Demonstration of altered signaling responses in bone marrow extracellular fluid during increased hematopoiesis in rats using a centrifugation method

Helge Wiig, Ellen Berggreen, Bengt Åge Sørby Borge, and Per Ole Iversen.

Innovative Methodology


The composition and characteristics of the bone marrow extracellular fluid supposedly modify the transport of cytokines, drugs, and other signaling molecules involved in the regulation of bone marrow function. Direct access to the bone marrow extracellular fluid surrounding hematopoietic cells is complicated by the virtually noncompliant surrounding bone tissue. We examined the applicability of a centrifugation method to obtain representative samples of bone marrow extracellular fluid from rats and humans. Perforated rat bones or human bone marrow biopsies were wrapped in nylon mesh baskets before being centrifuged at 180–239 g. In the rats, we found an only minor contribution of fluid from other sources than the bone marrow extracellular fluid as indicated by the average ratio of centrifugate-to-plasma activity of the extracellular tracer fluid 51Cr-labeled EDTA of 0.85. The colloid osmotic pressure in the centrifugate was consistently lower than that in the corresponding plasma in both species. In rats and humans, high-performance liquid chromatography showed a protein elution pattern from the bone marrow fluid similar to that of plasma, except for a peak eluting in the ~40-kDa molecular mass range. Western blotting of the cytokines erythropoietin and granulocyte colony-stimulating factor revealed generally higher amounts in the centrifugate than in the plasma. This difference was augmented during increased hematopoietic activity induced by inflammation or bleeding in rats. We conclude that the centrifugation method provides representative samples of bone marrow extracellular fluid and that extracellular signaling responses to altered hematopoiesis are more clearly reflected locally in the bone marrow interstitium than in plasma.

Interstitium in solid tumors is also difficult to access. Recently, we showed (22) that extracellular fluid can be isolated from tumors by exposing tumor tissue to centrifugation (G forces less than ~400 g). Having excluded wicks and microdialysis as alternative methods to gain access to bone marrow interstitium because of their more traumatic nature, these tumors experiments led to the hypothesis that a similar centrifugation method could also be applicable to isolate bone marrow extracellular fluid. Specifically, our aims were to study whether representative samples of extracellular fluid could be isolated from the tibial and femoral marrow and from human bone marrow biopsies by centrifugation. If so, we would expect that stimulation of hematopoiesis would be reflected in changed local cytokine levels. To further validate our isolation procedure, we therefore examined the levels of
hematopoietic cytokines in extracellular fluid sampled during increased erythro- or leukopoiesis.

MATERIALS AND METHODS

Experimental animals. The experiments were performed in Wistar rats (n = 44) of either sex (range 210–350 g, median 243 g) that were fed a standard laboratory diet. All experiments were performed in accordance with recommendations given by the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee. On the day when the experiment was terminated, the rat was anesthetized with pentobarbital sodium (50 mg/kg ip). During induction of hemorrhage (see Induction of sterile inflammation or hemorrhage), the rats were anesthetized with Equisit (42.5 mg/ml chloral hydrate, 21 mg/ml magnesium sulfate, and 9.7 mg/ml pentobarbital sodium; 0.4 ml/100 g ip) because of the shorter half-life of this anesthetic mixture. While anesthetized, body temperature was maintained at 36.5–37.5°C with a heating lamp. Unless otherwise specified, blood was sampled by cardiac puncture, and the rat was killed by intracardiac injection of saturated potassium chloride.

Centrifugation technique. Immediately after euthanasia, the femur and tibia were exposed and dislocated. With the use of stereomicroscopy, the femur and tibia were removed, transferred to centrifuge tubes, and handled as described above. Bone marrow fluid isolated in these experiments was pipetted into 0.5 ml saline in counting vials.

We determined the extracellular fluid distribution volume utilizing 51Cr-labeled EDTA in 12 other rats. The bone marrow content was isolated as described (8) except that compressed air was used for flushing the bone marrow cavity. Furthermore, for comparison and as a reference, samples taken from the bone marrow, spongy bone, and back skin for calculation of plasma equivalent distribution volumes [(cpm/g tissue)/(cpm/ml plasma)], where cpm is counts/min] were placed in tared, covered vials, and weighed. Samples were counted in a LKB gamma counter (model 1282 Compugamma; Turku, Finland).

HPLC of fluid isolated from bone marrow. The distribution of macromolecules in bone marrow fluid isolated by centrifugation, elution fluid of intact or homogenized bone marrow, and plasma was determined by HPLC using Superose 12 HR 10/30 size exclusion column (Pharmacia-Biotech; Uppsala, Sweden) with optimal separation range of 10–300 kDa, as previously described (22). The protein concentration in the elution fluid was measured by UV detection at 280 nm (SpectraSystem UV) (22).

