Correlation Between Erythrocyte CR1 Reduction and Other Blood Proteinase Markers in Patients With Malignant and Inflammatory Disorders

By Mark S. Currie, Marc Vala, David S. Pisetsky, Charles S. Greenberg, Jeff Crawford, and Harvey J. Cohen

Erythrocyte CR1, a C3b/C4b-binding complement-regulatory protein, is sensitive to proteolysis in vitro. To test the hypothesis that in vivo erythrocyte CR1 reduction results from intravascular proteinase activities, we used enzyme-linked immunosorbent assays to measure γ-crosslinked fibrin degradation products (D-dimers) as indicators of coagulation/fibrinolytic activity, and complexes of neutrophil elastase with α1 proteinase inhibitor (E/A) as indicators of neutrophil enzyme release in malignant and inflammatory disorders. Erythrocyte CR1, measured by monoclonal anti-CR1 antibody binding, was inversely related to disease activity and blood proteinase markers. Levels of erythrocyte CR1 were significantly lower for patients with active versus remittent squamous and small cell lung cancers, Hodgkin’s and diffuse large cell lymphomas, and acute myelogenous leukemias. In patients with active thoracic cancers, elevated D-dimer levels correlated with reduction of CR1. In patients with rheumatoid arthritis, CR1 reduction was correlated with elevated levels of elastase complexes. Our findings substantiate the relationship of acquired CR1 reduction to the activity of certain diseases and provide circumstantial support for the hypothesis that erythrocyte CR1 is lost to proteolysis in vivo. Although heritable differences in CR1 expression reduce the interpretability of single measurements of erythrocyte CR1 levels, disease-associated CR1 reduction may be a useful indicator of disorders with chronically increased blood proteinase activity.

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THE ERYTHROCYTE complement receptor, CR1, can bind C3b and iC3b, and acts as the physiologic cofactor for cleavage of iC3b to C3d,g with release of C3c by factor I.

Reduction of erythrocyte CR1 has been observed in a variety of disorders. In addition to inherited variations in CR1 number and molecular weights, erythrocyte CR1, whether measured by factor I cofactor function or by quantitating monoclonal anti-CR1 binding, can be reduced by processes associated with malignant, immune and inflammatory processes, including systemic lupus erythematosus, hemolytic anemias, and immune neutropenias.

In preliminary studies we reported that in patients with malignancies associated with reduced erythrocyte CR1, the number of receptors per cell returns toward normal with remission. CR1 in vitro is susceptible to cleavage by a number of plasma proteinases, so proteolytic degradation has been proposed as a mechanism of CR1 reduction in vivo. In this study we provide evidence that CR1 reduction is proportional to other evidence of proteinase activity in plasma from patients with certain disorders. We report that the reduction of erythrocyte CR1 is associated with elevation of crosslinked fibrin fragment (D-dimer) levels in small cell lung cancer, and elevation of elastase-α1 proteinase inhibitor (E/A) levels in rheumatoid arthritis. Thus, CR1 reduction may be a marker of plasma proteinase activities.

