Radioimmunoassay of Laminin in Serum and Its Application to Cancer Patients

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In this sensitive radioimmunoassay for laminin fragment P1 (purified from pepsin extracts of human placenta), monovalent antibody fragments obtained from rabbit antisera against the laminin component are used. The inhibition assay showed a low intra- and interassay variability, and the inhibition curves for various serum samples had parallel slopes. Molecular-sieve chromatography demonstrated heterogeneity of the laminin antigen in serum. For quantification we defined arbitrary units based on the amount of antigenic material present in normal human serum. Sera from 361 tumor patients showed increased values in about half of the cases. Correlation with increased concentration of carinoembryonic antigen was poor (r = 0.259). The assay may be useful for diseases involving basement-membrane metabolism.

Additional Keyphrases: basement membrane · monovalent antibody fragments · carinoembryonic antigen compared · reference interval

Basement membranes are abundant extracellular protein matrices that form characteristic structures within blood vessels, around nerves and muscle fibers, and within perineurial tissues (1,2). They have long been known to be involved in various clinical disorders, e.g., microangiopathic lesions in diabetes mellitus, potentially leading to severe renal complications (3), and various degradation patterns of basement membranes accompanying tumor growth and metastasis (4,5). Descriptions of a molecular pathology of basement membranes have recently become feasible owing to an extensive structural characterization of typical constituents such as collagen IV, laminin, and heparan sulfate proteoglycans (6-11). With the sensitive radioimmunoassays developed for several of these components (12), which were originally identified in mouse-tumor basement membranes, investigators have quantified circulating forms of basement membrane proteins and have demonstrated their increased concentrations in serum in experimental diabetes (13-15) and other disorders. Because of the restricted interspecies cross-reactivity of antibodies (16), however, the assays could not be used for analyzing human serum samples under normal or clinical conditions.

Another limitation on developing assays for human basement-membrane proteins is the scarcity of collagen IV and laminin in soluble form. To overcome this difficulty, radioimmunoassays for fragments of these proteins such as the 7S collagen (16) and NC1 domain (17) of human placenta collagen IV have been developed. A large (about 300 kDa) fragment (P1) of laminin has been identified in and purified from pepsin digests of human placenta (18). Radioimmunoassays for this fragment could detect low concentrations of laminin in human serum (18-20) but also demonstrated a nonparallel inhibition profile for serum samples as compared with that for the authentic fragment (20). Similar differences in inhibition profiles encountered in other protein assays (21) have, as shown recently for a procollagen peptide (22,23), been obviated by use of monovalent antibodies in the assays instead of bivalent antibody. Applying this approach to human laminin, we have developed a reliable assay for the P1 fragment in serum that may have some diagnostic potential for tumor patients.

Materials and Methods

Preparation of human laminin P1 fragment and other antigens. Fragment P1 of laminin was purified from pepsin digests of human placenta, as described previously (18), with the following modifications. We included a short digestion with collagenase (EC 3.4.24.3; “CLSPA,” Worthington, Freehold, N.J) for 2 h at 37°C (enzyme:substrate ratio, 1:200) before anion-exchange chromatography, and we used Biogel A5m (Biorad, Munich, F.R.G.) equilibrated in a solution containing 1 mol of CaCl₂ and 0.05 mol of Tris HCl (pH 7.4) per liter for molecular-sieve chromatography on a 5 × 140 cm column. We finally purified the laminin P1 fragment by binding to concanavalin A-Sepharose (Phar- macia, Uppsala, Sweden) equilibrated with (per liter) 0.05 mol of Tris HCl, 0.15 mol of NaCl, and 1 mmol of CaCl₂ (pH 7.4), then eluting with the same buffer to which α-methyl mannoside, 0.4 mol/L, had been added to displace the glycoprotein from the solid phase. The 7S (24) and NC1 domains (25) of human placenta collagen type IV were obtained from collagenase digests. Human collagens type I and II were purified by standard procedures (26). Human plasma fibronectin was obtained from Calbiochem, Frankfurt/Main, F.R.G. Procollagen type I was prepared from human fibroblast culture medium according to Uitto et al. (27). Bovine procollagen peptide type III was prepared as described earlier (28).

