Two Sensitive Time-Resolved Fluoroimmunoassays for Cellular Fibronectin

Jürgen Kropf and Axel M. Gressner

Two sensitive sandwich-type immunoassays for determination of cellular fibronectin (cFN) in cell culture supernatants and in human plasma were developed. Both assays used a monoclonal antibody with specificity against the EDA sequence, which is characteristic for the cellular form of human and rat FN. Assay 1 involves binding of FN on gelatin-coated microwells followed by reaction with the anti-cFN antibody, whereas in assay 2 the anti-cFN antibody was immobilized first and detection was with an anti-FN antisera. Time-resolved fluorescence spectrophotometry with measurement of an Eu³⁺ chelator after dissociation of the solid-phase complexes with urea/sodium dodecyl sulfate in the presence of excess Eu³⁺ was the detection method. The detection limit of the new assays was between 2.6 and 4.0 μg/L cFN. In serial dilution of human plasma samples, parallelism with the calibration curve was obtained over the whole measuring range (12-1000 μg/L) with assay 2, whereas assay 1 had deviations of the dilution curves at concentrations ≥100 μg/L. The between-run CVs for assays 1 and 2 were 11.4% and 7.2%, respectively, at a concentration of 200 μg/L (median value of 18 experiments). Respective within-series CVs of 4.3% and 4.7% were obtained at the same concentration. The recovery of added cFN from human plasma was between 90% and 96%.

Indexing Terms: rat fibronectin/monoclonal antibodies/europium chelators

Fibronectins (FN) are high-molecular-mass (Mr ~440 000) structural glycoproteins composed of two nearly identical subunits (1).² The plasma form of FN (pFN) is distinguished from the so-called cellular fibronectins (cFN), produced locally by various other cell types, by its lack of the specific extra domain sequences EDA, EDB, and V, which originate by differential splicing of FN precursor mRNA from a single gene (2).

Because FN occurs in relatively high concentrations in blood (~0.3 g/L), several analytical methods are suitable for its determination in plasma. In general, these methods, like most of the more sensitive immunoassays designed for determinations in other materials, measure the total FN (tFN) concentration, i.e., the sum of pFN and cFN. In many circumstances the selective determination of cFN in the presence of various amounts of pFN seems potentially more useful. Because in blood cFN constitutes only a minor fraction of tFN, determination of tFN mainly reflects hepatocyte function or alteration of FN consumption. Generally, the diagnostic usefulness of determinations of circulating tFN, especially as a tumor marker, seems only moderate (3, 4). The determination of cFN in other body fluids, such as ascites (5), amniotic fluid (6), bronchoalveolar fluid (7), urine (8), or bile (9), seems more promising, if we assume that cFN is the main isomer in most body fluids except blood. The same argument holds true for cell culture experiments, in which cFN secreted into the medium can be used for the assessment of cell stimulation and (or) transformation (10).

Here we describe two highly sensitive immunoassays for determination of cFN, using time-resolved fluorescence spectrophotometry with measurement of the Eu³⁺ chelator 4,7-bis(chlorosulfopheny1)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) after dissociation of the solid-phase complexes with urea/sodium dodecyl sulfate (SDS) in the presence of excess Eu³⁺ (11). Although we previously (11) exploited the binding of FN to gelatin-coated surfaces for measurement of tFN, the specificity of the new assays for cFN is achieved by using the DH1 monoclonal antibody. This antibody is directed against the EDA sequence, which is specific for the cellular form of human and rat FN. Assay 1 is based on the binding of FN on solid-phase coated gelatin followed by the specific detection of cFN with the DH1 antibody. In assay 2 the DH1 antibody is used first and a polyvalent anti-FN antisera is used in the second step. The biotin/streptavidin (SA) system, using SA labeled with BCPDA, is used for convenient detection.

Materials and Methods

Materials

Synthesis of BCPDA was performed as described (12). SA was labeled with BCPDA and separated from remaining BCPDA as described previously (11).

Rat cFN and human pFN were purified from culture supernates of rat fibroblasts grown in FN-free medium and from human plasma, respectively, by gelatin affinity chromatography on Gelatin-Sepharose 4B (Pharmacia, Uppsala, Sweden), according to Ruoslahti et al. (13). The purity of the preparations was assessed by silver staining after SDS-polyacrylamide gel electrophoresis.

