Decrees in Levels of Serum Fibronectin Predict the Severity of Vascular Leak Syndrome in Patients Treated with Ricin A Chain-containing Immunotoxins

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ABSTRACT

The major dose-limiting adverse effect of ricin A chain-containing immunotoxin (IT) therapy is vascular leak syndrome (VLS). Since plasma fibronectin (Fn) plays a role in maintaining microcirculatory integrity and since the gradient between plasma and tissue Fn can be altered in various pathological situations, we determined whether the administration of IT-ricin A chain to patients resulted in changes in the levels of serum Fn and, if so, whether these changes correlated with the severity of VLS. We also measured the serum levels of tumor necrosis factor α (TNFα), a proinflammatory cytokine which has been implicated in tissue damage and in interleukin 2-mediated VLS. Our results indicate that the most severe manifestations of VLS were associated with the highest pretreatment levels of Fn, the largest decreases in Fn immediately after starting IT therapy, increases in the levels of serum TNFα, higher concentrations of circulating IT, and the lowest numbers of circulating tumor cells. These parameters should, therefore, be useful for predicting which patients will have severe VLS.

INTRODUCTION

The major dose-limiting adverse effect of IT therapy in humans is VLS. VLS is characterized by an increase in vascular permeability accompanied by extravasation of fluids and proteins from the capillary vessels into the tissues, resulting in interstitial edema, a decrease in microcirculatory perfusion, and, in its most severe form, multiple organ failure. VLS occurs in patients treated with ITs containing RTA, blocked ricin, saporin, pokeweed antiviral protein, Pseudomonas exotoxin A, and diphtheria toxin (1–7). VLS is not unique to IT therapy and is a toxic side effect of therapy with cytokines, growth factors, antibodies, and of chemotherapy (8–16). VLS can also occur in a variety of different diseases (17–20). The mechanisms underlying VLS are not well understood, although in the case of IT therapy, it has been suggested that RTA alters EC functions required for vascular integrity (21). In this regard, ECs are 10-fold more sensitive to the in vitro nontargeted cytotoxic activity of dgRTA than most other cell lines (21). In addition, dgRTA binds to Fn in vitro, perhaps preventing it from binding to its receptors on ECs and promoting disruptions of EC monolayers (21A).

Fn is a multifunctional protein which exists in both a soluble form in the plasma and other body fluids and in an insoluble form in basement membranes, extracellular matrices, and connective tissues (22). Plasma Fn plays a role in microcirculatory integrity since it is incorporated into the extracellular matrix. It also augments the macrophage-mediated clearance of circulating microaggregates (23–28). The gradient between plasma and tissue Fn can be altered in various diseases, particularly as levels of plasma Fn decrease. One potential consequence is a deficiency in Fn-EC interactions, resulting in an increase in vascular permeability. Once the permeability of the vasculature increases, proteins and cytokines might enter the tissues, where further exacerbation of VLS may occur.

In this study, we determined whether the administration of IT-dgRTA to patients resulted in decreased levels of serum Fn and, if so, whether these decreases correlated with more severe VLS. We also correlated changes in Fn levels with the presence of circulating tumor cells and concentrations of IT in the same sera.

MATERIALS AND METHODS

Patients. Sera obtained from patients with histologically confirmed non-Hodgkin’s lymphoma of low, intermediate, or high grade and previously treated with RTA-based ITs were entered into these studies. The patients are described in detail elsewhere (2, 29–31).

IT Preparation and Administration. The three ITs (Fab’-RFB4-RTA, IgG-HD37-SMPT-RTA, and IgG-RFB4-SMPT-RTA) were prepared as described previously (2, 29). The ITs were administered by either bolus infusion (BI) or continuous infusion (CI). The CI was administered over 8 days at three dose levels. BI included 4-h infusions given every other day during treatment, and all adverse effects were recorded and graded from grades I to IV using criteria described previously.

