Development of Radioimmunoassay of Intact Laminin and Its Clinical Evaluation as a Tumor Marker

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The concentration of laminin including laminin variants in serum samples was measured by a double-antibody radioimmunoassay using intact laminin. The mean level in 92 normal subjects (47 men and 45 women) was defined as 1 unit (U)/ml, and the cut-off value (2 S.D. above the mean) was 1.37 U/ml. Mean laminin level in 391 patients with various malignancies was 1.50±0.86 U/ml. Laminin levels were elevated in various cancer patients, and in 45.0% (176/391) the values exceeded the cut-off level; in patients with cancer of the stomach or pancreas, positivity rate exceeded 60%. Mean laminin concentration for 130 pregnant women (2.06±0.65 U/ml) was also significantly higher than that of normal controls, but concentrations for patients with various benign diseases were within a low range (0.55±0.29—1.10±0.29 U/ml). In the stomach or pancreas cancer patients, a positive correlation between laminin level and tumor progression or course of the disease was observed. These findings indicate that serum laminin level is potentially useful in the diagnosis and monitoring of certain cancers.

Keywords intact laminin; radioimmunoassay; tumor marker; serum laminin concentration; cancer patient

Basement membrane is an extracellular matrix formed as a coassembly of its main components: collagen IV, fibronectin, laminin and heparan sulfate proteoglycan. It separates the epithelium from mesenchymal tissue, and its membrane proteins play an important role in cell adhesion, migration, differentiation and growth.

Laminin, a glycoprotein with a molecular weight of about 900 kilodaltons (kDa) and a major component of basement membrane, has been shown to be a multifunctional constituent.[1,2] Laminin is important in the adherence of collagen IV molecules to epithelial cells,[3-5] promotes the growth and differentiation of neurons,[6] and probably influences the metastatic capacity of tumor cells.[7] The structure of laminin has primarily been investigated using mouse Engelbreth-Holm-Swarm (EHS) tumor laminin. This is a cross-shaped molecule composed of A (440 kDa), B1 (225 kDa) and B2 (205 kDa) glycopeptide chains.[8,9] However, some structural differences between EHS laminin and laminins purified from several other cell lines or tissues are known.[10-12] Human placental laminin appears to contain a fourth component (called "M" chain). The M chain is detected between the A and B chains in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[13-15]; however, it is not clear whether the M chain is a degradation product of the A chain.[16] Moreover, recent investigations indicate that laminin is not a single distinct protein, but a family of several related proteins.[17-19]

Laminin immunoassay has recently been applied in the diagnosis of several diseases which are accompanied by alteration of basement membranes, *e.g.*, liver fibrosis and cirrhosis,[20] nephrotic syndrome,[21] diabetes mellitus,[22] and various cancers.[23-25] Serum levels of laminin in patients with various cancers are significantly elevated compared with healthy controls. In patients with carcinoma and leukemia, furthermore, it was reported that a high correlation could be found between serum laminin concentration and patient response to therapy or clinical course of the disease.[24] These results indicate that serum laminin level is a potentially useful parameter for the diagnosis of certain cancers and for monitoring the course of the disease.

The concentration of laminin in serum has been measured mainly using a double-antibody radioimmunoassay (RIA) for laminin P1 fragment (300 kDa) prepared by the limited pepsin-proteolysis of human placenta.[26] However, the alteration of laminin molecules in serum[23,27] and the isolation of some organ-specific laminin isoforms have been reported, and thus RIA for laminin P1 fragment is not sufficient to measure the concentrations of all the laminin variants in serum. In the present study we report the development of a new RIA system using intact laminin purified from human placenta and its application in pregnant women and numerous patients with various benign or malignant diseases.

