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Review

Considerations Regarding the Use of Blood Samples in the Proteomic Identification of Biomarkers for Cancer Diagnosis

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Abstract. *Blood is the most common source of biomarkers for use in the diagnosis and prognosis of malignant disease. Utilizing proteomic technology for biomarker identification offers greatly increased sensitivity. Such an increase in sensitivity requires precise sample preparation to eliminate any bias in analysis. Here, issues concerning the use of blood, plasma and serum for proteomic analysis are summarized.*

The development of technologies for the early identification of malignant disease would greatly assist in treatment. Ovarian cancer is a leading example of the case in point. Its early, definitive diagnosis would be a major benefit as most patients present with advanced disease (1); early diagnosis (Stage I disease) results in a far more favorable outcome than with advanced Stage III or Stage IV disease (2, 3). Success depends on the development of a technology that is both sensitive and specific (4).

Proteomic technology is critical for the identification of biomarkers for use in the diagnosis and prognostic assessment of malignancy (5). These biomarkers would serve as early indicators of malignant disease as well as markers for prognostic evaluation. Examples of some well-defined biomarkers are CA125 for ovarian cancer (6-8) and prostate specific antigen (9). While these biomarkers are important, there are issues with respect to their value in the early diagnosis of malignancy. It is critical for biomarkers to exhibit statistical sensitivity and specificity (10). Of equal importance is the ability to develop a facile and cost-effective laboratory assay for the biomarker(s), which can be validated from a regulatory perspective. In the case of CA 125, it has been possible to combine this biomarker with other biomarkers to improve sensitivity and specificity (11, 12).

Ideally, a diagnostic process should be quick, non-invasive, and relatively inexpensive. Few diagnostic tests have met these criteria with a significant proportion requiring an invasive process with either tissue and/or fluid collection. Blood is the most frequently used medium for clinical laboratory assays followed by urine, lymph, and cerebrospinal fluid. Our discussion focused on the use of blood as a sample for the identification of biomarkers.

Variance in analytical data can come from either the sample source (including sample handling) or from the analytical system. Both of these factors can have a major influence on the derived data (13, 14). The demonstrated ability of proteomic technologies such as multi-dimensional protein separation and mass spectrometric analysis, which enables extremely accurate identification of proteins in complex mixtures, is potentially of great importance in the identification of new biomarkers.

The purpose of this review is to discuss the critical factors associated with the use of blood as the selected sample for proteomic analysis. Similar considerations are valid for other biological fluids such as urine, lymph and saliva, although they are of less significance because of their acellular nature. Issues which may affect the integrity and influence the composition of the "raw" serum and plasma are of concern. There are additional sample issues with the pre-fractionation of serum or plasma primarily to remove high-abundance proteins (15, 16) which are beyond the scope of this article. We also recognize the need to validate such procedures with respect to yield and reproducibility (17) as well the fact that such procedures may remove potentially important biomarkers (18).

First, some general aspects of blood as a sample source are discussed. It is important to remember that blood is an organ and the removal of a sample is analogous to a biopsy. Next, general issues concerning the use of blood as a clinical sample are briefly discussed; most of this information is not

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novel but is not always addressed in proteomic studies. Additionally, several very recent studies are included which address some specific issues related to the use of blood as a sample for proteomics.

Blood is a complex fluid suspension composed of plasma, a humoral phase consisting of a protein-rich dilute salt solution, and a cellular phase. The bulk of the cellular phase consists of red blood cells (erythrocytes), "white cells" (leukocytes and macrophages) and platelets. Blood is contained within a closed circulatory system lined with endothelial cells which are considered non-thrombogenic.

The first issue is as much philosophical as it is pragmatic; is it possible to get a truly naïve sample of blood? Perhaps the answer is no and the best one can hope for is a representative, reproducible sample. The second issue, which is equally complex, concerns the effect of circadian variation (19), disease processes (20), and the influence of drugs/nutrients (21). The latter issue is not discussed, although the importance of such considerations is highlighted.

The initial blood sample is removed from the circulatory system *via* venipuncture (22). If the sample is withdrawn in the presence of an anticoagulant and centrifuged to remove cellular elements, a plasma sample is obtained; in the absence of an anticoagulant, the blood clots and can be centrifuged immediately to remove the fibrin clot and cellular elements or allowed to stand for period of time until clot retraction occurs (23). It is also possible to avoid the addition of any anticoagulant by withdrawing the blood through a resin-containing device which depletes the calcium, thus precluding coagulation (24). The addition of corn trypsin inhibitor to the blood as it is withdrawn (25) markedly slows the process of blood coagulation. This permits easy removal of the cellular elements and transfer, for example, into urea-detergent prior to two-dimensional gel electrophoresis. It is also probable that the presence of common protease inhibitor cocktails would also inhibit coagulation sufficiently to permit processing of the plasma samples.

