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Plasma fibronectin prepared from patients with metastatic breast cancer shows in vitro aggregation property

Laurent Vuillard, Andrew Miller and Philip J.H. Sizer

Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland

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Plasma fibronectin purified from the plasma of metastatic breast cancer patients has been studied by light scattering. It clearly shows abnormal self-aggregation properties; the possible significance of these findings to the in vivo situation is discussed.

Fibronectin; Cancer; Light scattering; (Plasma, Man)

1. INTRODUCTION

Fibronectins are a class of extracellular glycoproteins (520 kDa) (review [1]). Their primary structure can vary and depends on the alternative splicing of a unique gene which can generate at least ten different variants [2]. The molecules are composed of the concatenation of three types of homologous sequences [3] forming two similar, but not identical, chains linked near the carboxy-terminus end by two interchain disulphide bonds. One of the most striking features of the fibronectin molecules is that they are made of a succession of protease-resistant domains which can bind specifically to one or more of heparin, fibrin, factor VIIa, C1q, cell surface, DNA, gelatin collagen [1].

In solution, plasma fibronectin as studied by light scattering techniques does not show any significant self-aggregation properties [4], but fibronectins are present in the extracellular matrix in an insoluble, fibrillar, form [5].

Correspondence address: L. Vuillard, Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland

Receptors to fibronectin on the cell surface have been described [6] and a transmembranous continuity between cytoskeleton and fibronectins through the receptor has been demonstrated [7]. The association of fibronectin with the matrix assembly receptor has also been reported [8]. The position of fibronectin in the cell attachment process is therefore very important and it is probable that fibronectins are likely to be the extracellular matrix protein in closest contact with cells.

The involvement of fibronectin in cancer and metastatic processes has been investigated along three separate lines all related to early events of metastasis leading to the detachment of cells and their penetration into the blood stream:

(i) Synthesis of specific fibronectins by alterations of the primary structure [9] and carbohydrate side chains composition [10].

(ii) Modification at the cellular receptor level [11] and deposition rates of fibronectin in the matrix [12].

(iii) Degradation of fibronectin by tumour cells [13]. Specific enzymes have been described [14].

Surprisingly the role of fibronectins in the next stage, that is anchoring of the cell migrating in the blood stream, has been much less studied. De Petro et al. [15] have shown that transformation enhancement activity in the plasma of tumour pa-

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tients was linked with fibronectin. We report here evidence, from a light scattering study, that plasma fibronectin from breast metastatic cancer patients possesses significantly different hydrodynamic properties from normal plasma fibronectin.

2. MATERIALS AND METHODS

All affinity chromatography media were from Pharmacia (Sweden), and all other chemicals (analytical grade) were from BDH (England).

2.1. *Protein purification*

Blood samples were treated with 20% sodium citrate, 25 mM EDTA (1 vol. per 4 vols blood) and centrifuged at 200 × *g* for 5 min in a bench centrifuge. The supernatant was removed and was either frozen at −20°C or processed immediately as follows.

To remove any precipitates the plasma was centrifuged at 10000 × *g* for 1 h and then chromatographed on a 2 cm^2 × 1 cm gelatin-Sepharose column as described [16]. The sample was eluted with 4 M urea, 50 mM Tris-HCl (pH 7.4) but in order to test its possible influence on aggregation, 1 M arginine, 50 mM Tris-HCl (pH 7.4) was also used for one sample with no detectable difference. Exhaustive dialysis in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4 (dialysis buffer), was then performed to remove any urea traces.

To eliminate any possible artefactual contamination two samples were rechromatographed on a heparin-Sepharose column rinsed by dialysis buffer and then eluted by 1 M NaCl, 50 mM Tris-HCl, 1 mM EDTA (pH 7.4) and redialysed in dialysis buffer.

PMSF (0.05 mM) was present in all buffers and EDTA (5 mM) was added to all buffers used for purification. All specimens were examined by 5 or 6% discontinuous PAGE gels according to Laemmli [17].

