Neutralizing Monoclonal Antibody to Periostin Inhibits Ovarian Tumor Growth and Metastasis

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Abstract

Periostin (PN), an extracellular matrix protein, is reported to be overexpressed in a variety of human cancers and its functions appear to be linked to tumor metastasis. Our previous results show that engineered PN overexpression promotes ovarian tumor growth and dissemination in vivo. In the present study, we developed a neutralizing monoclonal antibody (mAb) to PN, named MZ-1, and investigated its effects on human ovarian tumor growth and metastasis. Our in vivo studies showed significant growth inhibition by MZ-1 on both subcutaneous (sc) and intraperitoneal (ip) tumors derived from the PN-expressing ovarian cancer cell line A2780. In addition, MZ-1 treatment led to a reduction of the metastatic potential of these A2780 ip tumors. The in vivo antitumor effects of MZ-1 were linked to its specific inhibition of anchorage-independent growth and survival of PN-expressing cells, as well as its neutralizing effects on PN-induced cancer cell migration and invasion. The data suggests that blocking PN expression may be a novel approach for treating the subset of invasive ovarian tumors that overexpress PN protein.
Introduction

Ovarian cancer is one of most lethal cancers in women and causes around 14,000 death annually in the U.S.(1). One major challenge of ovarian cancer treatment is the lack of reliable early diagnostic methods for this asymptomatic disease. As the result, a majority of patients are diagnosed with advanced disease characterized by widespread tumor metastasis in the peritoneal cavity (2). Despite high initial response rates to aggressive primary chemotherapy, this group of patients frequently develop drug resistance resulting in poor prognosis and the 5-year survival rate has not risen above 20-25% (3). Clearly, there exists a need for more effective therapeutic strategies for ovarian cancer treatment.

Biologically targeted therapy has become a main stream strategy for cancer treatment in the past two decades, due to an improved understanding of the genetic alterations underlying tumor progression (4, 5). Development of new treatments with trastuzumab (Herceptin) and bevacizumab (Avastin), two mAbs that selectively target Her2-neu and VEGF gene products have shown significant antitumor efficacy in clinical trials(6, 7). Compared to traditional treatment options that come with a price of toxicity to normal tissues, targeted therapy is more specific and less toxic thus more effective. In this regard, design of novel therapeutic agents against molecular targets that are essential for ovarian cancer metastasis represents an important step toward discovering more effective and rational therapy for this deadly disease.

Periostin (PN) has attracted increasing attention because it is frequently overexpressed in a variety of epithelial carcinomas including ovarian cancer and its function is implicated
in tumor metastatic growth. Analyses of clinical samples have revealed a correlation between PN overexpression and liver metastasis of colon cancer (8), distant metastasis of melanomas (9), lymph node metastasis of oral cancer (10), head and neck cancer (11), and cholangiocarcinoma (12). Moreover, PN overexpression or elevated PN serum levels have been demonstrated to be associated with other clinical parameters such as later stages, higher grade, and worse survival outcomes (13-20). Furthermore, the pro-metastatic role of PN also has been confirmed in experimental animal models derived from engineered PN-expressing cancer cells (8, 11, 21). Evidence indicates the modulation of this molecule in Epithelial-Mesenchymal Transition (EMT) that is a critical initiation step of the tumor metastasis cascade (22). As an adhesion molecule and chemoattractant, purified PN protein promotes cancer cell adhesion, migration and invasion with all 3 functions important for tumor metastasis (21, 23, 24). Exogeneous PN expression also results in increased cell invasive capabilities (11, 25); while silencing the PN gene by small interfering RNA suppresses cell migration and invasion phenotypes (25). It also has been reported that PN can promote cancer cell survival through activation of the PI3-k/Akt signaling pathway (8, 21, 23).