Plasma for preparation of human pFN was obtained...
from fresh samples of healthy blood donors. Potassium EDTA was used as anticoagulant for these samples. Specimen of patients submitted for routine analysis at our laboratory that were not analyzed immediately were stored at −80 °C until assay.

The monoclonal anti-cFN antibody DH1 (14) was obtained from ICN (Meckenheim, Germany) or Locus Genex (Helsinki, Finland). Polyclonal anti-human FN antiserum, raised in rabbit, was from Behring (Marburg, Germany). This antiserum was used in our previous report (11) for determination of tFN. Anti-mouse IgG antibody (Fₐₐₜ specific), biotinylated anti-mouse IgG antibody, biotinylated anti-rabbit IgG antibody, and SA were obtained from Sigma (Deisenhofen, Germany).

**Assay Procedures**

To coat the microwells, we used a solution of 0.05 mol/L NaHCO₃, pH 9.1. The washing solution contained 9 g/L NaCl, 0.5 g/L Tween 20, and 0.5 g/L NaN₃. The assay buffer was prepared by dissolving 5 g of bovine serum albumin, 0.05 g of NaN₃, and 0.05 mol of Tris in 1 L of H₂O, and adjusting the pH to 7.7.

The volume used for calibrators, samples, antibodies, and SA-BCPDA was 100 μL/well, whereas the urea/SDS/Eu³⁺ solution was used at 250 μL/well.

**Assay 1.** Microtiter strips (flat-bottom, 12 × 1 wells, MaxiSorp Immuno modules; Nunc, Wiesbaden-Biebrich, Germany) were coated overnight at 4 °C with 10 mg/L gelatin in coating solution. After washing, the wells were filled for blocking with assay buffer and stored at 4 °C until assay. Calibrators and samples were diluted in an assay buffer and incubated for 1 h. Anti-cFN antibody was added at 100 μg/L for 1 h followed by 30-min incubations with biotinylated anti-mouse IgG antibody, diluted 1:1000, and SA-BCPDA, at 1 mg/L, respectively.

**Assay 2.** Microwells precoated with anti-mouse IgG antibody, Fₐₐₜ specific, were incubated with anti-cFN antibody at 100 μg/L in assay buffer for 2 h. After washing, the wells were blocked with assay buffer and stored at 4 °C until assay. Calibrators and samples were diluted in assay buffer and incubated for 1 h. Anti-FN antiserum diluted 1:200 (a dilution of 1:1000 is possible with only minor loss of sensitivity) was added for 1 h, followed by incubations of biotinylated anti-rabbit IgG antibody, diluted 1:2000, and SA-BCPDA, at 1 mg/L, each for 30 min.

For both assays, the wells were washed three times between each incubation. Fluorescence was finally measured with the Arcus 1230 time-resolved fluorometer (LKB Wallac, Turku, Finland) after dissociation of the solid-phase complexes in a solution of 4 mol of urea, 10 g/L SDS, and 10⁻⁷ mol of Eu³⁺ for 30 min. Duplicate measurements of calibrators and samples were applied in all assays. Microwells precoated with gelatin or anti-mouse IgG antibody, filled with assay buffer, and sealed with an adhesive plastic sheet could be stored for several weeks at 4 °C without noticeable loss of analytical performance.

tFN was determined analogously to assay 2, but gelatin was used for coating instead of the DH1 monoclonal antibody. This assay was identical to that described in ref. 11.

**Calibrators**

Aliquots of a human pFN preparation obtained by affinity chromatography were dialyzed extensively against 0.15 mol/L NaCl plus 0.05 mol/L Tris, pH 7.5, and lyophilized. The mass of the aliquots was determined gravimetrically after subtraction of the mass of an equal volume of lyophilized dialysis buffer. Remaining aliquots of the same preparation were then used for calibration of the protein assay. Because by affinity chromatography essentially pure cFN is obtained, measurement of total protein could be used for subsequent calibration of both cFN assays. Aliquots of the cFN stock solution were diluted in assay buffer and stored at −80 °C until assay.

**Results**

**Assay Optimizations**

For optimization of the assays the concentrations of the primary and secondary antibodies were varied systematically. Taking low detection limits at practicable turnaround times and acceptable costs in terms of antibody consumption as main criteria, we selected the procedures outlined under Materials and Methods. The concentrations and incubation times used for the biotinylated anti-rabbit and anti-mouse antibodies, as well as for the subsequent steps, had already been optimized in previous investigations and were applied as described (11, 15).

