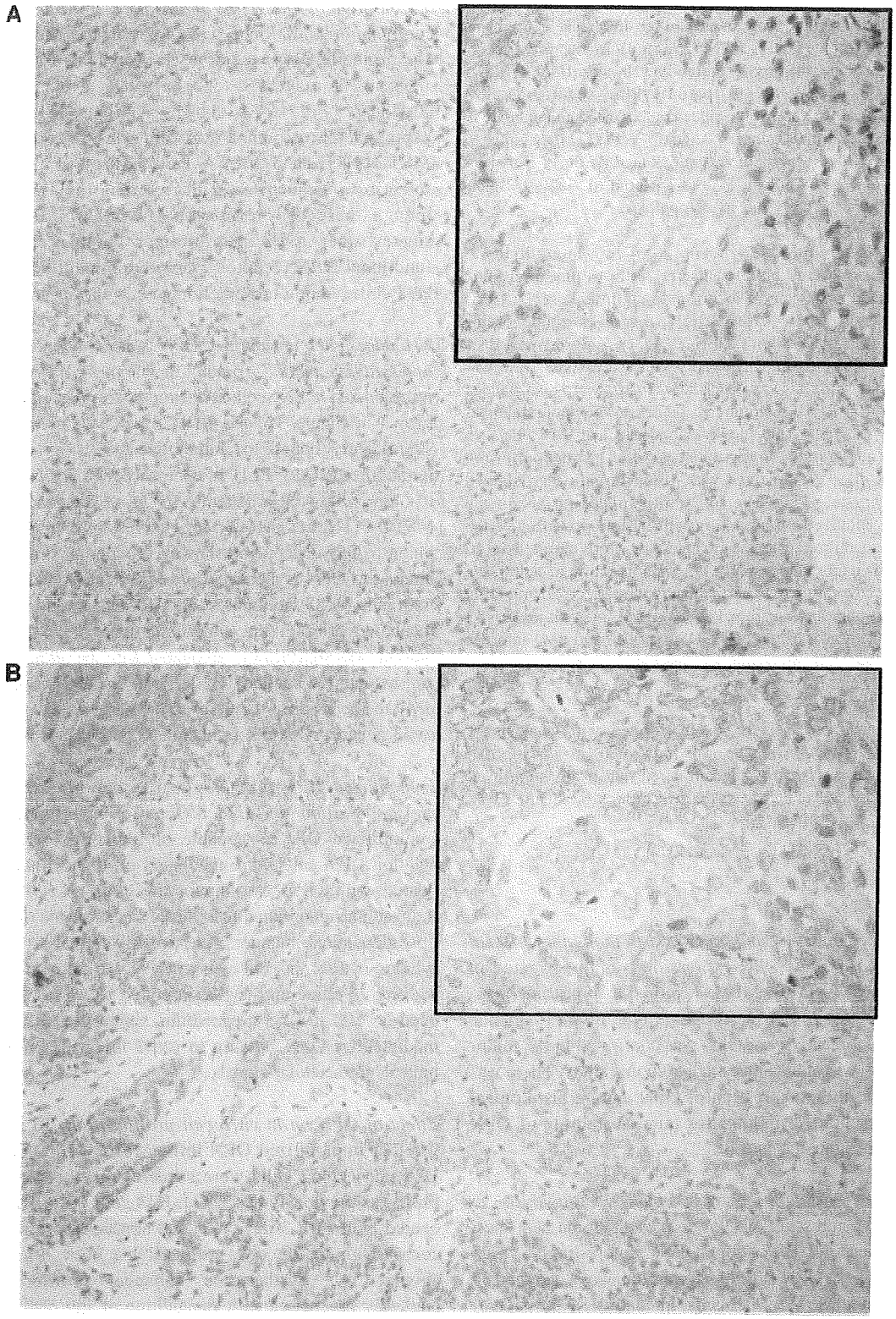

Figure 1. *Expression of OPN on mesothelioma tissues with immunohistochemical staining. A representative section of sarcomatoid (A) and epithelial (B) MPM tumor. OPN immunopositivity was localized within the tumor cells. Magnification for A and B: ×200, magnification for insets of A and B: ×400.*

Evaluation of apoptosis by Annexin V binding in H28 cells. H28 cells (2×10^5 cells/plate) were incubated for 48 h at 37°C on dishes that had been coated with OPN (5 μg/ml) in the presence or absence of either anti- αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM), OPN (1 μg/ml), PLL (0.001%), BSA (10 mg/ml) or HA (2 mg/ml). Cells were harvested and annexin-V binding was performed using an Annexin-V FITC kit (Sigma) as described by the manufacturer and stained with PI for flow cytometric analysis. Annexin-V does not bind to viable cells but binds to cells in the early stages of apoptosis.

