# 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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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 $3\times10^6$ 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.

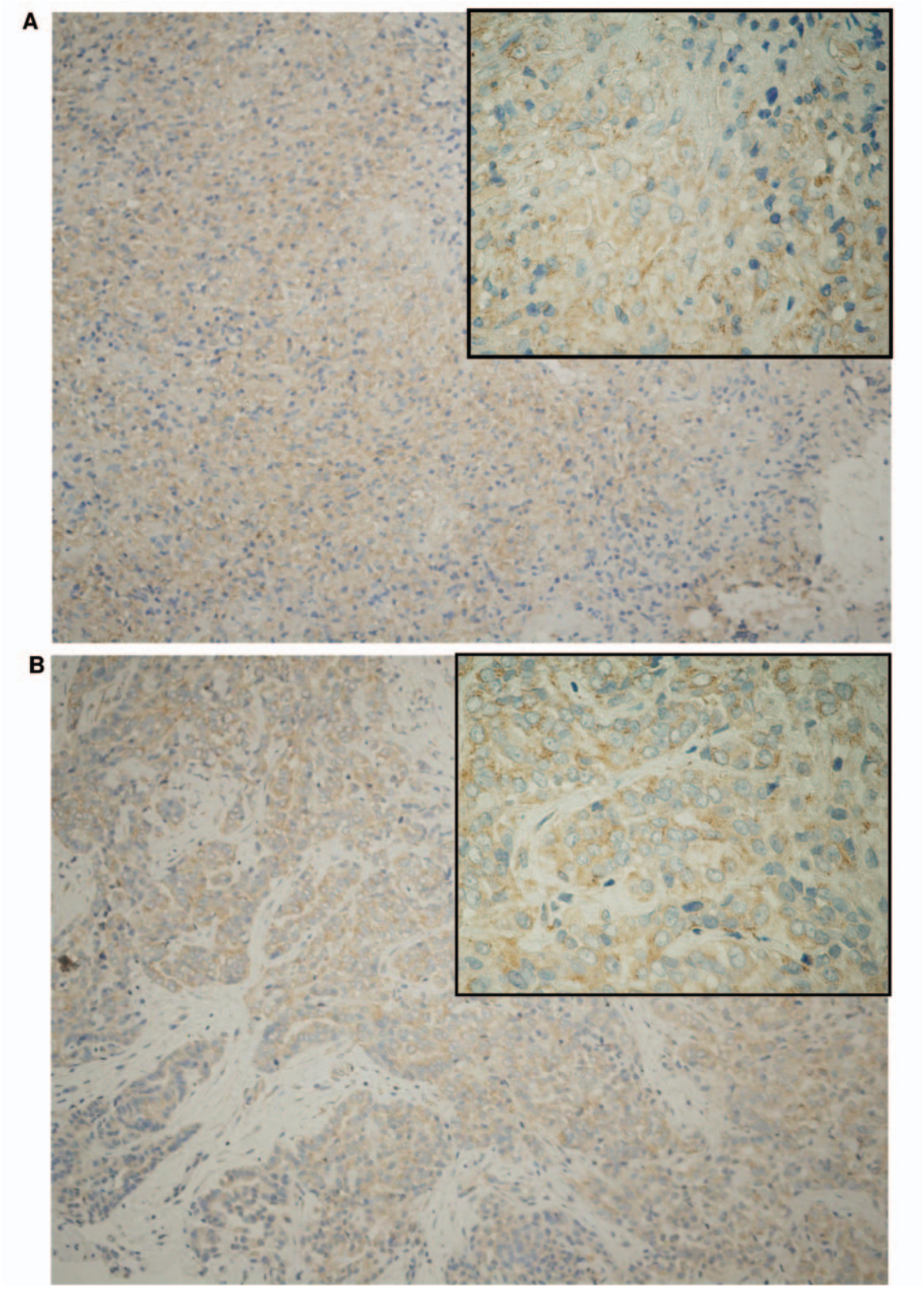


Figure 1. *Expression of OPN on mesothelioma tissues with immunohistochemical staining. A representative section of sarcomatoid (A) and epithelial (B) MPM tumor. OPN immunopositivity was localized within the tumor cells. Magnification for A and B: ×200, magnification for insets of A and B: ×400.*

*Evaluation of apoptosis by Annexin V binding in H28 cells*. H28 cells ($2\times10^5$ 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 $2\times10^4$ /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