Antiser and Fab fragments. Rabbits were immunized twice during four weeks, each time with 0.2 mg of fragment P1 in 1 mL of phosphate-buffered isotonic saline, pH 7.2 (PM16; Serva, Heidelberg, F.R.G.), plus complete Freund’s adjuvant. Sera were collected at various intervals during the four to eight weeks after the second injection. Monovalent antibody fragments were prepared from the IgG fraction, either by digestion with pepsin (EC 3.4.23.1) and reduction (Fab⁻), or by digestion with papain (EC 3.4.22.2) (Fab), according to established procedures (25,29). The Fab fragments could be separated into two populations by chromatography on carboxymethyl (CM)-cellulose (30).

Radioimmunoassays. We labeled laminin fragment P1 with ¹²⁵I by the Chloramine T method to a specific radioactivity of 10 000-50 000 counts/min per nanogram. We used
the labeled fragment in inhibition assays (sequential saturation type) according to a standard procedure (37) as follows: Dilute, with phosphate-buffered saline (PM16) containing 0.4 mL of Tween 20 polyethylene (20) sorbitan monolaurate surfactant per liter, the Fab preparations obtained after CM-cellulose chromatography to a concentration capable of binding 50% of the tracer. Incubate, overnight at 4 °C, 0.2 mL of the Fab preparation with 0.1 mL of unlabeled standard antigen solution or an aliquot of the unknown sample. Add to this mixture about 1 ng of 125I-labeled antigen in 0.1 mL and incubate for another 6 to 7 h. Then add 0.5 arb. unit (as defined by the supplier, Calbiochem) of goat antiserum to rabbit IgG in 0.5 mL, as secondary antibody for efficient precipitation of Fab fragments (overnight incubation at 4 °C). The comparison inhibitor was unlabeled laminin P1. To calculate the concentration of antigenic material in serum, we assumed that normal serum contained 1 arb. unit of laminin antigen per milliliter, based on the values measured in 55 sera. The molecular size of laminin antigen in 1.5-mL serum samples was determined by radioimmunoinhibition assays after molecular-sieve chromatography on another 1.5 × 130 cm Biogel A5m column, equilibrated in the buffered saline/Tween solution (see above).

The radioimmunoassay for carcinoembryonic antigen was done with a commercially available kit (Behringwerke AG, Marburg, F.R.G.).

Serum sources. Normal sera were obtained from 165 healthy blood donors (ages 18–60 years, both sexes). We also obtained sera from 361 patients with verified malignancy of the breast (124), lung (47), colon (46), rectum (26), stomach (34), prostate (15), bladder (13), ovary (eight), pancreas (six), esophagus (six), or ear-nose-throat (ENT) (11), and also 23 cases of cancer of miscellaneous origins.

Results
Radioimmunoassay of Human Laminin P1 Based on Fab fragments
Antisera raised in rabbits against human laminin fragment P1 showed distinct binding in radioimmunoassays (range of 50% binding titers, 1:10 000 to 1:50 000) and were sufficiently sensitive in inhibition assays (50% inhibition at 20–50 ng/mL) for analysis of biological samples. Human serum samples distinctly inhibited the binding but showed a less-steep inhibition profile than the comparison inhibitor P1 (Figure 1a), demonstrating that this assay is only of limited value for quantitative analyses of serum.

On the basis of previous experience (22, 23), we then replaced the primary antibody in this assay with monoclonal antibody fragments (Fab), which showed similar binding activity but a lower inhibition sensitivity than the whole antiserum. In this assay the slopes of inhibition curves produced by serum samples were, however, identical to those of the comparison inhibitor (Figure 1b). The assay was specific for laminin because several collagens, including collagen IV fragments and fibronectin, showed no inhibition (Figure 1b).