In assay 2, direct coating of microwells with the anti-cFN antibody was less satisfactory than the procedure suggested above, involving wells precoated with anti-mouse IgG antibody. Probably because of competition of albumin, which is present in the anti-cFN antibody preparation at 10 g/L, only relatively low signal intensities could be obtained. These could be improved by purification of the delivered antibody by Protein G affinity chromatography, but the overall losses of antibody during purification were such that we chose to selectively capture the anti-cFN antibody by introducing an additional coating step with anti-mouse IgG antibody.

The calibration curves for both assays involving concentrations between 12 and 1000 μg/L are shown in Fig. 1. An even higher upper measuring range could be obtained by using an additional calibrator of 3000 μg/L. No high-dose hook effects were observed, even with a large antigen excess (cFN >20 000 μg/L).

Unspecific binding of pFN to the surface of the microwells, which could occur in assay 2, was tested by omitting the primary (anti-cFN) antibody, which practically suppressed any signal above the assay blank in plasma samples. Because preparations of pure, cFN-free pFN are not available, lack of specificity could not be tested directly by addition of pFN to the samples.
Fig. 1. Calibration curves for cFN immunoassays and CVs at the calibrator concentrations for assays 1 (A) and 2 (B).

Median values of 18 assays.

Precision and Detection Limit

The median CVs of 18 assays performed on different days are shown at the concentrations of the calibrators within Fig. 1. The between-series CVs at 200 μg/L (n = 18) were 11.4% and 7.2% for assays 1 and 2, respectively. At the same concentration, within-series CVs of 4.3% and 4.7%, respectively, were obtained. Additional between-series and within-series CVs at a concentration of 80 μg/L are shown in Table 1.

The detection limit was computed as the concentra-

tion corresponding to the signal of the blanks plus 3 SD of the blank signal. The median values of the detection limits of 18 runs were 2.6 μg/L for assay 1 and 4.0 μg/L for assay 2.

Linearity

Human plasma samples were first prediluted 1:10 in assay buffer to meet the measuring range of the assays. By serial 1 + 2 dilution of the prediluted plasma samples, the results shown in Fig. 2 were obtained. Whereas in assay 1 a systematic deviation from the calibration curve occurs at higher concentrations, with assay 2 essentially parallel dilution curves of the same plasma samples were obtained over the whole calibration range.

By applying the same serial dilution to fetal calf serum but starting with undiluted samples, similar results were obtained as with the plasma samples, i.e., good agreement between the shapes of the calibration and dilution curves with assay 2, whereas deviations from parallelism were found at higher concentrations with assay 1.

Recovery

For assay 2 the recovery of cFN, tested by addition of various amounts of pure cFN into different human plasma samples, was between 90% and 96% of the

---

**Table 1. Analytical criteria for cFN immunoassays.**

<table>
<thead>
<tr>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Practical assay range, μg/L</td>
<td>12-1000</td>
</tr>
<tr>
<td>Detection limit, μg/L</td>
<td>2.6</td>
</tr>
<tr>
<td>CV, %</td>
<td></td>
</tr>
<tr>
<td>Between-run (200 μg/L, n = 18)</td>
<td>11.4</td>
</tr>
<tr>
<td>Between-run (80 μg/L, n = 10)</td>
<td>8.9</td>
</tr>
<tr>
<td>Within-series (200 μg/L)</td>
<td>4.3</td>
</tr>
<tr>
<td>Within-series (80 μg/L)</td>
<td>5.8</td>
</tr>
<tr>
<td>Recovery from human plasma, %</td>
<td>NC</td>
</tr>
<tr>
<td>Recovery from fetal calf serum, %</td>
<td>NC</td>
</tr>
<tr>
<td>Total incubation time, h</td>
<td>3.5</td>
</tr>
<tr>
<td>Sample volume, μL</td>
<td>100</td>
</tr>
</tbody>
</table>

(1: gelatin first, then anti-cFN antibody; 2: anti-cFN antibody first, then anti-FN antiserum.)

NC: Not computed because of nonparallelism of plasma dilutions (see text).
theoretical value. In fetal calf serum, diluted 1:5 in assay buffer, the recovery of different amounts of added cFN was between 90% and 105%. Because of the nonparallelism observed with plasma samples, recovery studies were not performed with assay 1.

**Human Plasma Samples**

Specimens anticoagulated with EDTA were used generally, although citrated plasma was used as well. Contrary to Ylätupa et al. (16), we did not observe any unsystematic variation with citrated plasma in our assays. Because unpredictable losses of FN occur during clotting, serum samples are not suited for the determination of FN.

