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OSTEOPROTEGERIN GENE EXPRESSION IN MULTIPLE MYELOMA PATIENTS

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ABSTRACT

Osteoprotegerin (OPG) is a novel secreted member of tumor necrosis factor receptor superfamily mainly secreted by marrow stromal cells. *In vitro*, Osteoclast formation can be inhibited by OPG in a dose-dependent manner. Multiple myeloma (MM) is a hematologic malignancy characterized by accumulation of plasma cells in the bone marrow (BM). Bone destruction is a complication of the disease and is usually associated with severe morbidity. The present study aimed to examine the expression of OPG at the serum protein level as well as the mRNA level in multiple myelomas (MM) in relation to the clinicopathological findings. Thirty five patients with Multiple Myeloma (MM) and 20 healthy subjects of matched age and sex were studied. Patients were subjected to complete clinical examination and laboratory investigations including protein electrophoresis to calculate monoclonal protein, Haemoglobin (Hb), Blood urea and serum level of B_2 microglobulin (B_2M).OPG concentration was estimated in serum by ELISA. A reverse transcriptase-polymerase chain reaction (RT-PCR) was used to investigate the expression of OPG m RNA. OPG concentration in sera of MM cases was lower than normal controls (mean value 0.10 versus 0.15 ng/ml) this difference was statistically highly significant (P=0.001). Bone Marrow aspirates were taken from patients (n=10) as well as controls (n=5) where, the OPG m RNA was expressed in MM Patients by the mean of 0.7± 0.5 fold while in the controls it was 4.5±1.1 fold increase in the intensity of expression (P=0.02).A highly positive significant correlation was found between serum OPG protein level and OPG m RNA expression in BM of MM patients and controls (r=0.12, p=0.01-r=0.98, p=0.002) respectively. It is concluded that OPG expression is reduced in bone marrow specimens from myeloma patients. The adhesive interactions of myeloma cells with bone marrow stromal cells inhibit OPG production both at the m RNA and Protein level. Down regulation of OPG expression in MM patients may be involved in pathogenesis of Myeloma bone disease and may provide a useful tool for prognosis.

INTRODUCTION

Multiple myeloma is a B-cell neoplasm characterized by clonal expansion of malignant plasma cells in the bone marrow, ultimately leading to osteolytic bone destruction, renal dysfunction and anemia (1).

Bone is a dynamic tissue in which the synthesis of bone matrix by osteoblasts and bone resorption by osteoclasts are coupled processes. Key regulators of osteoclastogenesis are members of the tumor necrosis family of receptors and ligands:

receptor activator of nuclear factor (NF-$_k$) B (RANK), receptor activator of NF-$_k$ B ligand (RANKL), and osteoprotegerin (OPG). RANKL is expressed by activated T cells, stromal cells, and osteoblasts. (2-4). When RANKL bind to RANK on osteoclast precursors, maturation and differentiation of the osteoclasts are induced, leading to bone resorption (5,6).OPG (TNFRSF11-B) is a soluble decoy receptor that is secreted by osteoblasts and bone marrow (BM) stromal cells and that competes with RANK for binding to RANKL. Binding of OPG to RANKL inhibits the development of osteoclasts (7, 8). OPG has documented effects on the regulation of bone metabolism (9-12). MM stimulates production of activated osteoclasts (OCs) from monocytic precursors, leading to severe osteoporosis and bone destruction in most patients (13-15). Osteoclasts are activated through interactions of myeloma cells with stromal cells, which result in increased resorptive activity without a comparable increase in bone formation, thus leading to the development of osteolytic lesions characteristic of myeloma bone disease. (16,17). The purpose of this study was to examine the expression of OPG at the serum protein level as well as the mRNA level in MM patients in relation to clinicopathological findings.

PATIENTS AND METHODS

MM patients and controls: This study was carried on 55 cases, 35 patients were diagnosed as MM patients (median age, 58 years; 25men, 10 women) and 20 healthy controls (median age, 57 years; 14 men, 6 women). Peripheral blood was aspirated from all cases and bone marrow sternal puncture from only 10 patients (median age, 58 years; 7men, 3 women) with MM (carefully selected MM cases from those who were previously diagnosed). 5 patients, who underwent BM aspiration for diagnostic purpose, but who had normal BM morphology and subsequently were not diagnosed as MM, were included as controls (median age, 54 years; 3men, 2 women). Patients' samples were obtained from Hematology unit, Alex. Main Univ. Hospital. After informed consent were obtained. The study was approved by the local ethics committee. All patients were diagnosed using routine procedures, including morphology, cytochemistry and immunophenotyping.