Materials and Methods

Purification of Intact Laminin Intact laminin was purified from human placenta by the method of Dixit[14] with modifications. Homogenized placenta was washed with chilled saline and centrifuged at 2300×*g* for 20 min. The precipitate was suspended in 0.02 M Tris–HCl buffer, pH 7.4, containing 0.2 M NaCl, 0.01 M ethylenediaminetetraacetic acid (EDTA) and 3% Triton X-100, and the suspension was kept for extraction for 30 h at 4 °C. The insoluble tissue was removed by centrifugation at 2300×*g* for 20 min. The final concentration of NaCl in the supernatant fluid was brought to 4 M by addition of solid NaCl, and then the solution was centrifuged at 10200×*g* for 30 min. The pellet was dissolved in a minimal volume of 0.2 M NaCl, 0.02 M Tris–HCl, pH 7.4 (Tris buffered saline; TBS) and dialyzed against the same buffer. The insoluble material was centrifuged out and the pH of the clear supernatant fluid was adjusted to 4.5 by titrating with 4 N HCl. The precipitated material was centrifuged (17500×*g*, 40 min) and washed with 0.2 M NaCl, 0.02 M acetate buffer, pH 4.5. The pellet was redissolved in TBS. The supernatant obtained by centrifugation (17500×*g*, 30 min) was applied to a Sepharose 4B gel filtration column (2.5×100 cm), and then eluted with TBS. The eluate containing laminin was concentrated, and the gel filtration was repeated again.

An immunodiffusion test for rabbit antiserum to human laminin (provided by Dr. E. Ruoslahti, La Jolla Cancer Research Center) was used to detect laminin in the fractions. The protein concentration was measured using the method of Lowry *et al.*[28] with bovine serum albumin as the standard. SDS–PAGE was performed according to Laemmli.[29]

Preparation of Antibody against Intact Laminin The purified intact laminin (100 μg/ml), emulsified with an equal volume of Freund's complete adjuvant, was injected intramuscularly into young male rabbits. Five injections were given at 2-week intervals. Two weeks after the last injection, antisera were collected and used for radioimmunoassay.

RIA Technique RIA was carried out by the double antibody method. The labeled laminin was prepared with ^{125}I by the Bolton–Hunter method.[30] Sample serum or standard laminin solution (0.1 ml), rabbit antiserum to intact laminin (diluted 1 : 20000, 0.2 ml), and labeled laminin

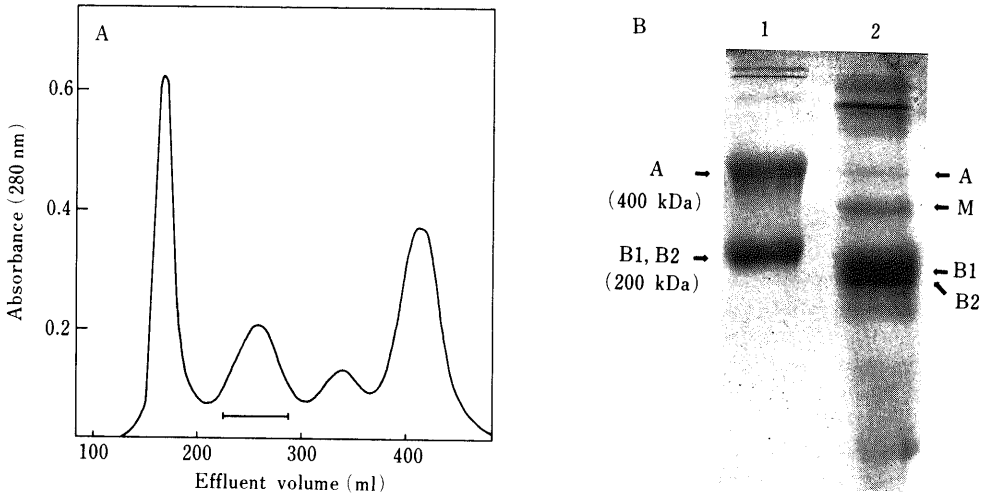

Fig. 1. Purification of Intact Laminin on a Sepharose 4B Gel Filtration Column (A) and SDS-PAGE of Purified Laminin (B)

(A) Homogenized placenta was suspended in 0.02 M Tris-HCl buffer pH 7.4, containing 0.2 M NaCl, 0.01 M EDTA and 3% Triton X-100. The extract was fractionated with solid NaCl, and then the materials precipitated at pH 4.5 were applied to a Sepharose 4B column (2.5 × 100 cm). The laminin-containing fractions (horizontal bar) were concentrated and the gel filtration was repeated once. (B) The final preparation was reduced with 2% mercaptoethanol and analyzed on 0.1% SDS-5% polyacrylamide gel, and separated proteins were stained with Coomassie brilliant blue (lane 2). In lane 1, the position of A and B chains of mouse EHS laminin (Iwaki Glass Co., Ltd.) is shown as the standard.