Our previous studies have shown that PN is frequently overexpressed in primary ovarian tumors (21, 24) and exogeneous expression of PN in ovarian cancer cells promotes tumor growth and metastasis in experimental animal models (21). These data suggest PN may be a promising target for anti-metastatic therapy of ovarian cancer. To test this more directly, we produced a specific anti-PN mAb, named MZ-1, that blocks the biological functions of PN, and now report its effect on human ovarian tumors grown in
immunodeficient mice. In addition, we investigated the \textit{in vitro} effects of MZ-1 mAb on ovarian cancer cell proliferation, survival, migration, and invasion.
Materials and Methods

**Materials.** Recombinant PN-His protein expressed in human embryonic kidney 293T cells was prepared as described previously(21). Recombinant PN-Gst and a non-related KJ-Gst expressed in bacterial BL21 cells were purified using BugBuster GST-Bind Purification Kit (Novagen, Madison, WI). Recombinant PN protein expressed in murine myeloma cells and human βig-H3 were purchased from R & D system (Minneapolis, MN). Fibronectin, vitronectin, laminin, collagen I and IV, keyhole limpet hemocyanin (KLH), complete adjuvant, Freund's incomplete adjuvant and anti-β-actin antibody were purchased from Sigma (St. Louis, MO). Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Isotype-matching control antibody mouse IgG was purchased from Innovative Research Co. (Novi, MI).

**Cell culture.** Human ovarian adenocarcinoma cell lines A2780, OAW-42, and SKOV-3 were obtained from the European Collection of Cell Cultures (ECACC). Human ovarian adenocarcinoma cell lines TOV-112D, ES2, and mouse myeloma SP 2/0 cell line were obtained from the American Type Culture Collections (ATCC). All cell lines were maintained in cell culture medium recommended by the company and incubated in a humidified 5% CO₂ atmosphere at 37°C. To authenticate cancer cell lines, mitochondrial DNA genotyping was performed. Furthermore, PN expression levels in the culture supernatants of these cancer cells were examined intermittently during the study by a PN ELISA.
Production of anti-PN mAbs. Recombinant PN-His protein secreted from 293T cells was purified and conjugated to KLH. The conjugated protein was used to immunize Balb/c mice three times over two month period with 20 μg PN per injection. Serum samples were collected after the second and third immunizations and screened by enzyme-linked immunosorbent assay (ELISA) for the presence of anti-PN antibodies. Spleen cells from mice that displayed high serum titers of the antibody were used to generate hybridomas via polyethylene glycol-based cell fusion with SP2/0 myeloma cells. Hybrid cells were selected in HAT culture media in 96-well plates and tested for secretion of anti-PN mAbs by ELISA before hybridomas from positive wells were cloned using the limiting dilution technique. Monoclonal antibodies were purified with protein A and finally dissolved in 20 mM sodium phosphate with 150 mM sodium chloride (pH 7.2). The mAbs were >95% pure by HPLC and endotoxin levels were < 20 EU/mg.

Capture ELISA. A capture ELISA was developed to screen anti-PN antibody titers in serum of immunized mice and those in culture supernatants of hybridomas. Briefly, 96-well Maxi-Sorp plates (Nunc, Rochester, NY) were coated overnight at 4°C with 5μg/mL of PN-His (293T cell), PN-Gst, or a non-related protein KJ-Gst. The wells were washed 3 times and blocked with PBS buffer containing 0.5% BSA and 1% casein (Sigma, St. Louis, MO) 2hr at room temperature( 22°C). 100μL of diluted serum or hybridoma culture supernatants were added to the plates and incubated for 2hr at 22°C. After washing, the plates were incubated with biotinylated-anti-mouse IgG (Pierce Chemical Company, Rockford, IL) for 1hr, followed by 1hr incubation with HRP-conjugated streptavidin (R&D System, Minneapolis, MN). After washing, substrate solution (R&D
System, Minneapolis, MN) was added for color development. Plates were read in an ELISA plate reader (Molecular Devices, Sunnyvale, CA) at 450nm.

To analyze mAb isotyping, the above capture ELISA was modified by employing isotype-specific antibodies from SBA Clonotyping System/HRP (SouthernBiotech, Birmingham, AL) as the secondary antibodies. To analyze the specificity of mAbs, 5μg/mL of recombinant PNs, βig-H3, or other adhesion molecules were used as coating antigens.