OPG measurements: Serum OPG was measured by enzyme- linked- immune-sorbent assay (ELISA); from Bender Medsystems, Austria-Europe). An OPG polyclonal coating antibody was used according to the manufacturer's instruct-ions. All samples were run in duplicate. The standard curve was linear between 0.01 and 1 ng/ml, and samples were diluted to concentrations within this range.

RNA extraction and Reverse transcription (RT): Total RNA was isolated from whole blood of 10 patients and 5 controls using the SV Total RNA isolation kit (Promega) according to the technical manual-TM048 as Oligotex direct mRNA.

Primer Design Program: Total RNA was subjected to reverse transcriptase polymerase chain reaction RT-PCR by using specific primers for the osteo-protegerin gene (OPG) that was detected Gene Bank data by www.nbci.com. RT-PCR Ready To Go kit (Bio Basic inc.) was used to isolate the cDNA fragment according to the manufacture system.

F, OPG 5'- CTGCTTATAACTGGAAATGGCC - 3'
R, OPG 5'- CTGTGGCAAAATTAGTCACTGG - 3'

These reagents contained 1 µg of total RNA and 10 µM of each specific primer. A total volume of 50ul reaction was completed using nuclease free water.

The PCR conditions were as following; the tubes were incubated at 42ºC for 15 minutes to activate the reverse transcriptase enzyme, and 94°C for 5 minutes to inactivate the enzyme, then followed by 35 cycles of 94°C for 1 min, 67°C for 1min, and 72°C for 1 min. finally 72º C for 10 min. The PCR products were electrophoreses in 1% agarose gel. A 1000 bp DNA marker was used range 100 to 1500 bp (Sib Enzyme, Russia co.) and photographed.

Statistical analysis: All statistical analysis were performed with the SPSSX/PC computer program (SPSS Inc., Chicago, IL) OPG levels were skewed and thus, logarithmically transformed before entering analysis in normality was required. Results were considered statistically significant when P values were < 0.05.

RESULTS

Patients' characteristics and clinical data

Our study group consisted of 35 patients with MM and 20 healthy subjects as a control group. Twenty five of our patients were males and ten were females; age range was between 40 and 70 years (mean 58 ± 6.6).

The mean concentration of Myeloma cell %, monoclonal protein, Haemoglobin (Hb), urea and β2 microglobulin (β2 m) of MM samples are listed in Table-1.

Table -1: Clinico-pathological characteristics of MM cases and controls.

	Groups	N	Mean	Std. Deviation
Myeloma cell %	cases	35	38.00	11.319
	control	20	------	------
monoclonal Protein g/dl	cases	35	3.25	.787
	control	20	------	------
Hb g/dl	cases	35	9.780	1.5785
	control	20	13.995	1.0226
Urea mg/dl	cases	35	69.03	34.428
	control	20	28.10	5.360
B_2m mg/ml	cases	35	5.8803	2.08535
	control	20	1.5735	1.03885

Serum OPG levels using ELISA: The OPG levels in MM patients were lower 0.10 ± 0.04 ng/ml (mean ± SD; n=35) than in controls 0.15 ± 0.07 ng/ml (n=20) as shown in Figure-1. The range of OPG concentrations in myeloma and control sera were 0.04-0.22 ng/ml and 0.07 -0.37 ng/ml respectively. This difference was statistically highly significant (P= 0.001) as shown in Table-2. There was a high significant difference between cases and control groups as regards to HB, urea and β2 macroglobulin (P= 0.000) as shown in Table-2.

