Quantification of tissue fibronectin from terminal villi of placenta

Bonnie Anderson BRAY

Department of Medicine, Columbia University, College of Physicians and Surgeons, 630 West 168th Street,
New York, NY 10032, U.S.A.

(Received 3 October 1984/Accepted 14 November 1984)

Tissue fibronectin (TFn) was solubilized from the terminal villi of perfused human placentas by sequential chemical extractions and plasmin digestion. Alternatively, plasmin digestion of intact tissue solubilized all the TFn, which amounted to 1.8–2.9% of the dry weight of the villi. Concomitantly, 69% of the tissue was solubilized. The non-equilibrium competitive e.l.i.s.a. (enzyme-linked immunoabsorbent assay), in which the TFn was immunologically identical with plasma fibronectin (PFn), was used for the quantification of TFn. This study demonstrates that the bulk of TFn can be obtained in a form that can be quantified by e.l.i.s.a. and that TFn is immunologically identical with PFn. Thus the fibronectin molecule is not significantly altered as it is incorporated into the connective-tissue matrix and could exchange with PFn.

Variants of fibronectin (Fn), a high- M_r glycoprotein, occur in plasma, on cell surfaces and in connective tissues in various organs [see Yamada (1983) and Pearlstein *et al.* (1980) for reviews]. Cellular Fn and plasma Fn (PFn) are similar but not identical (Atherton & Hynes, 1981; Yamada & Kennedy, 1979). The Fn that is present in connective tissue, tissue Fn (TFn), is obviously similar to both PFn and cellular Fn, since antisera prepared against Fn from plasma (Stenman & Vaheri, 1978) and from cell cultures (Mayer *et al.*, 1981) have been used for its detection in tissues by immunofluorescence techniques.

Since PFn may become incorporated into the extracellular matrix and supporting structures (Hayman & Ruoslahti, 1979; Oh *et al.*, 1981; Deno *et al.*, 1983), TFn may be a mixture of cellular and PFn. This possibility raises the question as to whether TFn can be mobilized to provide a source of PFn in situations in which PFn becomes depleted or bound to particles that are being cleared by the reticuloendothelial system (Saba & Jaffe, 1980; Deno *et al.*, 1984).

In this laboratory we have quantified Fn in connective-tissue fractions from human lung and placenta (Bray, 1978*a,b*) and have shown that heparin facilitates the extraction of TFn (Bray *et al.*, 1981). In the present study methods are

detailed for the solubilization of 69% of placenta and presumably all the TFn. The methods, sequential chemical extractions followed by plasmin digestion, were chosen so that all the solubilized TFn could be quantified by e.l.i.s.a. Therefore, use of these procedures will allow one to approach questions concerning the two pools of Fn, TFn and PFn.

Experimental

Tissue

Human placentas, obtained immediately after full-term normal births, were immediately perfused free of blood and then perfused with a mixture of proteinase inhibitors, as in our previous studies (Bray *et al.*, 1981). The use of human placentas was approved by the Institutional Committee on Human Investigation.

Solubilization of tissue and TFn

The sequential extractions and plasmin digestions are detailed in the legend to Table 1. To determine the amount of tissue that could be solubilized by plasmin digestion, larger samples (5mg) were digested in 0.5ml of phosphate-buffered saline (0.14M-NaCl/6.7mM-sodium phosphate buffer, pH7.4) containing 1.25 units of plasmin. After the third digestion the residues were washed with water to remove salt and then freeze-dried.

Abbreviations used: Fn, fibronectin; PFn, plasma fibronectin; TFn, tissue fibronectin; e.l.i.s.a., enzyme-linked immunosorbent assay.

E.l.i.s.a. for quantification of TFN

Fibronectin in all supernatants was quantified by the non-equilibrium competitive e.l.i.s.a. technique (Rennard *et al.*, 1980; Vuento *et al.*, 1981) in flat-bottomed wells of a polystyrene micro-titre plate (Costar tissue-culture clusters; Costar, Cambridge, MA, U.S.A.). The γ -globulin fraction from a monospecific polyclonal rabbit antiserum to human PFN was the primary antibody. Anti-Fn IgG bound to wells coated with PFN was detected with alkaline-phosphatase-conjugated goat anti-rabbit IgG and its substrate, disodium *p*-nitrophenyl phosphate, both of which were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