(about 12000 cpm, 0.1 ml) were mixed and incubated for 20 h at room temperature. Then, goat antiserum to rabbit immunoglobulin G (IgG) (diluted 1 : 50, 1.0 ml) was added to this mixture and incubated for 15 min at room temperature. After centrifugation (16000 × *g*, 15 min), the radioactivity of this precipitate was counted. Samples of intact laminin of 9 different known concentrations (15.6—4000 ng/ml) were used as standards.

Serum Sources Normal sera were collected from 92 healthy blood donors (47 men and 45 women; mean age, 30.2 years; range, 20 to 58 years). Serum samples were also collected from 130 women during the course of uncomplicated pregnancy (weeks of gestation, 8 to 40) and from 120 patients with various benign diseases. Samples were obtained from 391 patients with the following malignancies: cancer of the uterus (90), stomach (73), pancreas (54), colon (54), ovary (52), lung (17), liver (16), gallbladder (15), and bladder (11); and leukemia (9). All samples were stored at −20 °C until use.

Results

Development of RIA System and Laminin in Normal Controls Laminin was purified from human placenta as described under Materials and Methods. The elution profile on a Sepharose 4B gel filtration column is presented in Fig. 1A. The final preparation was analyzed on 5% polyacrylamide gel. Four laminin subunits, corresponding to A, M, B1 and B2 chains, were detected as described[14,16] (Fig. 1B). In double immunodiffusion test, the precipitation line formed by our laminin and the rabbit antiserum provided by Dr. Ruoslahti, completely fused to the line formed by our antiserum (data not shown). These results indicated that the purified laminin was the intact form.

The standard inhibition curve for intact laminin immunoassay is shown in Fig. 2. To evaluate the reproducibility of this assay system, the laminin concentration in normal human serum was measured. Serum samples from 71 normal healthy donors were assayed four times in the same assay run, and intra-assay variation was estimated to be 8.2%. Inter-assay variation was 5.9% for seven test runs using sera from 25 normal controls.

Because of the reported heterogeneity of the laminin antigen in serum, in this study serum laminin levels were expressed in arbitrary units (U)/ml with the mean value for 92 normal human serum samples being defined as 1 U/ml

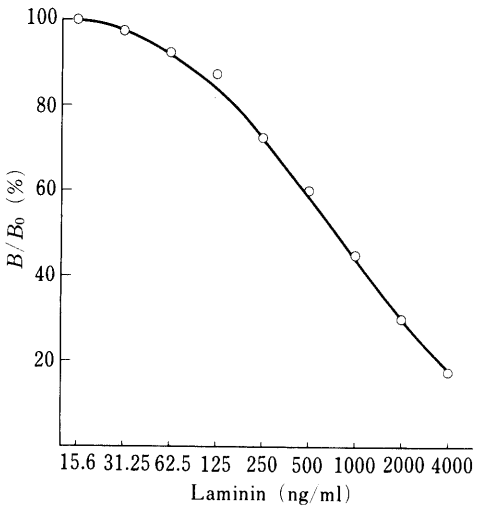

Fig. 2. A Typical Standard Inhibition Curve for Intact Laminin Immunoassay

The scale of laminin (ng/ml) indicates the concentration of standard solutions (15.6—4000 ng/ml). The detailed immunoassay technique is described in the text.

(656 ng/ml, $B/B_0 = 48$—55% in seven assay runs). The standard deviation (S.D.) was 0.18 U/ml, and the cut-off value (mean + 2S.D.) was 1.37 U/ml. Laminin-positive sera, *i.e.*, exceeding the cut-off level, constituted only 2.2% (2/92) of normal control serum samples (Fig. 3). Laminin levels of normal controls were not correlated with the age of the donor, and no significant difference was found between men and women (data not shown).

Laminin Levels in Sera of Pregnant Women and Patients with Various Benign Diseases Mean serum laminin level for 130 samples from pregnant women (8—40 weeks of pregnancy) was significantly higher than that of normal controls, and the positivity rate was 90.0% (117/130). As shown in Fig. 3, there was a remarkable rise in concentration beginning at the early stage (8th week of pregnancy; 1.80 ± 0.44 U/ml, $n = 12$). The concentration increased during the course of pregnancy and reached more than 3 U/ml in the 36th week (3.32 ± 1.22 U/ml, $n = 6$).