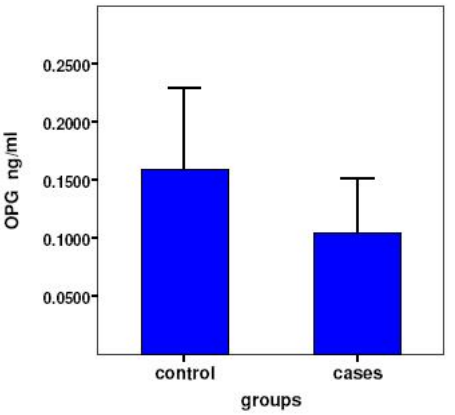

Figure-1: OPG in cases and controls groups.

Table -2: Statistical analysis of Hb, Urea, B_2m and OPG by student's t-test in MM patients compared to controls.

	M± SD		t	Sig.(2-tailed) (P)
	escas	control		
HB g/dl	35 ± 9.7	20 ± 13.9	10.70	0.000**
Urea mg/dl	35 ± 69.0	20 ± 28.1	5.26	0.000**
B_2m mg/ml	35 ± 5.8	20 ± 1.5	8.62	0.000**
OPG ng/ml	35 ± 0.10	20 ± 0.15	3.44	0.001**

P= high significance < 0.001**

OPG mRNA levels: Ten patients out of 35 MM cases were carefully chosen regarding the lower OPG protein level ranged from 0.046- 0.063 ng/ml. The OPG mRNA was studied in the bone marrows (BM) of these 10 patients. Also, bone marrow aspirated from 5 cases of non- MM patients considered as controls (OPG protein level ranged from 0.08 – 0.22 ng/ml) were evaluated for OPG mRNA expression.

The RT-PCR products of OPG gene of these patients and control are shown in Figure-2. The (135 Bp) fragment most probably candidate for the OPG gene according to gene bank.

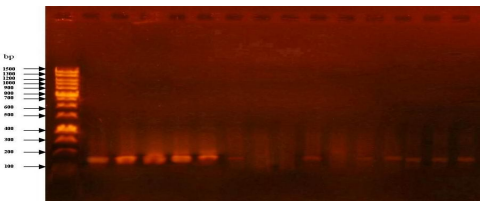

Figure -2: Agarose gel for RT-PCR amplified fragment of OPG- gene (s) for the present patients and controls, Lines 1 to 5 controls, lines 6 to 15 patients and M: molecular marker.

The expression of the gene was estimated as fold compared to the low level (analyzed using Phortix v.6 gel documentation software). The relative optical density scanning of agarose gel digital photos, using the algorithm dialogs of the computer program Phortix v.6, was used to compare the expression of OPG gene(s) in response to Myeloma cell disease, showed down regulation by exposure to Myeloma. The expression of the 135 bp fragments of patients levels of RT-PCR mRNA transcription (0.0 - 1.5 fold) than the controls levels of gene expression (3.0 -6.5 fold) as shown in Figure-3.

OPG expression in MM cases compared to controls was significantly different (P = 0.002) as statistically calculated by Mann-Whitney U test using data presented in table (3).

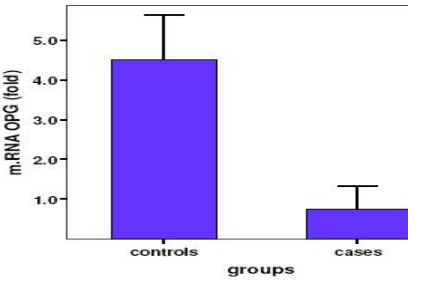

Figure-3: mRNA OPG (fold) in cases and controls group

Table-3: mRNA OPG fold as statistically calculated by Mann-Whitney U test

	N	Mean (fold)	Std. Deviation	Range	Asymp. Sig. (2- tailed) (P)
Cases	**10**	**0.77**	**0.554**	**0----2**	**0.002***
controls	**5**	**4.50**	**1.118**	**3----6**	

P is significant when < 0.05

Correlation between OPG levels, OPG mRNA expression levels and clinicpathological parameters: There were a correlation between the basic parameters of disease activity and serum OPG and mRNA-OPG as shown in Figure-4. In the controls, the correlations didn't reach the level of significance while in MM cases there was a negative highly significant correlations observed between urea and Hb (r = -0.65, P = 0.000), a positive significant correlations between Myeloma cell % and β_2 m (r = 0.43, P = 0.01), a positive highly significant correlations noted between the monoclonal protein and β2 m (r = 0.52, P = 0.001). There was a positive highly significant

correlation observed between urea and serum OPG protein (r = 0.49, P = 0.002). The correlations showed in figure (4) revealed a positive highly significant correlations betweens OPG mRNA and OPG protein in controls (r = 0.98, P = 0.002) as well as a positive significant correlations between OPG mRNA and OPG protein in MM patient (r = 0.72, P = 0.01).