There was no significant correlation between the concentrations of cFN and tFN determined simultaneously for the same samples ($n = 33$) obtained randomly from patients’ specimens submitted for routine analysis within our laboratory.

**Discussion**

Because of their high sensitivity, immunoassays based on time-resolved measurement of Eu fluorescence have recently received attention in clinical biochemistry (17, 18). Two main methodologies exist for time-resolved fluorescence measurements. The DELFIA method involves labeling of the immunoreactants with Eu$^{3+}$, which is liberated before measurement by an enhancement solution containing specific β-diketones that form intensely fluorescent complexes with the europium ion. The other method, which has been popularized by Diamandis (18), involves the phenanthroline derivative BCPDA for labeling. After complexation with excess Eu$^{3+}$, the solution is evaporated and fluorescence is measured by laser excitation of the solid-state complexes. In our method, BCPDA is used for labeling in analogy to the Diamandis method, but fluorescence is measured in solution in the presence of excess Eu$^{3+}$ with the DELFIA fluorometer, thus avoiding possible interference by Eu$^{3+}$ contamination. We used urea/SDS in the measurement solution for dissociating the solid-phase immunocomplexes. Additionally, the fluorescence signal is amplified by using high concentrations of urea, presumably by competition of urea with water molecules, which seem to have some quenching effect on the europium fluorescence. Recently a similar method that includes a dissociation solution containing detergents for measurement of BCPDA complexes in solution has been published (19).

Despite considerable theoretical and practical interest in the specific measurement of FN isoforms, few assays for determination of cFN have been reported until now (7, 16). By using time-resolved fluorescence as the detection method, we have developed two sensitive immunoassays for quantification of cFN. Most analytical criteria of the new assays compare well with those reported by Ylätupa et al. (16), who described a competitive enzyme immunoassay involving microtiter plates coated with purified cFN. The new assays are superior, however, with regard to sensitivity (2.6 vs 100 μg/L) and dynamic measuring range.

The generally high interspecies cross-reactivity found with most anti-FN antibodies (1) is also seen for the DH1 monoclonal antibody, as was demonstrated by application of the assays on specimens from different species, i.e., bovine, rat, and human samples. The cross-reactivity with bovine cFN has to be taken into account if medium enriched with fetal calf serum is used in cell culture experiments. Given that cell culture supernates are diluted 1:5 before assay, concentrations of >20 mL/L fetal calf serum will significantly contribute to the overall results.

An additional capturing step for the cFN antibody was introduced in assay 2. By precoating the micro-wells with anti-mouse IgG antibody, the purification of the DH1 preparation and its associated losses of antibody could be avoided. A further advantage of the antibody capturing technique might be the attachment of the antibody with its $F_\text{c}$ region at the solid phase, thus directing its antigen binding site into the solution and diminishing steric hindrance of antigen binding. However, a somewhat higher background signal was observed in assay 2 than in assay 1, which could not be removed by absorption with mouse IgG or addition of detergents to the assay buffer.

In view of the good agreement between the shapes of the calibration curves, assay 2 is especially suited for measurements of samples containing human plasma. The deviation from the calibration curve found for more concentrated plasma samples with assay 1 might be caused by a saturation of the gelatin layer by pFN, which occurs in large excess in relation to cFN in plasma. Under these conditions, low- and medium-strength associations between FN molecules/aggregates and gelatin that might bind at the solid phase during sample incubation might get washed out during subsequent steps, resulting in lower signal intensities.

One can hypothesize that both assays will recognize different fragments of cFN. Assay 1 should be most sensitive for intact cFN because both the gelatin binding site, which is located near the amino-terminal end of the molecule, and the EDA sequence, which is located about two-thirds of the way towards the carboxyl-terminal end, have to be present simultaneously. For assay 2, however, even fragments of the whole molecule that embrace little more than the EDA sequence should, in principle, be detectable if one assumes that the polyvalent anti-FN antiserum used in the sandwich assay will have some binding capacity for epitopes in the EDA sequence.

The diagnostic usefulness of determination of cFN in blood and in other body fluids and secretions is currently being investigated for different diseases in ongoing studies.

We appreciate the excellent technical assistance of Mathias Kothe and Michael Otto. This study was supported by Stiftung P. E. Kempkes, Marburg, Germany.
References