Osteopontin Modulates Malignant Pleural Mesothelioma Cell Functions in Vitro

Rina Ohashi¹,², Ken Tajima¹,², Ri Cui¹,², Tao Gu¹,², Okio Hino³;
Kazu Shiomi⁴; Hideaki Miyamoto⁵; Kazuto Nishio⁶; Kazuhisaka Takahashi¹,²

¹Department of Respiratory Medicine, ²Research Institute for Disease of Old Ages, ³Department of Pathology and Oncology,
⁴Department of General Thoracic Surgery, Juntendo University, School of Medicine, Japan; ⁵Department of General Thoracic
Surgery, Minami-Tohoku Hospital, Japan; ⁶Department of Genome Biology, Kinki University School of Medicine, Japan.

ABSTRACT — Objective. It has been reported that serum osteopontin (OPN) levels of persons with exposure to
asbestos who have malignant pleural mesothelioma (MPM) are increasing and useful for early diagnosis of MPM.
OPN contains binding sites for several receptors including αvβ3 integrins, which are thought to play various roles
in mediating cell-matrix interactions. The aim of this study is to evaluate roles of OPN in MPM cell line. Methods.
With MPM cell lines, we conducted reverse transcriptase-polymerase chain reaction (RT-PCR) to evaluate OPN
mRNA expression. Expression of integrins on the surface of this cell line were analyzed with a FACSscan™. To
evaluate cell adhesion and proliferation mediated by OPN, cells were added to 96-well flat bottom plates coated
with OPN, bovine serum albumin (BSA), poly-L-lysine (PLL) or hyaluronan (HA). Adherent and viable cells were
counted with cell counting kit-8™. To evaluate phosphorylation of focal adhesion kinase (FAK) in H28 cells on
OPN, we performed immunorecipitation and immunoblotting. Apoptotic cells cultured on these plates were
detected by the binding of annexin V. Results. OPN and αvβ3 expression was detected with both RT-PCR and
FACSscan™, respectively. H28 cells adhered to OPN, PLL, or HA to much greater extent than to BSA. However,
H28 cells cultured on OPN coated plates showed enhanced proliferation, but not on BSA, PLL or HA. In addition,
high level of phosphorylated FAK in H28 cells plated on OPN was observed. Furthermore, less apoptotic cells
were revealed cultured on OPN coated plates in comparison to others. Conclusion. In conclusion, OPN may play
an important role in the enhancement of adhesion and proliferation of H28 cells, presumably by interacting with
αvβ3 integrins.

KEY WORDS — Malignant pleural mesothelioma, Osteopontin, αvβ3 integrin


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はじめに
悪性胸膜中皮腫は体腔内面を覆う纖膜に発生し、局所的に浸潤・進行する予後不良の悪性腫瘍である。近年の新規抗腫瘍薬の登場にも関わらず、進行悪性胸膜中皮腫の予後は上皮型中皮腫患者で平均10～17ヶ月、肉腫型に至っては4～7ヶ月といまだ芳しくない。中皮腫細胞は細胞外マトリックス（extracellular matrix：ECM）分子と接触後、インテグリンを介して細胞外リガンドと結合することにより浸潤の転移、浸潤に関与している。また中皮腫細胞はヒアルロン酸、ファイブロネクチン、テネイシンなどのECMを産生し、接着・遊走・播種を生じていると報告されている。1-8
一方、2005年にはPassらはアスベストに曝露した胸膜中皮腫患者においてECM分子のひとつであるオステオポリン（osteonentin：OPN）の血清中濃度が上昇し、早期診断に有用であると報告している。9同様に胸膜中皮腫患者の胸水においてOPN濃度が高い傾向にある。10OPNは活性化マクロファージなどから産生分泌されるリン酸化糖蛋白で細胞接着や遊走、一酸化窒素合成酵素（iNOS）の抑制などの機能を有する多機能分子である。11-13分子構造のほぼ中央部に細胞接着配列のアルギニン・グリシン・アスパラギン酸のRGDドメインを有し、αvβ3などのインテグリンと結合することで機能発現する。14,16そのほか悪性細胞への形質転換、増殖、感染防御などに関与することが報告されている。12,17
しかしながら、悪性胸膜中皮腫細胞におけるOPNの役割については明らかでない。われわれは悪性胸膜中皮腫細胞株におけるOPNの発現、接着、増殖、アポトーシス、遊走における役割について検討した。

材料と方法
免疫組織化学染色
免疫組織化学染色はavidin-biotin complex（ABC）法にて行い、一時抗体は市販されているOPNエピトープ特異ラビット抗体（Spring-BioScience、Fremont、CA：希釈率50倍にて使用）を使用した。

