Naturally Occurring Anticoagulants and Bone Marrow Transplantation: Plasma Protein C Predicts the Development of Venocclusive Disease of the Liver

By Elena M. Faioni, Alessandro Krahmainooff, Scott I. Bearman, Augusto B. Federici, Adriano Decarli, Alessandro M. Gianni, George B. McDonald, and Pier Mannuccio Mannucci

Venocclusive disease (VOD) of the liver is the major dose-limiting complication of pretransplant regimens for bone marrow transplantation. Recent reports from different groups point to the involvement of the coagulation mechanism in the development of VOD. We measured the naturally occurring anticoagulants protein C, antithrombin III, and protein S in 45 patients undergoing bone marrow transplantation for hematologic malignancies before cytoreductive therapy and after transplant. The aim of this prospective study was both to evaluate the status of the naturally occurring anticoagulant pathway in patients who develop VOD compared with patients who do not, and to find a predictive marker of VOD. In transplant patients, protein C decreased from before cytoreductive therapy to posttransplant, whereas protein S and antithrombin III did not. In a multivariate analysis, protein C was the only variable that could independently discriminate between VOD and non-VOD patients at all times. Discriminant function analysis established that low protein C levels before cytoreductive therapy predicted the occurrence of VOD with good sensitivity and specificity.

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MATERIALS AND METHODS

Patient selection. We selected 50 patients for enrollment in this study during 1989 and 1990, based on their underlying diagnosis of hematologic malignancy, their scheduled regimen (cyclophosphamide [CY] and total body irradiation [TBI]), and their not being part of any other study protocol. All patients were treated at the Fred Hutchinson Cancer Research Center and gave informed consent to participate in this study. Forty-five patients were categorized as having either VOD of the liver or no liver disease after cytoreductive therapy and are reported here. Five patients were excluded from analysis because their liver disease was of uncertain cause, as previously defined.1 The 45 evaluable patients included 16 males and 29 females who received CY at 120 mg/kg followed by fractionated or hyperfractionated TBI at doses of 12 Gy (21 patients), 13.2 Gy (23 patients), or 15.75 Gy (1 patient). Intravenous vitamin K was administered to each patient at a dose of 10 mg weekly. The underlying diagnoses are given in Table I. Seven patients received autologous marrow grafts and 38 patients allogeneic grafts (22 HLA-identical family donor, 5 HLA-mismatched family donor, and 11 HLA-phenotypically identical unrelated donor marrow).

Diagnosis of VOD of the liver. VOD was diagnosed on the basis of clinical criteria, as previously described.3 Diagnosis required at least two of the following signs within 20 days of transplantation: hyperbilirubinemia (total serum bilirubin >2 mg/dL or 34.2 μmol/L), hepatomegaly or right upper quadrant pain of liver origin, and sudden weight gain (>2% of baseline body weight). No other explanation for these signs and symptoms could be present at the time of diagnosis. Severity of VOD was scored as mild, moderate, or severe, as previously defined.3

Blood sampling. Blood samples were drawn into sodium citrate (12.9 mmol/L) at the following four times: baseline (before the start of CY), post-CY (after the last dose of CY), post-TBI (after the last fraction of TBI but before marrow infusion), and post-BMT (7 days after marrow infusion). Blood was immediately centrifuged at.
Table 1. Patient Characteristics Relevant to Demographics, Risk Factors for VOD, and Signs of Liver Disease

<table>
<thead>
<tr>
<th>Age (yr, median and range)</th>
<th>All Evaluable Patients (n = 50)</th>
<th>Patients With No Liver Disease (n = 16)</th>
<th>Patients With Liver Disease (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>28/22</td>
<td>6/10</td>
<td>22/17</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>AML</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>CML-CP</td>
<td>17</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>CML-AP</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>10</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Pretransplant hepatitis</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>TBI ≥13.2 Gv</td>
<td>24</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Pretransplant acyclovir</td>
<td>9</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Vancomycin during</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytoreductive therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum total serum</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>bilirubin* (mg/dL, mean ± SD)</td>
<td>9.8 ± 7.1</td>
<td>1.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Percent weight gain*</td>
<td>(maximum, mean ± SD)</td>
<td>7.3 ± 5.2</td>
<td>2.8 ± 2.2</td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelocytic leukemia; CML-CP, chronic myelogenous leukemia in chronic phase; CML-AP, chronic myelogenous leukemia in acute phase; SD, standard deviation.

* Before day 20 posttransplant, where day 0 is the day of marrow infusion.