Assessment of Toxicity. The patient’s physical status, hematology, blood chemistry, and urinalysis were assessed daily during treatment, and all adverse effects were recorded and graded from grades I to IV using criteria described previously.
Table 1 Grading of immunotoxin-related toxicities

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
<th>Grade IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain/edema</td>
<td>Minimal ankle pitting edema</td>
<td>Ankle pitting edema and weight gain &lt;10 lb</td>
<td>Peripheral edema, weight gain &gt;10 lb, pleural effusion with no pulmonary deficit</td>
<td>Anasarca, severe pleural effusion with pulmonary function deficit, ascites, pulmonary edema ≤0.2</td>
</tr>
<tr>
<td>Hypoalbuminemia</td>
<td>&gt;2.8</td>
<td>2.4–2.7</td>
<td>2.0–2.3</td>
<td>&gt;6.0 × normal; required dialysis (≥8.0)</td>
</tr>
<tr>
<td>Renal</td>
<td></td>
<td></td>
<td></td>
<td>Irreversible loss of &gt;20% baseline</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>1.25–1.5 × normal (1.6–2.0)</td>
<td>1.5–3.0 × normal (2.1–3.9)</td>
<td>3.1–6.0 × normal (4–7.9)</td>
<td>Unable to perform test due to respiratory distress</td>
</tr>
<tr>
<td>Pulmonary function</td>
<td>70–80% of baseline</td>
<td>50–69% of baseline</td>
<td>30–49% of baseline</td>
<td>Severe symptoms at rest nonresponsive to Rx</td>
</tr>
<tr>
<td>Pulmonary function</td>
<td>FVC &gt;70–80% of predicted FEV1 or DLCO 60–80% of predicted; 15–25% decrease from normal baseline</td>
<td>FVC 50–69% of predicted FEV1 or DLCO 40–59% of predicted; 20–50% decrease from normal baseline</td>
<td>FVC &lt;50% of predicted FEV1 or DLCO 40–59% of predicted; 50% decrease from normal baseline</td>
<td>Severe, refractory CHF</td>
</tr>
<tr>
<td>Respiratory symptoms</td>
<td>Mild or transient, asymptomatic with pulmonary function tests abnormal</td>
<td>Dyspnea on significant exertion</td>
<td>Symptoms during normal activity, persistent dyspnea</td>
<td>Severe symptoms at rest nonresponsive to Rx</td>
</tr>
<tr>
<td>Chest X-ray</td>
<td>&lt;10% of lung fields show infiltrate or effusion</td>
<td>&lt;20% of lung fields show infiltrate or effusion</td>
<td>&lt;50% of lung fields shown infiltrate or effusion</td>
<td>&gt;50% of lung fields show infiltrate or effusion</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac function</td>
<td>Asymptomatic, ↓ ejection fraction by &lt;20% of baseline</td>
<td>Asymptomatic, ↓ ejection fraction by &gt;20% of baseline</td>
<td>Mild CHF, responsive to Rx</td>
<td>Severe, refractory CHF</td>
</tr>
<tr>
<td>Pericardial effusion</td>
<td>Asymptomatic, No Rx required</td>
<td>Pericarditis, (rub, chest pain, EKG changes)</td>
<td>Symptomatic; large effusion, drainage required no tamponade, resp to drainage</td>
<td>Large effusion, tamponade; drainage urgently required</td>
</tr>
<tr>
<td>Hypotension</td>
<td>10–20% ↓ systolic, no Rx required (includes transient orthostatic hypotension)</td>
<td>21–30% ↓ systolic, required fluids or other Rx but not hospitalization</td>
<td>31–40% ↓ systolic, required pressors and hospitalization, resolves within 48 h</td>
<td>&gt;40% ↓ systolic, required hospitalization, unresponsive to pressors, requires &gt;48 h to resolve after stopping agent</td>
</tr>
</tbody>
</table>

*FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; DLCO, diffusion capacity of the lungs for CO2; Rx, treatment; CHF, congestive heart failure; EKG, electrocardiogram.

