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Osteopontin is an adhesive factor for myeloma cells and is found in increased levels in plasma from patients with multiple myeloma

A B S T R A C T

Background and Objectives. Osteopontin (OPN) is a non-collagenous matrix protein produced by various cells including osteoblasts, osteoclasts and several types of tumor cells. It is involved in a number of physiologic and pathologic events including adhesion, angiogenesis, apoptosis, inflammation, wound healing and tumor metastasis. We wanted to investigate the potential role of OPN in multiple myeloma.

Design and Methods. Myeloma cells and stromal cells from myeloma patients were investigated as potential OPN-producers. Furthermore, OPN was tested in proliferation, migration and adhesion assays with myeloma cells. Serum and plasma OPN in myeloma patients were measured by enzyme-linked immunosorbent assay (ELISA). OPN levels were correlated to disease variables at diagnosis and to disease outcome.

Results. Myeloma cells produce OPN, and stromal cells from myeloma patients express higher levels of OPN than stromal cells from healthy controls. The myeloma cell lines ANBL-6 and INA-6 adhered to OPN. NOD/SCID mice inoculated with OPN-producing ANBL-6 cells had elevated levels of murine OPN in serum, whereas human OPN was not detectable. Plasma and serum levels of OPN were significantly higher in myeloma patients than in healthy individuals.

Interpretation and Conclusions. Myeloma cell lines adhere to OPN, indicating that elevated stromal expression of OPN may be one of the factors responsible for the retention of myeloma cells in the bone marrow. The elevated plasma OPN levels in myeloma patients could be due to both production of OPN by the tumor cells and tumor-induced production of OPN by non-tumor cells.

Key words: osteopontin, myeloma, adhesion, stromal cells.

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Osteopontin (OPN) is a secreted adhesive glycoprotein expressed by several cell types and is normally found in bone, teeth, kidney and epithelial lining tissues. It is involved in a number of physiologic and pathologic events including angiogenesis, apoptosis, inflammation, wound healing and tumor metastasis. Cells bind OPN via multiple integrin receptors. The binding can be either RGD-dependent or -independent.¹⁻³ Many tumor cell types produce OPN, and an association between plasma OPN and tumor burden, metastases to bone and survival has been found in breast cancer and prostate cancer patients.^{4,5}

In bone, OPN is produced by both osteoblasts and osteoclasts. Osteoclasts bind to osteopontin deposited in the bone matrix, through cell surface CD44 and $\alpha_v\beta_3$ -integrins. These interactions are essential for the migration, attachment, and resorptive activ-

ity of osteoclasts.⁶⁻¹⁰ Targeted disruption of the OPN gene in mice seems to have no effect on bone formation and development under physiologic conditions.¹¹ However, OPN plays an important role in mechanical stress-induced bone remodeling¹² and under circumstances of accelerated, pathologic bone loss.¹³ OPN knock-out mice are resistant to the bone loss usually observed after ovariectomy.¹⁴ Moreover, parathyroid hormone-induced bone resorption does not occur in the absence of OPN.¹⁵ Besides its effect on osteoclasts, OPN may also inhibit crystal growth by binding strongly to calcium phosphate crystals in mineralized tissues.^{16,17} Dysregulated expression of OPN may thus have important implications for bone homeostasis in several ways.

Multiple myeloma is a plasma cell malignancy in which the malignant cells are confined to the bone marrow. The disease is fre-

quently accompanied by bone destruction. Several reports indicate that there is an imbalance between osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) in favor of RANKL during the development of myeloma bone disease.^{18,22} OPN appears to be an important downstream factor in RANKL-mediated bone resorption,¹⁵ and OPN may thus be one of the factors involved in myeloma bone disease.

Why myeloma cells localize exclusively to the bone marrow is an open question, but it is reasonable to assume that there are factors in the myeloma marrow favorable for retention and growth of the cancer cells. In addition to the role of OPN in osteoclast activation, its role in adhesion and migration of normal and malignant cell types²³⁻²⁶ makes this glycoposphoprotein a molecule of potential importance in multiple myeloma.

We investigated whether myeloma cells themselves or stromal cells from myeloma patients could produce OPN. We also examined the potential role of OPN as an adhesion, migration and growth factor in myeloma. Furthermore, we determined plasma and serum concentration of OPN in myeloma patients and compared them with those in healthy controls.