Statistical analysis. Statistical analysis was performed on original data by parametric tests at the Istituto di Statistica Medica e Biometria of the University of Milano, using the software Statistical Package for Social Sciences (SPSS/PC+; SPSS Inc, Chicago, IL). If the distribution of the hemostasis measurements was not close to gaussian distribution, analysis was performed also after logarithmic transformation. The results did not differ from those obtained from the original data. For all tests, type I error was defined for $\alpha = 0.01$.

Analysis of variance for repeated measures was used to evaluate the changes with time of the hemostasis measurements. When a change was observed, a direct comparison between baseline values and day 7 post-BMT values was performed with the t-test for paired data. To evaluate whether or not any measurement could discriminate between the VOD and non-VOD groups, multivariate analysis of variance was performed for the different times, with the presence/absence of VOD as the independent variable. The same type of analysis was also performed with separate consideration of sex, type of graft, and malignancy as the independent variables.

To evaluate the classifying capacity of protein C, a two-by-two contingency table based on the cut-off level calculated by means of discriminant function was created. The sensitivity and specificity of protein C levels as a prognostic index were also calculated.

RESULTS

Patient characteristics. Table 1 illustrates the patient characteristics relevant to demographic aspects, risk factors for VOD, and signs of liver disease. The risk factors included in the table are pretransplant hepatitis, high-dose radiotherapy ($\geq 13.2$ Gv), and the need for pretransplant acyclovir or vancomycin during cytoreductive therapy. Of the 50 patients enrolled, 29 eventually developed VOD (58%), a prevalence consistent with recent experience at the Fred Hutchinson Cancer Research Center. Of 29 patients with VOD, 3 had severe, 17 had moderate, and 9 had mild VOD. All patients with severe VOD died of multiorgan failure.

Hemostasis measurements and VOD. Mean values and standard deviations of the naturally occurring anticoagulant function was calculated. The sensitivity and specificity of protein C levels as a prognostic index were also calculated.

All assays were run in duplicate and the operator was not aware of the final diagnosis or of the time of sampling. Values were calculated against plasma pooled from 40 normal donors (20 males and 20 females) given an arbitrary value of 100% and expressed as percent of normal controls. When an international standard was available, as for protein C (no. 86/622; NIBSC, London, UK) and factor VII (no. 84/665, NIBSC), values obtained were calculated against a plasma calibrated against the standard and expressed as percent thereof. Two control samples were included in all runs.

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Hemostasis measurements and VOD. Mean values and standard deviations of the naturally occurring anticoagulants and of factor VII are shown in Table 2. The functional amidolytic and the immunologic assays of protein C gave the same average results, whereas the functional anticoagulant assay gave lower values on average. Because this finding could have some pathogenetic relevance, the ratios of protein C values obtained by the two functional assays were compared at baseline and post-BMT; no significant difference could be found (mean ± one standard deviation of ratio of anticoagulant to amidolytic protein C values at baseline = 0.86 ± 0.27 and at post-BMT = 0.83 ± 0.27). Only measurements obtained by the functional amidolytic assay were used in the multivariate and discriminant analysis. The functional amidolytic assay was chosen because of its relative ease, possibility of automation, and greater accuracy. By analogy, for protein S only the cofactor activity was considered, because there was no significant difference between the functional and immunologic assays as judged by their ratio (not shown).

Analysis of variance for repeated measures performed on the whole population established that factor VII, protein S, and protein C changed significantly with time, whereas antithrombin III did not vary. The t-test for paired data on
these variables was then applied to the differences between baseline and day 7 post-BMT to look for a trend over the period of observation. The day 7 post-BMT time point was chosen because it is closer to the time of development of signs and symptoms of VOD; protein S increased over this period, whereas factor VII and protein C decreased ($P < .01$).