Colloid osmotic pressure. The colloid osmotic pressures (COP) in bone marrow fluid and in plasma were measured in a colloid osmometer designed for submicroliter samples (23), using membranes with a cutoff size of 10 kDa. Usually, samples of 0.5–1 μl were applied to the osmometer membrane. The coefficient of variation for this osmometer has been estimated to 3.2% for rat and 2.8% for human plasma (23).

Induction of sterile inflammation or hemorrhage. To induce a sterile peritonitis, we injected intraperitoneally a casein digest (4 ml, 12.5% in 0.9% NaCl; Bacto-Tryptone, Difco; Detroit, MI) into four awake rats. This treatment results in an approximately fivefold increased blood neutrophil number within about 5 h, mainly due to an enhanced release from the bone marrow storage (11). At this time point the rats were anesthetized, and bone marrow fluid was isolated, as described above.

Hemorrhage was induced in three other rats anesthetized with Equithesin. A PE-50 catheter was placed into one carotid artery, tunneled subcutaneously to the neck, and secured. When recovered and awake, these rats were bled 1.5% of their body weight (~3.5 ml). Plasma isolated during the bleeding served as control. Four days later the rats were anesthetized, and bone marrow fluid and plasma were isolated as described above.

Human studies. We wanted to extend the animal studies to examine whether the centrifugation method for bone marrow fluid isolation was also applicable to human bone marrow. The protocol was approved by the Regional Ethics Committee for Medical Research, Health Region II in Norway, and written consent was obtained from each study subject. We studied six adult, healthy males (age 33–57 y, body weight 56–83 kg). A blood sample (5 ml) was obtained from all subjects by puncture of an antecubital vein. After infiltration of the skin and perist with local anesthesia (5 mg/ml; Xylocain, AstraZeneca; London, UK), a cylindrical bone marrow biopsy was collected from one iliac crest. The centrifugation of the collected bone marrow biopsies was performed as outlined above.

Analysis of proteins in bone marrow fluid and plasma. We used species-specific monoclonal antibodies (rat: Research Diagnostics; Flanders, NJ; human: Pharmingen; San Diego, CA) raised against either erythropoietin or granulocyte colony-stimulating factor (G-CSF) to identify these cytokines in samples of bone marrow extracellular fluid obtained from either the rats or human subjects, using Western blotting as previously detailed (7). Briefly, the centrifugate proteins were separated on SDS-PAGE (7.5%), then transferred onto nitrocellulose membranes, and probed with relevant

BONE MARROW EXTRACELLULAR FLUID COMPOSITION
antibodies, before analysis with an enhanced chemiluminescence detection kit (ECL; Amersham, Little Chalfont, UK). Nonspecific binding was blocked with 10% skimmed milk. Equal volumes (usually 10–15 μl) of the extracellular fluid and plasma samples were added to the SDS-PAGE gels.

Concentrations of erythropoietin and granulocyte colony-stimulating factor (G-CSF) in human plasma were quantified in triplicates with ELISA-kits (Quantikine, R&D Systems; Minneapolis, MN).

Statistics. Values are reported as means ± SE. Differences were tested with two-tailed t-tests or ANOVA, followed by Tukey tests as appropriate, and were assumed significant for P < 0.05.

RESULTS

Typical bone marrow fluid samples were straw colored, and the amount isolated in the initial centrifugation (<239 g) was 0.5–1 μl, increasing to 1–2 and 2–4 μl at 307 and 424 g, respectively, in rat samples. There was no clear relationship between rat body weight and the amount extracellular fluid that was isolated by centrifugation. Assuming a density of 1.0 g/ml, the centrifugate volume corresponds to 1 ml for rats and 2.1 ml for the bone marrow isolated from femur (see below). We usually obtained larger volumes (<5 µl) after centrifugation of the human biopsies.