Flow Cytometric Analysis. The presence or absence of peripheral blood-circulating tumor cells was assessed according to previously described methods (2).

Statistical Analysis. All values are expressed as means ± SE. For statistical analysis of the data, Student’s t test and the Pearson correlation were used. Correlations between parameters were assessed by linear regression analysis. A two-tailed P < 0.05 was considered to represent a significant difference.

RESULTS

Patients. Sera from 56 patients with non-Hodgkin’s lymphoma were entered into this study. Patients had been treated by BI or CI with a total of 65 courses of IgG-HD37-RTA (30), IgG-RFB4-RTA (2, 31), or Fab'-RFB4-RTA (29). Seventeen patients had received CI and 9 had received BI of IgG-RFB4-RTA, and 7 had received CI and 18 had received BI of IgG-HD37-RTA. Five patients received BI of Fab'-RFB4-RTA.
VLS. The major clinical features of VLS in these patients are described in Fig. 1 and Table 2. VLS was mild in 28 courses, moderate in 25 courses, and severe in 12 courses. In patients with severe toxicity, the administration of IT was halted. The most severe manifestations of VLS were pulmonary edema and hypotension (Table 3). As shown in Fig. 2, patients who had mild VLS had lower levels of serum Fn prior to treatment than patients who had severe VLS. Serum Fn levels decreased in patients with moderate VLS (mean ± SE, −29 ± 3%, P < 0.01) and severe VLS (mean ± SE, −49 ± 4%, P < 0.001) and increased in patients with mild VLS (mean ± SE, 22 ± 3%, P = 0.07; Fig. 3A). The changes in levels of serum Fn occurred early and were sustained until the completion of therapy (Fig. 3B). Unlike Fn, serum albumin levels and weight gains showed fewer marked changes in patients with severe VLS. Thus, albumin levels decreased by 21, 25, and 28% in mild, moderate, and severe VLS, respectively, and weights increased by 2, 3.8, and 4.5%, respectively. Furthermore, reductions in the levels of serum albumin and weight gains were more pronounced late in the course of infusion, whereas changes in the Fn levels occurred early (data not shown). The most significant decreases in the levels of serum Fn were also apparent in patients with severe toxicity whose infusions were interrupted (Table 3). The decreases in the levels of serum Fn were observed during the first day of IT therapy in 83% of the patients with severe toxicity. Serious manifestations of VLS such as pulmonary insufficiency or hypotension were preceded by a decrease in the levels of serum Fn but not necessarily by either a fall in serum albumin or increases in body weight (Fig. 2). As shown in Fig. 4, in patients with severe VLS, there was a decrease in Fn on day 2 (1 day posttreatment) with little change in albumin levels or in weight. As shown in Fig. 5, there was also an inverse correlation (r = −0.50, P = 0.01) between the maximum concentration of IT achieved and the decrease in Fn levels observed. The largest decreases in Fn levels also correlated with the presence of the smallest numbers of circulating tumor cells (r = 0.44, P = 0.03). This suggests that a decrease in Fn levels may be as predictive a marker for severe VLS as the absence of circulating tumor cells and serum II concentrations greater than 1 μg/ml (2, 29, 31). The correlations between changes in the levels of serum Fn and levels of IT versus grades of VLS were r = −0.38, P < 0.001, and r = 0.40, P < 0.001, respectively.

Relationship between Decreases in Serum Fn and Changes in the Levels of TNF-α. Levels of serum TNF-α increased in patients with severe VLS (Fig. 6). There was a trend for correlations between maximal changes in the levels of Fn in patients with moderate and severe toxicity and increasing levels of TNF-α (r = −0.38, P = 0.02).
Fig. 2 Serum Fn levels in patients who developed severe VLS. The daily levels of serum Fn were determined in eight patients who had severe VLS 3–4 days after the initiation of IT therapy (●) versus patients with mild VLS (■). The levels of serum Fn were highest in patients with severe VLS prior to IT therapy. Following treatment with ITs, the levels of serum Fn were lower in patients with severe VLS than in patients with mild toxicity (195 μg/ml, P < 0.04). Bars, SE.