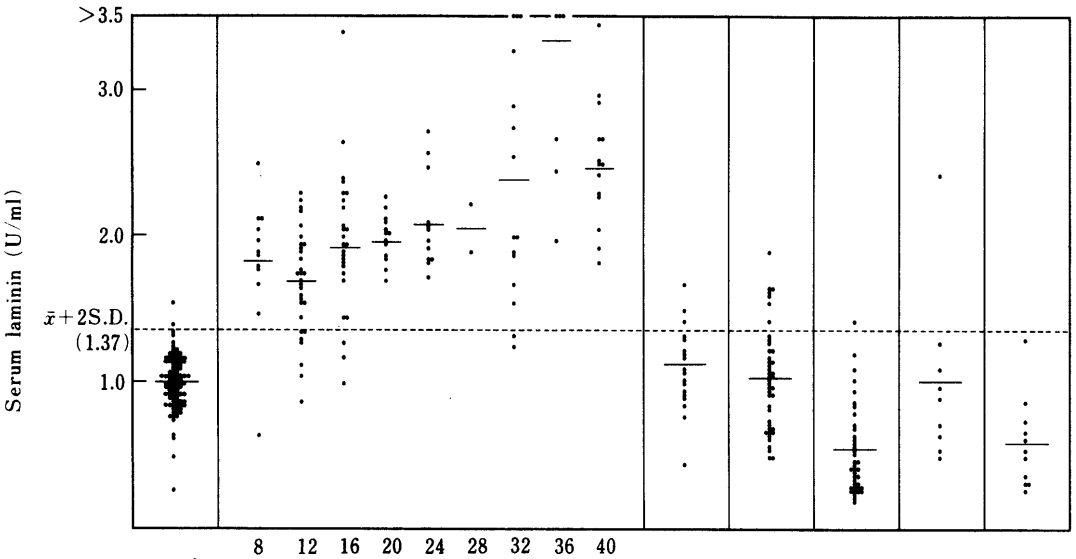

Fig. 3. Concentrations of Laminin in Serum of Normal Controls, Pregnant Women and Patients with Various Benign Diseases

The dashed line indicates the cut-off level (2 S.D. above the mean, *i.e.*, 1.37 U/ml), and the short line gives the median value of the individual group. Abbreviations: *n*, number of serum samples; PR, positivity rate (samples exceeding cut-off level/total samples); EM, endometriosis; HEPA, hepatitis; HYST, hysteromyoma; PANC, pancreatitis; OC, ovarian cystoma.

