Electron Spin Resonance Spectroscopy of Serum Albumin: A Novel New Test for Cancer Diagnosis and Monitoring

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Background: Proteins released by tumor cells can bind to serum albumin, leading to structural and functional modifications. We used electron spin resonance (ESR) spectroscopy to measure these changes in serum albumin and evaluate their utility for the diagnosis and monitoring of cancer.

Methods: We used an ESR spectrometer and 16-doxyl stearic acid as spin probe to measure conformational changes in albumin in blood samples from a population of healthy donors and volunteers (n = 349), patients with a wide variety of hematologic and nonhematologic malignancy (n = 135), and patients with chronic diseases such as gastrointestinal and pulmonary disease, diabetes, and cirrhosis (n = 91). We added differing amounts of 16-doxyl stearic acid spin probe in ethanol to 50 μL of serum from each patient to create 3 different aliquots that differed in concentration of spin probe and ethanol, then incubated the aliquots for 10 min at 37 °C with continuous shaking. We measured the ESR spectra of each aliquot in triplicate and used proprietary software (MedInnovation GmbH) to evaluate the ESR spectrum for differences between cancer patients and the other groups.

Results: The diagnostic sensitivity and specificity of this test were 87.4% and 95.7%, respectively, for differentiating healthy individuals from cancer patients and 87.4%, and 85.7% for differentiating cancer patients from chronic disease patients. Serial evaluation of albumin conformation changes in several patients followed during the course of their disease showed excellent agreement between the magnitude of abnormality in the ESR spectrum of albumin and clinical and pathologic estimates of disease severity.

Conclusions: ESR spectroscopy of serum albumin is a sensitive and noninvasive technique that clearly demonstrates diagnostic utility in patients with cancer. This test also enables monitoring of the disease course through use of serial measurements.

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Tumor cells release a variety of bioactive proteins and peptide fragments into the blood. These proteins are representative of diverse tissue and cellular origins and may reflect important disease-related information (1). These compounds may also reflect the physiologic state of the tumor and reveal the effects that the tumor has on the organism itself (2). Tumor-derived proteins that are produced in very low concentrations enter the circulation and bind to transport proteins (3, 4). Sequestration of these peptides by carrier proteins that are present in high concentrations and have long half-lives, such as albumin, protects these markers from clearance and helps amplify their concentration in the circulation. These albumin-sequestered peptides may prove to be a rich source of tumor-associated biomarkers. Investigators have used albumin binding of tumor-derived protein products to help identify candidate tumor markers (1).

Disease-associated biomarkers that bind to albumin can cause allosteric modifications to this protein, leading to changes to its binding and transport properties. One of the modifications that occur to albumin is its ability to bind and transport fatty acids. These structural and functional changes that occur to albumin can be readily
assessed with use of electron spin resonance (ESR) spectroscopy. Changes in albumin conformation, transport efficiency, and binding characteristics can be readily assessed. Use of this technique with 16-doxyl stearic acid (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinylxylo) as spin probe has previously demonstrated cancer-specific alterations in albumin conformation (5). The shape of the ESR spectrum reflects the status of the spin probe molecules, including characteristics of its molecular motion (i.e., rotation angle and speed) and properties of the surrounding medium, such as electric and magnetic field strength. We used ESR spectroscopy to investigate the diagnostic and prognostic utility of serum albumin conformation analysis in patients with cancer.

Materials and Methods
We obtained blood samples from a total of 575 individuals. Blood was obtained by standard venipuncture techniques and collected without any additive. After clotting, serum was separated by centrifugation (3000 g) for 10 min, removed, and then stored at −20 °C, for up to 6 months before analysis.

Samples were collected from 349 blood donors and other volunteers known to be in good health, 135 patients with a wide range of hematologic and nonhematologic cancers, and 91 patients with a variety of chronic diseases. Samples collected from blood donors were obtained immediately before donation. Blood samples from patients with cancer or chronic disease were obtained while in the hospital or clinic. The diagnosis of cancer was established on the basis of histological findings and encompassed all clinical stages of disease. All patients provided their consent for participation in this study.