**Table 3** Patients with interrupted infusions of ITs due to VLS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>IT/regimen</th>
<th>Duration of infusions (h) or no. of doses</th>
<th>Fn (μg/ml) prior to IT therapy</th>
<th>Fn (μg/ml) during IT therapy (daily measurement)</th>
<th>Symptoms of VLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60/F</td>
<td>IgG-RFB4-dgA/Cl</td>
<td>96</td>
<td>241</td>
<td>103, 148, 184, 55</td>
<td>Weight gain³, dyspnea, hypotension</td>
</tr>
<tr>
<td>2</td>
<td>66/F</td>
<td>IgG-RFB4-dgA/Cl</td>
<td>72</td>
<td>361</td>
<td>50, 50, 50</td>
<td>Weight gain 3, pulmonary edema, hypotension</td>
</tr>
<tr>
<td>3</td>
<td>71/F</td>
<td>IgG-RFB4-dgA/Cl</td>
<td>72</td>
<td>203</td>
<td>52, 52, 52</td>
<td>Weight gain, dyspnea</td>
</tr>
<tr>
<td>4</td>
<td>58/F</td>
<td>IgG-RFB4-dgA/Cl</td>
<td>72</td>
<td>139</td>
<td>50, 50, 74</td>
<td>Hypotension, respiratory failure, death</td>
</tr>
<tr>
<td>5</td>
<td>61/M</td>
<td>IgG-RFB4-dgA/Cl</td>
<td>2 doses</td>
<td>148</td>
<td>50, 50, 55</td>
<td>Pulmonary edema, oliguria, hypotension, death</td>
</tr>
<tr>
<td>6</td>
<td>44/F</td>
<td>IgG-RFB4-dgA/Cl</td>
<td>3 doses</td>
<td>299</td>
<td>94, 94, 241, 208, 94</td>
<td>Weight gain 3, dyspnea, pleural effusion</td>
</tr>
<tr>
<td>7</td>
<td>44/M</td>
<td>Fab-RFB4-dgA/BI</td>
<td>2 doses</td>
<td>515</td>
<td>500, 94, 139</td>
<td>Pulmonary edema</td>
</tr>
<tr>
<td>8</td>
<td>67/F</td>
<td>Fab-RFB4-dgA/BI</td>
<td>3 doses</td>
<td>471</td>
<td>139, 241, 188, 188, 500</td>
<td>Dyspnea, oliguria</td>
</tr>
<tr>
<td>9</td>
<td>63/M</td>
<td>IgG-HD37-dgA/CI</td>
<td>2 doses</td>
<td>485</td>
<td>500, 94, 55</td>
<td>Dyspnea, oliguria</td>
</tr>
<tr>
<td>10</td>
<td>44/F</td>
<td>IgG-HD37-dgA/CI</td>
<td>3 doses</td>
<td>515</td>
<td>130, 241, 241, 299, 188</td>
<td>Weight gain 3, dyspnea</td>
</tr>
<tr>
<td>11</td>
<td>72/M</td>
<td>IgG-HD37-dgA/CI</td>
<td>98</td>
<td>50</td>
<td>50, 50, 50, 50</td>
<td>Weight gain 3, dyspnea, pleural effusion</td>
</tr>
<tr>
<td>12</td>
<td>70/F</td>
<td>IgG-HD37-dgA/CI</td>
<td>151</td>
<td>188</td>
<td>94, 423, 500, 442, 50</td>
<td>Weight gain 3, dyspnea</td>
</tr>
</tbody>
</table>

* F, female; M, male.
* A CI regimen was planned for 192 h. A BI regimen was planned for four doses. The percentage of patients with decreases in the levels of serum Fn on days 1–3: dose 1, 83%; dose 2, 75%; dose 3, 83%.
* Numbers refer to grade.