Tracer recovery in rat bone marrow extracellular fluid. To determine the possible contamination of intracellular fluid in the isolated samples of bone marrow extracellular fluid, we measured the recovered centrifugate tracer (51Cr-labeled EDTA) that had equilibrated in extracellular fluid in rats (Fig. 1). At a centrifugation speed of <239 g, the average concentration of 51Cr-labeled EDTA in the bone marrow fluid relative to that of the plasma was 0.85 ± 0.03 (n = 13), which was significantly different from 1.0. Increasing the G force in consecutive centrifugations resulted in lower ratios than observed at <239 g. Thus for centrifugation speeds of 307 and 424 g, ratios of 0.64 ± 0.07 (n = 5) and 0.39 ± 0.072 (n = 5) were observed, respectively, both lower than that of <239 g (P < 0.01 and 0.001, respectively). The ratios for tibia and femur did not differ significantly, and thus the results from the two tissues were pooled. In some experiments, the tracer equilibration period was extended to 180 (n = 2) and 300 min (n = 2) without any further effect on the centrifugate to plasma 51Cr-labeled EDTA ratio (data not shown).

The amount of bone marrow isolated in the fluid volume determination experiments averaged 0.0289 ± 0.0022 g (range 0.0201–0.0377 g, n = 12). The extracellular fluid volumes measured as the 120-min distribution volume of 51Cr-labeled EDTA was 0.12 ± 0.01 ml/g wet wt (n = 12) in the bone marrow, with corresponding volumes of 0.14 ± 0.02 and 0.43 ± 0.03 ml/g wet wt (n = 12) in spongy bone and back skin, respectively.

COP in bone marrow fluid. COP in fluid isolated from the rat tibia and femur and human bone marrow are shown in Fig. 2. In rats (n = 8), COP averaged 19.4 mmHg in bone marrow, with a corresponding pressure in plasma of 24.1 mmHg (P < 0.05). In human controls (n = 6), the bone marrow fluid COP averaged 23.2 mmHg, which was clearly lower than the corresponding pressure in plasma of 33.0 mmHg (P < 0.05). The relative amounts of albumin and IgG did not change when the G force was increased.

Protein patterns in bone marrow fluid analyzed with HPLC. Figure 3 shows elution patterns of fluid isolated from rats with rat plasma (Fig. 3A) as a reference, showing the elution volumes of typical plasma proteins. Figure 3B depicts the pattern for femoral bone marrow fluid isolated by centrifugation at 239 g in control conditions. We observed that albumin dominated quantitatively and that IgG constituted a minor fraction of proteins in the eluent. The relative amounts of albumin and IgG did not change when the G force was increased. For molecules smaller than albumin, the elution pattern of bone marrow fluid and plasma differed significantly. In bone marrow, a macromolecule with molecular mass less than albumin consistently eluted at 13.4 ml (corresponding to ~40 kDa), and such a macromolecule was never seen in the plasma.

![Fig. 1. Ratio of bone marrow fluid-to-plasma concentration of the extracellular tracer as a function of the applied G force will reflect dilution of the centrifugate. An extracellular tracer (51Cr-labeled EDTA) was equilibrated in extracellular fluid and bone marrow fluid isolated by centrifugation. Increasing the G force resulted in a reduced bone marrow fluid-to-plasma ratio of the extracellular tracer, indicating increasing dilution of the centrifugate. All ratios differed significantly from 1.0 and from each other. Values are means ± SE; n = 13 for ≤239 g and n = 5 for 307 and 424 g.](www.ajpheart.org)
In addition, there were even smaller macromolecules in bone marrow fluid that were inconsistently found in plasma constituting a variable but small fraction (<8.3%), of the total area under the elution curve.

Induction of a sterile inflammation using Bacto-Tryptone (Fig. 3C) resulted in an increase of macromolecules eluting in the IgM and IgG region. Furthermore, this intervention markedly increased the 13.4-ml peak. In rat bone marrow exposed to repeated cycles of freeze thawing, the major fraction of proteins eluted in volumes larger than that of albumin (12.5 ml), with a major peak at 13.6 ml (Fig. 3D).

Figure 3E shows an elution curve from human plasma, and a representative curve for human bone marrow fluid isolated from a normal subject is shown in Fig. 3F. In the human samples, nearly all macromolecules eluted in the globulin and albumin fractions. The plasma concentrations of albumin and immunoglobulins did not differ between the controls and the patients (data not shown). The most notable difference between