使用細胞株
肉腫型：H28、上皮型：H2452、二相性：MSTO-211H、
中皮細胞：Met5Aを用いた（American Type Culture

Figure 1. Expression of OPN on mesothelioma tissues with immunohistochemical staining. A representative section of sarcomatoid (A) and epithelial (B) MPM case. OPN immunopositivity was localized within the tumor cells. Magnification for A and B: 200×, magnification for inserts of A and B: 400×.
Figure 2. (A) mRNA expression of OPN and β-actin by RT-PCR analysis. Total RNAs were extracted from each cell line and 1 μg of RNAs was subjected to RT-PCR analysis for OPN (top panel) and β-actin mRNA (bottom panel) expression. OPN expression was strongly detected in H28 cells (sarcomatoid cell type), MSTO-211H cells (biphasic cell type), and H2452 cells (epithelial cell type), while it was weakly detected in Met5A cells (normal mesothelial type) cells with RT-PCR. (B) Expression of integrins on MPM cell lines by flowcytometric analysis. To determine integrin expressions, cells were incubated with monoclonal antibodies and analyzed with a FACScan™. Note that αv and β3 integrin expressions were predominantly expressed on H28 cells. In contrast, αv and β3 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 of H28 cells (A) or H2452 cells (B) to 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 hr. The OPN/BSA ratio of adherence (percent specific adhesion to OPN/percent adhesion to BSA ×100) is described in the material and methods. H28 showed enhanced adherence 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 by 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. in triplicates.

Figure 4. In vitro cell proliferation assay. (A) Two thousand H28 or H2452 cells were added to the 96-well microtiter plate 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 number was assessed with the cell counting kit-8™. H28 cells cultured on OPN coated plates at the indicated concentration demonstrated 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 the 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 plate failed to demonstrate enhanced proliferation. OD: optical density.


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フローサイトメトリーによるインテグリン発現の検討
各中皮膚細胞株のインテグリン発現を抗ヒトαvインテグリン抗体(CD51)と抗ヒトβ3インテグリン抗体 (CD61)を用いて評価した。フローサイトメトリーはFACScan™(Becton-Dickinson Co., Mountain View, CA)を使用した。

細胞接着試験
OPN (0.1, 1.0, 5.0 µg/ml), ヒアルロン酸 (2 mg/ml), 0.001% ポリエチレングリコール, bovine serum albumin (BSA) 10 mg/ml を96ウェルプレートに固相化した後, 各細胞株を無血清培地で1時間反応させた。プレートを反転後, 遠心し付着細胞数の数をcell counting kit-8™で評価した。

細胞増殖試験
同様に各基質を固相化したELISAプレート上で各細胞株を培養し24時間後に細胞生存度を評価した。

免疫沈降ウエスタンプロットティング
OPN (0.1, 1.0, 5.0 µg/ml), ヒアルロン酸 (2 mg/ml), 0.001% ポリエチレングリコール, BSA (10 mg/ml)をポリエステルディッシュに固相化した後, H28細胞株を無血清培地中で1時間反応させた。そのcell lysatesを抗FAK抗体, 抗ホスホタイロシン抗体と反応させ, FAKのリン酸化を評価した。
Figure 5. Immunoprecipitation-western blotting analysis for focal adhesion kinase. Tyrosine phosphorylation of focal adhesion kinase (FAK) was assessed with immunoprecipitation-western blotting. H28 cells were incubated at 37°C for 60 min on dishes coated with OPN (0.1 μg/ml, 1 μg/ml, and 5 μg/ml), PLL, BSA (10 mg/ml) or HA (2 mg/ml). 5 μg of cell lysates were immunoprecipitated with anti-FAK antibody, and one-half of the precipitates were subjected to immunoblotting with anti-phosphotyrosine antibodies (top panel). The other half was probed with anti-FAK antibody to confirm the loading amount of total FAK (bottom panel). Note that the enhanced phosphorylation of FAK in H28 cells plated on OPN was observed in a dose dependent manner. In addition, enhanced phosphorylation of FAK in H28 cells toward OPN was abrogated by 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 on BSA, PLL, or HA (B).
Figure 6. OPN suppressed apoptosis in H28. H28 cells were placed for 48 hr at 37°C on dishes 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 flowcytometry. Normal viable cells are shown in the left lower quadrant, early apoptotic stage in the right lower quadrant, late apoptotic/necrotic stage in the right upper quadrant, and necrotic stage in the left upper quadrant. The percentage of gated cells in early apoptosis (annexin V positive, propidium iodide negative) in this representative experiment is indicated at the right lower corners. H28 cells cultured on OPN were more viable in comparison to cells cultured on the others. However, anti-apoptosis of H28 cells toward OPN was abrogated with the addition of either anti-αvβ3 antibody (10 μg/ml) or GRGDS peptide (100 μM) to the medium.
Figure 7. Migration of H28 cells toward OPN. Cells were placed in the upper chamber of a cell culture insert. The reverse sides of the membranes of cell culture inserts were coated with OPN (0.1, 1.0, or 5 μg/ml) or BSA (10 mg/ml). After 6 hr of incubation, cells that migrated through the porous filter were counted at ×400 magnification. (A) H28 cells migrated toward immobilized OPN to much greater extent than the cells toward the immobilized BSA. Data are presented as the mean ± SD. *p<0.0001 vs BSA. (B) Enhanced migration of H28 cells towards 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 towards 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<0.0001 vs upper chamber (OPN 5 μg/ml).

REFERENCES