The most common anticoagulants are EDTA (ethylenediamine tetraacetic acid), or sodium citrate and heparin: EDTA and citrate function as anticoagulants by chelating calcium ions, while heparin functions by a different mechanism and "activates" antithrombin. Banks and coworkers (26) have recently published a study comparing plasma with EDTA, citrate or heparin as anticoagulants and serum using SELDI-MS protein chip technology (surface-enhanced laser desorption/ionization-mass spectrometry). These investigators also examined platelet-rich and platelet-poor plasma, as well as purified platelets. Several different types of protein chips where examined. The cluster analysis of the mass spectrometric profiles obtained with SELDI-MS showed that sample preparation did have a marked effect. The most significant differences were observed with immobilized metal affinity chips (IMAC). In this analysis, serum and plasma differed markedly. There are other interesting comparisons which could be useful when applied to specific studies. Space does not allow a more thorough consideration of this work, but these workers provide some useful baseline information for future studies.

The investigators using proteomic technology to identify biomarkers come from diverse backgrounds and it is apparent that there is a genuine lack of understanding of the fact that serum and plasma are not equivalent biological specimens, though this situation is gradually changing as more investigators begin to appreciate the differences (26, 27). Serum is qualitatively different from plasma in that the bulk of the fibrinogen has been removed by conversion into a fibrin clot together with the platelets, which have either been physically bound in the fibrin matrix or activated to form aggregates or both. Varying amounts of other proteins are removed into the fibrin clot either by specific or non-specific interactions. There are a number of other changes which occur (28), including the formation of a protease-serpin complex and protein fragments such as D-dimer and prothrombin fragment 1. The protein concentration of serum is less than that of plasma (29, 30). Lum and Gambino (29) reported an average value of 7.45 gm/dL for heparinized plasma *versus* 7.21 gm/dL for serum. The authors suggested that the difference was largely due to the removal of fibrinogen. Conversely, these investigators did observe an increase in albumin in serum. A similar difference in the mean protein concentration between serum (7.29 gm/dL) and plasma (7.58 gm/dL) was observed by Ladenson and coworkers (30).

In the process of whole blood coagulation, the cellular elements (erythrocytes, leukocytes, platelets) can secrete components. In particular, the platelets contribute a variety of components to the blood serum such as vascular endothelial growth factor (VEGF) (31, 32). In one study (31), normal individuals had a serum VEGF concentration of 250 pg/mL with a plasma concentration of 30 pg/mL; however, breast cancer patients with thrombocytosis had a median VEGF concentration of 833 pg/mL compared to 249 pg/mL in other patients. The studies suggested that platelets can contribute to the VEGF levels in both plasma and serum, but more markedly in the serum. It was suggested that the immediate separation of plasma or serum from the cellular elements provided optimal analyte stability (33). The time of contact of serum with the cellular elements will variably influence the composition, but sufficient time must be allowed to permit the completion of fibrin formation. One frequently ignored point is that the quality of the *in vitro* blood clotting process relates to the composition of the container; there is a marked difference in serum obtained from clotting in plastic *versus* glass (34).

This is consistent with the observations of Banks and coworkers (26). There are likely to be a range of differences depending on the analyte that is being measured.

The choice of anticoagulant and temperature can influence the storage stability of an analyte (35-40). Critical process variables for serum are the process/storage containers, the time of clot retraction/removal of the fibrin clot with associated platelets and other cellular elements, the centrifugation speed and the storage temperature.

There are many factors, other than the underlying biology which can influence a blood sample. It is difficult, if not impossible, to eliminate all of these factors. As a result, it is most important to document the conditions of blood processing and to optimize such conditions depending on the specific analyte. It is strongly recommended that a Standard Operating Procedure (SOP) be established for the process of obtaining a blood sample. In this way, one is able to assure the reproducibility of the samples and to allow rational comparison of intra-laboratory and inter-laboratory data, permitting the development of a robust, accurate process. While there may well be greater than a million discrete protein species in a plasma or serum sample, the significance of such compositional heterogeneity is not clear. In particular, there is concern regarding the role of sample processing in the production of such heterogeneity. Finally, the longer the circulatory half-life of a plasma protein, the greater the chance of in vivo modification including oxidation and glycation, which also introduces heterogeneity.

While each biomarker search will provide its own challenge, the following recommendations are presented as fundamental for the initiation of a study:

i) A proactive protocol for sample processing should be developed and used in the study. Samples should be obtained at the same time to avoid possible complications from diurnal or circadian variation.

ii) Serum is preferred to plasma and must be collected in a glass container and placed in storage within a uniform period of time; one hour at 25˚C is recommended. A rigorous study on analyte stability is recommended after initial studies.

iii) If plasma is used, EDTA is the recommended anticoagulant and an absolutely uniform process should be established for obtaining the plasma.

iv) In the absence of other information, storage of samples at –80˚C is recommended; samples must not be used more than once because of freeze/thaw problems. It is recommended that, given a sufficient initial sample size, several portions of a given sample be prepared and frozen.

v) Normal values for potential biomarkers must be established prior to an experimental series; it is recommended that 30 normal samples be used. The individual investigator must establish whether normal samples need to be age-matched, gender-matched, *etc.*

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