Design and Methods

Cell lines

The human myeloma cell lines U-266 and RPMI-8266 were purchased from American Type Culture Collection (ATCC, Rockville, Maryland, USA) and cultured according to the manufacturer's instructions. The human myeloma cell line JJN-3 was a kind gift from Jennifer Ball (Department of Immunology, University of Birmingham, UK) and was maintained in RPMI 1640, 10% fetal calf serum (FCS). The human myeloma cell line INA-6 was a gift from Dr. Martin Gramatzki (University of Erlangen-Nuremberg, Erlangen, Germany) and was maintained in RPMI 1640, 10% FCS, 1 ng/mL interleukin (IL)-6. The human myeloma cell line ANBL-6 was generously provided by Dr. Diane Jelinek (Mayo Clinic, Rochester, MN, USA) and was maintained in RPMI 1640, 10% FCS, 1 ng/mL IL-6. The human myeloma cell lines OH-227, and IH-128 were established in our laboratory and were routinely grown in RPMI 1640, 10% human serum (The Blood Bank, St. Olav's Hospital, Trondheim, Norway) and 1 ng/mL IL-6. All culture media contained 2 mmol/L glutamine and 40 μ g/mL gentamycin. The cell lines were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

Primary myeloma cells

We studied myeloma cells from four patients admitted to the Section of Hematology, St. Olav's Hospital, Trondheim. Myeloma cells from bone marrow aspirates

were purified by immunomagnetic separation using Macs CD138 MicroBeads (Miltenyi Biotec, CA, USA). From 200,000 to 250,000 cells were seeded in a total volume of 500 μ L. The OPN content was measured after three days of culturing. Informed consent from the patients and approval from the Regional Ethics Committee was obtained.

Generation of long-term bone marrow cultures (LTBMC)

Bone marrow mononuclear cells (BMMNC) obtained from 9 patients with multiple myeloma and 4 normal donors were suspended in Iscove's modified Dulbecco's medium including 10% horse serum, 10 FCS, penicillin 100 U/mL, streptomycin 100 μ g/mL (Gibco BRL, Tastrup, Denmark) and 1% hydrocortisone (Sigma, St. Louis, Missouri, USA) as previously described.²⁹ Adherent cell layers were harvested with trypsin after 5-7 weeks, washed with PBS and collected for RNA extraction. The isolation of BMMNC and RNA was performed as previously reported.³⁰

Flow cytometry and single-cell sorting

Cell samples were stained with 3 or 4 colors with the monoclonal antibodies CD19 FITC, CD38 FITC/APC, CD45 PERCP, CD56 PE (Becton Dickinson Immunocytometry Systems (BDIS), San José, California, USA) and CD19 Cy5 (Dako, Glostrup, Denmark). IgG₁ FITC, IgG₁ PE, IgG₁ PerCP and IgG1 APC (BDIS) were used as negative controls. A minimum of 100,000 cells were collected and analyzed using a FACSCalibur (BDIS). Flow sorting of single cells directly to polymerase chain reaction (PCR) tubes was performed using a FACS Vantage (BDIS) as previously described.³⁰

Global reverse transcriptase (RT)-PCR on FACS sorted cells

For generation of a cDNA archive global RT-PCR was performed on flow-sorted myeloma plasma cells as previously reported.³¹ Normal (n = 3) and myelomatous (n = 31) plasma cells were FACS sorted according to their characteristic immunophenotype.

Multiple myeloma plasma cells were identified as having either a CD38⁺⁺/CD19⁻/CD45⁻ⁱ/CD56⁺⁺ or CD38⁺⁺/CD19⁻/CD45⁻ⁱ/CD56⁻ aberrant immunophenotype, whereas normal plasma cells were identified as CD38⁺⁺/CD19⁺/CD45ⁱ⁺/CD56⁻.

Real-time RT-PCR assays

For the determination of mRNA levels in flow-sorted cells, real-time RT-PCR assays for OPN and β -actin were designed. OPN, forward: 5-TGAGTCTGGAAATAACTAAT-GTGTTTGA-3, reverse: 5-GAACATAGAC-ATAACCCT-GAAGCTTTT-3, Probe: 5-TTTGTGGCTTCATGGAACTCCCT-GTAAAC-3. β -actin, forward: 5-CCTTTTGTCCCCAACT-

TGA-3, reverse: 5-TGGCTGCCTCCACCCA-3, Probe: 5-ATGTATGAAGGCTTTGGTCTCCC-TGGGA-3. Absolute numbers of β -actin transcripts were determined generating a standard curve using the TaqMan™ DNA Template Reagents Kit (PE Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed essentially as previously described³² on cDNA (LTBMC) or global RT-PCR amplified products (all FACS sorted cells). The identity of the PCR products was determined by sequencing as previously reported.³⁰ All samples were run in triplicate and three control samples with no template were included for each run.