**PN ELISA.** PN ELISA was performed as described previously(21). Briefly, 96-well Maxi-Sorp plate was pre-coated with 8μg/mL of a PN polyclonal antibody at 4°C overnight and blocked for 2hr at 22°C. PN standard protein (PN-His from 293T cells), along with culture supernatants of ovarian cancer cells were diluted serially and incubated in plates for 2hr at 22°C. Plates were washed five times and then incubated with a biotinylated-anti-PN mAb (100μL/well) for 1hr.

**Animal experiments.** 6-8 week old female SCID/beige mice were obtained from the UCLA DLAM breeding facility and housed in laminar flow cabinets. Procedures involving animals and their care were conducted in conformity with the guidelines established by the American Association for Laboratory Animal Care (AALAC). Mice were implanted subcutaneously (sc) (A2780 or TOV-112D cells) or intraperitoneally (ip)(A2780) with 1 million cells. For sc experiments, mice with established tumors were randomly divided into 2 groups and treated with mAb MZ-1(20mg/kg) or an isotype-matching control antibody(20mg/kg) twice weekly. The tumor was measured in two
dimensions, and the volume was calculated using the formula, width$^2 \times$ length/2. For ip experiments, antibody treatments started 3 days after tumor cell injection to give the tumor cells time to attach to the inner side of the abdominal wall and the surfaces of the ip organs. To monitor the development of peritoneal carcinomatosis, the body weights of the mice were routinely measured. In all experiments, the mice were sacrificed and necropsied when the first mouse in either control or treatment group became moribund. The following were recorded after the necropsy: (a) presence of ascites, (b) cumulative weight of all excised tumor nodules, (c) the total number of tumor nodules and (d) tumor dissemination to other organs. Tumor burden was defined as cumulative weight of tumor tissues.

**Anchorage-independent growth assay.** Five or ten thousands ovarian cancer cells were resuspended in 0.3 mL 0.3% Noble Agar (Difco laboratories, Detroit, MI, diluted with corresponding culture medium) in the presence of MZ-1 or control antibody at a final concentration of 100μg/mL. Embedded cell mixtures were overlaid on 0.5 ml of 0.7% Noble Agar in 48-well plates, and a top layer of 0.5 mL of cell culture medium was added to each well to prevent evaporation. Plates were maintained at 37°C in 5% CO$_2$ for 2-3 weeks. Colonies composed of more than or equal 50 cells were counted manually under the microscope. All experiments were carried out in triplicate three times.

**In vitro apoptosis assay.** Cells were grown in 24-well plates in serum-free medium (SFM) plus 1%BSA overnight to reach 50-60% confluency. 20μg/mL of MZ-1 or control antibody was added into the wells. Cell apoptosis was analyzed two and four
days later by using Annexin V-FITC Apoptosis Detection Kit (MBL, Nagoya, Japan).
The cell flow cytometry was carried on Cell Lab Quanta SC (Beckman Coulter Inc.,
Fullerton, CA).

**Anoikis assay.** To measure cell anoikis which is a kind of apoptosis induced upon cell
detachment, cells were grown in ultra-low attachment plates (Corning Inc., Corning, NY)
in SFM containing 20μg/mL of MZ-1 or control antibody for four days. The percentage
of dead cells was determined by trypan blue exclusion method using a hemocytometer.

**In vitro migration/invasion assay.** Cell migration and invasion assays were performed
as previously described(21). For migration, one hundred thousand tumor cells in 100
μL SFM were added to the culture plate inner chamber and allowed to migrate overnight
in a 37°C incubator. PN (or BSA, 1μg/mL), or MZ-1 (or control antibody, 20μg/mL)
was added to an outerchamber. Cell migration results were quantitated in terms of the
average number of cells/microscopic field at a 20-fold magnification. In all cases, at least
6 separate fields were counted per membrane, and all experiments were carried out in
triplicate two times. For invasion, cells were seeded into inner chambers in which
membranes were pre-coated with growth factor reduced (GFR) Matrigel (BD
Biosciences, San Jose, CA) and the cells reaching the bottom chambers were quantitated
by direct visual cell count after 24 hr, as described previously (26).
Results

Characterization of anti-PN mAb MZ-1. A panel of candidate anti-PN mAbs (13) was produced by hybridomas derived from mice immunized with His-tagged PN protein, a recombinant form of human PN secreted by mammalian 293T cells. All the mAbs including MZ-1, were able to detect PN-His (from 293T cell) and PN-Gst (from bacteria) by ELISA (data not shown) as well as by Western blotting and represented by mAb MZ-1 as shown in Fig. 1a.