In vitro cell migration assay. In vitro cell migration was performed using cell culture inserts with 8 μm micropore membrane (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) as described elsewhere (21). Briefly, the reverse side of the membrane was coated with OPN (0.1, 1, 5 μg/ml) or BSA (10 mg/ml). After 15 min incubation, the excess substrate was removed by washing twice with PBS. H28 cells were resuspended in 0.1% BSA in RPMI medium and seeded to the upper chamber at a density of 2×10^4 /200 μl. Five hundred μl of 0.1% BSA in RPMI were added to the lower chamber. After incubation for 6 h at 37°C, the filters were fixed with 10% formalin and stained with 0.2% crystal violet. The cells on the upper surface of the filters were removed by swabbing with a cotton swab and the cells that had migrated to the reverse side were counted in 10 random fields under a microscope at a magnification of x 400. We also performed additional experiments by treating cells with OPN at concentrations ranging from 1 to 10 μg/ml, or with anti-αvβ3 antibody (10 μg/ml), or with GRGDS peptide (100 μM) in order to confirm that cell migration was mediated by the interaction between OPN and its receptor.

Statistics. Statistical analysis was performed with analysis of variance (ANOVA). All data are presented as mean±S.D. Differences between means were considered statistically significant at $p<0.05$. Statview version 5.0 (Abacus Corporation, Seattle, WA, USA) was used for all analyses.

Results

Immunohistochemical staining of OPN in malignant pleural mesothelioma tissues. OPN expression was investigated in tumor tissues from 6 MPM (epithelial type in three, desmoplastic type in two, sarcomatoid type in one) patients. Strong immunoreactivity of OPN was confirmed in the tumor cells of all MPM patients investigated in this study. There was no difference in the expression of OPN among histological types. Representative findings for immunostaining of OPN are shown in Figure 1A and B.

Expression of OPN and β actin mRNA by RT-PCR analysis. To verify mRNA expression of *OPN* in mesothelioma cell lines, we conducted RT-PCR for OPN. Interestingly, high concentrations of OPN mRNA expression were detected in H28, H2452 and MSTO-211H cells. In contrast, expression of *OPN* mRNA to a much lesser degree was detected in Met5A (Figure 2A).

Expression of integrins on MPM cell lines and normal mesothelial cells. Since αvβ3 integrin has been reported as the principle OPN receptor, we investigated whether αvβ3 integrin is expressed on the surface of cells with a FACScan™ (22). As shown in Figure 2B, H28 cells expressed all αv, β3 and αvβ3 integrins. In contrast, H2452, MSTO-211H, and Met5A cells expressed β3 and αvβ3 integrin to a much lesser degree than the H28 cells even though these cells expressed αv integrin. These results suggest that αvβ3 hetero-dimer complex, which is a functional OPN receptor, was predominantly expressed in H28 cells, among mesothelioma cells and mesothelial cells.

Cell adhesion to immobilized OPN is mediated by αvβ3 integrin and GRGDS peptide. To confirm whether MPM cells bind to immobilized OPN, cell adhesion assay was performed. As shown in Figure 3A and B, H28 cells were revealed to be significantly bound to immobilized OPN as compared to immobilized HA or PLL. In contrast, H2452 cells did not bind to immobilized OPN (Figure 3B). As expected, Met5A and MSTO-211H cells, which do not express αvβ3 integrin, did not bind to OPN (data not shown). To demonstrate that H28 binding to OPN is mediated by αvβ3 integrin, cell suspension with anti-αvβ3 antibodies or GRGDS peptide were pre-incubated prior to the adhesion assay. As expected, OPN binding was significantly abrogated with the addition of either anti-human αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium (Figure 3C). These results suggest that αvβ3 integrin serves as a principle OPN receptor in H28 cells.

Immobilized OPN promotes focal adhesion kinase (FAK) phosphorylation in H28 cells. To investigate whether immobilized OPN is capable of inducing FAK phosphorylation, H28 cells were incubated on dishes that had been coated with OPN. As shown in Figure 4, OPN binding induced phosphorylated FAK in H28 cells plated on OPN in a dose-dependent manner. Additionally, enhanced phosphorylation of FAK in H28 cells to OPN was abrogated with the addition of either anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium, suggesting that the signal mediated by OPN binding to αvβ3 integrins on H28 cells induces intracellular signals.