Adhesion assay

Ninety-six-well plates were coated overnight at 4°C with 10 μ g/mL human milk OPN in PBS, 50 μ L/well (Kamiya Biomedical Company, Seattle, WA, USA) or BSA (10 mg/mL in PBS, 50 μ L/well). The plates were then washed and blocked with BSA (10 mg/mL, 100 μ L/well) for one hour at room temperature. Before use the plates were washed three times and dried completely. The cells were washed twice and resuspended in RPMI/0.1%BSA at a concentration of 1×10^6 cells/mL and labeled for one hour at room temperature with 5mM acetoxymethyl ester-2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF-AM, Sigma). Subsequently, the cells were washed four times and 80,000 cells per well were seeded and incubated in the dark for one hour at 37°C in 5% CO₂. After incubation, non-adherent cells were carefully removed by washing. The cells were thereafter solubilized by adding 1% Triton X-100, 50 μ L/well. Fluorescence intensity at 538 nm was measured with a Fluoroskan II fluorescence reader (Labsystems, Helsinki, Finland). The excitation wavelength was 485 nm.

Animal experiments

Four to six-week old NOD/SCID mice (Taconic M&B, Ry, Denmark) were housed in a specific pathogen-free facility with 12-hour night and day cycles with controlled humidity and temperature conditions. The mice were housed in microisolator cages placed in laminar flow hoods and were fed *ad libitum* with sterile water and autoclaved rodent chow. All handling of mice was performed in a sterile manner. The animals received 2Gy total body irradiation, and were injected with cells the following day under analgesia after subcutaneous injection of Hypnorm/Dormicum. Mice were exsanguinated by cervical clip in narcosis with Hypnorm/Dormicum, and blood was collected. All animals appeared well throughout the experiments. At autopsy, we found no sign of thymic lymphoma, a frequent condition in this mouse strain.

Twelve animals were injected intraosseously (right tibia) either with 100,000 JJN-3T1³³ cells in 10 μ L of PBS or with PBS only (six animals in each group). Animals

were sacrificed after 21 days when most tumor-injected animals had small palpable masses in the right popliteal fossa (maximally 600 μ L). All tumor-injected animals had take of tumor in the tibia, as verified by histology and measurements of human immunoglobulin kappa light chain. There was no sign of disseminated spread of myeloma at autopsy.

Five mice were injected intraosseously with 100,000 serially passaged ANBL-6 cells (Hjorth-Hansen, manuscript in preparation). Mice were sacrificed after 12 weeks. All tumor-injected animals had take of tumor as evaluated by histology, flow cytometry with human markers and measurements of human immunoglobulin lambda light chain. In short, injection of passaged ANBL-6 cells resulted in a myeloma-like disease, characterized by disseminated spread of myeloma cells throughout the bone marrow with lytic bone disease, without sign of growth in lymphoid tissue or solid organs.

Plasma samples

After informed consent, EDTA plasma samples from 68 myeloma patients (40 men and 28 women), were collected. Their median age was 57.5 years (range 46–86). Plasma samples from 21 patients with monoclonal gammopathy of undetermined significance (MGUS) (8 males, 13 females) were also collected. The median age of these patients was 66 years (range 50–79). Plasma was collected from 30 healthy blood donors who formed the control group. This group was formed of 16 males and 14 females with a median age of 62 years (range 46–70).

Serum samples

Serum samples were collected from patients entered in a study on high dose therapy with autologous stem cell support conducted by the Nordic Myeloma Study Group. Fourteen centers in Denmark, Norway and Sweden participated in the study. In the regions served by the centers, all newly diagnosed, symptomatic myeloma patients less than 60 years old were registered during the inclusion period. A total of 348 patients were registered. Of these, 274 patients were entered in the specified intensive-therapy protocol in the period from March 1994 until June 1997. Two hundred and fourteen of the 274 entered patients followed the intended high-dose protocol with autologous stem cell support. The diagnostic and eligibility criteria, reasons for non-entry, treatment and results are described elsewhere.³⁴ Serum samples from 114 of the 274 entered patients were available, and these constitute the study material. The median age of the patients in the study population was 52 years (range 28–59). There were 73 males and 41 females. Registered parameters at diagnosis were age, sex, Durie-Salmon stage, WHO performance status,