MZ-1 was then tested for blocking of PN-induced cell adhesion and migration (Fig. 1b and 1c). As an adhesion molecule, PN efficiently induced adhesion and migration of ovarian cancer cells SKOV-3. Addition of an isotype-matching antibody did not have any influence, while adding MZ-1 significantly reduced SKOV-3 cell adhesion and migration by 45% (p=0.004) and 80%(p<0.001), respectively, with magnitudes comparable to those of an anti-PN polyclonal antibody.

MZ-1 belongs to the IgG1 isotype. A study utilizing BIAcore demonstrated that the dissociation constants of MZ-1 to our own and commercially purchased recombinant PN proteins were 1.9x10^{-8} and 2.0x10^{-8}, respectively, confirming that this mAb has high affinity for PN. A capture ELISA was employed to determine the antigen specificity of MZ-1 (Fig. 1d). MZ-1 was capable of recognizing all three recombinant PN proteins produced in human cells 293T, murine myeloma cells and insect cells Sf9. However, it was unable to recognize human βig-h3 which is a homologue of PN in the fascilin-I
protein family (27). In addition, MZ-1 did not show any crossreactivity with other adhesion molecules including fibronectin, vitronectin, laminin, collagen I and IV.

**Establishment of endogenous PN-expressing ovarian tumor models.** Through cDNA microarray and PN ELISA analyses, 2 out of 33 ovarian cancer cell lines, A2780 and TOV-112D, were identified that expressed relatively larger amount of PN mRNA and protein (data not shown). PN expression in the culture supernatants of these two lines and three other ovarian cancer cell lines were further examined by PN ELISA (Table 1). A2780 cells expressed significant amounts of PN, reaching the concentration of 271.6ng/mL after 6 days in culture. TOV-112D cells produced a moderate amount of PN, while the other three cell lines, ES2, OAW-42 and SKOV-3, expressed PN at very low or non-detectable levels. A2780 and TOV-112 both established subcutaneous (sc) and intraperitoneal (ip) tumors in severe combined immunodeficiency (SCID) mice with PN expression in xenografts confirmed by PN western blot and immunohistochemistry (Supplemental Fig. S1). Thus, these two PN-expressing cells, along with 3 non-PN expressing lines, were used to provide in vitro and in vivo models for evaluating the anti-cancer effects of mAb MZ-1.