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**Figure 3.** Characterization of plasma and bone marrow fluid in rats and humans using size exclusion high-performance liquid chromatography (HPLC). Representative patterns for plasma and bone marrow fluid from rats (A–D) and humans (E–G) eluted in a Superose 12 HR column. A: rat plasma indicating elution volumes (Vₑ) for typical plasma proteins. B: bone marrow centrifugate isolated from control rats. A reduced IgM and IgG fraction and a defined peak at 13.4 ml (arrowhead) can be observed. C: sterile inflammation induced by Bacto-Tryptone, resulting in an increase in IgM and IgG fractions and area under the peak at 13.4 ml (arrow). D: hemolyzed bone marrow, where most of the macromolecules eluted in a volume larger than albumin (Alb; 12.5 ml) with a major peak at 13.6 ml. The difference between bone marrow centrifugate and hemolysate clearly indicates that the centrifugate is of extracellular origin. E: human plasma. F: bone marrow centrifugate isolated from a normal subject. The pattern for F resembled plasma except for a reduced fraction of albumin relative to that of immunoglobulin (albumin-to-globulin ratio) (see text). G: hemolyzed human bone marrow. As for hemolyzed rat marrow, the majority of macromolecules eluted in volumes higher than albumin.
human bone marrow fluid and plasma was the smaller albumin-to-globulin ratio for the marrow fluid in normal subjects, averaging 1.50 ± 0.01 (n = 6) compared with the corresponding ratio of 1.81 ± 0.09 (n = 3) obtained in plasma. Similar to the rats, after freeze thawing of human bone marrow, the major part of the macromolecules eluted in volumes higher than that of albumin (Fig. 3).

**DISCUSSION**

Our hypothesis was that the seemingly inaccessible bone marrow extracellular fluid could nevertheless be sampled by exposing bone marrow to centrifugation. We argue that the present centrifugation method is applicable to isolate bone marrow extracellular fluid, because 1) the concentration of the extracellular tracer E14Cr-labeled EDTA in bone marrow fluid approximated that in plasma, 2) the protein profiles in bone marrow fluid and plasma showed striking similarities and clearly different from that induced by bone marrow lysis, and 3) the levels of the two hematopoietic cytokines erythropoietin and G-CSF changed differently in the two compartments upon altered increased hematopoiesis. The major conclusion of the present study is then that samples representative for bone marrow extracellular fluid can be isolated from rat and human bone marrow by low-speed centrifugation (<239 g) and that responses to hematopoietic challenges are more clearly reflected at the local level than in plasma. To the best of our knowledge this is the first report describing a method for direct sampling of extracellular fluid from the bone marrow, thereby demonstrating local regulatory changes during altered hematopoiesis.

**Fig. 4.** Hematopoietic cytokines in plasma and bone marrow fluid in control rats and during stimulated hematopoiesis. A: erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) were present in both plasma and isolated bone marrow fluid (centrifugate), as shown with Western blotting. The albumin signal demonstrated equal loading of protein on the gel. Data from one rat are shown. B: pooled data from densitometric measurements of samples from 5 rats. The values are calculated as the percentages of the respective albumin recordings and shown as means ± SE. Upon bleeding, erythropoietin increased in both plasma and centrifugate as shown in the Western blot of samples from one rat in C and the pooled data from samples of 3 rats in D. Similarly, peritonitis induced a substantial elevation of G-CSF in both compartments, as demonstrated in the Western blot from samples of one rat in E and in the pooled data from 4 rats in F.
Evaluation of the centrifugation method. Bone marrow is a highly specialized cell-rich tissue enclosed in a rigid compartment, and cell compression during centrifugation may lead to extrusion of cellular fluid resulting in a mixture of extracellular and cellular fluid. As in the recent study in chemically induced mammary tumors (22), we decided to use $^{51}$Cr-labeled EDTA as a probe in rats to test whether other sources than the bone marrow interstitium contributed to the isolated bone marrow fluid. $^{51}$Cr-labeled EDTA (molecular weight 341) is a substance that is not metabolized and is not taken up by cells (13). Addition of cellular fluid to the centrifuged volume should thus be mirrored by a reduced $^{51}$Cr-labeled EDTA concentration in the centrifugate relative to plasma. For tissue centrifugation at $\geq 239$ g, we found that the ratio of $^{51}$Cr-labeled EDTA between centrifugate and plasma was 0.85. By modifications of the centrifugation procedure, we aimed at an extracellular tracer ratio between centrifugate and plasma of 1.0, as found for other compartment than the extracellular fluid. A further reduction of this ratio when we exposed the tissue to even higher G forces, indicates that an increased dilution occurred, which may be a result of further extrusion of cellular fluid and/or cell damage. Difference in characteristics between the interstitium within the bone marrow and within the tumor and skin may explain why such dilution occurs during centrifugation. As shown here, the amount of extracellular fluid in the bone marrow is 11% of wet weight, which is approximately one-fourth of the corresponding volumes in 9,10-dimethyl-1,2-benzanthracene-induced mammary tumors and skin (22). Another special feature of the bone marrow is the relatively high content of immature cells. Although mature blood cells are able to resist high G forces, e.g., in ordinary separation from plasma, this may not be the case for blood precursor cells. In line with this, isolation of bone marrow extracellular fluid with our method required centrifugation of bone marrow tissue in situ, i.e., within the bone cavity. For obvious reasons we were not able to quantify the contribution to the centrifugate from nonextracellular fluid in humans.

