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High level of circulating platelet derived microvesicles in bone tumors

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ABSTRACT

Co^{II}, Ni^{II}, Cu^{II} and Mn^{II} complexes of Mannich base, as ligand, was prepared by condensation of aqueous semicarbazide, morpholine and pyridine-3-carboxaldehyde. The structure of the newly synthesized Mannich base was investigated by UV-Vis, IR, ^{1}H-NMR, ^{13}C-NMR, molar conductance and magnetic susceptibility studies. The antimicrobial activities of the ligand and metals complexes have been screened in vitro against the organisms E.faecalis, Proteus mirabilis, Bacillus cereus, E.aerogens, ESBL E.coli, ESBL K.pneumoniae, by disc diffusion and well diffusion techniques. It is observed that the coordination of metal ions has pronounced effect on the microbial activities of the ligand. The metal complexes have higher antimicrobial effect than the free ligand.

KEYWORDS : microvesicles, bone tumor, platelet

Extracellular vesicles (EVs) are heterogeneous population of small, membrane-coated vesicles with diverse biological functions (György et al, 2011a). EVs are released by various cells to the extracellular space and play a major role in the cellular cross-talk. Although as yet there is no consensus regarding their classification, we can distinguish apoptotic bodies and microvesicles (MV) or microparticles (MP) and exosomes. Exosomes, the smallest EVs (50-100nm) are generated by exocytosis; apoptotic bodies (1-5 m) are released from blebs of cells undergoing apoptosis (György et al, 2011a). MVs (100nm-1m) are generated by budding/blebbing of the plasma membrane. The existence of MVs was described almost fifty years ago (Wolf, 1967), however, these structures have been considered for many decades as irrelevant 'platelet dust'. According to our earlier observations, immune complexes share biophysical properties (size, light scattering, and sedimentation) with MVs (György et al, 2011b). MVs are secreted upon activation or apoptosis from several cell types such as platelets, red blood cells, lymphocytes, monocytes, endothelial cells, tumor cells and trophoblasts. The majority of circulating MVs in the blood are derived from platelets or shed directly by megakaryocytes (Italiano et al, 2010). MVs generally display the characteristics of the mother cell, contain cell surface receptors that can be used to identify the cell types from which they originate, they also contain cytoplasm proteins, nucleic acid (mRNA, microRNA and DNA) and cytokines (György et al, 2011a). | Although recently published data clearly indicate that MVs play a central role in a number of diseases including systemic autoimmune diseases (Distler et al, 2010, Boillard et al, 2010) and tumors (Galindo-Hernandez et al, 2013), the potential role in metabolic bone diseases and bone tumors have not been studied yet. | In our present study we compared the circulating levels of MVs in patients with bone tumors, active and inactive Paget's disease, spondylosisand osteoporosis. | While an extremely high number of platelet-derived CD41a+ MVs were detected in the plasma of patients with bone tumor, total number of (Ax+) MVs, and bone-derived (Rank+ and RankL+) MVs counts showed no difference in patients' groups. | ***Patients*** | Twenty six patients with Paget's disease, twenty two patients with osteoporosis and twenty two patients with spondylosis were recruited in the National Institute of Rheumatology and Physiotherapy, Budapest, Hungary. Eight patients with bone tumor were included in the Department of Ortopedics, SemmelweisUniverisy, Budapest, Hungary. The bone tumors were as follows: three patients had metastasis of the femur; two patients had Ewing sarcoma and one patient had osteosarcoma, one patient had adamantinoma and one patient had chondrosarcoma. Informed consent was obtained from all patients and the experiments were approved by the Hungarian Scientific and Research Ethics Committee (TUKEB). Laboratory parameters potentially correlating with the MV count were considered: beta crosslaps levels, serum alkaline phosphatase, serumosteocalcin. Eight out of twenty six patients with Paget's disease had blood samples before and after zolendronic acid infusion. Thirteen patients were defined Paget active, with bone pain. Thirteen patients were inactive without pain. The mean age ± standard deviations and the male/ female ratios were: 69.1 ± 8.1 and 14/12 in the Paget; 40.3 ± 23.7 and 0/8 in the tumor; 68.7 ± 8.18 and 5/17 in the spondylosis; 70.6 ± 9.21 and 3/19 in the osteoporosis patient groups, respectively. Routine laboratory tests incuded: complete blood count, serum osteocalcin, beta crosslaps, alkaline phosphatase and erythrocyte redimentation rate