MATERIALS AND METHODS

The subjects for this study were healthy volunteers stratified by age (young as less than 40 years, mean of 25 years; and elderly as greater than 60 years, mean of 78 years), and patients seen in the outpatient Hematology/Oncology and Rheumatology clinics of the Durham Veterans Administration Medical Center (VAMC), from whom samples were obtained in accordance with the policies of the local Human Subjects committee. Blood that had been drawn into EDTA-containing lavender stopped vacutainers (Becton-Dickinson, Rutherford, NJ) was refrigerated and centrifuged, and the plasma was removed and frozen within 2 hours of phlebotomy to avoid leakage of granulocyte contents. After removal of the buffy coat, erythrocytes were stored at 4°C in Alsever’s buffer. Erythrocyte CR1 was assayed within 7 days, as previously described. Mean binding per erythrocyte of a radioiodinated monoclonal anti-CR1 antibody (E11; provided by Dr Gordon D. Ross, University of North Carolina, Chapel Hill, NC) was calculated by centrifuging through phthalate oils 104 erythrocytes that had been incubated for 20 minutes at 20°C with 3 ng/μL labeled antibody, and comparing the radioactivity of the pellet and supernate. The same method was used to assess erythrocyte-bound C3d,g, substituting radioiodinated monoclonal anti-C3g (provided by Dr Gordon D. Ross). Plasma was stored in aliquots at −70°C until enzyme-linked immunosorbent assays (ELISA) for D-dimers or elastase/α1 proteinase inhibitor complexes (E/A) were performed. Assay for D-dimers was according to the instructions provided with the Dimertest EIA kit from American Diagnostica (Greenwich, CT), in which different monoclonal antibodies to the D fragment of fibrin serve for capture (DD-3B6/22) and detection (DD-4D2/182). This assay provides sensitive quantitation of coagulation/fibrinolytic activity below the range detected by agglutination methods. Plasma E/A levels were measured by a sandwich ELISA analogous to those described previously. Polyclonal sheep anti-human, neutrophil elastase (Products for Bioscience, Indianapolis, IN) diluted 1:1000 in phosphate-buffered saline (PBS) was coated on microtiter plates (overnight at 4°C) to capture elastase/antiproteinase complexes. After a

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blocking step with 3% sheep albumin in PBS:O.I% Tween, and incubation of samples diluted 1:5 or 1:20 in 3% sheep albumin or 5% sheep serum for 2 hours at 20°C, peroxidase-conjugated polyclonal sheep anti-human-a1 proteinase inhibitor (Products for Bioscience) diluted 1:400 in PBS was used to detect E/A complexes. After 1 hour incubation at 20°C followed by three washes with PBS:O.I% Tween, the peroxidase-conjugated antibody bound was assayed by the increased absorbance at 414 nm using substrates 0.015% azino-diethyl-benzthiazoline-sulfonate (ABTS) in 0.1 mol/L citrate buffer, pH 4, with 0.03% hydrogen peroxide. Mean of duplicate patient samples were compared with the best fit curve for duplicate standards included on each plate. Standards were prepared by sonating normal neutrophils at 10^7/mL in autologous plasma with 1% Triton X-100, and making serial dilutions in plasma from a patient with aplastic anemia. Aliquots of the standards were frozen immediately and stored at −70°C until use. Subsequent comparison with E/A formed by incubation of purified elastase (Elastase Products, St Louis, MO) with an excess of a1 proteinase inhibitor (Sigma, St Louis, MO) demonstrated a linear relationship, so that log elastase ng/mL = (0.85 × log lysed neutrophils/mL plasma) − 1.6, but for the current assay, values for E/A are expressed in units of log neutrophil-equivalents/mL. Sheep serum was used as diluent to circumvent spurious elevation of results by rheumatoid factors that could bind the sheep antibodies to elastase and a1 proteinase inhibitor used in the assay. Results were excluded from three rheumatoid arthritis patients’ samples whose rheumatoid factor activity could not be overcome by 5% sheep serum, as examined by an identical ELISA procedure in which sheep anti-human a2-macroglobulin (Products for Bioscience) replaced sheep anti-human neutrophil elastase as the rheumatoid factor “capture” antibody, and parallel samples diluted in either 3% sheep albumin or 5% sheep IgG-containing serum were compared.

Differences between patient groups were tested for significance at the 0.05 probability level, using the Students t-test, and for sample sizes less than 15 using the Mann-Whitney U test.

RESULTS

Mean erythrocyte CR1 level was similar for healthy young (mean age 25, n = 58) and elderly (mean age 78, n = 27) individuals, but was significantly reduced in many malignant disorders (Table 1 and Fig 1). Erythrocytes from patients with active Hodgkin’s disease, large cell lymphomas, and small cell or squamous cell lung cancers had about half as much detectable complement receptor as cells from healthy donors. Significantly higher erythrocyte CR1 levels were found in patients in remission compared with those with active disease, demonstrating the acquired nature of the defect.