The Fab assay showed a low intra-assay variation 0.9–2.6%, n = 5) and also a low interassay variation (1.2–3.9%; see Figure 1b) when tested 10 times during three months. We noted some larger differences in the 50% inhibitory potency of fragment P1 when comparing two forms of antibody Fab fragments (Fab I and Fab II) obtained after treatment with papain and of Fab' fragments obtained after treatment with pepsin (Table 1). We found much lower differences, all within the range of interassay variability, among the three assays when we compared several human sera as inhibitors (Table 1). Because the assay was designed

![Fig. 1. Comparison of laminin fragments P1 and human sera in radioimmuno inhibition assays with use of intact antibodies (a) or Fab fragments (b) Inhibitors of the reactions were laminin fragment P1 (I) and Individual normal sera (shorter curves at right of a and b). Several other antigens (7S and NC1 domain of collagen IV, procollagen I, collagen I and II, procollagen III peptide, fibronectin) were noninhibitory (II). The scales refer to the concentrations of protein antigens (ng/mL) or the amounts of serum (μL) used as inhibitors in a total volume of 0.1 mL. Arbitrary units refer to the mean value of a panel of normal human sera and were used to calculate the relative amounts of laminin antigen in unknown serum samples (see Figs. 2–4); the scale is matched with that of protein antigen. Vertical bars in b indicate the range of interassay variation (n = 10).](image-url)
We estimated the molecular size of laminin in serum by molecular-sieve chromatography (Figure 3) and found, for that in normal serum, a bimodal size distribution. About 50% of the antigenic activity eluted coincident with an apparent relative molecular mass of standard laminin P1; the remaining material eluted in a position that indicated a distinctly higher molecular mass. The latter material predominated in tumor sera (Figure 3) as shown in the analysis of three different patients’ sera.

Application to Tumor Patients

Quantitative determinations of laminin in the serum of 361 patients with various tumors demonstrated a much broader range than in normal sera, the mean (and SD) being 1.49 (0.49) arb. units/mL. Laminin concentration in about 50% of the tumor sera exceeded the upper normal range (Figure 3). The panel of tumor patients studied included a large variety of different neoplasms (see Materials and Methods), the largest patient groups being those with tumors of the breast, lung, rectum, colon, stomach, prostate, and bladder.

For all these groups the mean values and proportions of increased values were similar to those for the tumor population as a whole (Figure 2).

When we compared values for laminin and carcinoembryonic antigen (considered to be a relevant tumor marker) in serum, we found increased values for both in 37% of 351 patients (Figure 4). About 14% and 33% of the patients, respectively, had increased values for either laminin or carcinoembryonic antigen. The two assays together identified 84% of the tumor patients as having increased values for one or both analytes.

**Table 1. Sensitivity of Radiolmmunoinhibition Assays with Different Antibody Fragments against Laminin P1**

<table>
<thead>
<tr>
<th>Monovalent antibody fragment</th>
<th>Laminin P1, ng/mL</th>
<th>Normal sera, µL</th>
<th>Conc for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fab'</td>
<td>40</td>
<td>363 (85)</td>
<td></td>
</tr>
<tr>
<td>Fab (I)*</td>
<td>74</td>
<td>448 (94)</td>
<td></td>
</tr>
<tr>
<td>Fab (II)*</td>
<td>110</td>
<td>348 (68)</td>
<td></td>
</tr>
</tbody>
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*Av. of two analyses. †Av. and SD for 5 normal sera. *I and II refer to Fab fragment obtained from the first and second peak after chromatography on CM-cellulose.

to measure the relative contents of serum laminin, we did all further studies with a pool of Fab I and Fab II. To allow precise comparisons, we standardized the assay with a panel of 55 normal human sera, whose mean antigen content we defined as 1 arb. unit of laminin antigen per milliliter.