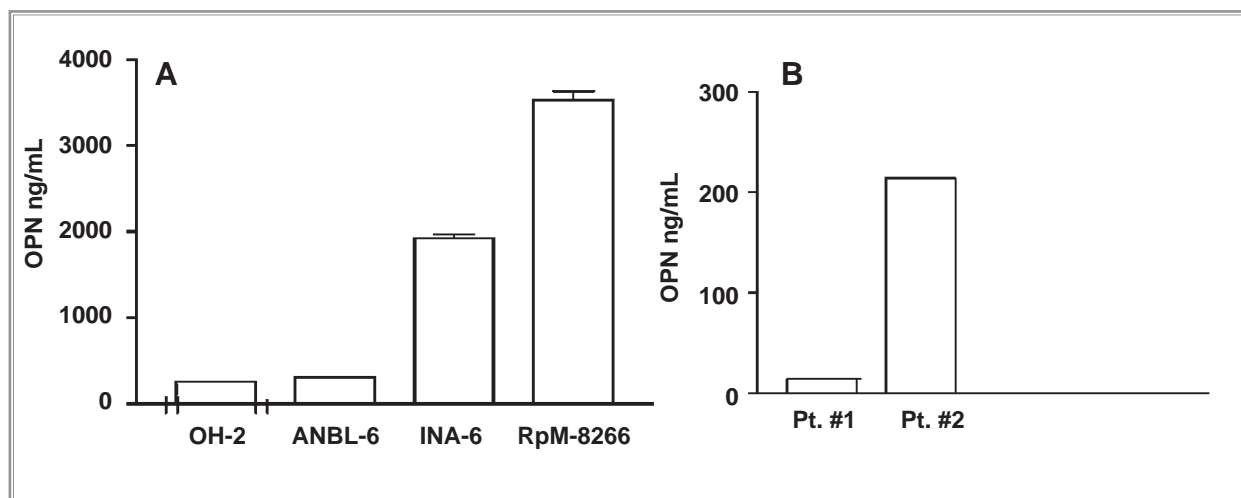


Figure 1. OPN production by myeloma cells. (A) Cells from seven different myeloma cell lines were seeded at the same concentration, and conditioned media were harvested after three days of culturing. Data are means + SEM. Similar results were obtained in two different experiments. The cell lines J2N-3, IH-1 and U266 produced undetectable amounts of OPN (not shown). **(B)** OPN production by primary myeloma cells. Approximately 200,000 cells were seeded, and conditioned media were harvested after three days of culturing. Primary cells from two out of four patients secreted detectable amounts of OPN.

grade of bone morbidity (three severity levels as judged by X-ray analyses), percentage of plasma cells in the bone marrow, immunoglobulin class, urine immunoglobulin/24 hours and serum M-protein concentration, blood hemoglobin, serum albumin, serum calcium, serum creatinine, serum lactate dehydrogenase, serum β -2 microglobulin, C-reactive protein in serum (CRP), number of leukocytes and thrombocytes and hepatocyte growth factor (HGF). Serum samples from 30 healthy blood donors served as controls. The median age of the controls was 48 years (range 40–59). There were 18 males and 11 females in the control group.

OPN measurement

Human serum and plasma OPN was measured by enzyme-linked immunosorbent assay (ELISA) (Assay Designs Inc, MI, USA). This assay detects uncleaved OPN (*personal communications, AssayDesign*), and the recommended sample material is EDTA plasma. Detected levels of OPN in serum are lower than in plasma due to proteolysis of OPN during blood clotting. We therefore did a small study ($n = 12$) comparing plasma and serum levels of OPN from the same patients/controls. We found that serum levels reflected plasma levels (Pearson's correlation coefficient $r = 0,80$, $p = 0,02$), indicating that it would be meaningful to analyze OPN in our serum sample material. The detection limit in the ELISA was 5 ng/mL. All samples were run in duplicate. Plasma samples were diluted 1:10 and serum samples were diluted 1:5. Plasma samples outside the range of the standard curve were re-analyzed after appropriate dilution. Due to restricted amounts of sample material serum measurements could not be repeated and there-

fore samples with OPN concentrations below 25 ng/mL or above 1600 ng/mL were given the values 25 ng/mL and 1600 ng/mL, respectively. Heparin up to 100 μ g/mL or nine times repeated freeze/thawing of plasma/serum did not influence OPN detection.

Murine serum OPN was measured by ELISA (Assay Designs Inc, MI, USA). This assay is valid for both serum and plasma samples. The detection limit was 1 ng/mL and samples were diluted to concentrations within the range of the standard curve (1:100 dilution).

Statistical analyses

All statistical analyses were done with the SPSSX/PC computer program (SPSS Inc. Chicago, IL, USA). Results were considered statistically significant when p values were $\leq 0,05$. Skewed variables were transformed by the natural logarithm before entering analyses requiring normal distribution. Comparisons between groups were performed with Mann-Whitney U-test.