As shown in Fig 2, there was a moderate (but statistically significant versus normal) reduction of erythrocyte CR1 in patients with rheumatoid arthritis without neutropenia (white blood cell count greater than 3,000/μL) and a marked reduction in patients with elevated granulocyte binding IgG and neutropenia.

Erythrocyte-bound C3d,g was measured in some patients, because a relationship has previously been reported between erythrocyte-bound C3d,g and CR1 reduction in disorders associated with deposition of complement on red blood cells.5 No significant increase in erythrocyte-bound C3d,g was observed in patients with lung cancer or other malignancies.5 In patients with rheumatoid arthritis (RA), a weak inverse relationship was observed between erythrocyte CR1 and C3d,g (R = .15) as shown in Fig 3.

Plasma from the same patient blood samples used for erythrocyte CR1 determinations were then assayed for blood proteinase markers. Coagulation/ fibrinolytic activity was assessed by measuring plasma D-dimer levels, and release from neutrophil primary granules was assessed by measurement of plasma E/A. The four graphs in Fig 4 show the relationships between erythrocyte CR1 and plasma levels of E/A or D-dimers in patients with small cell carcinoma of the lung and RA. As shown, for patients with small carcinoma of the lung (and also other thoracic malignancies, not shown), reduction of erythrocyte CR1 was more closely related to plasma D-dimer levels (R = .87) than to granulocytic enzyme release as measured by E/A (R = .59). Conversely, in RA the stronger correlation was to E/A (R = .87), with a weaker relationship to D-dimer levels (R = .5). In RA patients with normal neutrophil counts, a relationship was observed between D-dimer and E/A (Fig 5A), but E/A's were substantially higher in patients with neutropenia, without a proportionate increase in D-dimer levels. A relationship was also observed between D-dimer and E/A in plasma from

### Table 1. Mean Erythrocyte CR1 Levels

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>CR1 (Molecules/Cell ± SD)</th>
<th>Statistical Difference (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
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<tr>
<td>Younger than 40 y (58)</td>
<td>804 ± 192 Normal</td>
<td></td>
</tr>
<tr>
<td>Older than 60 y (27)</td>
<td>848 ± 160 NS v young</td>
<td></td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td></td>
<td></td>
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<tr>
<td>Active (6)</td>
<td>440 ± 136 &lt;.0005 v remission</td>
<td></td>
</tr>
<tr>
<td>Remission (16)</td>
<td>720 ± 168 &lt;.05 v normal</td>
<td></td>
</tr>
<tr>
<td>Diffuse LCL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active (7)</td>
<td>400 ± 116 &lt;.0005 v remission</td>
<td></td>
</tr>
<tr>
<td>Remission (19)</td>
<td>764 ± 160 NS v normal</td>
<td></td>
</tr>
<tr>
<td>Diffuse poorly differentiated lymphoma (7)</td>
<td>564 ± 220 &lt;.005 v normal</td>
<td></td>
</tr>
<tr>
<td>Nodular poorly differentiated lymphoma (4)</td>
<td>396 ± 160 &lt;.01 v normal</td>
<td></td>
</tr>
<tr>
<td>CLL (36)</td>
<td>592 ± 240 &lt;.0005 v normal</td>
<td></td>
</tr>
<tr>
<td>MM (37)</td>
<td>524 ± 160 &lt;.0005 v normal</td>
<td></td>
</tr>
<tr>
<td>Benign monoclonal gammopathy (12)</td>
<td>648 ± 232 &lt;.01 v normal,</td>
<td></td>
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<tr>
<td>AML</td>
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<tr>
<td>Active (13)</td>
<td>356 ± 68 &lt;.0005 v remission</td>
<td></td>
</tr>
<tr>
<td>Remission (8)</td>
<td>682 ± 176 &lt;.05 v normal</td>
<td></td>
</tr>
<tr>
<td>Chronic myeloproliferative disorder (18)</td>
<td>520 ± 164 &lt;.005 v normal</td>
<td></td>
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<tr>
<td>SLC</td>
<td></td>
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<tr>
<td>Active (24)</td>
<td>452 ± 160 &lt;.0005 v remission</td>
<td></td>
</tr>
<tr>
<td>Remission (10)</td>
<td>728 ± 90 NS v normal</td>
<td></td>
</tr>
<tr>
<td>SLC</td>
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<tr>
<td>Active (6)</td>
<td>456 ± 160 &lt;.0005 v normal</td>
<td></td>
</tr>
<tr>
<td>Remission (6)</td>
<td>788 ± 90 NS v normal</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC &gt; 3000 (9)</td>
<td>600 ± 120 &lt;.05 v normal</td>
<td></td>
</tr>
<tr>
<td>Neutropenic (5)</td>
<td>316 ± 104 &lt;.005 v other RA</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: LCL, large cell lymphoma; CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia; SCLC, small cell lung cancer; SLC, squamous lung cancer; MM, multiple myeloma; NS, not statistically significant.
patients with small cell lung cancer (Fig 5B), but the slope was less (0.3 versus 0.5), and the ratio of D-dimer to E/A levels was higher. Because the apparent relationship between CR1 reduction and E/A in samples from RA patients was strongly influenced by the very low CR1 and high E/A values found for patients with suspected immune neutropenia, we compared E/A and CR1 levels in samples from 45 RA patients with leukocyte count greater than 3,000/μL. A line of regression identical to that of Fig 4D was calculated for this group.