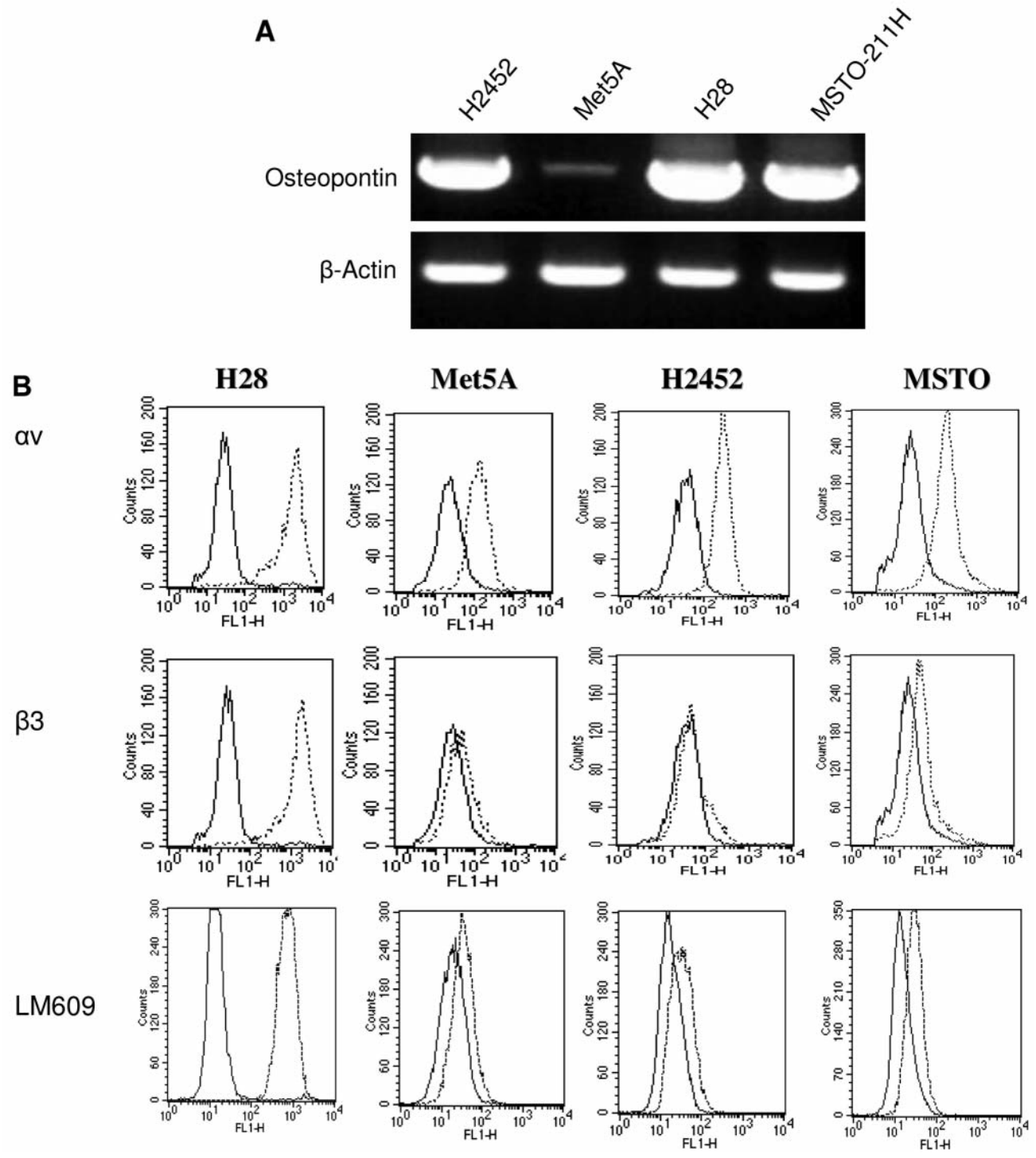


Figure 2. *A, RT-PCR analysis of mRNA expression of OPN and β-actin. Total RNAs were extracted from each cell line and 1 μg of RNA was subjected to RT-PCR analysis for OPN (top panel) and β-actin mRNA (bottom panel) expression. Strong OPN expression was confirmed in H28 cells (sarcomatoid cell type), 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.*