Effect of OPN on in vitro cell proliferation. To investigate whether immobilized OPN influences *in vitro* cell growth, H28 and H2452 cells were seeded on the coated 96-well plates, as previously described. H28 cells cultured on OPN-coated plates for 3 days revealed enhanced proliferation in comparison to the cells cultured on BSA, PLL, or HA (Figure 5A). Furthermore, enhanced proliferation was markedly suppressed with the addition of anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium (Figure 5B). In contrast, H2452 cells, which do not bind to

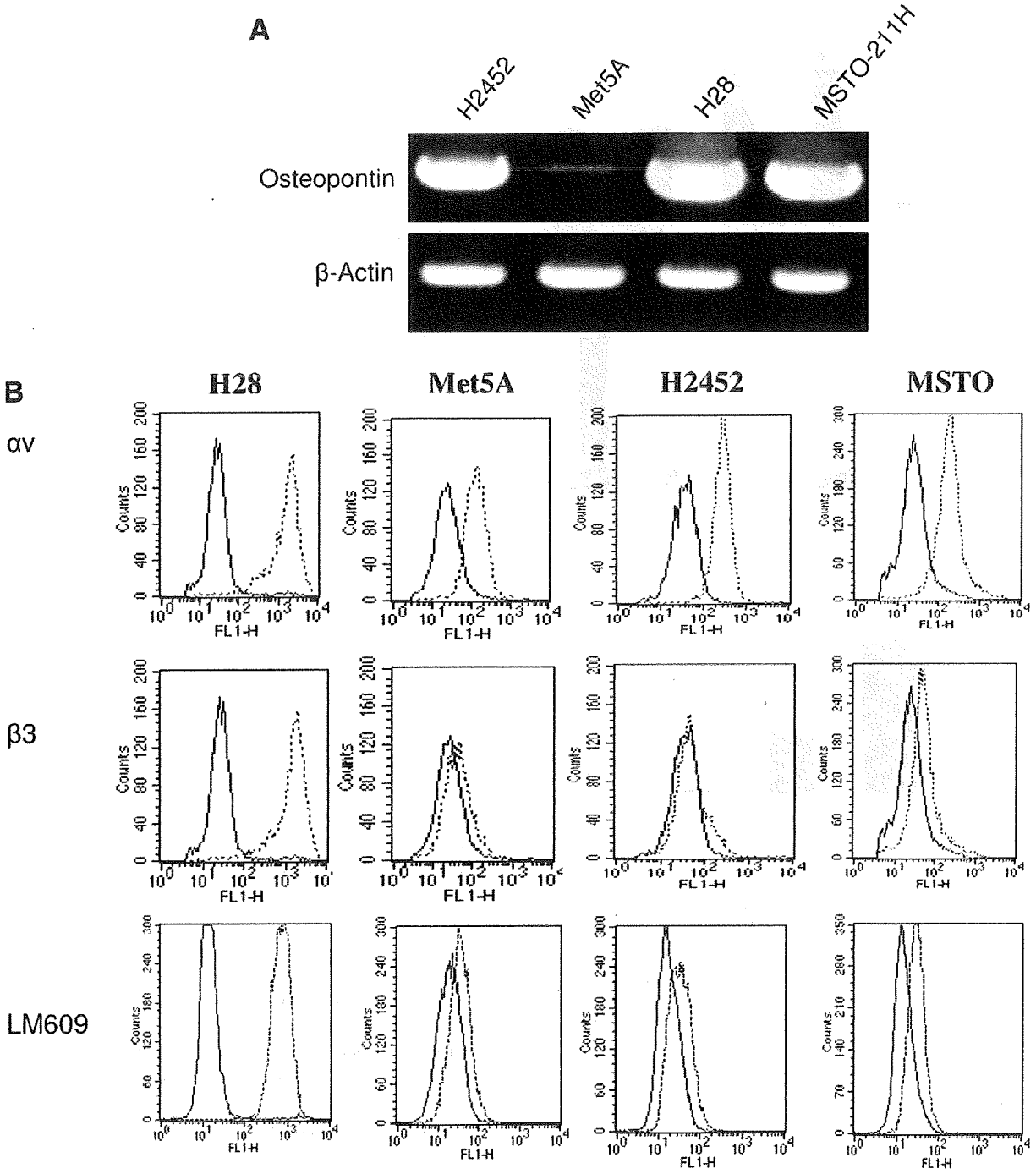

Figure 2. *A, RT-PCR analysis of mRNA expression of OPN and β-actin. Total RNAs were extracted from each cell line and 1 μg of RNA was subjected to RT-PCR analysis for OPN (top panel) and β-actin mRNA (bottom panel) expression. Strong OPN expression was confirmed in H28 cells (sarcomatoid cell type), MSTO-211H cells (biphasic cell type) and H2452 cells (epithelial cell type), while it was weakly expressed in Met5A cells (normal mesothelial type) with RT-PCR. B, Expression of integrins on MPM cell lines with flow cytometric analysis. To determine integrin expressions, cells were incubated with monoclonal antibodies and analyzed with FACScan™. Note that αv, β3 and αvβ3 integrin expressions were predominantly found on H28 cells. In contrast, β3 and αvβ3 integrins were weakly expressed on other cells. Solid lines indicate background immunofluorescence, while dotted lines indicate the fluorescence intensity of integrins.*