Correlation between two parameters was estimated by Spearman's rank correlation analysis. Survival was modeled with Cox regression. The method of Kaplan and Meier was used to compute the survival curves and to estimate the median survival. To compare the survival curves (test for significance) the logrank test was used.

Results

OPN production by myeloma cells

To investigate whether myeloma cells produce OPN, we measured OPN in conditioned media from seven myeloma cell-lines (Figure 1A). Two of them (INA-6 and

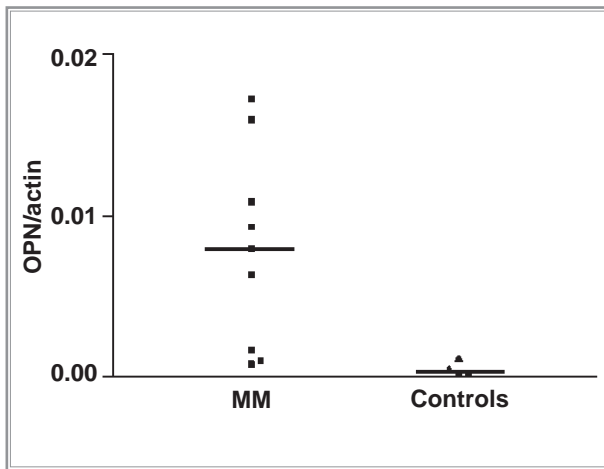


Figure 2. LTBM from myeloma patients (n=9) express more OPN than do LTBM from healthy controls (n=4). Values represent numbers of OPN transcripts relative to β -actin transcripts. Bars indicate median values.

RPMI-8266) produced substantial amounts of OPN, two of the cell lines (OH-2 and ANBL-6) produced OPN in intermediate levels and three of the cell lines (JJN-3, IH-1 and U266) did not secrete detectable amounts of OPN (<5 ng/mL).

We also measured OPN in conditioned media from primary myeloma cells from four different patients. OPN was not detectable in media from primary cells from two patients, whereas cells from the two other patients secreted low but detectable amounts of OPN (14 ng/mL and 214 ng/mL) (Figure 1B). OPN transcript was detected in 2 out of 31 FACS-sorted myeloma plasma cells.

Increased OPN expression in long-term bone marrow cultures derived from MM patients

OPN expression in long-term bone marrow cultures (LTBM) derived from myeloma patients was assessed by real-time quantitative RT-PCR and compared to OPN expression in LTBM obtained from healthy donors. OPN expression in LTBM derived from myeloma patients was significantly higher (median 0.0080, Mann Whitney U test, $p = 0.014$) than OPN expression in LTBM derived from healthy individuals (median 0.0004) (Figure 2).

Myeloma cells adhere to OPN

Since the myeloma cells produced OPN, and stromal cells from myeloma patients expressed higher levels of OPN than did stromal cells from healthy individuals, we wanted determine whether OPN had any effect on the cancer cells. We investigated whether myeloma cells adhered to OPN. Both the myeloma cell lines INA-6 and ANBL-6 adhered significantly more ($p < 0.001$) to OPN than to BSA (Figure 3), indicating that OPN may be functionally important for the myeloma cells.

We also tested whether the myeloma cells migrated towards OPN in a modified Boyden Chamber assay, but this seemed not to be the case (*data not shown*). Furthermore, proliferation assays indicated that OPN is not a growth factor, at least not as a single factor, for myeloma cells (*data not shown*).

Elevated host-derived serum OPN in xenografted NOD/SCID mice

By applying OPN assays that are specific for either human or murine OPN it was possible to discriminate between tumor-derived OPN and host-derived OPN in