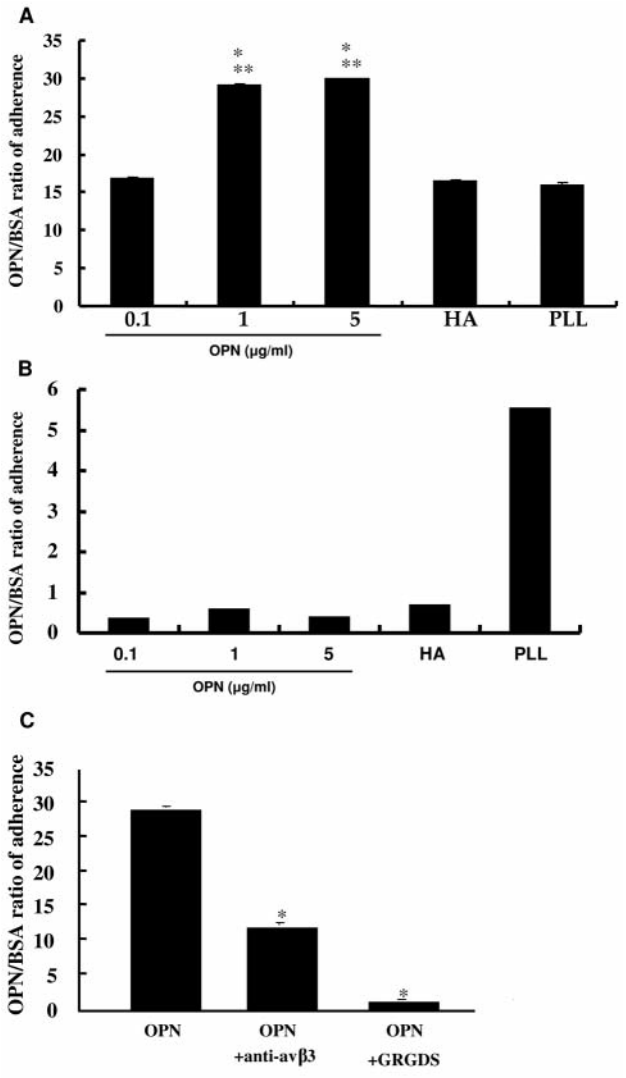


Figure 3. *In vitro cell adhesion activity of H28 cells (A) or H2452 cells (B) with OPN, PLL, BSA or HA. Cells were allowed to adhere to wells coated with OPN (0.1 μg/ml, 1 μg/ml, or 5 μg/ml), HA (2 mg/ml), PLL (0.001%) or BSA (10 mg/ml) at 37˚C for 1 h. The OPN/BSA ratio of adherence (% specific adhesion 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.*

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.

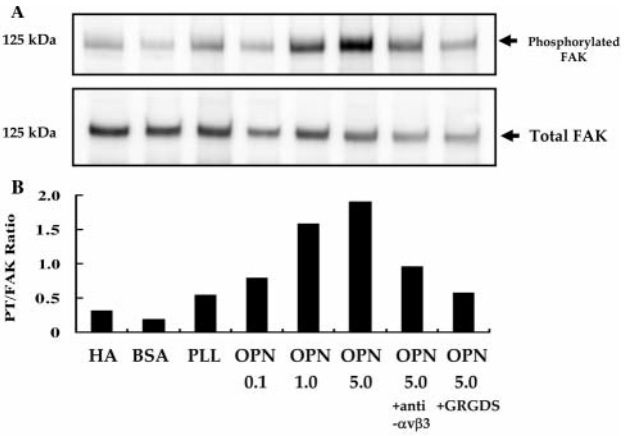


Figure 4. *Tyrosine phosphorylation of focal adhesion kinase (FAK) was assessed with immunoprecipitation and Western blotting. H28 cells were incubated for 60 min at 37˚C on dishes coated with OPN (0.1 μg/ml, 1 μg/ml, and or 5 μg/ml) PLL, BSA (10 mg/ml) or HA (4 mg/ml). Cell lysates (5 μg) were immunoprecipitated 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).*