**Anti-PN mAb MZ-1 inhibits ovarian tumor growth and metastasis in mice.** We first examined the in vivo effect of MZ-1 using a sc tumor model derived from A2780 cells. Treatment with MZ-1 was initiated when tumors were already established one week after cell implantation. When compared to control antibody, MZ-1 treatment significantly reduced tumor growth by 53% (p=0.03) after 2 weeks (Fig. 2a). MZ-1 treatment also
led to 30% growth inhibition on sc tumors derived from a second PN-expressing cell line, TOV-112D, but the effect was not statistically significant (data not shown). We next investigated the effects of MZ-1 on tumor metastatic growth by using an experimental metastasis model derived from A2780 cells. One million cells were implanted ip into the SCID mice and treatment was started 3 days later with ip injections of either mAb MZ-1 or a control antibody twice weekly. The study was halted when the first mouse of the control group became moribund with all of the mice sacrificed and examined via necropsy as described in the Materials and Methods. Necropsy revealed a similar tumor distribution for all mice implanted with A2780 cells (Fig. 2b), including an extensive tumor mass beneath the liver and omentum as well as small tumor nodules scattered in pelvic areas, on abdominal walls, intestines, and diaphragm. However, noticeably less tumor foci were observed in these areas of mice treated with MZ-1 compared to those treated with control antibody. In addition, at the end of the experiment, ascites were present in four out of nine control mice, while absent from all nine MZ-1-treated mice (Fig.2d). Compared to control antibody, MZ-1 treatment significantly reduced tumor burden and nodule number by 51% (p= 0.01) and 58% (p=0.0002), respectively, suggesting an efficient inhibitory effect of MZ-1 on A2780 ip tumor growth. We further examined the PN expression in A2780 ip xenografts by western blot. PN levels were much lower in xenografts treated with MZ-1 in comparison to those treated with control antibody (Supplemental Fig. S2). No side effects or severe toxicity of MZ-1 treatment were observed during the whole course of treatment.
MZ-1 inhibits proliferation of ovarian cancer cells in soft agar. We first examined the effect of MZ-1 on proliferation of A2780 cells growing as a monolayer on cell culture plates. Compared to medium and antibody controls, four days of treatment with MZ-1 (40μg/mL) led to a mild (5-10%), but not statistically significant reduction of proliferation over a period of six days (Fig. 3a). The inhibition was independent of serum concentrations. Similar results were obtained when growing A2780 cells on ultra-low attachment plates (Fig. 3b). We then examined the influence of mAb MZ-1 on A2780 cell proliferation in soft agar. As shown in Fig. 3c (left column), treatment with MZ-1 (100μg/ml in the middle layer) significantly suppressed the colony formation when compared to medium control (52% reduction, p<0.001) and antibody control (44% reduction, p<0.001). MZ-1 also showed significant inhibitory effects (36% reduction, p<0.001 vs. control antibody) on another PN-expressing cell, TOV-112D. However, MZ-1 did not show any effects on ES2 and SKOV-3 (Fig. 3c), two cell lines with low or non-PN-expression levels. Another PN-negative cell line OAW-42 did not grow in soft agar in our experiments.

MZ-1 induces apoptosis of ovarian cancer cells in vitro. We employed two approaches to determine the effects of mAb MZ-1 on ovarian tumor cell survival. First, cells were grown on plastic surfaces and exposed to MZ-1 (40μg/mL) for four days in SFM. Tumor cell apoptosis, including both early and late apoptosis, was evaluated by the Annex V/ PI (propidium iodide) system. As shown in Fig. 4a, compared to antibody control, four days of MZ-1 treatment increased A2780 cell apoptosis by 69% (p=0.01). The effect of MZ-1 on TOV-112D cells was much weaker than that on A2780 cells and
no effect was seen on the other three ovarian tumor cell lines. Second, cells were grown on ultra-low-attachment plates and exposed to MZ-1 in the absence of serum for four days. Tumor cells anoikis, a form of apoptosis that is caused by cell detachment from the ECM (28), was assessed by trypan blue exclusion method. In contrast to control antibody, MZ-1 treatment efficiently induced the anoikis of A2780 cells by 2.3 fold (p=0.04). Similar to the apoptosis seen on plastic surfaces, treatment with MZ-1 had a weaker effect on TOV-112 cells and none on other cell lines (Fig. 4b). It has been reported that PN promotes cell survival through activation of Akt/PKB signaling pathway in colon(8), pancreatic(23) and non-small lung cancer cells(29). We thus examined activated Akt status in response to MZ-1 treatment in ovarian cancer cells. Compared to control antibody, MZ-1 markedly reduced the phosphorylation of Akt1 on serine 473 in two PN-expressing cells A2780 and TOV-112D, but not in non-PN-expressing cell SKOV-3 (Fig.4c).

**MZ-1 inhibits PN-mediated ovarian cancer cell migration and invasion in vitro.**

We investigated effects of PN and anti-PN mAb MZ-1 on the metastatic potential of A2780 and TOV-112D cells by in vitro migration and Matrigel invasion assays. As shown in Fig. 4a, PN efficiently elicited a substantial increase in migration of A2780 (2.6 fold) and TOV-112D cells (1.6 fold) when compared to a negative control using BSA. Addition of an isotype-matching control antibody did not affect PN-induced cell migration, while addition of MZ-1 significantly mitigated the effect of PN by 45.7% (p<0.001) and 57.9% (p<0.001) for A2780 and TOV-112D cells, respectively. Specific invasion towards PN as a chemoattractant was 1.6-fold (A2780) to 2.1-fold (TOV-112D).
greater than for BSA. In contrast to control antibody that showed no effect, MZ-1 significantly suppressed PN-induced invasion of A2780 cells ($\approx 44\%$ reduction) and TOV-112D cells ($\approx 47\%$ reduction) (Fig. 5b).
Discussion

