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Serum Osteopontin, an Enhancer of Tumor Metastasis to Bone, Promotes B16 Melanoma Cell Migration

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Abstract Tumor malignancy is associated with several features such as proliferation ability and frequency of metastasis. Since tumor metastasis shortens patients' lifetime, establishment of therapy for anti-metastasis is very important. Osteopontin (OPN), which abundantly expressed in bone matrix, is involved in cell adhesion, migration, extracellular matrix (ECM) invasion and cell proliferation via interaction with its receptor, that is, αvβ3 integrin. OPN is believed to be a positive regulator of tumor metastasis in vivo. However, how OPN regulates metastasis is largely unknown. Here, we explore the role of OPN in cell migration. Serum from wild-type mice induced cell migration of B16 melanoma cells, while serum from OPN-deficient mouse suppressed this event. The presence of recombinant OPN significantly enhanced cell migration compared to albumin containing medium. OPN-induced cell migration was suppressed by inhibiting the ERK/MAPK pathway indicating that OPN-induced cell migration depends on this pathway. Overexpression of OPN in these cancer cells per se promoted cell proliferation and tended to increase B16 cell migration suggesting that OPN promotes bone metastasis by playing dual roles both in host microenvironment and in tumor cell itself. In conclusion, the elevated OPN expression in host tissue and tumor cell itself promotes tumor cell migration reading to tumor metastasis, suggesting that neutralization of OPN-induced signal might be effective in suppression of tumor metastasis. J. Cell. Biochem. 101: 979–986, 2007.

Key words: osteopontin; melanoma; migration; metastasis; bone

Tumor cell metastasis to bone involves various steps related to cell adhesion. osteopontin (OPN) has been implicated in metastatic cell adhesion [Senger et al., 1989; Brown et al., 1992; Denhardt and Chambers, 1994] and is one of the extracellular matrix (ECM) molecules present in bone. Bone is a preferred site for the metastasis of several types of tumors including breast and prostate cancer [Yin et al., 2005; Yoneda and Hiraga, 2005]. Melanoma is also known to metastasize frequently to bone in both human and mouse models [Gokaslan et al., 2000; Winkelmann et al., 2006]. In addition to adhesion, OPN also acts as a cytokine to modulate cell functions [Denhardt and Noda, 1998]. OPN binds to several types of integrins

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and CD44 to promote the attachment of the cells to the ECM and also to modulate the functions of these cells through inside out and outside in signaling [Zohar et al., 2000].

OPN is secreted by osteoblasts and osteoclasts during remodeling of bone. OPN facilitates the action of osteoclasts to resorb bone and its presence modulates the function of hormonal signals such as PTH [Ihara et al., 2001; Kitahara et al., 2003]. OPN is also known to be one of the constituents of the niche for the hematopoietic stem cells in endosteal microenvironment [Stier et al., 2005].

With respect to cancers, high plasma OPN levels were observed in patients with multiple metastatic tumors [Senger et al., 1989]. The levels of serum OPN concentration in prostatic cancer patients are inversely correlated to the survival rate of patients [Singhal et al., 1997; Hotte et al., 2002]. Also, expression levels of OPN in breast cancer tissues were observed to be proportional to the levels of malignancy of the tumor [Tuck et al., 1998; Thalmann et al., 1999]. It was further found in experimental models that overexpression of OPN in cancer cells increases their ability to metastasize in animal models [Tuck et al., 1999]. These features suggested that OPN is involved in the mechanisms of metastasis of the cancer cells in both clinical cases and experimental models.

As OPN is one of the most abundant noncollagenous proteins in bone, we previously investigated the possible correlation between the present OPN in host mice to the frequency of the experimental metastasis of a mouse B16 melanoma cells. Our results indicated that in addition to the observations that OPN in 'tumor cells' plays a role in metastasis, OPN in the 'host' also enhances metastasis of B16 melanoma cells in several organs in the body including bone [Nemoto et al., 2001]. In bone tissue, OPN could be involved in the invasion of these cells into the matrix [Ohyama et al., 2004].

However, OPN is also present in the circulation, and the role of OPN in the circulation per se with respect to its function in supporting migration of tumor cells is not well understood. Here, we investigated the effects of OPN in the serum on the migration of B16 cells in vitro by using the serum obtained from either wild-type or OPN-deficient mice. Our data indicated that in the context of the serum, OPN promoted the migration of B16 cells in vitro.