Assay and Molecular Size of Laminin in Serum

For serum samples from 165 adult healthy donors we found a median of 1.11 arb. units of laminin per milliliter, with respective 97.5 and 2.5 percentiles of 1.42 and 0.83 arb. units/mL (Figure 2). Only three values were slightly larger than the upper normal range of 1.42 arb. units/mL. When we tested 50 samples at different concentrations (range 20–80 µL/100 µL), we saw no significant deviation from the slope of the curve given by the standard. For routine tests, therefore, we used single duplicates of 50 or 100 µL with an estimated analysis error of ±6%. The interassay variation, tested for four sera in 17 assays during six months, was within the range 6–11%. The analytical recovery of laminin fragment P1 added to normal serum samples was within the range 104–110%.

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\begin{array}{ccccccccc}
\text{n} & 165 & 361 & 124 & 47 & 26 & 46 & 34 & 15 & 13 \\
\text{mean ± SD} & 1.11±.15 & 1.49±.49 & 1.49±.42 & 1.47±.37 & 1.33±.34 & 1.55±.35 & 1.61±.51 & 1.27±.58 & 1.44±.68 \\
\end{array}
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Fig. 2. Concentrations of laminin in serum of normal subjects and various tumor patients, as determined by RIA.

Number of sera (n) and mean values ± SD for each group are indicated at the top. The dashed horizontal lines indicate the normal range.
Discussion

We have described here a reliable and reproducible radioimmunoassay for measuring circulating forms of human laminin, which is known to be a relatively abundant basement membrane protein (32). The assay is based on laminin fragment P1, which originates from the central portion of this cruciform molecule and accounts for about a third of the molecular mass of the protein (7). This fragment is easily prepared from available tissue sources such as human placenta (18). The assay was further improved by use of monovalent antibody fragments instead of intact antibodies, but the slopes in the inhibition curves produced by the standard laminin antigen and the cross-reacting antigens present in serum samples were identical with either. The theoretical basis of this phenomenon is not completely understood (22), but it has also been observed in radioimmunoassay analyses of serum and urine for the procollagen III aminopropeptide (23). As a practical consequence, the assay for laminin as described here allows the reproducible quantification of the antigen by use of a single dilution of the serum samples.

With molecular-sieve chromatography of several serum samples we demonstrated at least two different size classes of the laminin antigen. One had an apparent molecular size comparable to that of fragment P1; the other was larger and could correspond to intact 900 kDa laminin or some oligomeric variant(s) (7). Different size classes have been also found for collagen IV antigens in rat serum (15) and may be attributed to intact and catabolic forms of the protein. The apparent heterogeneity of laminin in serum, together with the possibility that different forms of the antigen may vary in their affinity for antibodies, precluded our expressing the radioimmunoassay data in quantitative terms. We circumvented this problem by standardizing the assay against a restricted panel of normal human sera, which we defined to contain, on the average, 1 arb. unit of laminin antigen per milliliter. The subsequent analysis of a larger normal panel gave then a mean value of 1.11 arb. units/mL, which was within the range of variation of the assay. With this panel we determined the normal range (95% confidence range) to be 0.83–1.42 arb. units/mL.

To evaluate possible clinical applications of the laminin radioimmunoassay, we analyzed a large assortment of tumor patients and compared the data for them with data on the concentrations of the carcinoembryonic antigen in the serum. About half the patients showed values for serum laminin that exceeded the normal range, some values being two- to threefold the normal mean. Even though larger increases are more commonly observed for carcinoembryonic antigen (see Figure 4), 14% of the patients with normal values for carcinoembryonic antigen had above-normal values for laminin. Further studies by ourselves and others will be needed to evaluate the possible diagnostic and (or) prognostic value of the laminin assay in tumor patients. These may reveal whether an increased concentration of laminin in serum correlates with the stage of disease or the presence of metastases and whether one can use the assay in monitoring therapeutic treatments. It is also premature to speculate on the possible causes of the increase of laminin in serum as being increased basement membrane production or degradation by the tumor cells (4, 5). Other possible applications of the laminin radioimmunoassay are obvious and may include clinical studies of alcoholic liver diseases (20), and of diabetic microangiopathy and other vascular diseases.

We are grateful to Cornelia Steinert, Elke Bernhardt, and Manfred Quint for excellent technical assistance and to Claudia Müller for preparing this manuscript.