Multivariate analysis of variance at each time, with the independent variable the type of graft, malignancy, or sex, showed that no hemostasis measurement could distinguish between groups. On the other hand, when the independent variable was the presence/absence of VOD, the analysis yielded significant differences. Protein C was the only measurement that could discriminate between the VOD and non-VOD groups at all times, being significantly lower in patients who developed VOD (baseline, $P < .001$; post-CY, $P < .001$; post-TBI, $P = .004$; post-BMT, $P = .001$). On the day 7 post-BMT, antithrombin III was also significantly different in the two groups ($P < .001$). Figure 1 shows amidolytic protein C levels at the four times for patients in the VOD and non-VOD groups. All 9 patients who had protein C values at baseline equal to or below 66%; developed VOD. At day 7 post-BMT, protein C values were below 66% in 16 of 29 patients who developed VOD (baseline, $P < .001$; post-CY, $P < .001$; post-TBI, $P = .004$; post-BMT, $P = .001$). The two-by-two contingency table based on the cut-off level of
Protein C derived from the discriminant function (88%) is shown in Table 3. Based on this level, 22 patients were predicted to develop VOD, misclassifying 2 patients (positive predictive capacity = 91%). On the other hand, 23 patients were predicted not to develop VOD, and 9 of these were misclassified (negative predictive capacity = 61%). These results were statistically significant ($\chi^2 = 13.16, P = .0003$). The specificity of baseline protein C for predicting VOD was 87%, whereas the sensitivity was 69%. The inclusion of the other naturally occurring anticoagulants and factor VII in the discriminant analysis did not substantially improve the ability to predict VOD over that of protein C alone. There was no correlation between baseline protein C levels less than 88% and severity of VOD, and the frequency of this finding was similar among patients who developed severe VOD (2 of 3), moderate VOD (12 of 17), and mild VOD (6 of 9).

Comparison of protein C with other known risk factors for VOD indicated that 5 of 7 patients with no known risk factors who developed VOD were identified only by low baseline protein C levels. Furthermore, although 7 of 16 patients who did not develop VOD had one or more risk factors, protein C levels in these patients were normal.

**DISCUSSION**

Protein C plays a pivotal role in the regulation of hemostasis. After its activation at the cell surface, it modulates thrombin formation by proteolytically inactivating factors V and VIII. Patients with congenital deficiency of protein C are at risk of developing thromboembolism at a young age. Recent experimental evidence in vivo suggests its involvement in inflammatory responses. Haire et al reported low protein C levels after transplant in 75% of patients undergoing BMT and a discrepancy between amidolytic and anticoagulant levels of protein C, which they attributed to incomplete carboxylation and consumption. In contrast, we found no significant discrepancy between protein C anticoagulant and amidolytic levels after transplant, although a trend could be observed. Perhaps differences between the two studies are due to the fact that Haire et al’s sampling time was 14 days after transplant (1 week later than our sampling time), when consumption and/or impaired hepatic synthesis might be more prominent.

The major finding of our study was that plasma protein C predicts the development of VOD in patients undergoing BMT. We observed that patients undergoing BMT who subsequently developed VOD had lower baseline protein C levels than patients who did not develop this complication. Low levels of protein C can be due to decreased synthesis or increased consumption. The greater defect in VOD patients might be due to a more severe impairment of liver function, with protein C being a sensitive and early index of liver dysfunction due to its very short plasma half-life. The liver dysfunction, in turn, may be related to the chemotherapy used to treat the underlying hematologic malignancies or to posttransfusion hepatitis. However, liver dysfunction cannot be the only explanation for lower protein C in VOD patients because factor VII, another vitamin K-dependent protein with a similar half-life, was low to the same degree in patients with and without VOD, and did not predict the occurrence of the complication. Furthermore, another protein synthesized by the liver, such as protein S, was normal or high. Because low protein C has been observed during disseminated intravascular coagulation, consumption of protein C might contribute to its decrease in VOD patients.

In our study, the prevalence of VOD was higher among males than females, an observation not confirmed by others, and the five patients with pretransplant hepatitis (a well-established risk factor) did not develop VOD. We think the explanation for these findings is that we studied a very small population, which does not allow us to draw conclusions regarding the distribution of some of the risk factors for VOD. The pretransplant characteristics that represent

<table>
<thead>
<tr>
<th>Predicted</th>
<th>Non-VOD</th>
<th>VOD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-VOD</td>
<td>14</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>VOD</td>
<td>9</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>22</td>
<td>45</td>
</tr>
</tbody>
</table>

$\chi^2 = 13.16, P = .0003$; specificity = 87%; sensitivity = 69%; positive predictive value = 91%; negative predictive value = 61%.
risk factors for VOD have been extensively evaluated on larger populations. In a recent prospective study of 355 patients undergoing transplant, significant risk factors for severe VOD included hepatitis before transplantation, high-dose cytoreductive therapy, or the requirement for acyclovir or vancomycin therapy during cytoreduction. As shown in Table 1, several of the patients we studied had one or more of these risk factors for VOD. Of the patients who developed VOD, the majority could also be identified by protein C levels at baseline. Furthermore, 5 patients who developed VOD could be identified only by their protein C levels. Therefore, the measurement of protein C may give additional information for predicting the development of VOD and in some patients it may be the only marker.

Low levels of protein C might play a role in the pathogenesis of VOD. A clinical trial for the prophylaxis or treatment of VOD with protein C concentrates in selected, high-risk patients undergoing BMT would test this hypothesis.

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