MATERIALS AND METHODS

Animals

OPN-deficient mice with a C57B16/129sv F2 background were produced as described previously [Rittling et al., 1998]. OPN-deficient mice and wild-type mice from 28 to 40 weeks of age were used. All animal experimental designs and procedures were approved by the Animal Ethic Committee of Tokyo Medical and Dental University.

Migration Assay

Cell migration assays were conducted using modified Boyden chamber, Transwell® cell culture plates (0.33 cm^2/well; Corning), according to the standard procedure as described previously [Das et al., 2003]. B16 melanoma cells were plated at density of 5×10^3 cells/well in serum free medium in top chambers and bottom wells were filled with medium supplemented with serum (2.5% v/v) obtained from wild-type mice or OPN-deficient mice. In other experiments, we used similar transwell system containing either albumin (5 μg/ml or 50 mg/ml) (control) or recombinant mouse OPN (5 μg/ml) (rmOPN) (Immuno-Biological Laboratories Co., Ltd.). For inhibitor experiments, MEK inhibitor, PD98059 (MAPK/ERK kinase inhibitor; Promega, Tokyo, Japan) was examined. The inhibitor stock solution was prepared at 2.5 mM in DMSO and used at 50 μM as final concentration in the bottom well (0.2% v/v). The concentration of DMSO in the medium was about 28 mM (0.2% v/v). After 24 or 48 h, migrated cells on the reverse side of the filters were fixed and stained with crystal violet. These migrated cells on the filters were counted under microscope.

Serum Preparation

Blood was obtained from wild-type and OPN-deficient mice through the cardiac puncture. Blood was allowed to clot for 30 min at the room temperature before centrifugation. Blood was then centrifuged at 1,500 rpm for 10 min and the resulting supernatant was taken. To inactive the serum, serum was incubated for 15 min with the 37°C water bath. Then, raise the temperature to 55°C in the water bath, the serum was incubated for 30 min. Serum was removed from water bath and cooled at room temperature. Serum was stored at 4 or −20°C.

Transfection

The expression vector was constructed by inserting 1.35 kb full length of mouse OPN into the pTriEx-1.1 Neo Vector. B16 melanoma cells (5×10^5 cells/well in ϕ10 cm culture dish) were transfected with OPN expression vector (6 μg) or an empty vector as a control using the FuGene6 transfection reagent according to the manufactures protocol (Roche).

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using iQ SYBR Green Supermix by iQ5 system (Bio-Rad). The quantification was conducted as described previously [Pfaffl, 2001].

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) when the examined experimental groups exceeded three groups. Tukey's multiple comparison test (Figs. 2 and 3) or Dunnett's test (Fig. 4) were applied as post-hoc test. Other experiments were analyzed by Student's *t*-test with Bonferroni's approximation. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

We first examined the expression levels of OPN mRNA in B16 F1 and F10 cells which metastasize at relatively low and high frequency, respectively. As shown in Figure 1, OPN expression levels in these two types of B16 lines were undetectable at least within the limit of the PCR sensitivity. OPN mRNA was also undetectable in the parental B16 cell line we used previously (Fig. 1a,b) [Nemoto et al., 2001] indicating that OPN is undetectable regardless of the levels of metastasis in B16 cells. As control, MC3T3-E1 cells exhibited OPN expression as known before [Nemoto et al., 2001]. Therefore, in the following experiments, we chose parental B16 cell line, which was used in our experimental metastasis assay previously [Nemoto et al., 2001; Ohyama et al., 2004].

Based on the fact that B16 cells did not express detectable levels of OPN mRNA, we tested whether OPN in the serum but not in these cells would affect the migration in transwell chambers. For comparison, sera from wild-type and OPN-deficient mice were used. In this assay, sera were placed at a volume of 2.5% v/v in total medium at the bottom well and no serum