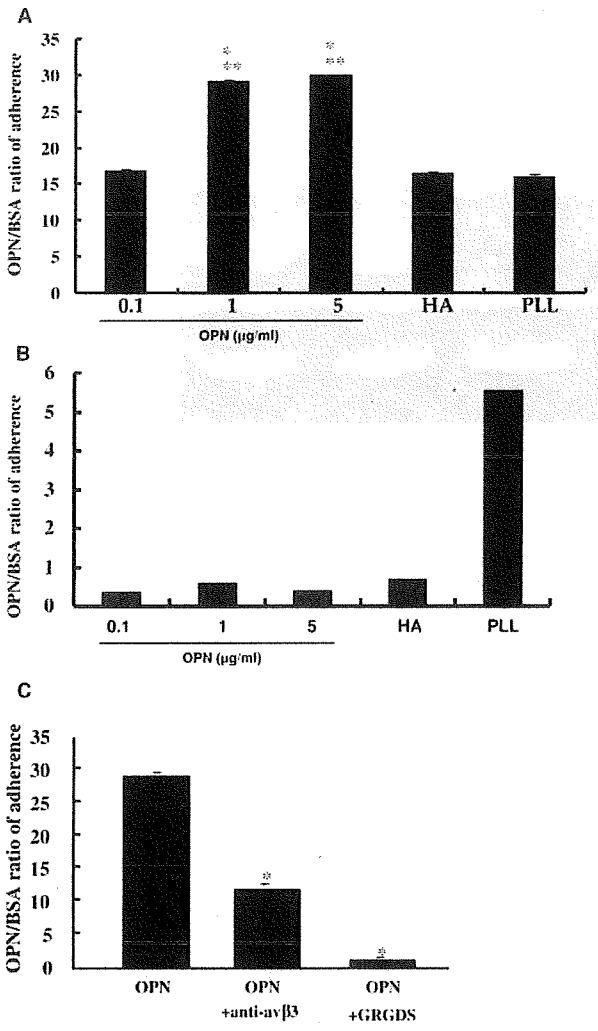

Figure 3. *In vitro cell adhesion activity of H28 cells (A) or H2452 cells (B) with OPN, PLL, BSA or HA. Cells were allowed to adhere to wells coated with OPN (0.1 μg/ml, 1 μg/ml, or 5 μg/ml), HA (2 mg/ml), PLL (0.001%) or BSA (10 mg/ml) at 37°C for 1 h. The OPN/BSA ratio of adherence (% specific adhesion to OPN/% adhesion to BSA/×100) was described in the Material and Methods. H28 revealed enhanced adhesion to OPN in a dose-dependent manner, while H2452 did not. *p<0.0001 vs. HA, **p<0.0001 vs. PLL. C, Effect of anti-human αvβ3 antibody or GRGDS peptide on H28 binding to OPN. Enhanced adhesion of H28 cells to OPN (1 μg/ml) was abrogated with the addition of either anti-human αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium. *p<0.0001 vs. OPN. Data are presented as the mean±S.D. of triplicates.*