Although contradictory conclusions have been reported regarding the functions of PN in progression of human malignancies, a majority of studies suggest PN plays an oncogene-like role through promoting tumor angiogenesis and metastasis. Our previous work using PN overexpression models have found a pro-metastatic role of PN in ovarian tumor progression(21). In the present study, we have revisited this issue by investigating the effects of our newly-developed specific anti-PN mAb MZ-1 on ovarian tumor cell growth both in vivo and in vitro. The in vivo data are particularly important, as no similar results have been reported previously for ovarian cancers nor any other PN-overexpressing human tumors. In addition, our study has relevance in evaluating this mAb as a therapeutic approach for ovarian cancer, particularly given the ineffectiveness of current clinical treatment protocols.

Exogenous PN expression models have been previously employed to demonstrate the in vivo pro-metastatic role of PN in tumorigenesis (8, 11, 21). Such models may not be suitable for evaluating the effects of PN mAbs. Instead, a more appropriate model appears to require tumor cells or xenografts with endogenous PN expression. Although our previous studies did not detect PN expression in established ovarian cancer cell lines by western blot analysis, the current study employed a newly-developed and more sensitive approach, PN ELISA, to examine PN expression in culture medium of 33 established ovarian cancer cell lines. A2780, an adenocarcinoma cell line, was found to secret the highest amount of PN into the culture medium which was comparable to the physical PN concentration in malignant ascites from patients. TOV-112D, an epithelial
ovarian carcinoma line, produced lesser levels of PN, whereas all other lines examined expressed PN at very low or non-detectable levels. Furthermore, both sc and ip implantation of A2780 or TOV-112D cells established PN-expressing tumor xenografts in SCID mice. In addition, the mice implanted ip with these cells showed metastatic patterns similar to that seen in ovarian cancer patients and may provide an in vivo model to evaluate anti-PN neutralizing properties of mAb MZ-1 in tumor progression and metastasis.

We first tested the effects of MZ-1 in sc tumor models. Both PN cell lines, A2780 and TOV-11D, grow very aggressively in mice, establishing visible sc tumors in one week and reaching the maximum volume allowed by the institute guideline in 2-3 weeks. As a result, the treatment could be only given three to four times before we terminated these experiments. Despite this narrow therapy time window, MZ-1 treatment showed over 50% and 30% growth inhibition of A2780 and TOV-112D tumors, respectively. The lesser effect of MZ-1 on TOV-112D tumors might be due to its much weaker inhibition on the survival of TOV-112 cells that was demonstrated by our in vitro apoptosis assay.

We then evaluated the anti-metastatic effect of MZ-1 in an ip tumor model derived from A2780 cells. Similar to the sc experiments, MZ-1 treatment reduced tumor burden by 51% in comparison to control antibody. Moreover, mice treated with MZ-1 exhibited many less metastatic foci in the peritoneal cavity than those treated with control antibody, which is consistent with our previous data that shows the pro-metastatic role of exogenous PN expression. Interestingly, compared to control antibody, MZ-1 treatment also eliminated ascites formation. The inhibitory effect of MZ-1 on tumor metastatic
growth is likely related to the reduced PN levels observed in treated tumors. In a separate experiment, we did not observe significant benefits of MZ-1 treatment on survival (data not shown), which may be due to the incomplete blockade of PN by MZ-1, short treatment time, or activation of additional survival signaling pathways.