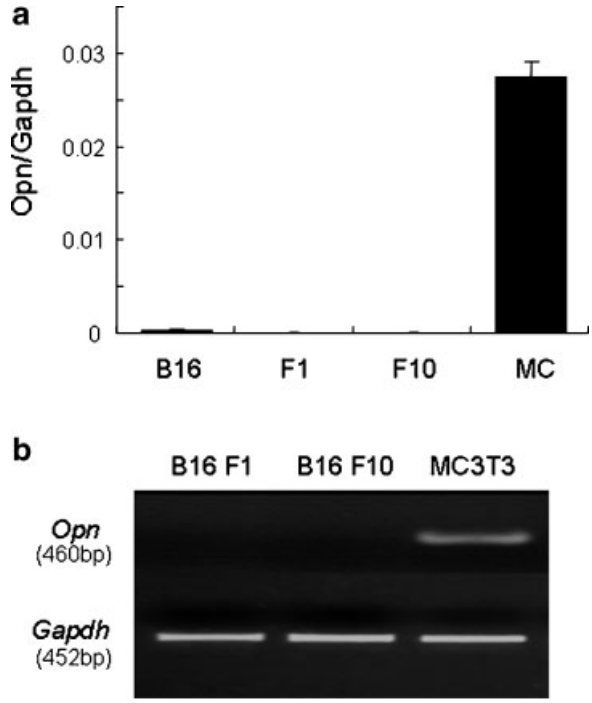

Fig. 1. OPN was not expressed in B16 melanoma cells. RNA was prepared from B16 F1, B16F10 and MC3T3-E1 cells. RT-PCR was performed as described in Materials and Methods. **a**: Real-time PCR analysis of mRNA level of OPN in B16, B16F1, B16F10, and MC3T3-E1 cells. **b**: RT-PCR analysis of mRNA levels of OPN. OPN was not expressed in B16 melanoma cells. Data are expressed as means and standard deviations (SDs).

was included in the media of top well. Wild-type serum promoted the migration of B16 melanoma cells over 20 times more than the serum free control (Fig. 2a,b). In the sera of OPN-deficient mice, the migration activity of B16 cells was reduced about 1/3 of that in the wild-type serum. These data indicated that the serum of OPN-deficient mice exhibited significantly less promotion of B16 cell migration (Fig. 2b).

The sera of wild-type and OPN-deficient mice contain numerous proteinaceous or nonproteinaceous factors whose presence or whose levels could be affected by the presence or absence of OPN. Therefore, indirect effects rather than direct OPN effects may have affected in the serum assay shown in Figure 2. In addition, OPN in serum could be modified posttranslationaly by phosphorylation or glycosylation. We therefore asked whether recombinant OPN protein per se could affect the degree of migration of B16 cells.

When recombinant mouse OPN was placed in the serum free medium in the bottom well, B16

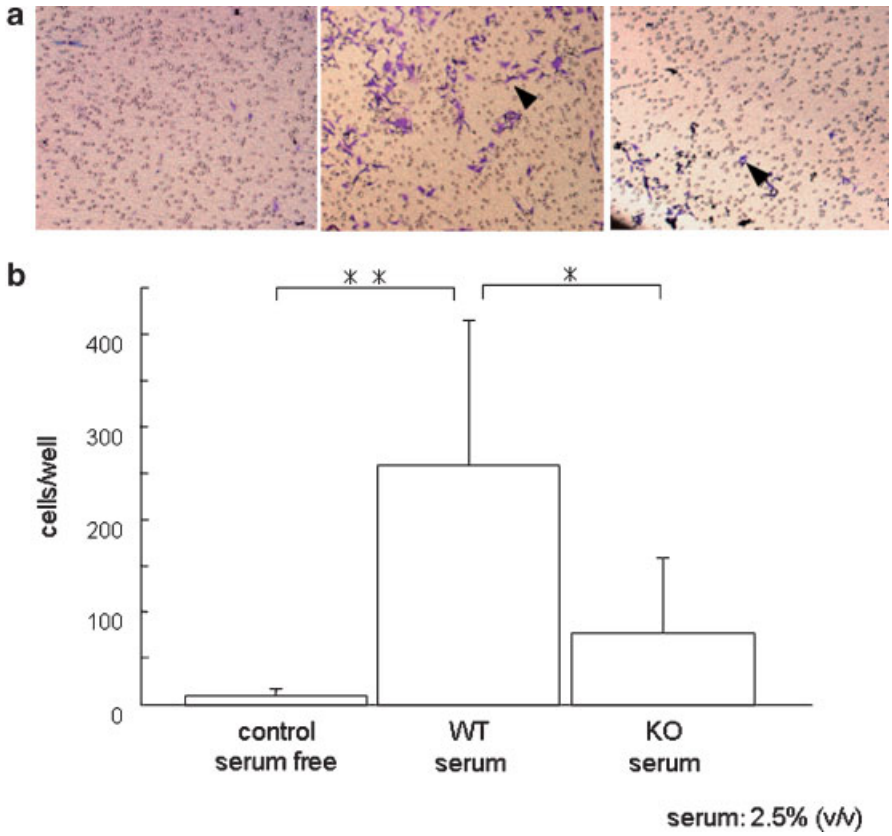

Fig. 2. Cell migration was suppressed in the absence of OPN. The migration assay was performed using B16 melanoma cells. Sera (2.5% v/v) from wild-type mice or OPN-deficient mice (KO) were added in the lower chamber. After 48 h, the migrated cells were stained by crystal violet. **a**: Arrow heads indicated migrated cells. **b**: The number of migrated cells. Cell migration was suppressed in the absence of OPN. Data are expressed as means and SDs. Asterisk and double asterisk indicates *P*-value less than 5% and 1% ($P < 0.05$; ANOVA, Tukey's test).