Each sample was measured with an ESR spin probe technique. We measured the ESR spectra of each sample with a commercially available ESR spectrometer (ESR-Analyzer/MMS, MedInnovation GmbH). The principle of this technique is the measurement of albumin binding variables, achieved by a fatty acid spin probe. We measured binding variables of the spin probe at different permutations of ethanol concentration, and the ratio of spin probe and albumin concentration. Differences in ethanol concentration allow binding variables of spin probe to albumin to be assessed under different hydrophilic conditions. Changes in the ratio of spin probe to albumin enable the binding affinity of albumin to spin probe to be measured.

Commercial 16-doxyl stearic acid (Sigma-Aldrich GmbH) was used as spin probe. This compound was chosen because of the extremely high binding constant of albumin for stearic acid ($6.9 \times 10^8$ L/mol), which produces >99.9% binding of spin probe to albumin. Ethanol, extra pure, (Merck KG) was used for modifying the binding affinity of the fatty acid spin probe to albumin. The final concentrations of ethanol (mol/L) and spin probe ($10^{-3}$ mol/L) in each of the 3 aliquots was 2.9, 0.83 in aliquot 1; 3.4, 1.61 in aliquot 2; and 3.8, 2.34 in aliquot 3.

The previously frozen serum samples were assayed within 4 hours after thawing. Three separate aliquots were prepared from each sample. To prepare each aliquot, 50 µL of serum was mixed in a microtiter plate well with a specified amount of ethanol containing spin probe to achieve aliquots differing in the concentration of spin probe and ethanol. The serum/ethanol mixture was covered with Parafilm and then incubated for 10 min at 37 °C under continuous shaking. Immediately after incubation, the serum/ethanol mixture was placed into a glass capillary tube and analyzed in the ESR spectrometer. The spectrometer maintained the capillary temperature at 37 °C (0.1 °C, and each aliquot was analyzed in triplicate; each reading taking 1 min to perform. Measurement values of the spectrometer were microwave power of 15 mW at a frequency of 9.5 GHz and magnetic field strength of 0.34 T with a scan range of 12 mT and a modulation amplitude of 0.07 mT.

Analysis of the ESR spectra was performed with computer simulation of its components on the basis of a Hamilton spin function with axial anisotropy (6). ESR spectra were analyzed through simulation, which was performed with a least-square fitting of a model spectrum to the one measured experimentally. The ESR spectrum is comprised of a large set of data points, which can be approximated by an ideal spectral curve. During simulation, values for variables of the simulated spectrum that provided the best fit to the ideal spectrum were estimated. These estimated values included g-factors, hyperfine structure constants, and line widths that characterized the shape and intensity of each spectral component.

Selected values for variables such as g-factors, hyperfine structure constants, and line widths can then be used to estimate the biophysical characteristics of the 16-doxyl stearic acid spin label. These biophysical characteristics include angle of the spin-labeled molecule axis’ precession, polarity of the environment surrounding the spin label, and the rotation speed of the spin label.

A discriminant variable (DR) was calculated by use of squared discriminant analysis to differentiate patients with cancer from healthy individuals and those patients with chronic disease. The DR was calculated as a linear function with the following general equation:

$$ DR = F(C1^A, C2^A, C3^A, C1^B, C2^B, C3^B, C1^C, C2^C, C3^C), $$

in which $C1$ is the relative intensity of the ESR spectrum of spin probe bound to the primary binding site of albumin, $C2$ is the relative intensity of the ESR spectrum of spin probe bound to the secondary binding site of albumin, and $C3$ is the relative intensity of the ESR spectrum of unbound spin probe. The indexes $A$, $B$, and $C$...
refer to the ESR spectra of each of the 3 aliquots measured in each individual.

Sensitivity and specificity of ESR spectral analysis for differentiating patients with cancer from healthy individuals and those with chronic disease was calculated with generally accepted statistical methods.

Results

The demographics of the 3 groups of patients involved in this study are summarized in Table 1, which also gives a summary of the primary types of cancer for patients in group 2 and the types of chronic disease affecting patients in group 3. Mean (SD) serum albumin concentrations were significantly different ($P<0.001$) between patients with cancer [38.8 (4.3 g/L)] and the individuals in good health [43.6 (1.9 g/L)]. Data on albumin concentrations in patients with chronic disease were not available.