To assess the effect of urea and heparin on the e.l.i.s.a., PFN was diluted to a concentration of 200 $\mu\text{g/ml}$ in phosphate-buffered saline containing heparin (5mg/ml) and urea (2M) to approximate the concentrations of Fn, heparin and urea in tissue extracts. This mixture, as well as PFN and a tissue extract, were diluted serially and carried through the e.l.i.s.a. PFN treated with dithiothreitol was compared with untreated PFN. To 0.5ml of PFN (100 $\mu\text{g/ml}$ in 0.05M-phosphate buffer, pH 7.1, containing 2mM-phenylmethanesulphonyl fluoride) were added 60mg of urea and 4mg of dithiothreitol. The sample was flushed with N_2 and left at room temperature for 4h. This treated sample and untreated PFN (100 $\mu\text{g/ml}$) were diluted serially and carried through the e.l.i.s.a. procedure as was a urea/dithiothreitol extract of tissue. Likewise, plasmin-digested PFN was compared with untreated PFN. To 350 μg of PFN in 50 μl of buffer was added 50 μl of plasmin (0.6 unit) in phosphate-buffered saline. The mixture was incubated for 4h at 37°C. As a control, 350 μg of PFN in 50 μl of buffer was mixed with 50 μl of phosphate-buffered saline, and this control mixture was kept at 4°C. The control, plasmin-digested PFN and a plasmin digest of villi were diluted serially and carried through the e.l.i.s.a. procedure.

Results

Solubilization of tissue

In two experiments in which 2g wet wt. of placental villi was extracted three times with heparin/urea followed by an extraction with dithiothreitol to disrupt disulphide bonds, the weights of the freeze-dried insoluble residues were 84 and 81 mg. The dry weight obtained by washing and freeze-drying intact placental villi was 116mg. Therefore the chemical extractions solubilized 29% of the tissue by weight.

Exhaustive digestion with plasmin solubilized 56% of the extracted residue. Since this extraction

residue represented 71% of the intact villi, this amounted to solubilizing an additional 40% of the villi. Therefore 69% of the tissue was solubilized by the combined chemical and enzymic treatments. Plasmin digestion alone solubilized 68% of intact villi.

E.l.i.s.a. for TFN

Human PFN in the non-equilibrium competitive e.l.i.s.a. produced the inhibition curve shown in Fig. 1 (○ symbols). Inhibition was proportional to concentration of Fn over the range 50–250 ng of Fn/ml. The addition of heparin and urea to PFN did not affect the inhibition curve (Fig. 1a, ● symbols). Fn in tissue extracts (Fig. 1b, ■ symbols) produced an inhibition curve that could be superimposed on the PFN curve. These data show that the presence of heparin and urea does not interfere with e.l.i.s.a. and that TFN extracted with heparin and urea is identical with PFN in its

Fig. 1. Inhibition of binding of antibody in a non-equilibrium competitive e.l.i.s.a. obtained with purified PFN (○), purified PFN in the presence of heparin and urea (●), and heparin/urea extracts of placenta (■). For experimental details see the text.

interaction with this antibody. Thus untreated PFn can be used as a standard for quantifying TFn extracted with heparin and urea.

In Fig. 2(a) is shown a comparison of the inhibition curves obtained with PFn (○ symbols) and with PFn pretreated with dithiothreitol (8mg/ml) in 2M-urea to disrupt disulphide bonds (● symbols). The two curves are essentially superimposable, indicating that disrupting disulphide bonds in 2M-urea did not affect the antigenicity of fibronectin towards this antibody. TFn solubilized with dithiothreitol gave the inhibition curve shown in Fig. 2(b) (▲ symbols). In the region of interest this curve could be superimposed on the curve given by PFn pretreated with dithiothreitol (● symbols). The data indicate that TFn solubilized from the terminal villi of placenta by disruption of disulphide bonds is immunologically identical with human PFn and can be

quantified by e.l.i.s.a. with the use of PFn as a standard.

A noticeably flattened inhibition curve and incomplete inhibition were obtained when PFn was digested with plasmin before competing for anti-Fn (Fig. 3a, ▽ symbols). The inhibition curve given by plasmin digests of placental villi (Fig. 3b, ▲ symbols) was the same shape as that given by plasmin-digested PFn. Thus TFn solubilized by plasmin digestion is qualitatively the same as plasmin-digested PFn, which can be used as a standard in the e.l.i.s.a.

Quantification of TFn (Table 1)

The Fn solubilized by three successive urea/heparin extractions followed by a dithiothreitol extraction was 1.9 and 1.7% (mean 1.8%) of the dry weight of the tissue. The values on urea/heparin extracts are in good agreement with those obtained

Fig. 2. Inhibition of binding of antibody in a non-equilibrium competitive e.l.i.s.a. obtained with purified PFn (○), dithiothreitol-treated purified PFn (●) and TFn solubilized from placenta with dithiothreitol (▲)

For experimental details see the text.