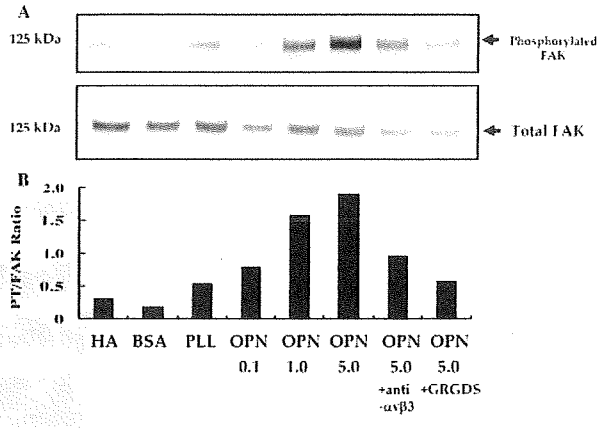

Figure 4. *Tyrosine phosphorylation of focal adhesion kinase (FAK) was assessed with immunoprecipitation and Western blotting. H28 cells were incubated for 60 min at 37°C on dishes coated with OPN (0.1 μg/ml, 1 μg/ml, and or 5 μg/ml) PLL, BSA (10 mg/ml) or HA (4 mg/ml). Cell lysates (5 μg) were immunoprecipitated with anti-FAK antibody and one-half of the precipitates were subjected to immunoblotting with anti-phosphotyrosine antibodies (A, top panel) and the other half with anti-FAK antibody to confirm the loading amount of total FAK (A, bottom panel). Note that increased phosphorylation of FAK in H28 cells plated on OPN was observed in a dose-dependent manner. Additionally, increased phosphorylation of FAK in H28 cells with OPN was abrogated with the addition of either anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium (A). The ratio of phosphorylated FAK/total FAK (PT/FAK) of H28 cells cultured on OPN was greater than that of BSA, PLL, and HA (B).*

OPN, when cultured on OPN-coated plates did not reveal enhanced proliferation (Figure 5C).

Evaluation of apoptosis by Annexin V binding in H28 cells. To evaluate the effect of OPN binding to H28 cells on apoptosis, we performed flow cytometric analysis using Annexin V kit. As shown in Figure 6, fewer apoptotic cells were identified on OPN-coated plates in comparison to BSA, HA and PLL. Interestingly, inhibition of apoptosis by OPN binding was observed in a dose-dependent manner. As expected, anti-apoptosis of H28 cells with OPN was abrogated with the addition of either the anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) (Figure 6).

Migration of H28 cells toward OPN. H28 cells migrated toward immobilized OPN to a much greater degree than they did toward the immobilized BSA (Figure 7A). Enhanced migration of H28 cells towards OPN was abrogated with the addition of either the anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the upper chambers (Figure 7B). Enhanced migration of H28 cells towards OPN was abrogated with the addition of OPN (1, 5 or 10 μg/ml) to the upper chambers (Figure 7C). These results suggest that OPN acts as a chemoattractant for H28 cells.

Discussion

In this study, we first revealed that i) OPN clearly regulates mesothelioma cell function, and ii) the signal transduction *via* αvβ3 integrin is required to modulate mesothelioma cell