migration through the membrane was increased about twofold more than the control albumin (5 μg/ml) group (Fig. 3). We conducted migration assay using 50 mg/ml albumin in medium. The data shown below indicated that 50 mg/ml albumin in the bottom well did not change cell migration and the results were similar to those using 5 μg/ml albumin (data not shown). Thus, recombinant OPN protein could promote per se migration of B16 cells in this setting. These data supported the idea that reduction of B16 cell migration in the serum of OPN-deficient mice would be at least in part due to absence of OPN protein.

Since recombinant OPN has been shown to activate migration of B16 melanoma cells through its binding to αvβ3 integrin followed by the activation of MEK and ERK [Das et al., 2004, 2005], we asked whether the migration

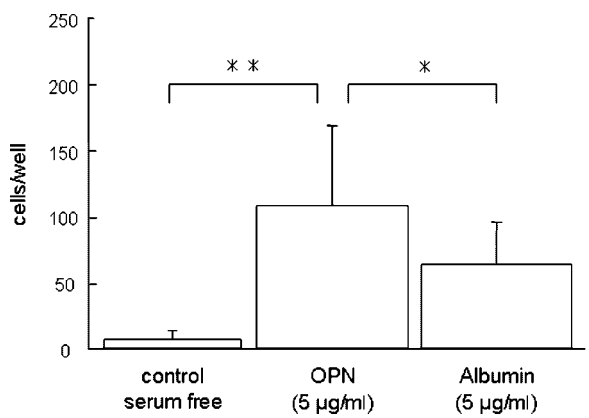

Fig. 3. Recombinant mouse OPN induced B16 cell migration. Recombinant mouse OPN (5 μg/ml) was added in the lower chamber. Albumin (5 μg/ml) was added as a control. Recombinant mouse OPN induced B16 cell migration. Asterisk and double asterisk indicates *P*-value less than 5% and 1% ($p < 0.05$; ANOVA, Tukey's test).

induced by the serum of wild-type and OPN-deficient mice would respond differentially to inhibitors of the MAP kinase pathway. The presence of the inhibitor PD98059 in the culture media reduced the B16 cell migration induced by the wild-type serum in this transmigration assay (Fig. 4a). In contrast, transmigration activities of B16 melanoma cells induced by serum of OPN-deficient mice were not significantly altered by the presence of PD98059 (Fig. 4b). DMSO was served as vehicle control. These data further indicated that at least one of the signaling events underlying the migration of OPN in the wild-type serum would be through the MAP kinase signaling pathways and such activity was significantly less in the serum from OPN-deficient mice.

To test the role of endogenous OPN expressed in the B16 cells, we overexpressed OPN in the B16 cells. As shown in Figure 5a, overexpression (O/E) resulted in increased levels of OPN mRNA expression, which were several fold more than those in MC3T3-E1 cells. The migration of the OPN-O/E-B16 cells was slightly enhanced as compared to vector-transfected B16 cell when tested in transwell chamber containing wild-type serum (Fig. 5b). In contrast, OPN-O/E-B16 cells migrated about two-folds more than vector-transfected B16 cells when tested in the transwell chamber containing serum from OPN-deficient mice (Fig. 5c).