measurements. | ***Isolation of microvesicles*** **|** The isolation and storage procedure as well as preparation for transmission electron microscopy of microvesicles has been described earlier in detail (György et al, 2011b). | ***Flow cytometry*** **|** Samples were analyzed by using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), equipped with 20-mW argon laser (emission at 488 nm). The FC instrument settings were adopted from our previous works (György et al, 2011b, György et al,2012). To identify and characterize MVs, annexin V-(AX) fluorescein isothiocyanate (FITC); anti-receptor activator of nuclear factor kappa B (RANK)-phycoerythrin (PE) and anti-RANK ligand (RANKL)-PE were used (all from BD Biosciences). We applied our earlier protocol for staining MVs. Briefly, 1 ìg of antibodies was added to 20 µl of plasma/SF, and incubated for 30 minutes at RT. To reduce background event numbers, samples were diluted in 1:30 with 0.01 µm pore size membrane filtered PBS (Millipore). Event numbers of equal sample volumes were counted for 60 seconds. Annexin staining was carried out in the presence of 2.5 mM Ca^{2+}. Event numbers of equal sample volumes were counted for 30 s. | ***Statistical analysis*** **|** Differences between MV subpopulations were estimated by Mann-Whitney test and a p-value of <0.05 was considered statistically significant. The correlation of MVs and different clinical and laboratory parameters were assessed using Spearman's rank correlation test (confidence interval of 95%). | Representative EM images of the MV preparations showed vesicular structures (**Fig 1.**). The number of CD41+ MVs was significantly elevated in the bone tumor group (Fig 2.B), however, the total counts (AX^+) of MVs (Fig.2A) were similar in all patient groups and the number of bone-derived circulating MVs were not significantly different in bone tumor and in other patients with various non-malignant bone disorders. No correlation was found with the disease activity in the Paget's patient group, either (data not shown). We could detect bone derived RANK and RANKL bearing MVs in all patient cohorts, but there was no significant difference between the patient groups studied (**Fig 2. C, D**). Zolendronic acid effectively inhibits osteoclast activation, thereafter we studied its effect on MV levels. Zolendronis acid treatment of patients with clinically active Paget's disease did not alter the AX^+, $RANK^+$ or $RANKL^+$ MV levels significantly (data not shown). | MVs are subcellular structures released from the plasma membrane of the cells, the release of MVs appears to be a uniform ability of both prokaryotic and eukaryotic cells (Gyorgy et al, 2011a). Although several recent publications suggest that MVs are newly recognised, important players of the intercellular communication, their precise physiological role is not known, yet. MVs of different origin were widely investigated recently, however the potential role of MVs in bone diseases were not studied yet. The aim of our present work was to study the MV production in bone tumor and in metabolic bone diseases. Our present data suggest that number of total (AX^+), and bone-derived $RANK^+$ and $RANKL^+$ MV counts are not significantly different in patients' cohorts and did not correlate with the serum alkaline phosphatase activity, osteocalcin and collagen crosslink levels in Paget's disease. On the contrary, a strong increase of platelet-derived CD41a+ MVs were deteced in the circulation of patients with bone tumors. | Bone is continuously renewed by the process of bone remodeling, in which process osteoclasts resorb bone, subsequently replaced by new bone produced by osteoblasts. Bone remodeling is perturbed in a number of pathologic conditions including: bone tumors, Paget's disease, osteoporosis and rheumatoid arthritis Ralston et al, 2013, Pisetsky et al, 2012). Interestingly merely in bone tumors the platelet derived (CD41a+) MVs were highly elevated merely. Simultaneously, we could readily detect $RANK^+$ and $RANKL^+$ MVs in all patient groups studied, however the lack of difference between the patient groups suggest that altered bone remodeling does not necessarily lead to altered bone-derived MV production. | Therefore one my suggest, that platelet MVs may serve as peculiar biomarkers in bone tumor. Further studies are required to clarify the relation of selective platelet activation and elevation of CD41a+ microvesicles as well as bone malignancy. | Acknowledgement | This work has been supported by grants OTKA K77537 and OTKA K84043. | **Figure 1.**Transmission electron micrograph of blood plasma MVs from a patient with Paget's disease. Original magnification was 30,000x. | |

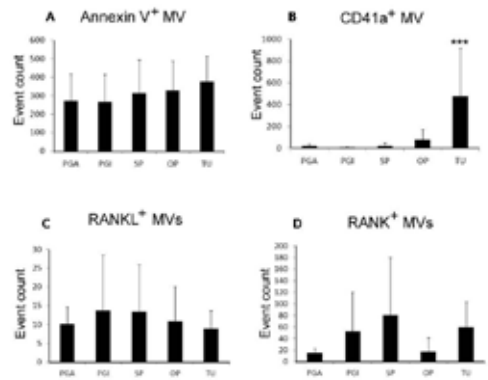
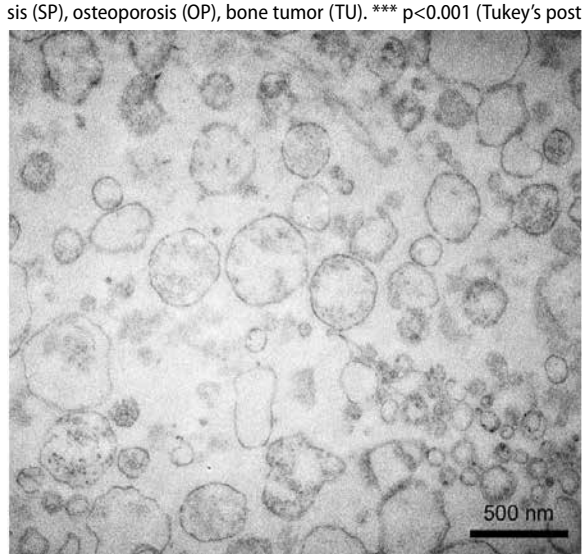


Figure 2. Flow cytometricimmunophenotyping of MVs in blood plasma of patients with active (PGA), inactive (PGI) Paget's disease, spondylosis (SP), osteoporosis (OP), bone tumor (TU). *** p<0.001 (Tukey's post



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