2.2. *Photon correlation spectroscopy*

All work presented here was performed on a type 4700c Malvern system (Malvern Instruments, England). The argon-ion laser (Innova 4, USA) was used at 514.5 nm with a power of approx. 150 mW.

For measurements on the fibronectin molecule the experimental time was set between 5 and 15 s with no noticeable difference between results. Each measurement involved the accumulation of a number of runs; the software calculated the mean value of total counts for each run and rejected any run with a significant difference in integrated counts.

To remove any possible dust or contaminant from the 1 cm diameter quartz cells (ALV Lasers, FRG), they and their teflon caps were stored in pure sulphuric acid. Prior to use they were washed 20 times with double-distilled water to remove any traces of acid and three times further with filtered sample buffer to ensure that the buffer composition of the sample would not be altered by some remaining water. All buffers and all but one sample were filtered through 200 nm pore size Nucleopore filters (USA). These are well known for their highly constant pore size as well as low retention of the sample because of their design as flat surfaces as opposed to ordinary filters which have mesh-like surfaces.

To test the influence of filtration one sample was centrifuged and showed no difference in properties from filtered samples. When filtering the samples care was taken to avoid any disturbance leading to aggregation. We avoided the use of metal needles which leads to metal contamination thereby triggering fibronectin polymerisation, as well as creating high-speed flows possibly leading to aggregation.

The first-order correlation function was calculated from the measured second-order and related to the diffusion coefficient *D* and polydispersity index (review [18]). No correction was made for buffer viscosity which was assumed to be the same as pure water. The analysis by the software has a specific feature to estimate the diffusion coefficient distribution for polydisperse solutions. The software retains in the memory the shape of correlation functions for a large number of diffusion coefficients. It then calculates a multiexponential curve and fits it with the observed one. It is important to note that no assumption about the distribution of particles is made.

2.3. *Molecular mass determination by light scattering*

When the concentrations were high enough to produce permissible signal-to-noise ratio, molecular mass estimations were made using the classical method of Zimm [19].

3. RESULTS

Fig.1 shows the diffusion coefficient/scattering intensity profile for normal plasma fibronectin (open squares) and for one sample of gelatin affinity-purified fibronectin from metastatic cancer patient plasma (black squares).

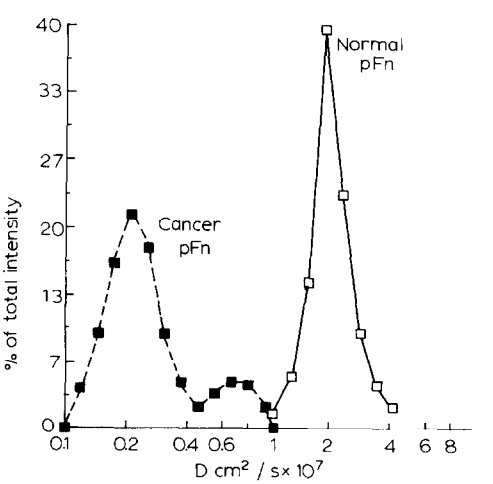

Fig.1. Diffusion coefficient/scattering intensity profile of (□—□) normal plasma fibronectin and (■---■) one sample of gelatin-affinity purified fibronectin from plasma of metastatic cancer patient.

Table 1

Sample		Polydispersity (%)	Molecular mass (kDa)
(1) Samples purified by gelatin affinity only (4 samples):			
D population 1 (cm^2/s)	D population 2 (cm^2/s)		
$0.29 \pm 0.07 \times 10^{-7}$	$0.97 \pm 0.4 \times 10^{-7}$	55	4000
(2) Samples repurified on heparin-Sepharose (2 samples):			
D population 1 (cm^2/s)	D population 2 (cm^2/s)		
$1.12 \pm 0.2 \times 10^{-7}$	$2.80 \pm 0.3 \times 10^{-7}$	38	
(3) Normal plasma fibronectin: Monodisperse distribution $D = 2.2 \times 10^{-7}$ (cm^2/s)		10	500

4. DISCUSSION

The most immediate result of our study is that plasma fibronectin prepared from metastatic cancer patients, when studied by dynamic light scattering, presents a polydisperse distribution, with two populations with markedly different diffusion coefficients. This contrasts with fibronectin purified from healthy individuals which presents a monodisperse distribution as shown in fig.1 as well as in the literature [4].