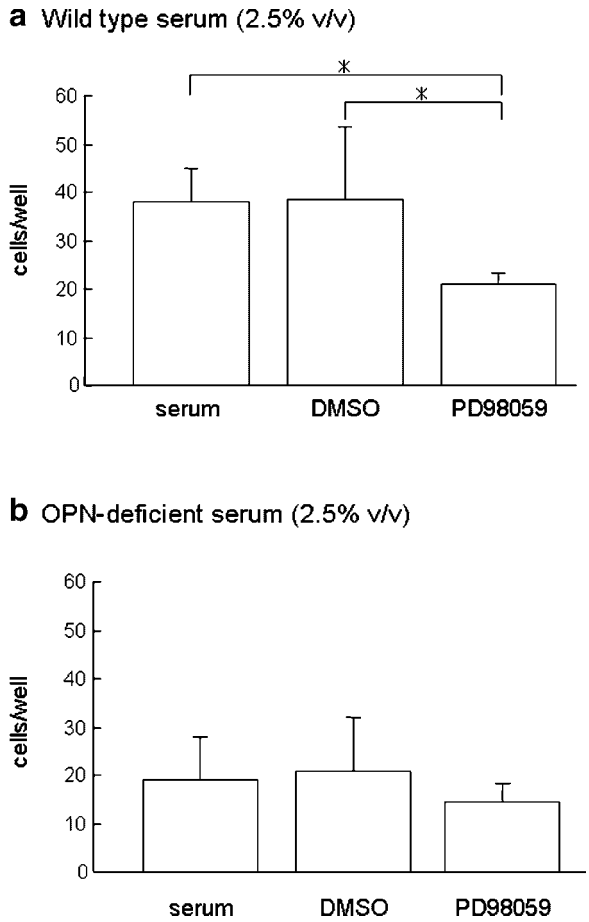

Fig. 4. Wild-type serum-induced B16 cell migration was blocked by MEK inhibitor. **a**: Serum (2.5% v/v) from wild-type mice were added in the lower chamber with PD98059 (50 μM). DMSO (0.2% v/v), 28 mM was added as a control. **b**: Serum (2.5% v/v) from OPN-deficient mice (OPN KO) were added in the lower chamber with PD98059 (50 μM). Stimulatory effects of OPN + serum on B16 migration were blocked in the presence of inhibitor of ERK1/2 upstream kinase MEK1/2. Asterisk indicates *P*-values less than 5% ($p < 0.05$; ANOVA, Dunnett's test).

DISCUSSION

Our data indicated that OPN in mouse serum can affect in vitro migration of B16 cells. OPN-deficiency in the host was shown to reduce efficiency of metastasis of B16 cells which were injected either through left ventricle of heart or through the femoral vein [Nemoto et al., 2001]. Previous data indicated that host OPN would be involved in the metastatic efficiency of B16 cells. However, since OPN could be involved in either the migration through the vessels and survival of the cells in the blood stream and attachment and migration during the extravasations of the tumors and/or the migration in the extravascular environment, the role of OPN in circulation in each of these events was not certain in the previous experiments. Since OPN levels in the serum has been reported to correlate with the metastatic activity of tumors in human patients, it was possible that the serum OPN in circulation may be involved in the regulation of the B16 cells migration in vivo. Our current data supported this notion.

Since we simply compared the levels of migration between the sera prepared from wild-type and OPN-deficient mice, it is still possible that other factors, whose levels could be altered by OPN deficiency, may influence the migration of B16 cells. Furthermore, OPN form in the plasma would differ than OPN form in serum as it could be cleaved during the preparation of serum. Although we do not completely exclude such possibilities, recombinant OPN protein stimulated migration of the same B16 cells more than albumin. Thus, at

