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Bone matrix vesicle-bound alkaline phosphatase for the assessment of peripheral blood admixture to human bone marrow aspirates

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ABSTRACT

Purpose: Peripheral blood (PB) admixture should be minimized during numerical and functional, as well as cytokinetic analysis of bone marrow (BM) aspirates for research purposes. Therefore, purity assessment of the BM aspirate should be performed in advance. We investigated whether bone matrix vesicle (BMV)-bound bone alkaline phosphatase (ALP) could serve as a marker for the purity of BM aspirates.
Results: Total ALP activity was significantly higher in BM serum (97 (176–124) U/L, median (range)) compared to PB serum (63 (52–73) U/L, $p < 0.001$). Agarose gel electrophoresis showed a unique bone ALP fraction in BM, which was absent in PB. Native polyacrylamide gel electrophoresis revealed the high molecular weight of this fraction, corresponding with membrane-bound ALP from bone matrix vesicles (BMV), as evidenced by electron microscopy. A serial PB admixture experiment of bone cylinder supernatant samples, rich in BMV-bound ALP, confirmed the sensitivity of this proposed quality assessment method. Furthermore, a BMV ALP fraction of ≥15% is suggested as cut-off value for minimal BM quality. Moreover, the BM purity declines rapidly with larger aspirated BM volumes.
Conclusion: The exclusive presence of BMV-bound ALP in BM could serve as a novel marker to assess purity of BM aspirates.
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1. Introduction

Relatively large amounts of bone marrow (BM) are aspirated for cellular analysis and cytokinetic profiling of BM in both diagnostic and research settings. However, inevitable peripheral blood (PB) contamination during BM aspiration could lead to misinterpretation of the obtained results. In the setting of autologous stem cell therapy for cardiovascular diseases for example, numerical and functional differences between BM could explain the magnitude of response to stem cell therapy [1], but ideally should take PB contamination into account. Therefore, quality assessment of the BM aspirate prior to BM analysis in research settings is essential and should be reinstated into the field [2].

The current method in daily clinical practice to assess purity of a BM aspirate is a qualitative method based on the observation of spicules in the BM smear. Quantitative assessment on the other hand is challenging and is often neglected, and to date, no consensus is reached on an acceptable gold standard.

A first quantitative approach dating from the 80s was based on the difference of nucleated cells in S-phase index between BM biopsies (i.e. bone cylinders) and BM aspirates, which was considered as

Abbreviations: ALP, alkaline phosphatase; BC, bone cylinder; BM, bone marrow; BMV, bone matrix vesicle; Hb, hemoglobin; MW, molecular weight; NA, neuraminidase; PAGE, polyacrylamide gel electrophoresis; PB, peripheral blood; PI-PLC, phosphatidyl-inositol-phospholipase C; SN, supernatant; TEM, transmission electron microscopy.

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a consequence of PB cell contamination exclusively [3]. Secondly, flow cytometric quantification of the percentage CD45+ gated lymphocytes and monocytes in the BM aspirate was introduced to evaluate BM quality, since this percentage is inversely related to the percentage of cells in the S-phase for the total BM cell population [4]. A total lymphocyte–monocyte count of >30% was used as cut-off value to exclude BM aspirates with an unacceptable degree of PB contamination [4]. Nowadays, the most common technique is the Holdrinet method [5,6]. By means of Cr^{15} labeled autologous erythrocytes, Holdrinet et al. showed that 97% of the hemoglobin (Hb) content in BM is derived from PB. Based on the assumption that a proportional number of PB cells is present, the fraction of PB admixture could be calculated by determining the Hb levels in blood and bone marrow.

However, the aforementioned methods for the quantification of PB admixture to BM aspirates are based on *cellular* assessments and should not be used in conditions that possibly influence cellular function of BM such as heart failure [7–9]. Therefore, we searched for an alternative stable marker, which is exclusively present in BM and is independent of BM cellular characteristics.

To this purpose we investigated bone alkaline phosphatase (ALP), an important bone turnover marker produced by bone forming osteoblasts from BM [10]. The enzyme ALP consists of different isoenzymes and, in humans, four ALP genes are identified: tissue-nonspecific, intestinal, placental and germ-cell ALP [11,12]. After posttranslational modifications of the tissue-nonspecific ALP gene product, bone and liver ALP isoforms are formed [11].

Whereas the tertiary structure of ALP in PB promotes dimerization, tetrameric ALP and high molecular weight (MW) isoforms have also been described for all the ALP isoenzymes/isoforms [11]. The high MW isoforms consist of ALP anchored to the plasma membranes of shedded microvesicles and incubation of these microvesicles with a non-ionic detergent results in the release of anchor-bearing ALP from the membrane which, in the presence of detergents, has a MW that is consistent with tetrameric ALP [11]. Phospholipases, such as phosphatidyl-inositol-specific phospholipases, are responsible for the conversion of this tetrameric ALP into the soluble dimeric form of ALP [11,13–15].

In PB serum from healthy subjects, liver and bone ALP mainly circulate as soluble dimers, and tetrameric liver and bone ALP are absent. In pathological conditions, the finding of membrane-bound bone ALP in PB is extremely rare, in contrast to membrane-bound liver ALP [11].