The analysis of the measured diffusion coefficients shows that: (1) Fibronectin from metastatic cancer patients which was purified solely on gelatin-Sepharose comprises two populations of material larger than normal plasma fibronectin. (2) Heparin-repurified samples comprise one population of larger objects and one population of slightly smaller objects.

(i) Proof that the material scattering light is fibronectin:

– The presence of protein contaminating the fibronectin preparation would be detected on the PAGE control gels.

– If contaminating material had been present, it would have been removed by the combination of various preparation procedures: for example, if the contaminants were eluted by 4 M urea only and they would not appear with material eluted from the gelatin column by 1 M arginine or repurified on the immobilised-heparin column, otherwise contaminants would have been eluted by high ionic strength, and therefore be eluted before fibronectin during the 1 M NaCl wash of the gelatin column.

– Furthermore, fibronectin alone binds to gelatin at high salt concentration and heparin at physiological salt concentration.

(ii) How much material is in an aggregated form?

The intensity of light scattering is proportional to the square of the mass of the scattering particles and thus to the sixth power of their size; so, clearly to allow detection of the higher diffusion coefficient values (i.e. those particles with smaller sizes) the high molecular mass material must represent a small number fraction of the total sample.

(iii) Why does the high diffusion coefficient population of fibronectin from cancer patients have a translational diffusion coefficient value (D) different from that of normal plasma fibronectin?

This is not too surprising, since the diffusion coefficients have to be calculated from a correlation function containing two decay rates, therefore the resolution is low. It is important to note that this is not a problem of noise but simply that the resolution is low when the distributions of two populations are close which means that the decays in the correlation function are not sufficiently dissimilar to estimate them precisely (the same problem occurs with Scatchard plots when two binding sites have similar dissociation constants). In addition, this loss in resolution is a direct consequence of the polydispersity and it lowers the quality of the diffusion coefficient measurements but the presence of aggregation is clearly established. This is confirmed by the very high values of the polydispersity index.

(iv) The important point established by our measurements is the presence of a low D population (i.e. large aggregates) in plasma fibronectin purified from metastatic cancer patients. As shown above in point (i), identification with fibronectin is clear but it is more difficult to determine whether such aggregates exist in vivo:

– They clearly exist at concentrations similar to the in vivo situation.

– The exclusion limit of the gelatin affinity column (Sepharose 4B) is smaller than that of the heparin affinity column (Sepharose CL6b). Despite this fact a considerable reduction in the size of the aggregates occurs after heparin affinity repurification. This would suggest that aggregates cannot easily dissociate after formation.
– Because of their size they would be excluded from the gelatin column, being directly eluted in the first preparation step. So, the more likely hypothesis is that they form during the preparation where the use of urea or 1 M arginine buffer leads to unfolding of the protein. The presence of these large aggregates in vivo cannot be stated with certainty.
– They are not the consequence of filtration because one sample that had been centrifuged still presented abnormal aggregation.

(v) We can only speculate on the origin of fibronectin presenting this abnormal aggregation property in vitro.

The presence in the blood stream of fibronectin synthesised by tumour tissues seems unlikely because tumour cells have been shown to degrade fibronectin in tissues [13] and fibronectin produced in tissues would have considerable difficulty migrating considering its binding properties. Furthermore, the amount produced would be very low compared to existing normal plasma fibronectin. Our conclusion is that the aggregates detected are genuine fibronectin with a different structure from that of normal plasma fibronectin. It could be present in plasma from confirmed metastatic cancer patients through modification in the blood of a small fraction of preexisting plasma fibronectin or could represent a form used by the metastatic cells to anchor at the surface of a blood capillary in their target organ.

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