D-dimer and E/A levels have been measured in different groups of young and elderly control subjects. Despite variability in the (upper) range of log E/A levels, a median value near 4.2 (about 100 ng/mL) was found for young and healthy elderly plasma samples. The distribution of D-dimer levels is higher in plasma from elderly compared with young subjects, with a median value for log D-dimer (ng/mL) of 1.5 for volunteers aged 18 to 37, and 1.8 for volunteers older than 65. No significant relationship between E/A and D-dimer levels was observed in control samples, nor did these proteinase markers relate to variability in erythrocyte CR1 levels in blood from healthy volunteers. As can be seen in Figs 4 and 5, plasma from patients with small cell carcinoma of the lung and rheumatoid arthritis tended to have increased amounts of D-dimer and E/A. Plasma from almost every patient with small cell lung cancer was above the normal median for D-dimers, and plasma from almost every patient with rheumatoid arthritis was above the normal median for E/A.

DISCUSSION

Previous studies have shown that erythrocyte CR1 declines as erythrocytes age in the circulation, and that CR1 is susceptible to cleavage by several proteinases, including thrombin, plasmin, and elastase. Susceptibility to proteolysis may be increased by both the clustering of CR1 molecules and the extended and exposed structure of the molecule. In preliminary studies, we found that while erythrocyte CR1 and C3d,g were inversely related in patients with immune hemolytic anemia, equally marked CR1 reductions were seen in patients with malignancies and no measurable increase in erythrocyte C3d,g. Markers of plasma proteinase activities other than red blood cell bound C3d,g were therefore investigated.

This study provides evidence for the in vivo relationship between erythrocyte CR1 reduction and exposure to proteinases associated with malignant and inflammatory disorders. Small cell lung cancer is associated with elevated D-dimer levels.
levels, and rheumatoid arthritis with elevated elastase-α1 proteinase inhibitor complexes. Of course, since complement may be activated by neutrophil and coagulation enzymes, and complement anaphylatoxins can activate granulocytes, the present study does not exclude the complement cascade as the major common mediator of acquired CR1 reduction. Characterization of specific cleavage fragments in plasma and the portion of CR1 remaining in the erythrocyte membrane might establish the responsible enzymes but was beyond the scope of this investigation.

The elevation of D-dimer levels in patients with small cell carcinoma is consistent with previous findings of local fibrin production. Increased serine proteinase activity has been inferred from studies of β-2-microglobulin degradation in the serum of patients with small cell lung cancer. It is intriguing to note that administration of anticoagulant drugs, such as warfarin, has been associated with prolongation of survival in small cell carcinoma of the lung. Dvorak et al and others have suggested that tumor-induced activation of the coagulation cascade is key to metastatic and invasive properties, and D-dimer levels may provide a sensitive measure of such activity. The procoagulant activity of monocytes is also upregulated in cancer as in other inflammatory processes.