Fig. 3. Inhibition of binding of antibody in a non-equilibrium competitive e.l.i.s.a. obtained with purified PFn (○), plasmin-digested purified PFn (▽) and TFn solubilized from placenta with plasmin (▲)

For experimental details see the text.

Table 1. *Quantification of placental TFN*

Intact placental villi (2g wet wt.) was extracted with 4ml of 0.05M-sodium phosphate buffer, pH 7.1, containing urea (2M) and heparin (5mg/ml). After 4h extraction the supernatant was separated from the insoluble material by centrifugation at 7700g. The residue after three extractions was extracted under N₂ (fourth extract) with 4ml of urea (2M)/dithiothreitol (8mg/ml) in the phosphate buffer for 16h (Expt. 1) or for two sequential 4h extractions (Expt. 2). All extraction solutions contained phenylmethanesulphonyl fluoride at a final concentration of 2mM to inhibit proteolysis. To prepare the extraction residues for plasmin digestion, they were washed with water and freeze-dried. The dry weights were 84mg (Expt. 1) and 81mg (Expt. 2). Since the dry weight of 2g of wet tissue was 116mg, this represented 71% of the intact tissue, and the Fn content of plasmin digests of these residues was multiplied by 0.71 to correct them back to intact tissue. Duplicate 1mg samples of residues and of intact freeze-dried tissue were digested for 24h at 37°C in a total volume of 0.1 ml of phosphate-buffered saline containing 0.25 unit of plasmin. Digestion was carried out in an atmosphere of toluene to prevent bacterial growth. Two digestions were performed, and the residue was washed between digestions. Plasmin digestion was stopped by the addition of phenylmethanesulphonyl fluoride (final concn. 2mM) to the supernatants. For all supernatants Fn was quantified by the non-equilibrium competitive e.l.i.s.a. technique, with appropriately treated PFN as a standard.

		Fibronectin in supernatants			
		Expt. 1		Expt. 2	
A. Sequential treatments					
I. Chemical extractions		(μg/g wet wt. of tissue)			
(a) Urea/heparin	1	340		368	
	2	310		280	
	3	160		86	
(b) Urea/dithiothreitol	4	280		190	
				52	
Total, all extractions		1090		976	
		(% of dry wt. of intact tissue)			
		1.9		1.7	
II. Plasmin digestion of residue					
		(μg/mg dry wt. of residue)			
Digest 1		12	13	11	12
Digest 2		2.3	2.8	3.8	3.9
Total, 2 digests		14.3	15.8	14.8	15.9
		(% of dry wt. of residue)			
		1.5		1.5	
		(% of dry wt. of intact tissue)			
		1.1		1.1	
Total, I and II		3.0		2.8	
B. Plasmin digestion of intact tissue					
		(μg/mg dry wt. of tissue)			
Digest 1		24.7	23.0	23.1	22.2
Digest 2		6.0	7.2	6.9	4.7
Total, 2 digests		30.7	30.2	30.0	26.9
		(% of dry wt. of intact tissue)			
		3.0		2.8	

previously on a different placenta with the use of heparin at 10mg/ml for the extraction and the electroimmunoassay for the quantification of Fn (Bray *et al.*, 1981). Also, as was shown in the earlier

study for heparin alone, the third heparin/urea extraction was less effective than the second, which was less effective than the first, as one would expect in an extraction of a finite amount of a

substance. Extraction of the residue from three heparin/urea extractions with dithiothreitol/urea, which would disrupt disulphide bonds, solubilized additional Fn amounting to 0.42–0.48% of intact tissue. The extraction with dithiothreitol was essentially complete after the first 4 h (second column).

By exhaustive digestion of the extracted freeze-dried residue with plasmin, additional Fn amounting to 1.1% of the dry weight of intact tissue was solubilized. Total Fn comprised 3.0 and 2.8% of the dry weight of the terminal villi in two experiments. The portion of the Fn solubilized by the chemical procedures alone was 63% in one experiment and 61% in the other. Digestion of intact tissue from the same placenta with plasmin directly gave values of 3.0 and 2.8% Fn, which agreed with the total obtained by the sequential extraction and digestion procedures. Terminal villi from two other placentas were digested with plasmin. Fn in the supernatants was 1.8 and 2.4% of the dry weight. The average for the three placentas was 2.4%.