By centrifugation we sampled from the whole extracellular phase, including the vascular volume. Bone marrow plasma volume is low, accounting for <1% of the extracellular fluid volume (5). The fraction of the isolated bone marrow fluid deriving from plasma will accordingly be negligible.

Another potential problem associated with the present method might be cell damage during centrifugation, resulting in contamination of the sample with intracellular proteins. That such contamination occurred to a minor degree is indicated by the peak eluting at 13.4 ml not found in rat plasma. This peak was, however, always <7.2% of the total under the elution curve, suggesting that possible contamination by intracellular proteins was low. To address this question further, we passed through the HPLC column extracts from lysed bone marrow and fluid used for elution of intact marrow. Whereas bone marrow eluate had a HPLC pattern similar to that of plasma except for the peak at 13.4 ml discussed above, marrow lysate had a much higher fraction of low-molecular-mass macromolecules. This is to be expected because a large fraction of the latter consists of intracellular proteins. These experiments, together with the $^{51}$Cr-labeled EDTA results, suggest that intracellular fluid without proteins was the fluid that was added to the centrifugate.

Our HPLC data showing that the fraction of large plasma proteins was lower in the bone marrow fluid than in the plasma might indicate that the larger molecular mass immunoglobulins were sieved to some extent during centrifugation. If such sieving occurred, we might have expected an even lower fraction of large molecular mass proteins at increased extracellular matrix compaction likely to occur with increasing G force. Such a change in relative protein content was not observed. Furthermore, in skin, a similar ratio between immunoglobulins and albumin content as that of bone marrow was found in fluid isolated by centrifugation and from wick fluid (22), the latter not likely to be a result of sieving. Taken together, these observations suggest that sieving did not affect the relative distribution of proteins in fluid isolated by centrifugation from bone marrow. This assumption is supported by comparable studies on rat tail tendon where sieving was excluded for various probes spanning a wide range of molecular mass (1).

In rats as well as in the human subjects, the colloid osmotic pressures in plasma were similar to those found in previous studies (17, 22). COP relative to plasma, however, was significantly higher in bone marrow fluid than observed for subcutaneous extracellular fluid (22) but comparable to the corresponding relative pressure in tumors (21, 22), a tissue with high capillary permeability and dysfunctional lymphatics (10, 18), i.e., similar to the bone marrow with its leaky sinusoids and lack of lymphatic vessels.
Local levels of cytokines reflect hematopoietic challenges. To further evaluate centrifugation as a valid method to isolate bone marrow extracellular fluid, we specifically examined its content of erythropoietin and G-CSF. The levels of these two hematopoietic cytokines vary according to the prevailing hematopoietic activity and the presence of cytokines, which are known to regulate hematopoiesis. Inflammation, induced as aseptic peritonitis, led to a substantial increase in the cytokine levels of these two cytokines, which is consistent with the assumption that the cytokine levels in the extracellular fluid are lower than in plasma. If anything, the cytokine content of the extracellular fluid may represent an underestimate due to the possible dilution of the samples, as discussed above. This assumption is supported by the increase in the peak eluting at 13.4 ml during sterile inflammation (Fig. 3C), a finding suggesting a higher proportion of cell fluid in the centrifugate during this condition. Although being outside the scope of the present study, a further characterization of the protein(s) eluting in this peak may be of interest.

Functional aspects of bone marrow extracellular fluid composition. Being situated between the vascular compartment and the bone marrow, the composition of the bone marrow interstitium probably affects locally both the structure and the transport of nutrients, growth factors, and signaling molecules. This transport of molecules is essential for an adequate hematopoietic activity. For example, the hematopoietic cytokines erythropoietin and G-CSF, which play a role in hematopoiesis, are known to be released locally to stimulate hematopoiesis and to regulate the differentiation of hematopoietic progenitors. The cytokine levels were apparently larger in bone marrow extracellular fluid than in plasma. If anything, the cytokine content of the extracellular fluid may represent an underestimate due to the possible dilution of the samples, as discussed above. This assumption is supported by the increase in the peak eluting at 13.4 ml during sterile inflammation (Fig. 3C), a finding suggesting a higher proportion of cell fluid in the centrifugate during this condition. Although being outside the scope of the present study, a further characterization of the protein(s) eluting in this peak may be of interest.

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