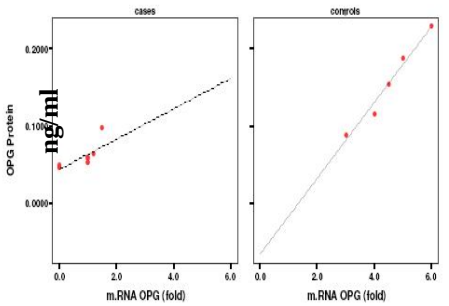

Figure-4: Correlation between OPG protein (ng/ml) and mRNA OPG (Fold) in both cases and controls groups.

DISCUSSION

Pathological osteolyses are considered a consequence of a disturbance in the mechanisms that govern the bone remodeling mainly the communication between osteoclasts and osteoblasts. Osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) are newly discovered molecules that play a key role in these communications (18). Any disturbance between these effectors leads to the development of skeletal abnormalities characterized by osteoporosis. Increased osteoclast activity is observed in many osteopathic disorders, including multiple myeloma (19-21). In the present context OPG has been implicated as a member of a ligand-receptor system that directly regulate osteoclast differentiation and bone resorption (2,7,8,22).OPG was expressed as mRNA and proteins in multiple myeloma patients and controls. Moreover, OPG expression was down regulated in MM patients while it was notably expressed in controls both at the transcript and serum protein levels. These results of the present study are in agreement with that reported by Standal *et al.*, (23) which demonstrated a reduction of OPG levels in the BM compartment of patients with MM compared to patients without myeloma. Furthermore, they presented evidence that the myeloma cells bind, internalize and degrade OPG, thereby providing a mechanism that may contribute to the reduced BM plasma OPG levels observed in myeloma patients.

Similar results were noticed by two independent groups (24, 25) and they have shown reduced expression of OPG by stromal cells in BM of myeloma patients compared to non-myeloma patients. Further more, data were presented indicating that myeloma cells reduced stromal cell OPG production *in vitro* (24, 25), and this was suggested as a cause of the reduced OPG levels in myeloma marrow. It should be noted that the various proposed mechanisms for OPG reduction are not mutually exclusive. It remains to be determined if they all operate simultaneously within the BM microenvironment, and if any of them play a dominant role, for instance, in certain phases of the bone disease.

Serum levels of OPG are shown to be reduced in patients with MM compared to healthy individuals (26). This is in contrast to other diseases associated with increased bone loss, such as postmenopausal osteoporosis (27), where serum OPG levels are increased. The results of the present study are in agreement with that reported by Nicola Giuliani *et al.*, (25) regarding the production of OPG by myeloma cells seems to be an uncommon event (cells of

only 3 of 26 MM patients tested expressed OPG mRNA and produced a small amount of this protein). In these patients there were no correlations between OPG productions and the presence or absence of bone lesions.

The bone resorption modulated by Receptor activator of nuclear factor –Kappa B ligand (RANKL) and OPG in murine calvarial organ culture, leads to changes in osteoblast proliferation, suggesting a feedback mechanism from osteoclasts to osteoblasts. It was found that RANKL and OPG have more potent effects on osteoclastogenesis than on the activity of mature osteoclasts (28). In newly diagnosed patients with multiple myeloma by Goranova-Marinova *et al.*, (29) OPG showed a tendency to decrease in advanced clinical stages and high grade myeloma bone disease (MBD), while RANKL/OPG ratio were significantly increased. Renal failure modified the expression of OPG.

The gene expression of myeloma cells by quantitative reverse transcription polymerase chain reaction of 10 candidate genes was studied for osteolytic bone disease. TNFRSF11B (OPG) was very rarely expressed in MM patients. (30).