Discussion

Direct demonstration of Fn functions in tissues and the possible interaction of Fn pools has not been feasible because of the difficulties in quantitatively extracting TFN. Additionally, such studies require that contamination with PFN be ruled out and that degradation of Fn by tissue proteinases be prevented. The present study has addressed these technical issues successfully in that a large portion (69%) of a perfused tissue has been solubilized concomitantly with the release of TFN in a form that could be quantified. Since the e.l.i.s.a. procedure is so sensitive, the methods are also applicable to tissues with much less Fn than placenta, and I have reproducibly analysed both human and dog lung tissue, which contain around 0.4% Fn, by these methods (B. A. Bray, unpublished work).

Plasmin digestion of the tissue has proved to be a useful procedure because it solubilized a major portion of the tissue (68%). Since it cleaves Fn near its cross-linking region (Pearlstein *et al.*, 1980), even that portion of Fn which is cross-linked to collagen or to fibrin or to itself should be released, leaving a tissue residue whose Fn content is negligible. The large Fn fragments resulting from plasmin cleavage of TFN were immunologically similar to those from PFN similarly treated. This allowed their quantification by the e.l.i.s.a. technique.

Two recent studies (Isemura *et al.*, 1984; Zhu *et al.*, 1984) concerned only that portion of Fn which

could be extracted from placenta with urea and which represents a small part of the total Fn. This urea-extractable Fn was immunologically identical with PFN and had a larger subunit than PFN owing in part to an increased carbohydrate content. Additionally, the structure of the carbohydrate chains differed between the two types of Fn. Thus the immunological comparisons in the present study are significant in that they demonstrated that all fractions of TFN from placenta, including that portion which required plasmin digestion for solubilization, and was therefore probably cross-linked in the matrix, were very similar immunologically to PFN. These data allow the conclusion that TFN, whether derived from plasma or cellular sources, is incorporated into this tissue unchanged in any way detectable by this polyclonal antibody in the non-equilibrium competitive e.l.i.s.a. technique. This supports the concept that TFN could be mobilized to replenish PFN concentrations in the circulation.

It is a pleasure to acknowledge the technical assistance of Ms. Lillian Rodriguez. This work was supported by U.S. Public Health Service Grant HL 15832.

References

- Atherton, B. T. & Hynes, R. O. (1981) *Cell* **25**, 133–141
- Bray, B. A. (1978a) *J. Clin. Invest.* **62**, 745–752
- Bray, B. A. (1978b) *Ann. N.Y. Acad. Sci.* **312**, 142–150
- Bray, B. A., Mandl, I. & Turino, G. M. (1981) *Science* **214**, 793–795
- Deno, D. C., Saba, T. M. & Lewis, E. P. (1983) *Am. J. Physiol.* **245**, R564–R575
- Deno, D. C., McCafferty, M. H., Saba, T. M. & Blumenstock, F. A. (1984) *J. Clin. Invest.* **73**, 20–34
- Hayman, E. G. & Ruoslahti, E. (1979) *J. Cell Biol.* **83**, 255–259
- Isemura, M., Yamaguchi, Y., Munakata, H., Aikawa, J., Kan, M., Yamane, I. & Yosizawa, Z. (1984) *J. Biochem. (Tokyo)* **96**, 163–169
- Mayer, B. W., Jr., Hay, E. D. & Hynes, R. O. (1981) *Dev. Biol.* **82**, 267–286
- Oh, E., Pierschbacher, M. & Ruoslahti, E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3218–3221
- Pearlstein, E., Gold, L. I. & Garcia-Pardo, A. (1980) *Mol. Cell. Biochem.* **29**, 103–128
- Rennard, S. I., Berg, R., Martin, G. R., Foidart, J. M. & Robey, P. G. (1980) *Anal. Biochem.* **104**, 205–214
- Saba, T. M. & Jaffe, E. (1980) *Am. J. Med.* **68**, 577–594
- Stenman, S. & Vaheri, A. (1978) *J. Exp. Med.* **147**, 1054–1064
- Vuento, M., Salonen, E., Pasanen, M. & Stenman, U.-H. (1981) *J. Immunol. Methods* **40**, 101–108
- Yamada, K. M. (1983) *Annu. Rev. Biochem.* **52**, 761–799
- Yamada, K. M. & Kennedy, D. W. (1979) *J. Cell Biol.* **80**, 492–498
- Zhu, B. C., Fisher, S. F., Pande, H., Calaycay, J., Shively, J. E. & Laine, R. A. (1984) *J. Biol. Chem.* **259**, 3962–3970