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

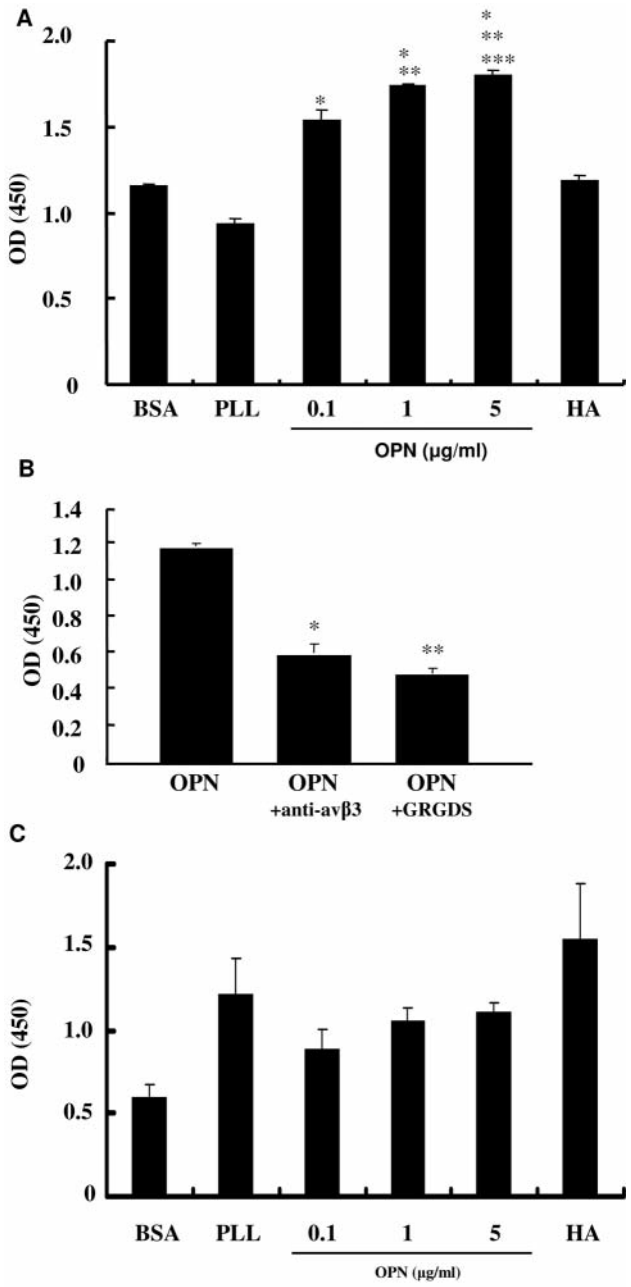


Figure 5. *In vitro cell proliferation assay. A, Two thousand H28 or H2452 cells were added to 96-well microtiter plates coated with OPN (0.1 μg/ml, 1 μg/ml, or 5 μg/ml), PLL (0.001%), BSA (10 mg/ml) or HA (2 mg/ml) in triplicate, and allowed to grow for 3 days. Cell numbers were assessed with a Cell 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,