We hypothesized that a high activity of membrane-bound bone ALP, originating from bone matrix vesicles (BMV) formed by budding of osteoblasts that reside in the BM [16], is present in BM aspirates but absent in PB. As such, the activity of BMV-bound ALP could serve as a novel marker to assess BM purity.

2. Materials and methods

2.1. Patients and collection of BM and PB samples

After approval of the Antwerp University Hospital Ethics Committee and written informed consent, patients undergoing elective cardiac surgery were included in the study. Exclusion criteria were chronic inflammatory or malignant disease, chronic kidney disease (Creatinine clearance <30 ml/min), severe liver failure and disorders with abnormal bone turnover. Table 1 shows the patient characteristics and indicates in which cohort the experiments were performed.

Prior to sternotomy and under general anesthesia, BM samples were aspirated by sternal puncture (15G Jamshidi BM aspiration needle) as described previously [17] and arterial PB was sampled. Both BM and PB were collected in Vacutainer® serum tubes (Becton Dickinson (BD) Benelux, Erembodegem, Belgium). Serum of BM and PB was obtained by 15 minute centrifugation at 1500 *g*. For the quantification of PB admixture by the Holdrinet method, PB and BM samples were collected in Vacutainer® EDTA tubes (BD Benelux) to determine Hb levels.

Table 1
Patient characteristics and indication in which cohort the different experiments were performed. The results are expressed as mean ± SD or as percentage (%) of patients.

Characteristic	Cohort 1 N = 55	Cohort 2 N = 20	Cohort 3 N = 9	Cohort 4 N = 4
Age (yrs)	63 ± 10	64 ± 11	70 ± 11	42 ± 15
Male (%)	84	90	89	50
BMI (kg/m^2)	27 ± 4	27 ± 4	28 ± 3	N.A.
Creatinine clearance (ml/min)	77 ± 18	74 ± 22	74 ± 20	N.A.
Left ventricular ejection fraction (%)	53 ± 19	52 ± 18	44 ± 12	N.A.
Framingham risk score (%)	14 ± 8	15 ± 8	19 ± 10	N.A.
CRP (mg/l)	5.7 ± 7.8	15.1 ± 33.3	27.7 ± 46.3	N.A.
Diabetes (%)	5	5	22	N.A.
Pulmonary disease (%)	9	3	22	N.A.
Surgery				
- CABG (%)	71	68	67	N.A.
- Aortic valve surgery (%)	7	11	33	N.A.
- Mitral valve surgery (%)	24	11	0	N.A.
- VAD implantation (%)	7	5	0	N.A.
- Heart transplantation (%)	2	5	0	N.A.
Medication				
- Statins (%)	66	63	44	N.A.
- B-blockers (%)	64	68	89	N.A.
- ACE-inhibitors (%)	31	21	33	N.A.
- Diuretics (%)	35	37	33	N.A.
Total aspirated BM volume (ml)	10	10	20	N.A.
Sampled arterial PB volume (ml)	10	10	10	N.A.
Experiment				
- Total ALP activity quantification	X			
- ALP isoenzymes/isoforms differentiation	X			
- MW ALP isoforms determination	X			
- In vitro PB admixture experiment of BC SN				X
- BMV ALP versus Holdrinet comparison		X		
- Influence of aspirated BM volume on BMV ALP			X	

Abbreviations: ALP, alkaline phosphatase; ACE-inhibitors, angiotensin-converting-enzyme inhibitors; BC SN, bone cylinder supernatant; BM, bone marrow; BMI, body mass index; BMV, bone matrix vesicle; CABG, coronary artery bypass grafting; CRP, C-reactive protein; VAD, ventricular assist device.

2.2. Quantification of total ALP activity

Total ALP activity (U/L) of BM and PB serum was measured spectrophotometrically on the Dimension Vista® System (Siemens), using the ALP Flex® reagent cartridge (Siemens). This colorimetric assay uses p-nitrophenylphosphate (p-NNP) as substrate, which is catalyzed into p-nitrophenol (p-NP) by all ALP isoenzymes/isoforms. For this IFCC calibrated method, the reference interval for healthy individuals in PB serum is 42–98 U/L in women from 15 to 59 years old, 53–141 U/L for women older than 59 years, 53–128 U/L in men from 20 to 59 years old and 56–119 U/L for men older than 59 years [18,19]. Coefficients of variation for total ALP activity quantification were 4.5% for a mean ALP activity of 27 U/L (n = 86 PB serum samples) and 2.7% for a mean activity of 137 U/L (n = 85 PB serum samples).

2.3. ALP isoenzyme/isoform differentiation

To separate the different ALP isoenzymes and isoforms present in PB and BM serum, agarose gel electrophoresis was performed with the Isopal Plus® kit (Beckman Europe, Analis S.A, Suarlée, Belgium) as described previously [20]. In addition, samples were subjected to a set of treatments for ALP isoenzyme differentiation. Treatment with neuraminidase (2 U/ml, purified from Clostridium perfringens, Analis S.A.)

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