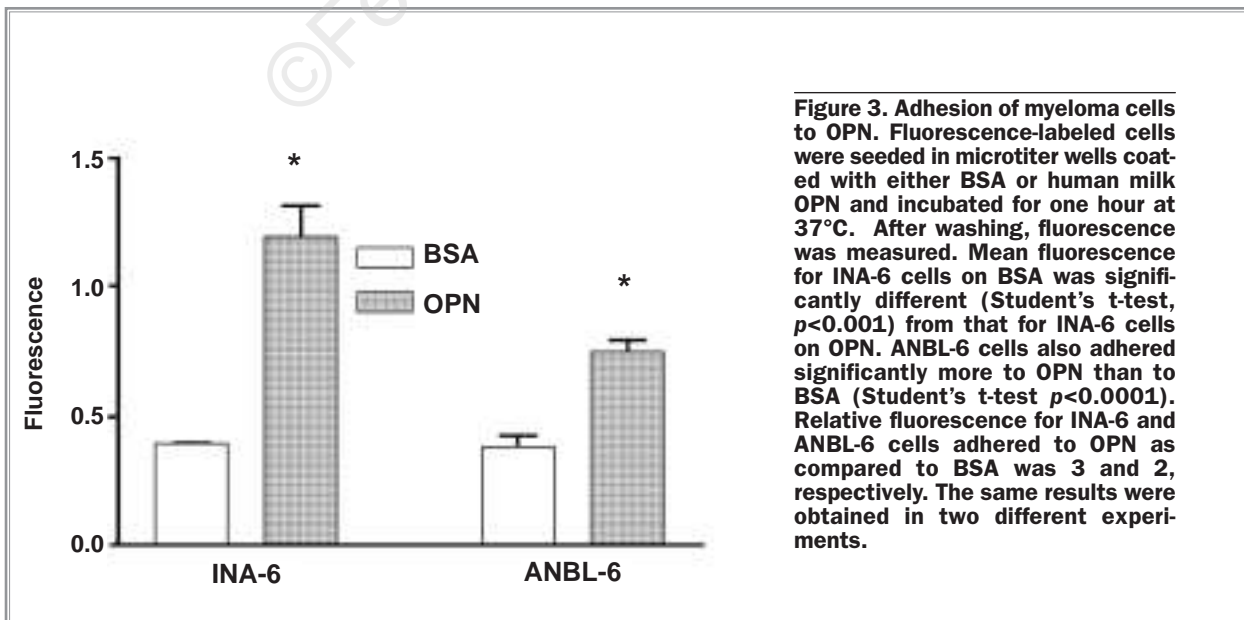


Figure 3. Adhesion of myeloma cells to OPN. Fluorescence-labeled cells were seeded in microtiter wells coated with either BSA or human milk OPN and incubated for one hour at 37°C. After washing, fluorescence was measured. Mean fluorescence for INA-6 cells on BSA was significantly different (Student's t-test, $p < 0.001$) from that for INA-6 cells on OPN. ANBL-6 cells also adhered significantly more to OPN than to BSA (Student's t-test $p < 0.0001$). Relative fluorescence for INA-6 and ANBL-6 cells adhered to OPN as compared to BSA was 3 and 2, respectively. The same results were obtained in two different experiments.

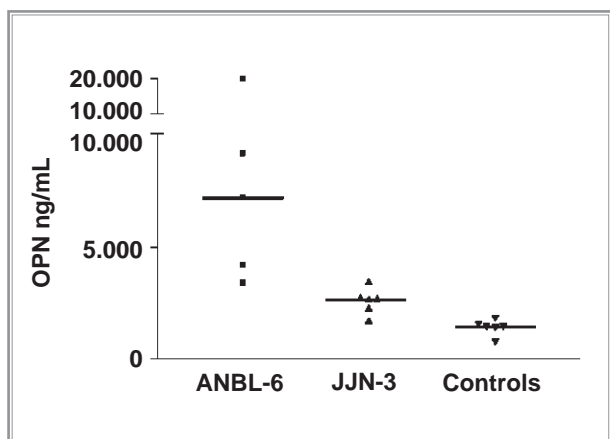


Figure 4. Xenografted NOD/SCID mice have a higher concentration of murine OPN in serum than do control mice. Bars indicate median values.

our xenograft myeloma mouse models. In mice with multiple myeloma derived from OPN-producing human ANBL-6 cells, the serum concentration of murine OPN (median 7,210 ng/mL, range 3,426–20,000) was significantly higher (Mann-Whitney U test, $p=0.009$) than the serum concentration of murine OPN in the control mice (median = 1469, range 789–1,836). However, we could not detect human OPN in mouse serum, indicating that the increased serum OPN was tumor-induced and not tumor-derived. Also, the serum concentration of murine OPN in mice injected with non-OPN-producing JJN-3 cells (median = 2,712 ng/mL, range 1,759–3,488) was significantly higher (Mann-Whitney U test, $p = 0.006$) than murine OPN serum concentration in the control mice (Figure 4). The difference in OPN levels between ANBL-6 mice and JJN-3 mice could be due to differences in tumor burden in the two models. ANBL-6 mice had a higher tumor burden than did the JJN-3 mice (*data not shown*). Taken together, these data indicate that myeloma cells are able to induce OPN production in host cells.