**DISCUSSION**

The major findings to emerge from this study are: (a) Patients with severe VLS have the highest levels of serum Fn prior to treatment. (b) Following treatment with ITs, levels of serum Fn decreased most significantly in patients who developed severe VLS. (c) The development of severe VLS was preceded by several days by decreases in the levels of serum Fn, but not by decreases in the levels of serum albumin or increases in body weight. (d) The largest decreases in the levels of serum Fn and the most severe VLS occurred in patients with the smallest numbers of circulating tumor cells and the highest levels of serum IT. (e) The decreases in the levels of serum Fn were accompanied by increases in the levels of serum TNF-α.

VLS is the major dose-limiting toxicity in patients treated with RTA-based ITs (1). Manifestations of VLS include fluid retention, increase in body weight, peripheral edema, pleural and pericardial effusions, ascites, anasarca, and, in severe form, renal, pulmonary, and cardiovascular failure. Symptoms are highly variable among patients for reasons which are not understood. During VLS, the levels of serum albumin decrease in IT-treated patients. In the patients treated with RIA-ITs previously (2, 29, 31), decreases in albumin ranged from 4 to 40%. However, the consistency and magnitude of these decreases were less marked and did not always precede the manifestation of clinical symptoms. By contrast, the early and rapid decrease in Fn levels observed in patients with most severe VLS suggest a causal relationship. Similar early decreases in the levels of serum Fn have been reported in patients with sepsis, burns, trauma, and disseminated intravascular coagulation (22, 23, 32). Persistent decreases in Fn levels in patients with these diseases...
Nevertheless, we chose the criteria described by Sausville et al. VLS had the highest levels of Fn prior to treatment. Further-

"Results." Using these criteria, patients with the most severe (2) to define mild, moderate, and severe VLS as discussed in etiology-based grading criteria for different manifestations of VLS. One problem in determining this relationship is that there are no "standard" VLS because of its variable and complex clinical presentation. The decrease in the levels of serum Fn is more significant in patients with severe VLS than in those with moderate VLS (P < 0.001). The changes in the levels of Fn in patients with moderate and severe VLS are significantly different from the changes in patients with mild VLS (P < 0.000001). Means are presented. B. early versus late changes in the levels of serum Fn. The changes in serum Fn levels after 4 h (E), on the first day (F), and on the last day (I) of IT therapy are presented in patients with mild, moderate, and severe VLS. Significant differences in the decreases in the levels of serum Fn in severe VLS versus moderate VLS were found on the first day (P = 0.01) and the last day (P = 0.001) of therapy. Bars, SE.

Fig. 3 A. changes in the levels of serum Fn in patients with different grades of VLS. The levels of serum Fn were determined in samples obtained before therapy and daily during infusion of IT (average of all days) in 56 patients. The mean Fn levels before therapy ranged from 175 to 300 μg/ml. The percentage of change versus pretreatment levels of serum Fn are shown in patients with mild, moderate, and severe VLS. The decrease in the levels of serum Fn is more significant in patients with severe VLS than in those with moderate VLS (P < 0.001). The changes in the levels of Fn in patients with moderate and severe VLS are significantly different from the changes in patients with mild VLS (P < 0.000001). Means are presented. B. early versus late changes in the levels of serum Fn. The changes in serum Fn levels after 4 h (E), on the first day (F), and on the last day (I) of IT therapy are presented in patients with mild, moderate, and severe VLS. Significant differences in the decreases in the levels of serum Fn in severe VLS versus moderate VLS were found on the first day (P = 0.01) and the last day (P = 0.001) of therapy. Bars, SE.