In RA patients we also found D-dimer levels to be elevated. A similar relationship between D-dimer elevation and CR1 reduction was observed, although the coefficient of correlation was lower (r = .5 versus r = .8) for patients with rheumatoid arthritis compared with lung cancer. Plasma elastase/α1 proteinase inhibitor complex levels were more increased in the RA patients and also appeared to be more strongly related to the degree of CR1 reduction (r = .8 versus 0.6) in our initial study groups. Plasma E/A levels were particularly elevated, and CR1 was reduced in patients whose blood bound increased amounts of IgG to paraformaldehyde-fixed neutrophils in an indirect antibody-binding assay system. Although not shown here, we have also observed CR1 reduction in other patients with anti-neutrophil antibodies and increased E/A levels, eg, idio-
The correlation between D-dimer and E/A levels in non-neutropenic patients might indicate a common relationship to activation of neutrophils in the plasma space (vascular and perivascular inflammation), whereas in patients with antibody-mediated destruction of neutrophils in the spleen and bone marrow, the release of neutrophil enzymes could be greater and activation of coagulation relatively less than that which occurs at sites of inflammation. Conversely, it may be that release of elastase is related to neutrophil-binding antibodies and/or complement activation in both neutropenic and non-neutropenic RA patients, and that neutrophil enzymes then activate the coagulation and or complement cascades. Release of neutrophil enzymes outside the vasculature and reticuloendothelium should not cause erythrocyte CR1 reduction due to neutralization of enzymes by plasma proteinase inhibitors before contact with erythrocytes. Areas of direct contact, eg, in the bone marrow, and slow circulation areas of the spleen represent the most propitious areas for potential CR1 proteolysis. In the setting of neutrophil degranulation and or destruction, many proteinases are released and/or activated, and proteinase inhibitors are locally disabled. Thus, CR1 might be cleaved by granule enzymes, or activated plasma proteinases. Moreover, in immune neutropenia, complement fixation by anti-neutrophil antibodies may cause C3 coated neutrophils to become bound to erythrocytes via CR1.

Previous studies have documented CR1 reduction in disorders resulting in increased C3 fixation to erythrocytes, a situation in which the erythrocytes may bind to and cause degranulation of phagocytic cells. No significant increase in erythrocyte-bound C3d,g was observed in patients with malignancies, and while patients with RA had slightly increased erythrocyte-bound C3d,g, the relationship to CR1 reduction was weak. Thus, while C3 deposition on erythrocytes may contribute to CR1 reduction, it is unlikely to be the only cause of acquired erythrocyte CR1 reduction, especially in the setting of malignancy.

Study of the causes of CR1 reduction in hematologic malignancies is complicated by the fact that malignant cells may contribute to hematopoiesis (eg, clonal myeloid disorders) and/or the bone marrow may be infiltrated by malignant cells. We observed a significant elevation of plasma D-dimers in all patients studied with acute myelogenous leukemia, but we did not see D-dimer elevations in patients with lymphoproliferative disorders. The striking correlation between CR1 reduction and disease activity in these disorders invites further investigation.

In summary, this study documents a relationship between acquired CR1 reduction and plasma proteinase activities, consistent with the hypothesis that reduced erythrocyte CR1 function is the result of proteolytic cleavage, although the actual mechanism remains to be determined. The evidence presented here suggests the possibility of differing proteolytic pathways in different disorders. The study of erythrocyte CR1 reduction is important not only because of the role of CR1 in the kinetics of complement-activating immune complexes, but also as a model for the effects of plasma proteinase activities on other proteins including cell surface proteins of leukocytes, platelets, and endothelium. Changes in erythrocyte CR1 levels may be useful markers of disease activity in malignant and inflammatory disorders.

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