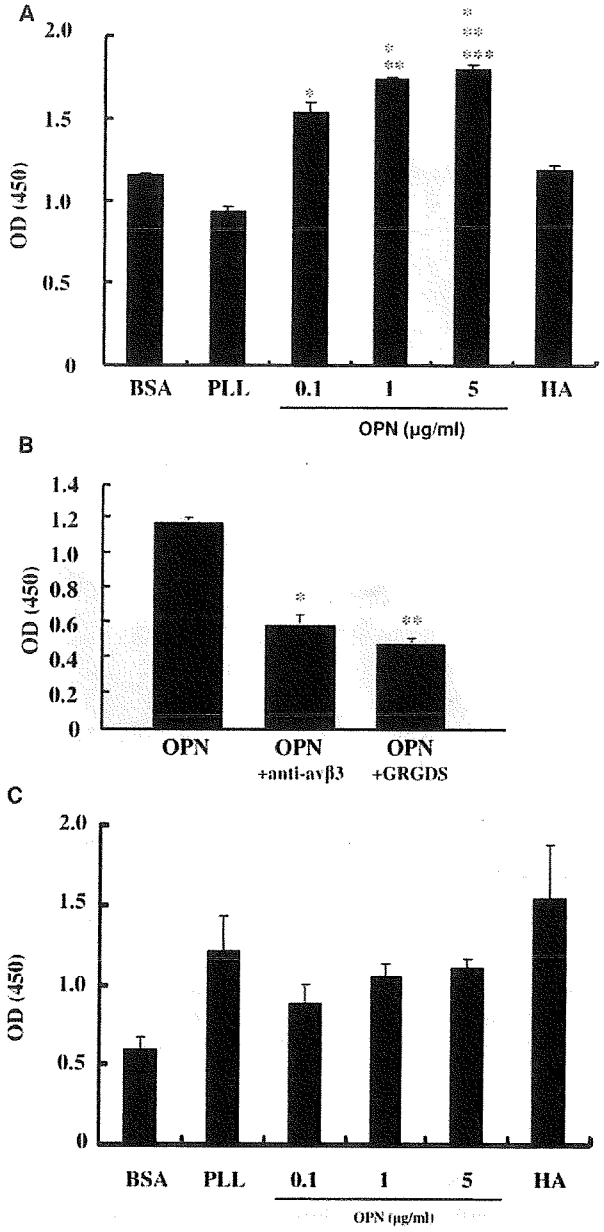

Figure 5. *In vitro cell proliferation assay. A, Two thousand H28 or H2452 cells were added to 96-well microtiter plates coated with OPN (0.1 μg/ml, 1 μg/ml, or 5 μg/ml), PLL (0.001%), BSA (10 mg/ml) or HA (2 mg/ml) in triplicate, and allowed to grow for 3 days. Cell numbers were assessed with a Cell Counting Kit-8™. H28 cells cultured on OPN-coated plates at the indicated concentration revealed enhanced proliferation in a dose-dependent manner in comparison to the cells cultured on BSA, PLL, or HA. *p<0.001 vs. PLL. **p<0.001 vs. BSA ***p<0.001 vs. HA. B, Inhibitory effect of anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) on H28 cell proliferation mediated by coated OPN at a concentration of 1 μg/ml. Enhanced proliferation was markedly suppressed with the addition of anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium. *p, **p<0.0001 vs. OPN 1 μg/ml. Data are presented as the mean±S.D. in triplicates. C, In contrast, H2452 cells cultured on OPN-coated plates failed to reveal enhanced proliferation.*

function. The interaction between OPN and the H28, MPM cell line, is involved in the enhancement of cancer cell adhesion, proliferation, anti-apoptosis and migration. Higher concentrations of OPN induced increased levels of phosphorylated FAK in H28 cells. Although OPN has already been reported to be involved in tumorigenecity of a variety of cancer types, there are few reports investigating the role of OPN in the progression of MPM. As already described in the Introduction, Pass *et al.* revealed that serum OPN could be a useful marker in the early detection of MPM (17). However, they did not report on distinct OPN expression in different histological types, nor the functional property of OPN in the pathogenesis of MPM. In this study, the immunoreactivity of OPN in the tumor cells of MPM cases was not significantly different among the histological types. This result is consistent with that reported by Frey *et al.* (23).

Furthermore in this study, we revealed that the mesothelioma cell line, H28, adhered to OPN, migrated toward OPN, and demonstrated enhanced proliferation and anti-apoptosis functions when cells were cultured on OPN-coated plates. In contrast, these findings were not consistent with the results from other MPM cell lines, which did not demonstrate any OPN binding, indicating cell adhesion is essential to carry out these functions. OPN exerts various functions by interacting with adhesion molecules such as integrins αvβ3, αvβ5, αvβ1 and αvβ9, and CD44 in an arginine-glycine-aspartic acid (RGD) sequence-dependent or independent manner (24, 25). Among these receptors, αvβ3 appears to be responsible as a functional OPN receptor because: i) anti-αvβ3 antibody strongly inhibits adhesion, migration and *in vitro* cell proliferation of H28 cells, and ii) MPM cell lines which do not express β3 integrin did not bind to OPN, although these cells express the αv integrin. Interestingly, Giuffrida *et al.* reported that integrin β3 was predominantly expressed in invading mesothelioma with immunohistochemical analysis (26). These results suggest that αvβ3 integrin may play a crucial role in the progression of MPM and the role of OPN in the pathogenesis of MPM is variably dependent upon the expression of its functional adhesion receptor, αvβ3, regardless of the histological type.

It has been reported that the interaction of αvβ3 with the ECM has been identified to play an important role in cell survival in nascent vessels. In some types of cancer, αvβ3 expression correlated with the aggressiveness of the disease. In fact, the αvβ3/αvβ5 integrin antagonist S247 demonstrated significant anti-metastatic functions and anti-angiogenic activity. S247 caused detachment and apoptosis and inhibited *in vitro* cell growth. Moreover, S247 therapy inhibited metastases of colon cancer to the liver and increased survival, *in vivo*. Interestingly, combined treatment with S247 and an Arg-Gly-Glu peptidomimetic antagonist of αvβ3 integrin, and external beam radiotherapy have revealed its benefit in localized tumor treatment (27-29). Moreover,