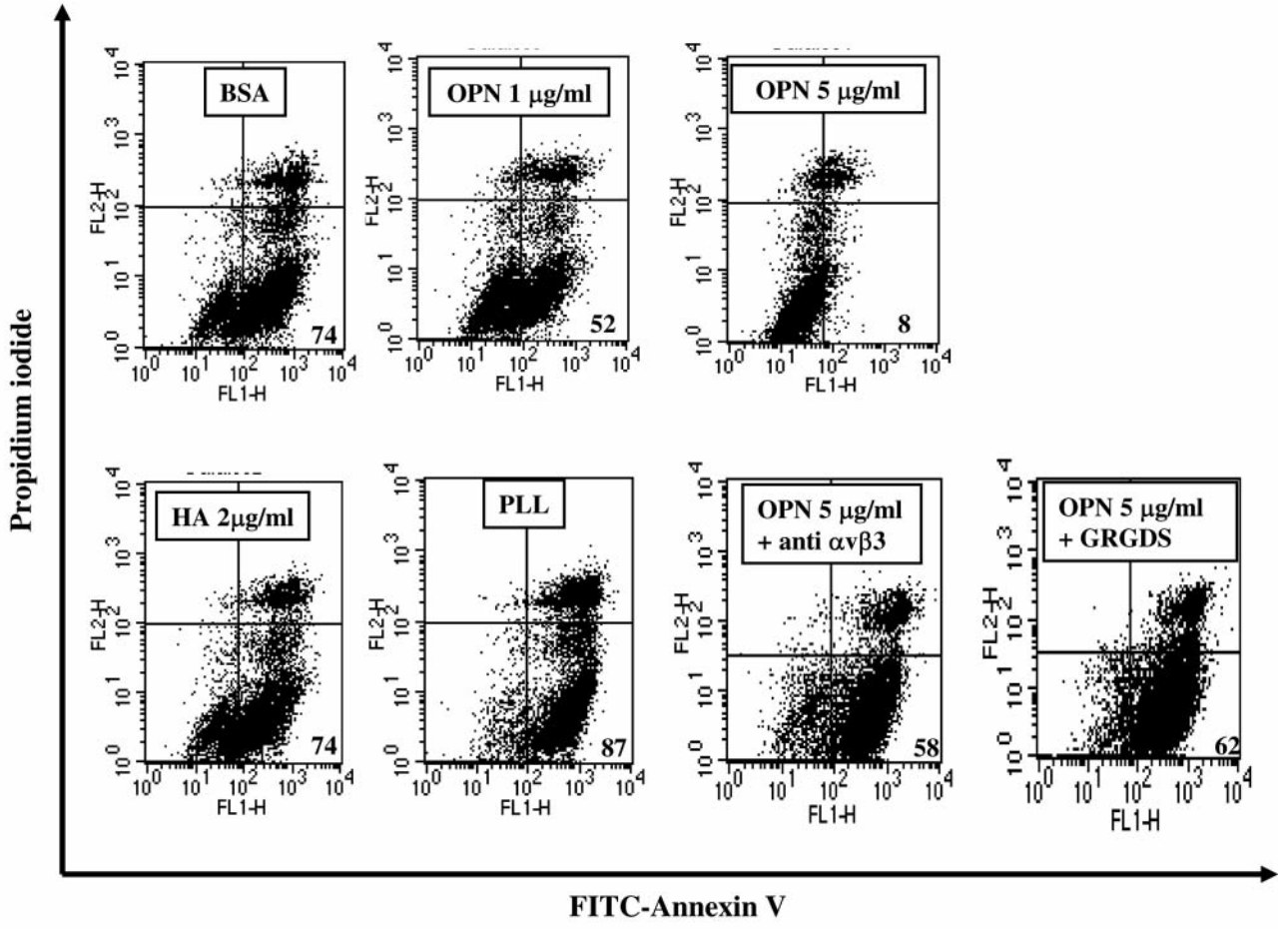


Figure 6. *OPN suppressed apoptosis in H28. H28 cells were incubated for 48 h at 37˚C on dishes that had been coated with OPN (5 μg/ml), OPN (1 μg/ml), 0.001% PLL, BSA (10 mg/ml) or HA (2 mg/ml). Cells were harvested and stained with FITC-annexin V and propidium iodide for analysis with flow cytometry. 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.

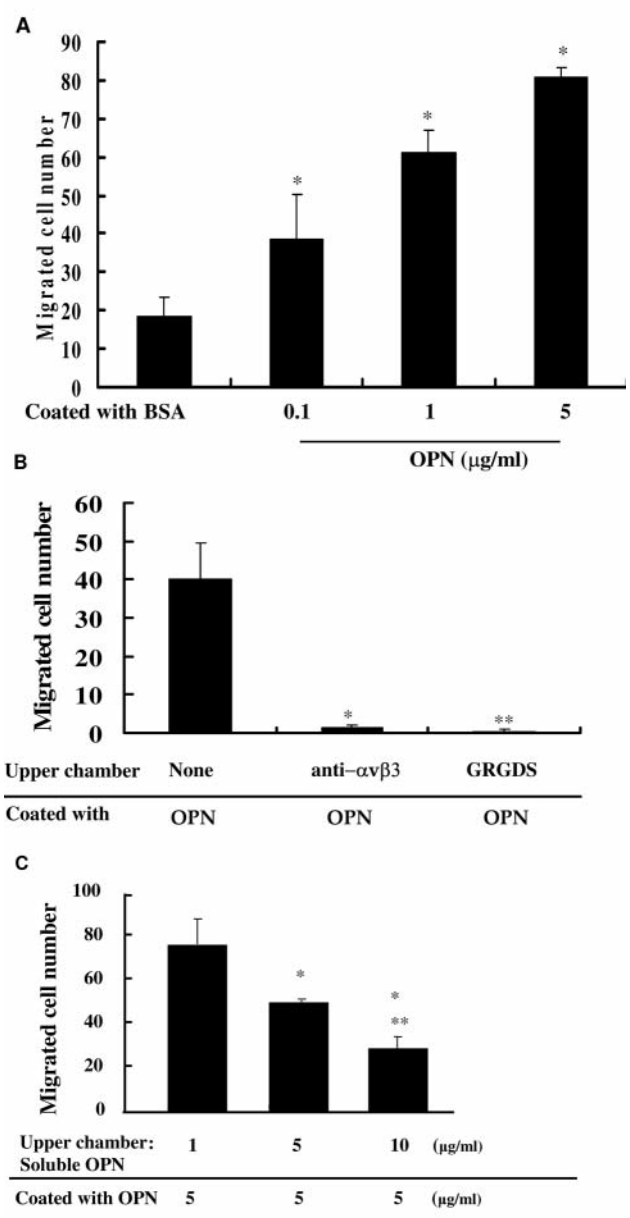


Figure 7. *Migration of H28 cells toward OPN. Cells were placed in the upper chamber of the cell culture insert. The reverse sides of membranes of cell culture insert were coated with OPN (0.1, 1.0, or 5 μg/ml) or BSA (10 mg/ml). After 6 h of incubation, cells that migrated through the porous filter 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.

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