Finally, a very recent study performed on the bone marrow sera from 21 MM patients. They showed that Wnt 3 a - induced OPG expression was diminished and enhanced RANKL expression in osteoblasts cultured with a myeloma-derived Dickkopf-1 (DKK1)- expressing MM cell line or primary MM cells. These results suggest that osteoclastogenesis via DKK1 mediated increase in RANKL/OPG ratios (31).

A study was performed on myeloma cell lines were cocultured in different culture system. RT-PCR was used to evaluate the expression of RANKL and OPG of myeloma cells. It showed in mouse primary bone marrow stromal cells (PBMSC), some myeloma cells which expressed neither RANKL nor OPG, up regulated RANKL expression and decrease OPG expression. Concluding that myeloma cells disrupting the balance of RANKL/OPG, thus maintaining a vicious circle between myeloma cells and osteoclasts (32).

The OPG values did not differ significantly by age, but serum levels were significantly higher than levels in plasma (33). Other studies, pretreatment of OPG serum levels were found to be increased in myeloma patients in comparison to healthy controls (34 -36).

It is concluded that OPG expression is reduced in bone marrow from myeloma patients. The adhesive interactions of myeloma cells with bone marrow stromal cells inhibit OPG production both at the m RNA and protein level. Down regulation of OPG expression in MM patients may be involved in pathogenesis of Myeloma bone disease and may provide a useful tool for prognosis.

Table-4: Correlation between OPG protein (ng/ml) and mRNA OPG (Fold) in both cases and controls groups.

	Myeloma cell%	Monoclonal band	Hbg/dl	Urea mg/dl	OPG ng/ml	B2m mg/ml	mRNA OPG (Fold)
pg/dl	-- -- 20	-- -- 20	1 -- 20	-0.189 0.425 20	-0.050 0.833 20	-0.127 0.594 20	-0.569 0.317 5
rea mg/dl	-- -- 20	-- -- 20	-0.189 0.425 20	1 ---- 20	0.276 0.239 20	-0.115 0.629 20	0.536 0.352 5
PG ng/ml	-- -- 20	-- -- 20	-0,050 0.833 20	0.276 0.239 20	1 ---- 20	-0.145 0.541 20	0.987** 0.002 5
2m mg/ml	-- -- 20	-- -- 20	-0.127 0.594 20	-0.115 0.629 20	-0.145 0.541 20	1 ---- 20	0.214 0.729 5
RNA PG(Fold) (r)	-- -- 5	-- -- 5	-0.569 0.317 5	0.536 0.352 5	0.987** 0.002 5	0.214 0.729 5	1 ---- 5
yeloma cell%	1 -- 35	0.447** 0.007 35	-0.172 0.322 35	0.022 0.899 35	0.095 0.588 35	0.432** 0.010 35	0.392 0.263 10
onoclonal nd (r)	0.447** 0.007 35	1 -- 35	-0.132 0.451 35	0.081 0.643 35	0.214 0.217 35	0.521** 0.001 35	0.463 0.178 10
pg/dl	-0.172 0.322 35	-0.132 0.451 35	1 -- 35	-0.654** 0.000 35	-0.129 0.459 35	-0.243 0.159 35	0.394 0.260 10
rea mg/dl	0.022 0.889 35	0.081 0.643 35	-0.654** 0.000 35	1 ---- 35	0.497** 0.002 35	0.230 0.184 35	-0.470 0.170 10
OPG ng/ml (r) (p) (n)	0.095 0.588 35	0.214 0.217 35	-0.129 0.459 35	0.497** 0.002 35	1 ---- 35	0.252 0.145 35	0.722* 0.018 10
2m mg/ml	0.432** 0.010 35	0.521** 0.001 35	-0.243 0.159 35	0.230 0.184 35	0.252 0.145 35	1 ---- 35	0.052 0.886 10
RNA OPG old) (r)	0.392 0.263 10	0.463 0.178 10	0.394 0.260 10	-0.470 0.170 10	1 ---- 10	0.052 0.886 10	1 ---- 10

level (2-tailed) Pearson correlation (r), significance (2-tailed) (P), number of cases (n)

***correlation is significant at the 0.05 level (2-tailed) **correlation is significant at the 0.01**

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