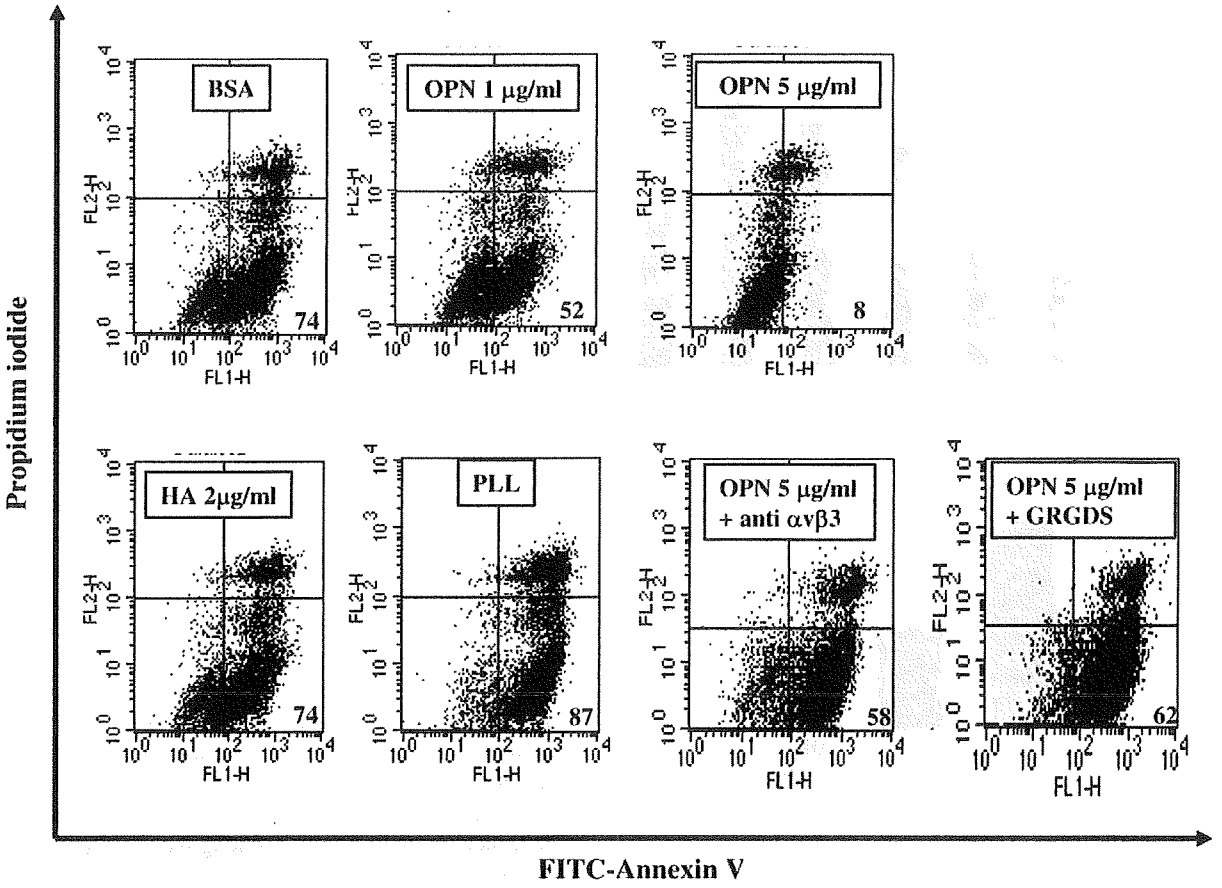

Figure 6. *OPN suppressed apoptosis in H28. H28 cells were incubated for 48 h at 37°C on dishes that had been coated with OPN (5 μg/ml), OPN (1 μg/ml), 0.001% PLL, BSA (10 mg/ml) or HA (2 mg/ml). Cells were harvested and stained with FITC-annexin V and propidium iodide for analysis with flow cytometry. Normal viable cells are in the lower left quadrant, early apoptotic cells in the lower right quadrant, late apoptotic/necrotic cells in the upper right quadrant and necrotic cells in the upper left quadrant. The percentage of gated cells in early apoptosis (annexin V-positive, propidium iodide-negative) in this representative experiment is indicated at the lower right corners. H28 cells cultured on OPN were more viable in comparison to cells cultured on the PLL, BSA and HA. However, anti-apoptosis of H28 cells to OPN was abrogated with the addition of either anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium.*

the humanized monoclonal antibody, Abegrin™ , has been used to achieve selective targeting of the many tumor cells that express the αvβ3 integrins, and is currently in phase II trials for treatment of solid tumors (30,31). Cai *et al.* suggested that chemotherapeutics or radiotherapeutics using Abegrin™ as the delivering vehicle is effective in treating integrin αvβ3-positive tumors (32). These results indicate that S247 and/or Abegrin™ may be a potential candidate for the treatment of patients with MPM.