Elevated plasma OPN concentration in myeloma patients

The above *in vitro* and *in vivo* findings led us to study OPN levels in patients with multiple myeloma. Plasma samples from 68 myeloma patients and 30 healthy controls were analyzed by OPN ELISA. OPN levels were higher in myeloma patients (median 745 ng/mL, range 0–10,780) than in controls (median 525.5 ng/mL, range 131–1,009) (Figure 5A). This difference is statistically significant (Mann-Whitney U-test, $p = 0.001$). Forty-one percent of the patients had plasma OPN levels above the mean $\pm 2SD$ in the control population (940 ng/mL) and these plasma levels could therefore be considered abnormal by conventional criteria.

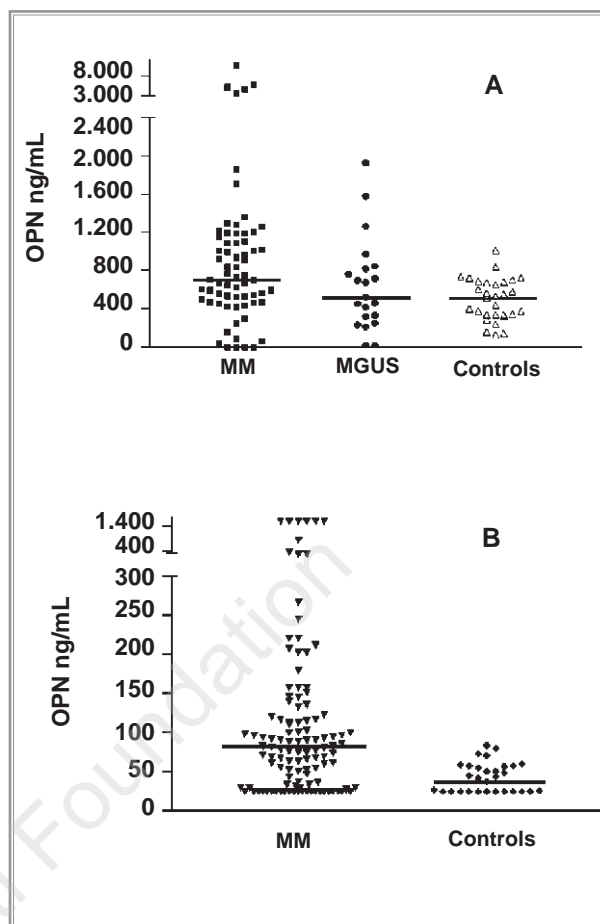


Figure 5. Elevated OPN concentration in myeloma patients. (A) Plasma OPN measured by ELISA in myeloma patients (MM, $n=68$), MGUS patients ($n=21$) and healthy controls ($n=30$). (B) Serum OPN measured by ELISA in myeloma patients ($n=114$) and healthy controls ($n=30$). Bars indicate median values.

OPN levels were also measured in plasma from 21 MGUS patients. Plasma OPN (median 532 ng/mL, range 30–1,933 ng/mL) did not differ significantly between myeloma patients and MGUS patients (Mann-Whitney U-test $p = 0.082$), or between controls and MGUS patients (Mann-Whitney U-test $p = 0.450$) (Figure 5A).

Elevated serum OPN concentration in myeloma patients

Since we found that plasma OPN levels were higher in myeloma patients than in healthy controls we wanted to do a more thorough analysis in a larger, better defined group of patients. We therefore analyzed OPN content in serum samples of 114 myeloma patients and 30 controls. Also in this material, we found that OPN levels were higher in myeloma patients (median 80 ng/mL, range 25–1,600) than in control patients (median 43 ng/mL, range 25–84) (Figure 5B). This difference is statistically significant (Mann-Whitney U-test, p

<0.001). Forty percent of the patients had serum levels higher than mean+2SD in the control population (81 ng/mL). In this material, the OPN concentration was correlated significantly with the concentrations of creatinine ($r=0.278$, $p = 0.003$), β_2 microglobulin ($r=0.382$, $p < 0.001$), corrected calcium ($r=0.264$, $p = 0.005$), hemoglobin ($r= -0.266$, $p = 0.015$), albumin ($r= -0.199$, $p = 0.033$) and white blood cell count ($r=0.332$, $p < 0.001$). OPN levels did not correlate with age, sex, number of osteolytic lesions, percentage of plasma cells in the bone marrow, immunoglobulin class, urine immunoglobulin/24 hours and serum M-protein concentration, serum lactate dehydrogenase, CRP, platelet counts or HGF. Furthermore, OPN concentration did not significantly predict prognosis in this population.

Discussion

In the present study we demonstrate that myeloma cells produce OPN, and that LTBM from myeloma patients express higher levels of OPN than do LTBM from healthy controls. Investigations on the biological role of OPN in multiple myeloma revealed that OPN might be an adhesion factor for myeloma cells. In keeping with these findings, we show that myeloma patients as a group have a higher concentration of OPN in the circulation than do healthy controls.