The ESR spectral line shape allows several different values to be deduced, including the capacity of albumin to bind spin probe, the polarity of the spin probe binding

Table 1. Demographic information for patients evaluated in this study.

<table>
<thead>
<tr>
<th>Group number</th>
<th>Median age, years</th>
<th>Age range</th>
<th>Percent Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>38</td>
<td>9–92</td>
<td>46</td>
</tr>
<tr>
<td>II</td>
<td>68</td>
<td>19–89</td>
<td>42</td>
</tr>
<tr>
<td>III</td>
<td>67</td>
<td>20–82</td>
<td>53</td>
</tr>
</tbody>
</table>

Demographic information on all study patients was not known.

Group I. Blood donors, healthy volunteers ($n=349$; demographic information known for 310).

Group II. Patients with cancer ($n=135$; demographic information known for 118). Primary sites of cancer included the following: oral cavity, bronchial, stomach, colon, bladder, breast, cervix, uterus, prostate, kidney, brain, melanoma, Hodgkin and non-Hodgkin lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, and multiple myeloma.

Group III. Patients with chronic disease ($n=91$; demographic information known for 87). Chronic diseases affecting patients in group III: diabetes, chronic obstructive pulmonary disease, gastrointestinal bleeding, hepatic cirrhosis, and renal insufficiency.

Fig. 1. Example of ESR spectrum obtained from a single analysis. (a), composite spectrum of 16-doxyl stearic acid in serum. (b), spectrum component corresponding to 16-doxyl stearic acid bound to high affinity fatty acid binding site on albumin. (c), spectrum component corresponding to 16-doxyl stearic acid bound to second, low affinity fatty acid binding site on albumin. (d), spectrum component corresponding to unbound 16-doxyl stearic acid in serum. (e), spectrum component corresponding to 16-doxyl stearic acid micelle aggregates. This spectrum component is shown at 500 times actual intensity.
site, the mobility of the spin probe, and the distance between different spin probes. Analysis of the ESR spectra revealed 4 distinct spectral components. Two components represented binding of spin probe to different fatty acid binding sites on the albumin molecule, 1 component represented spin probe that was free or weakly bound to the surface of albumin, and the 4th component corresponded to minute amounts of fatty acid spin probe that had aggregated into micelles. A typical ESR spectrum of 16-doxyl stearic acid in albumin solution or serum is shown in Fig. 1.

Analysis of the ESR spectra revealed substantial differences in spectrum variables between patients with cancer and healthy individuals and those with chronic disease. For example, fraction d (see Fig. 1) corresponding to unbound spin probe was ~2 times greater in cancer patients, and increased width of the spectral line of component c indicated an alteration in albumin conformation that limited the movement of spin probe bound to this site. A representative ESR spectra of 16-doxyl stearic acid bound to albumin from a patient with cancer compared with that obtained from a healthy blood donor is shown in Fig. 2.

DR was calculated as described above. We used a threshold value of 1.0 to differentiate patients with cancer from healthy individuals or patients with chronic disease but without cancer. The DR value [mean (SD); range] in participants in good health, [4.66 (2.17); −0.71–8.83] and in patients with chronic disease [3.85 (2.25); −1.25–7.09] were significantly higher than those in patients with cancer [−0.27 (1.49); −2.45–7.54]. Diagnostic sensitivity of the test for differentiating patients with cancer from those in good health and those with chronic disease was 87%. Diagnostic specificity was somewhat better, at ~94%. Sensitivity and specificity of the test according to the 3 groups of patients are summarized in Table 2.

The prognostic utility of this test was assessed by monitoring the ESR spectrum of albumin in several patients with cancer who provided serial samples over the course of their disease. The magnitude of the DR showed excellent correlation with the clinical condition of the patient (Fig. 3).