Interestingly, the extent of abrogation of adhesion to OPN by anti-αvβ3 antibody was one third of that with GRGDS peptide in H28 cells. In contrast, anti-αvβ3 antibody completely inhibited the enhanced migration activity as demonstrated with GRGDS peptide. For the proliferation assay, pretreatment of H28 cells with either anti-αvβ3 antibody or GRGDS peptide inhibited approximately 50% of the proliferative activity. These results suggest that other αv-containing receptors, such as αvβ5, or other RGD-dependent receptors may also be involved in cell adhesion to OPN, while αvβ3 integrin is the principal OPN receptor for cell migration. In fact, it has been reported that αvβ5 integrin plays a crucial role in the uptake of vitronectin or serum-coated asbestos in mesothelial cells (33). These findings suggest that αvβ5 integrin could be involved in the malignant transformation of mesothelioma cells. For cell proliferation, other RGD-independent receptors may also be partially involved, although we were unable to determine these receptors. Further investigations are needed to determine the role of αvβ5 receptor and other OPN receptors in the pathogenesis of MPM.

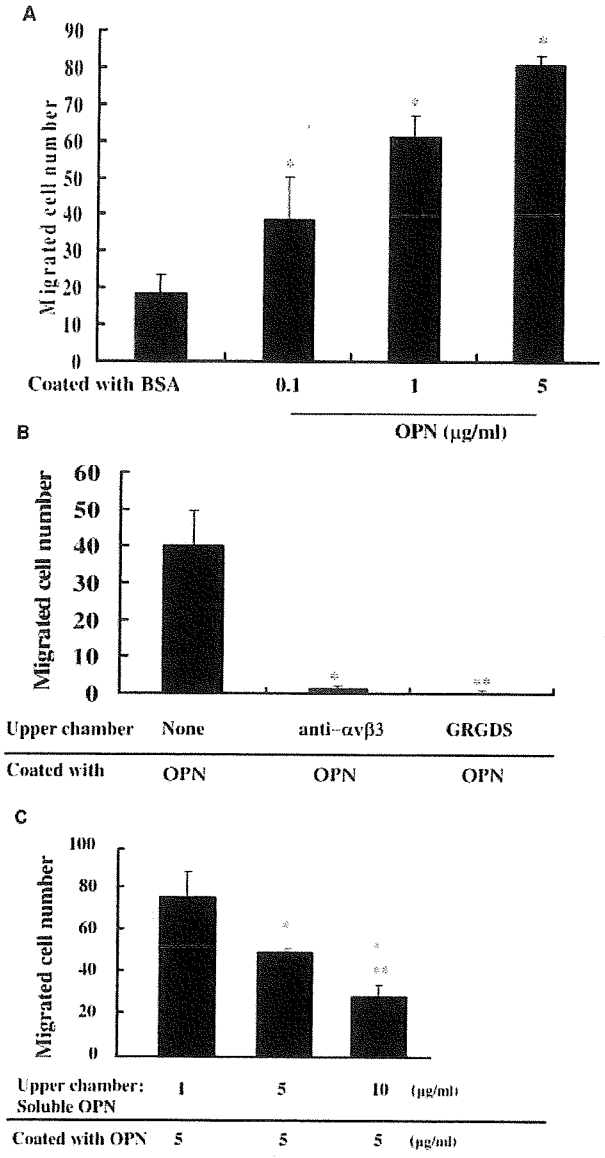

Figure 7. *Migration of H28 cells toward OPN. Cells were placed in the upper chamber of the cell culture insert. The reverse sides of membranes of cell culture insert were coated with OPN (0.1, 1.0, or 5 μg/ml) or BSA (10 mg/ml). After 6 h of incubation, cells that migrated through the porous filter were counted at ×400 magnification. A, H28 cells migrated toward immobilized OPN to a much greater extent than they did toward the immobilized BSA. Data are presented as the mean±SD. *p<0.0001 vs. BSA. B, The increased migration of H28 cells toward immobilized OPN (5 μg/ml) was abrogated with the addition of either anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the upper chambers. Data are presented as the mean±SD. *p<0.0001 vs. none. C, Inhibitory effect of soluble OPN in the upper chamber on the migration of H28 cells toward the reverse side of the filters coated with OPN (5 μg/ml). Enhanced migration of H28 cells was abrogated with the addition of soluble OPN to the upper chambers. Data are presented as the mean±SD. *p<0.0001 vs. upper chamber (OPN 1 μg/ml), **p<0.001 vs. upper chamber (OPN 5 μg/ml).*

In conclusion, we were clearly able to reveal that OPN is involved in mesothelioma cell function and αvβ3 integrin is the functional receptor for OPN in H28 cells. Moreover, the signal induced by αvβ3 integrin binding OPN may play an important role in the regulation of mesothelioma cell motility and tumor cell growth. Finally, αvβ3 integrin could be a novel molecular target for the treatment of patients with MPM with positive αvβ3 expression. Determination of αvβ3 integrin expression on tissue specimens is required for the selection of a potential candidate for this novel targeting therapy.

Acknowledgements

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