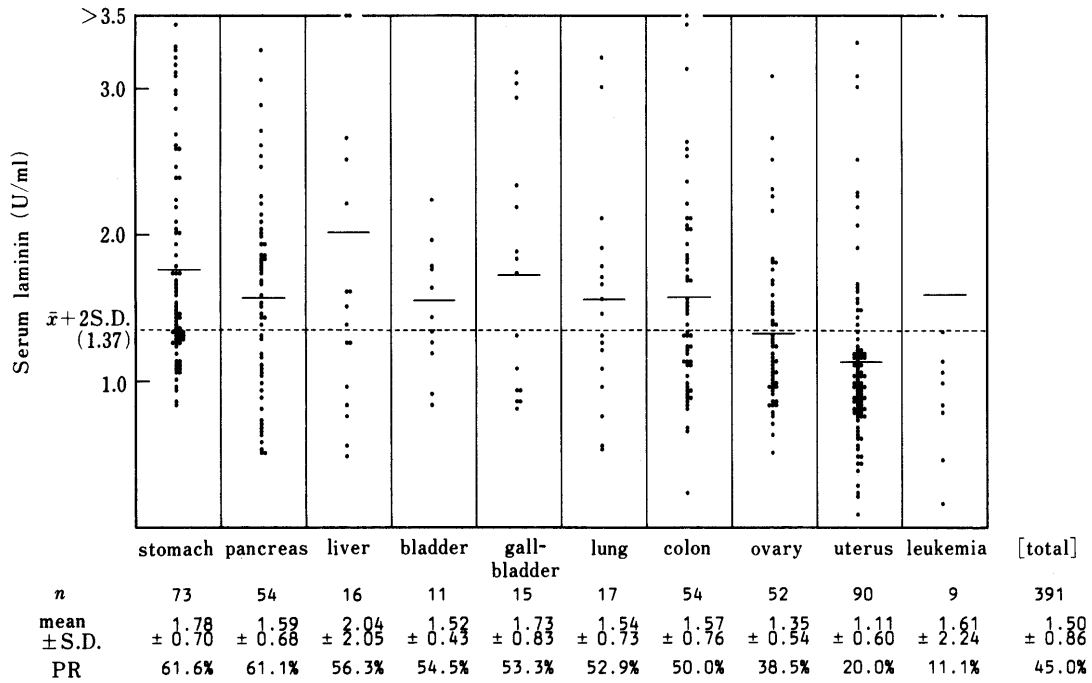

Fig. 4. Concentrations of Laminin in Serum of Patients with Various Cancers

Total, all cancer patients.

Serum laminin concentrations of patients with various benign diseases are also shown in Fig. 3. The laminin levels of this group lay in the low range, 0.55±0.29—1.10±0.29 U/ml. The positivity rates for patients with hysteromyoma (2.6%), pancreatitis (1.1%) and ovarian cystoma (0%) were within the normal range, but those of patients with endometriosis (16.7%) and hepatitis (16.3%) were somewhat high.

Serum Laminin Level of Cancer Patients Concentrations of laminin in sera of 391 patients with various malignancies are shown in Fig. 4. A significant increase was observed (overall serum laminin level, 1.50±0.86 U/ml; overall positivity rate, 45.0%). Laminin concentrations were elevated in various cancer patients, especially in those with cancers of the digestive system, *e.g.*, the liver (2.04±2.05 U/ml) and stomach (1.78±0.70 U/ml).

The relationship between changes in laminin levels and course of the disease was investigated in patients with stomach or pancreas cancer because the sample number was

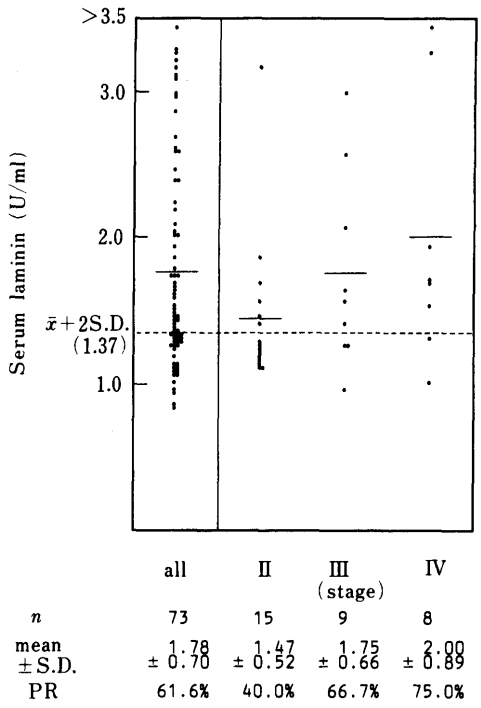

Fig. 5. Correlation between Serum Laminin Level and Tumor Progression in Stomach Cancer Patients

Some stomach cancer patients were classified into three groups (stages II—IV). All, all stomach cancer patients.

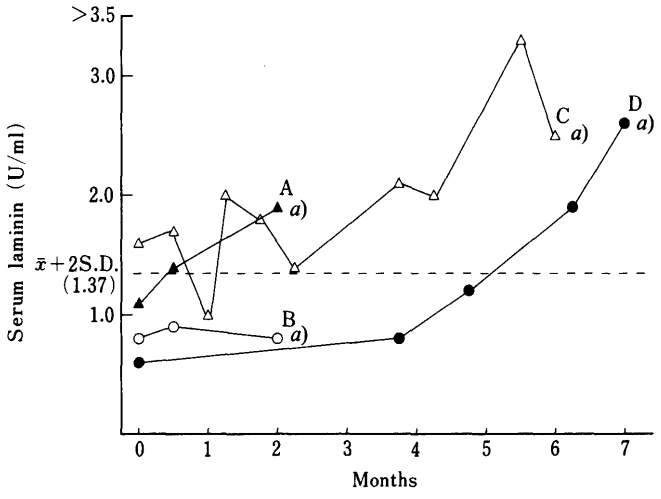

Fig. 6. Follow-up Laminin Levels in Four Patients (A—D) with Pancreatic Cancer

a) Death of the patient.