Variable results have been reported regarding the role of PN in cancer cell proliferation. Addition of recombinant PN induces the proliferation of colorectal cancer cells(30) and cholangiocarcinoma cells(12). In contrast, ectopic PN expression in ovarian and breast cancer lines shows no influence on or even inhibition of cell proliferation (21, 31). A recent study employing pancreatic cancer cells suggests PN may exert biphasic effects on tumor development (32). These authors hypothesized that PN might display a biphasic dose dependence in tumor cell proliferation with low doses from exogenous expression levels being anti-tumorigenic. Our in vitro proliferation data show that anti-PN mAb MZ-1 specifically and efficiently inhibits the colony formation of PN cells in soft agar, which clearly indicates a pro-proliferation role of PN. Moreover, our data also exclude the possibility of a biphasic role of PN in ovarian cancer cell proliferation since PN levels in A2780 and TOV-112D cells are equivalent and 10-fold lower than those obtained through exogenous expression, respectively (our unpublished data). Also our soft agar results are consistent with a previous study in which two PN mAbs inhibited the colony forming capability of a colorectal cancer cell line MIP101(30). Although these authors did not detect PN expression in cell lysates of MIP101 by western blot, this result may not exclude the possibility of the cell line secreting PN at lower levels. In support of this
possibility, PN expression in culture medium of our TOV-112D cells was not detected by our western blot (data not shown), but was detected by a more sensitive PN ELISA.

Our findings that treatment with PN mAb MZ-1 triggers both ovarian cancer cell apoptosis and anoikis are consistent with previous results from our and other groups. Addition of recombinant PN into culture medium promotes in vitro survival of malignant cells including ovarian cancer (21), colon cancer (8) and pancreatic cancer (23). In addition, treatment with a specific PN mAb induced the apoptosis of colorectal cancer cells (30). It has been reported that PN exerts a pro-survival role through interaction with cell surface integrin receptors, which in turn trigger intracellular PI-3k (Phosphoinositide 3-kinase)/AKT signaling pathway (8, 21, 23). Consistent with these findings, MZ-1 specifically reduced the activation of Akt in PN-expressing cells, which may account for increased PN cell apoptosis.

In summary, our current work shows that a specific anti-PN mAb, MZ-1, efficiently inhibited PN-expressing ovarian tumor growth and metastasis in animal models, which is at least partially the result of its in vitro anti-proliferation and anti-survival cytotoxicity as well as its anti-metastatic effects on cell migration and invasion. Our research findings demonstrate for the first time, to our knowledge, that antibody blocking the action of PN may have a significant inhibitory effect on metastasis of PN-positive ovarian tumors.

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References


Table 1. Comparison of PN levels detected by ELISA in the conditioned medium of human ovarian tumor cell lines.

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<th>Cell Line</th>
<th>1d</th>
<th>3d</th>
<th>6d</th>
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<tr>
<td>A2780</td>
<td>1</td>
<td>18.8</td>
<td>271.6</td>
</tr>
<tr>
<td>TOV-112D</td>
<td>N.D.</td>
<td>0.3</td>
<td>30.6</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>0.2</td>
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<tr>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>SKOV-3</td>
<td>N.D.</td>
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NOTE: Cells were cultured in the regular medium supplemented with 10% FBS. Subconfluent cultures were washed with PBS and then incubated in serum-free medium. At the indicated time points, aliquots of medium were collected and subjected to PN ELISA, as described in Materials and Methods. The endpoint limit of sensitivity for the assay was 0.15ng/ml. N.D. : non-detected.
Legend

Fig. 1. Characterization of PN mAb MZ-1. (a). Western blot analysis indicated MZ-1 recognized recombinant PN-His produced by mammalian cell 293T and PN-Gst produced by bacteria. A specific PN polyclonal antibody and a mouse IgG were used as positive and negative controls, respectively. (b-c), mAb MZ-1 efficiently blocked the PN-induced adhesion (b ) and migration (c) of ovarian cancer cell SKOV-3. *p<0.05. (d). The antigen specificity of MZ-1 as determined by ELISA. FN: fibronectin; VN: vitronectin; LN, laminin; CN: collegen.

