Effect of Anticoagulants and Cell Separation Media as Preanalytical Determinants on Zymographic Analysis of Plasma Matrix Metalloproteinases

To the Editor:

Matrix metalloproteinases (MMPs) are calcium/zinc-dependent endoproteinases involved in physiologic and pathologic processes, modulating extracellular matrix degradation (1). MMP-2 (EC 3.4.24.24) and MMP-9 (EC 3.4.24.35) circulating in the peripheral blood (PB) of patients with neoplasia showed contrasting results, revealing the preanalytical issue of whether the method of PB sampling influences MMP concentrations (2) and their zymographic profiles (3). We therefore analyzed the effects of anticoagulants and cell separation media on PB gelatinolytic profiles.

PB samples from 30 healthy volunteers were collected into Vacutainer™ Tubes with clot activator (SST), lithium heparin (LH), dipotassium EDTA (K₂E), sodium fluoride/potassium oxalate (NaF/KOx), and buffered/acidic citrate [natrium citrate (9NC), acid-citrate-dextrose (ACD), and citrate-phosphate-dextrose-adenine (CPDA); Becton Dickinson]. After centrifugation at 500 g for 15 min at 4 °C, the supernatants and buffy coats were collected and analyzed. Leukocyte subpopulations were obtained after Lympholyte® gradient (5.64% Nycograde™ Polysucrose 400, 9.65% sodium diatrizoate; Cedarlane), and their subset recovery was tested through cytometric analysis (4). Gelatinases from leukocytes and plasma samples were analyzed by gelatin zymography, with 150 μg of total protein loaded on the gel (3). Calibrators were prepared from capillary PB (5). MMP-2 and MMP-9 were measured by ELISA (5). Differences were compared using the Mann–Whitney U-test and the paired t-test; P values <0.05 were statistically significant.

The present work was carried out in accordance with the ethics standards of the Helsinki Declaration of 1975, as revised in 1983.

We found in plasma a 72-kDa constitutive gelatinase that was produced by nonproteolytic activation of MMP-2 with sodium dodecyl sulfate, and additional MMP-9 forms at 92, 130, and 225 kDa. Western blot analysis, Ca²⁺/Zn²⁺ dependence, and p-aminophenyl-mercuric acetate activation (data not shown) identified the plasma gelatinases as fibroblast-derived proMMP-2 and neutrophil-derived proMMP-9, circulating as latent activatable proenzymes.

MMP-9 was significantly higher in
Plasma than in LH plasma [14 (3) vs 202 (15), Fig. 1A, lane 4 vs lane 5]. The concentrations of MMP-9 forms decreased significantly with increasing amounts of K2E during PB collection, whereas MMP-2 was increased (P <0.01; Fig. 1B). When we added anticoagulants to the zymography buffer (to mimic the conditions in Vacutainer Tubes), only K2E inhibited the gelatinolytic activities (data not shown). Although EDTA may alter MMP expression (7), the reasons for the contrasting K2E effects remain unknown.

To minimize interindividual variability, we collected PB from the same individual into different buffers. We found mainly proMMP-2 in the buffered/acidic citrate plasma [202 (15) μg/L], whereas there were no statistically significant differences among the 9NC, ACD, and CPDA plasmas. We found additional proMMP-9 in the K2E, LH, and NaF/KOx plasmas (Fig. 1A, lanes 1–3 vs lanes 4–6). Our observations revealed that anticoagulants can act as preanalytical determinants of PB MMPs.

LH and 9NC plasmas collected after Lympholyte gradient (Fig. 1C, lanes 3 and 4 vs lanes 1 and 2), as well as after 9.6% sodium diatrizoate alone (Fig. 1C, lanes 5 and 6 vs lanes 3 and 4), showed increased concentrations of all MMPs. Polysucrose 400 alone did not affect MMP concentrations (data not shown) or isoform profiles.

Cytometric analysis revealed differences in MMP composition between leukocytes from LH PB vs leukocytes from 9NC PB (data not shown). Physiologic buoyant coasts from 9NC PB showed only MMP-9 forms, had lower gelatinase activity, and had a different zymographic profile with respect to LH PB (Fig. 1C, lane 7 vs lane 8). The MMP differences between LH vs 9NC plasma could be caused by differential release of MMPs from, e.g., platelets and leukocytes, with a changed MMP content/profile depending on the anticoagulant used (6, 8).

Although previous reports suggested heparin as the anticoagulant of choice to study circulating MMPs (2, 3), to optimize the diagnostic validity of PB MMPs as cancer biomarkers (1), we recommend the use of buffered/acidic citrate (9NC, ACD, and CPDA), whereas LH, K2E, and NaF/KOx, which affect the MMP content and zymographic profiles of plasma and leukocytes, should be avoided.

Measurement of Immunoglobulin Free Light Chains in Serum

To the Editor:

In a recent issue of this journal, Tate et al. (1) reported studies using assays for free immunoglobulin light chains (FLCs) that we have been instrumental in developing (2). Although in general agreement with their findings, we would like to highlight some additional data that are pertinent to several of their comments.

Tate et al. (1) concluded that more clinical data were required before the assays are adopted for routine clinical use. Since the acceptance of their report, however, several relevant studies have been published. Bradwell et al. (3) presented data showing that 224 of 224 patients with Bence Jones myeloma could be identified based on abnormal serum concentrations of FLCs at presentation, without the requirement for urine testing. They also demonstrated that serum FLC measurements were more sensitive than urine, presumably because of the kidney’s high capacity for protein catabolism. For the diagnosis of AL amyloidosis, Lachmann et al. (4) reported that serum FLC assays were more sensitive than electrophoresis or immunofixation of both serum and urine. Of 262 patients with primary amyloidosis, 257 had abnormal serum FLC concentrations, whereas only 207 had monoclonal proteins in their serum and/or urine detectable by immunofixation electrophoresis or protein electrophoresis (χ² = 45.19; P <0.0001). Changes in the concentrations of serum FLCs were also found to be the best marker for disease monitoring and prognosis. Preliminary results from a study at the Mayo Clinic indicate the existence of “free light chain monoclonal gamopathy of undetermined significance (MGUS),” which can be identified only by use of serum FLC assays (5).

Additional confirmation of all of these results is appropriate, and many additional studies are underway. All patients entered in the Med-