OPN was first characterized as a transformation-related phosphoprotein,³⁵ and its expression is increased in several forms of cancer.^{4,36-38} However, when measuring circulating OPN, the source of the protein is not defined. In our study, OPN protein was detected in supernatants from the tumor cells. Furthermore, microarray gene expression studies from our laboratory (*data not shown*) and from others³⁹ have indicated that myeloma cells express OPN mRNA. Based on such data it is tempting to conclude that elevated plasma levels are a direct consequence of tumor cell production of OPN. However, it seems as if only a subgroup of patients has tumor cells expressing OPN. The high frequency of myeloma cell lines expressing OPN (4/7) and the low frequency of primary myeloma cells expressing OPN (2/31) could be due to differences between extramedullary myeloma cells, from which most cell lines are generated,⁴⁰ and primary cells confined to the bone marrow.

In our animal study, injection of OPN-producing human ANBL-6 cells did not lead to detectable levels of circulating human OPN in the mice. This does not exclude the possibility of myeloma cells being responsible for elevated serum OPN in myeloma patients, since human OPN may be rapidly eliminated in the mouse. However, the increase of murine serum OPN in our two xenograft models demonstrates that myeloma cells induce OPN from host cells. This mechanism may repre-

sent a substantial contribution to the elevated OPN-levels observed in myeloma patients. The finding of higher expression of OPN in LTBM from myeloma patients than in LTBM from healthy individuals further strengthens this hypothesis.

When we analyzed OPN in patients' sera, we found that the distribution of OPN levels was the same as that in the plasma samples (approximately 40% of the patients had OPN levels that were above the normal range). The fact that the results were similar in two different study populations strengthens the reliability of our findings. High levels of OPN have been reported in other tumor types and are often associated with short survival, increased tumor burden and metastasis.^{4,5,41} We analyzed OPN in our well-defined sample material to determine whether levels of OPN predicted survival of the patients and to look for correlations indicating a biological role of OPN in multiple myeloma. None of the correlations was strong (although several were statistically significant), however, the correlation of OPN to serum calcium is of particular interest in relation to myeloma bone disease. On the other hand, there was no correlation between serum OPN and the crude estimate of bone morbidity as judged by the number of osteolytic lesions in the patients. We did find a correlation with β_2 microglobulin levels. β_2 microglobulin is a tumor marker and a prognostic factor in multiple myeloma.⁴² This correlation indicates that there is a correlation between OPN and tumor activity, but in our study OPN was not a statistically significant prognostic factor in itself.

Myeloma cells preferentially grow in the bone marrow, and it is reasonable to believe that the myeloma cells in some way instruct the bone marrow environment to help the expansion of the malignant clone. By causing the stromal cells to produce more OPN, the myeloma cells possibly make the bone marrow environment favorable for retention and growth of the tumor cells. Several adhesion molecules are present on myeloma plasma cells, including known ligands for OPN such as CD44 variant isoforms, and $\alpha 4\beta 1$ - and $\alpha \nu \beta 3$ -integrins.⁴³⁻⁴⁵ These receptors are potential candidates engaged in binding myeloma cells to OPN.

OPN has been shown to protect myeloma cells from complement-mediated killing,⁴⁶ and it was recently reported that OPN has growth-stimulating effects on myeloma cells.⁴⁷ In our experiments, neither soluble nor immobilized OPN had any favorable effect on growth of primary myeloma cells or myeloma cell lines (*data not shown*). In the study by Yaccoby *et al.* the myeloma cells were co-cultured with osteoclasts for 14 days and the proliferation of myeloma cells was inhibited by adding neutralizing antibodies against OPN. It is thus possible that other factors, perhaps secreted by osteoclasts, are needed in addition to OPN to give a proliferative effect.

Based on the observed levels of circulating OPN in myeloma patients, its well known activating effect on osteoclasts and our data showing that it is an adhesion molecule for myeloma cells, OPN could be a protein of importance in multiple myeloma. Myeloma-induced production of OPN by stromal cells, as well as the synthesis of OPN by tumor cells, may be components of the vicious circle between the malignant plasma cells and the environment.

ØH, TS, HHH, MB and AS designed the study. TS, HHH, TR, VB and ATB did the experimental work. IMD, SL and AW collected the patient's sample material. TS prepared the manuscript. All authors were involved in critically revising the manuscript and gave final approval for its submission. After we submitted this work Saeki et al.⁴⁹ published data which are essentially similar to the data shown in Figures 1 and 5 of this manuscript. The authors indicated no potential conflicts of interest.

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