large and positivity rate was high. Some of the patients with stomach cancer were classified into three groups on the basis of stage of the disease, and laminin concentration at each stage was measured. As shown in Fig. 5, there was a positive correlation between laminin level and tumor progression (1.47 ± 0.52 U/ml at stage II; 1.75 ± 0.66 U/ml at stage III, and 2.00 ± 0.89 U/ml at stage IV).

Long-term follow-up was performed in four patients with pancreatic cancer (Fig. 6). In these patients as well, there was a positive correlation between change in laminin level and the course of the disease.

Discussion

We have described the development of an immunoassay system using intact laminin, and its application in human subjects, including normal controls and patients with various diseases. Significantly increased serum laminin concentration was observed in pregnant women and patients with certain cancers, especially those of the digestive system.

The increased amount and degradation of placental basement membrane seems to be the main reason for the rise in serum laminin level in pregnant women; however, various hypotheses could account for the increased levels in cancer patients. (i) Laminin molecules are synthesized abundantly by the tumor cells. In fact, mouse EHS tumor, which has been used for many studies of laminin, produces abundant amounts of basement membrane components. (ii) The amount of laminin molecules in serum increases with the degradation of basement membranes by tumor cells. (iii) Laminin level rises with the decreased incorporation of laminin molecules into the extracellular matrix. We believe the increased laminin levels in serum of cancer patients to result mainly from causes (ii) and (iii). Thus, factors affecting the serum laminin levels of cancer patients appear to be the absolute quantity of basement membrane contained in the organ or tissue affected by tumor cells, the extent of infiltration into the basement membranes, and the extent of the influx of laminin molecules into blood. Basement membranes are found throughout the body, especially under the epithelial cells of the skin and digestive tract. This may be one reason for their high level in patients with cancer of the digestive system.

TABLE I. Elevated Levels of Serum Laminin in Various Cancer Patients

Cancer	Positivity rate[a] (%)	
	Present work[b] (Intact laminin)	Brocks *et al.*[c] (Laminin P1 fragment)
Stomach	61.6 (45/73)	61.8 (21/34)
Bladder	54.5 (6/11)	61.5 (8/13)
Lung	52.9 (9/17)	48.9 (23/47)
Colon	50.0 (27/54)	67.4 (31/46)

a) Samples exceeding cut-off level/total samples. *b*) RIA of intact laminin. Cut-off level was 1.37 U/ml (2 S.D. above the mean for 92 normal controls). *c*) RIA of laminin P1 fragment. Cut-off level was 1.42 U/ml (97.5 percentile of the concentrations in 165 normal controls).[23]

Table I shows our data on intact laminin and other data of the laminin P1 fragment.[23] Considerable agreement was found in the positivity rate for each cancer (stomach, bladder, lung, and colon). However, as we have stated, heterogeneity of laminin molecules in serum and organ-specific isoforms has been reported. Therefore, increased specificity for particular cancers could be expected if not only the antibody against the intact form but the antibodies specific for individual chains (A, B1, B2 and M) or the monoclonal antibodies are used as the combination assay.

It has been noted that laminin probably influences the metastatic capacity of tumor cells,[7] and we therefore compared serum laminin concentrations of patients with and without metastases. The results expected were not obtained, perhaps owing to the limited number of patients; however, laminin concentration in the serum of pancreas or uterine cancer patients with metastases was significantly higher than that of patients without metastases (data not shown). Further work using a larger number of patients is

needed.

It has been reported that the correlation between laminin and carcinoembryonic antigen is poor,[23] and our recent studies suggest that the correlation of laminin with other tumor markers (CA 19-9, CA 125 and AFP) is also poor (data not shown). More extensive investigation of laminin assay and an combined assay systems with other tumor markers may yield an increased positivity rate.

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