The decrease in the levels of serum Fn was the earliest event that preceded manifestations of severe VLS. More, the largest decreases in the levels of serum Fn occurred in patients whose infusions were interrupted or terminated for any one of several clinical criteria and who typically had pulmonary and cardiovascular problems. In these patients, Fn concentrations decreased before clinical manifestations of severe VLS, thus providing a measurable parameter to predict severe VLS. Thus, in 83% of the patients with severe VLS, serum Fn levels decreased during the first day of therapy, and, in 75% of the patients, the decrease was observed daily during the first 3 days (Table 3). However, in patients with mild VLS, 35% had decreases in the levels of serum Fn on day 1 and only 14% maintained this decrease over 3 days. These data suggest that a persistent decrease in the levels of serum Fn during the first 3 days of IT therapy might be most predictive for the development of severe VLS. Furthermore, a concomitant increase in the levels of serum IT might strongly suggest that therapy should be terminated.

Based on studies of Fn depletion associated with sepsis, trauma, and other diseases, it has been proposed that as Fn is consumed by opsonic utilization and rapidly bound to sites of tissue injury, levels of serum Fn are acutely depleted (17, 22, 23). In our IT-treated patients, the early decreases in the levels of Fn in the sera of patients who later developed VLS also suggest a rapid consumption of Fn. This might occur in several ways: (a) Fn might bind to the RTA portion of the circulating IT and, as a result, bind less effectively to its receptor on ECs. This could lead to EC damage by virtue of a disruption of the extracellular matrix and, subsequently, further consumption of serum Fn by the injured cells. (b) The IT might bind to ECs nonspecifically, and because these cells are highly sensitive to RTA, they would be damaged. The damaged cells would consume more Fn and, in addition, be unable to produce and replenish the Fn. Previous studies have failed to identify a specific IT-binding site on HUVECs (21) but such sites could be present in low abundance. (c) In either of the above situations, cytokines released by damaged ECs and/or other tissues could correlate with organ failure and, in particular, respiratory distress and cardiac failure. In this regard, the administration of a Fn cryoprecipitate or purified Fn to patients with decreased levels of serum Fn frequently reduces the symptoms of VLS (33-35).

To examine further the relationship between decreased levels of serum Fn and the development of VLS, we analyzed Fn levels in patients with different grades of VLS. One problem in determining this relationship is that there are no "standard" etiology-based grading criteria for different manifestations of VLS because of its variable and complex clinical presentation. Nevertheless, we chose the criteria described by Sausville et al. (2) to define mild, moderate, and severe VLS as discussed in "Results." Using these criteria, patients with the most severe VLS had the highest levels of Fn prior to treatment. Further-
Fig. 5 Changes in the serum Fn levels versus levels of IT and circulating tumor cell levels. A, maximum changes in serum Fn levels correlate inversely with serum IT levels ($r = -0.50$, $P = 0.01$). B, changes in the levels of serum Fn on the first day of therapy correlate with the number of circulating tumor cells ($r = 0.44$, $P = 0.03$).

Further increase vascular permeability. Although it is not clear why the higher pretreatment levels of Fn correlate with the most severe VLS, it is possible that prior therapies alter the Fn profile in the sera of some patients, and these patients are in some way predisposed to IT-mediated damage of the vasculature.

The correlations between the severity of VLS and both the decrease in the levels of serum Fn and the absence of circulating tumor cells further suggest that if circulating tumor cells are present, they may act as a blood-bone “sink” for the IT and, hence, decrease its binding to Fn and/or ECs. The damaged ECs in combination with released cytokines and inflammatory mediators might cause a variety of clinical symptoms. Indeed, it has been shown that TNF-α and prostaglandins inhibit the synthesis of Fn (36–39).
ACKNOWLEDGMENTS

We thank C. Patterson, C. Self, P. Lodes, and S. Richardson for excellent secretarial help.

REFERENCES

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