The assay technique was relatively simple to perform. It took ~20 min to perform triplicate measurements of all 3 aliquots from an individual patient. Previous work by our group relied on analysis of 8 samples per patient; 3 samples containing different spin probe/albumin ratios but constant ethanol concentration, and 5 samples containing various permutations of ethanol concentration vs spin probe/albumin ratios. Extensive evaluations of this data showed that analysis of 3 samples, as in the current

### Table 2. Diagnostic sensitivity and specificity of ESR spectroscopy of serum albumin for differentiating patients with cancer.

<table>
<thead>
<tr>
<th>Group comparison</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer vs healthy</td>
<td>87.4</td>
<td>95.7</td>
</tr>
<tr>
<td>Cancer vs chronic disease</td>
<td>87.4</td>
<td>85.7</td>
</tr>
<tr>
<td>Cancer vs healthy and chronic disease</td>
<td>87.4</td>
<td>93.6</td>
</tr>
</tbody>
</table>

Fig. 2. ESR spectra of 16-doxyl stearic in a healthy blood donor (A), a patient with cancer (B), and the spectral difference (C).
study, provided the same information as that provided by analysis of 8 samples (unpublished data). Thus, the current study was performed with analysis of 3 aliquots per individual patient.

Although we manually performed all dilutions of serum with spin probe, this part of the procedure could easily be automated if necessary. Automation of the pipetting and dilution step would also probably enhance the precision of the procedure. Even when we performed this procedure manually, however, multiple repeated measurements of samples over a period of several days revealed intraassay and interassay precision to be 3% and 6%, respectively.

Discussion

Albumin is the single most abundant protein in non-pathogenic plasma, comprising approximately two thirds of total plasma proteins. Major physiologic functions of albumin include maintaining plasma oncotic pressure, serving as a reservoir of amino acids for incorporation into other proteins, and transporting a wide variety of low–molecular-weight molecules. The long half-life of albumin, 15–19 days, helps prolong the half-life of these bound, low–molecular-weight substances that otherwise would be metabolized or eliminated via renal clearance (7). Another important function of albumin is as a transporter of fatty acids. Although 5 primary binding sites have been discovered in the crystal structure of fatty acid–albumin complexes, under typical physiologic conditions, albumin carries 1 to 2 long-chain fatty acids (8, 9).

Spin labeling combined with ESR spectroscopy is a powerful tool for detecting structural changes in proteins. The shape of the ESR spectrum reflects the state of the spin probe molecules, such as characteristics of its molecular motion and electrical and magnetic fields in the surrounding environment (6). Changes in the mobility and accessibility of the spin labels, and the distances between spin labels, enables changes in the secondary and tertiary structure of proteins to be discerned. Results of recent application of ESR spectroscopy in animal models and humans suggest that ESR has great diagnostic potential (10). Some promising initial applications include noninvasive, continuous measurement of partial oxygen pressure in tissue and measurement of clinically significant exposure to ionizing radiation (11). Another useful application of ESR spectroscopy is in the measurement of structural and functional changes in both soluble and membrane proteins (12). Although x-ray crystallography gives insight into the 3-dimensional structure of a protein, spin labeling combined with ESR spectroscopy is a powerful tool for detecting changes in protein structure and function. The sensitivity of ESR spectrometers makes it possible to work with samples in the 50–100 pmol range, and there is no upper limit on the molecular weight of proteins that can be investigated.

The binding of biologically important proteins and peptides, representative of diverse tissue and cellular origins, may reflect important disease-related information. Recent studies have demonstrated that carrier proteins such as albumin may be a rich source of disease-associated biomarkers, and provide new opportunities for
expanding the knowledge base for the molecular composition of the circulation (1). Our study, which investigated the ESR spectral changes of albumin in a diverse group of patients, showed this technique to be very promising in the identification and monitoring of patients with cancer. Previous investigations in patients with stomach cancer clearly demonstrated decreases in albumin binding reserve in patients with operable vs those with nonoperable disease (13).

A deficiency of the current study was the significant difference in age distribution between healthy individuals in group I vs patients in groups II and III with cancer and chronic diseases, respectively. However, the age distributions of patients with cancer and those with chronic diseases were not significantly different. ESR spectroscopic analysis of albumin showed good diagnostic discrimination for separating patients with cancer from those with chronic disease. Studies are currently underway to further characterize the diagnostic and prognostic utility of this test.

References