Fig. 2. Anti-PN mAb MZ-1 inhibited ovarian tumor growth and metastasis in mice. (a). One million A2780 cells were injected sc into SCID mice. Established tumors were treated biweekly with 20mg/kg of MZ-1 or an isotype-matching control antibody. Experiments were terminated after 2 weeks’ treatment when the first tumor reached the maximum size limit allowed by university guidelines. *p=0.03 (MZ-1 vs. control antibody). (b). One million A2780 cells were injected ip into SCID mice. Mice were treated three days later with MZ-1 (20mg/kg) or the control antibody. Experiments were terminated after sixteen days when the first mouse in the control group became moribund. Necropsy was performed on all mice and representative pictures of treated and control mice were shown. White arrows: tumor mass and nodules. (c). Compared to control antibody, treatment with MZ-1 not only significantly reduced tumor burden (*p =0.01) and tumor nodule dissemination (**p=0.002), but also decreased the incidence of formation of ascites and abdominal metastases. Results of tumor burden and nodule number are expressed as mean ± SE.
Fig. 3. Effects of mAb MZ-1 on ovarian cancer cell proliferation.  (a-b). A2780 cells were grown on plastic surfaces (a) or in ultra-low attachment plates (b) in the culture medium supplemented with 10%, 1% or 0% (SFM) of FBS.  MZ-1 mAb or control antibody was added at the final concentration of 40\(\mu\)g/mL.  After 2, 4, and 6 days, triplicate wells were exposed to trypsin and cells were counted using a Z1 Coulter counter (Beckman Coulter, Fullerton, CA).  Representative data of 4 day treatments are shown.  In all these experiments, treatment with MZ-1 only led to marginal inhibition (5-15%).  (c). Soft agar colony formation assay was performed using 100\(\mu\)g/mL of MZ-1 or control antibody in the middle layer.  MZ-1 specifically and significantly inhibited the proliferation of two PN-expressing cell lines, A2780 and TOV-112D; but had no effects on the other two cell lines, ES2 and SKOV-3, which expressed PN at very low or undetectable levels.  *P<0.001.

Fig. 4. Effects of PN mAb MZ-1 on ovarian cancer cell survival.  Cells were grown on plastic surfaces (a) or in ultra-low attachment plates (b) in the absence of FBS.  MZ-1 or control antibody was added at a final concentration of 20\(\mu\)g/mL.  Four days later, cell apoptosis (a) or anoikis (b) were examined as described in the Material and Methods.  *p<0.05.  (c). Akt phosphorylation on serine 473 was analyzed in ovarian cancer cells grown in SFM on plastic surface.  Cell lysates were prepared after one day treatment with MZ-1 or control antibody at a final concentration of 20\(\mu\)g/mL.

Fig. 5. Effects of PN or anti-PN mAb MZ-1 on ovarian cancer cell migration and invasion.  *p<0.001 vs control Ab; **p<0.05 vs control Ab.
Fig. 1
MZ-1 inhibited metastatic growth of A2780-derived ovarian tumors *in vivo*

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<tr>
<th>Mice#</th>
<th>tumor burden (g)</th>
<th>nodule#</th>
<th>ascites</th>
<th>Metastasis</th>
<th>abdominal wall</th>
<th>liver</th>
<th>diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl Ab</td>
<td>9</td>
<td>0.94 ± 0.1</td>
<td>31.7 ± 3.1</td>
<td>4/9</td>
<td>5/9</td>
<td>2/9</td>
<td>2/9</td>
</tr>
<tr>
<td>MZ-1</td>
<td>9</td>
<td>0.46 ± 0.13*</td>
<td>13.2 ± 1.6**</td>
<td>0/9</td>
<td>1/9</td>
<td>0/9</td>
<td>0/9</td>
</tr>
</tbody>
</table>

*Fig. 2*
Fig. 3
a. Apoptosis

![Graph showing apoptosis](image)

b. Anoikis

![Graph showing anoikis](image)

c. Phospho-Akt and β-actin levels

![Western blots](image)

Fig. 4
**Fig. 5**
Molecular Cancer Therapeutics

Neutralizing Monoclonal Antibody to Periostin Inhibits Ovarian Tumor Growth and Metastasis

Min Zhu, Romaine E Saxton, Lillian Ramos, et al.

Mol Cancer Ther Published OnlineFirst June 13, 2011.

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