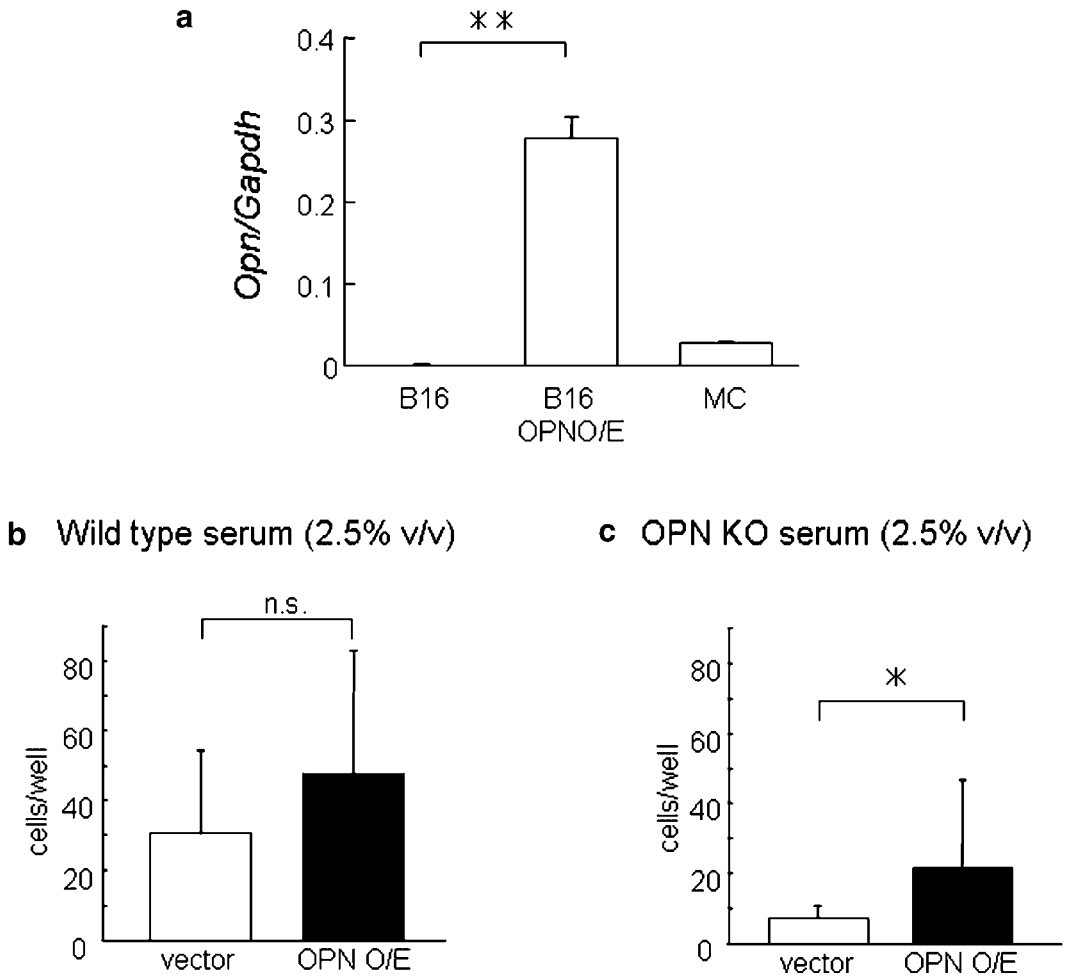

Fig. 5. Effects of OPN O/E on B16 cells on B16 cell migration. **a**: B16 cells were transfected with OPN or control vector. Real-time PCR was performed. **b**,**c**: Migration assay was performed in transfected cells with either control vector or OPN expression vector. Either serum from wild-type mice or OPN-deficient mice (OPN KO) were added in lower chamber. O/E of OPN tended to increase B16 cell migration. n.s., non significant. Asterisk and double asterisk indicates *P*-value less than 5% and 1% ($P < 0.05$; Student's *t*-test).

least these B16 cells are capable of recognizing recombinant OPN signals. Previous experiments suggested that B16F10 cells, which are highly metastatic, would respond to OPN signaling. However, this experiment utilized purified OPN. Therefore, it was not sure whether OPN present in the context of serum would work similarly. Since OPN activity was reported to be inhibited by the binding of the factors present in the serum such as factor H [Fedarko et al., 2000; Jain et al., 2002], regulation of OPN signaling in terms of its activity to stimulate cell migration would have been controlled by several layers of a regulatory mechanisms possibly present in the serum. It was shown that some of the OPN receptors would differentially respond to phosphorylated OPN and nonphosphorylated OPN [Chambers et al., 1992]. Phosphorylation could be increased based on the intracellular signaling of the particular cells such as tumors. This phosphorylation pathway could modulate the function of OPN in terms of its regulation of the migration.

In our previous models of a experimental metastasis, B16 cells were injected either into the left ventricle or into the femoral vein to go into the veinous circulation [Nemoto et al., 2001]. In both of the arterial and venous experimental metastasis models, B16 cells were exposed to the OPN in the blood of the host mice. Therefore, our observations in this paper that serum OPN could regulation of migrate the cells would be related to the possibility that OPN in blood may be involved in the migration of the

cells inside the vessels to attach inner the vascular walls or to go through the vascular walls in the process of extravasations. As mentioned previously, tumor cell metastasis requires several steps from the detachment from the primary tumors to attachment to the distant metastatic sites. Whether OPN is involved in these several steps other than the migration events examined in this paper is still to be elucidated.

Our observations revealed that serum OPN regulates the migratory activities of B16 melanoma cells in vitro. This model would be useful for the future analysis to examine possible associating factors which may positively or negatively control the migration of the tumor cells in association with OPN, and to establish bases for contemplating model therapies for metastasis.

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