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MODIFICATION OF SHEAR MODULUS AND CREEP COMPLIANCE OF FIBRIN CLOTS BY FIBRONECTIN

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Shear moduli and creep compliances have been measured for four types of clots of human fibrin (about 7 mg/ml) clotted with and without human plasma fibronectin (usually 1.2 mg/ml). Fine clots (with little lateral aggregation of the fibrin protofibrils) were formed at pH 8.5, ionic strength 0.45; coarse clots (with substantial lateral aggregation) were formed at pH 7.5, ionic strength 0.15; in both cases with and without ligation by fibrinoligase. In fine clots, the addition of fibronectin without ligation scarcely affected the shear modulus; with ligation, the modulus was decreased by a factor of 0.48. In coarse clots, the shear modulus was increased by addition of fibronectin. The increase was by a factor of 2.0 without ligation and by a factor of 2.4 with ligation. Creep and creep recovery in clots formed with and without fibronectin were similar except for the scale factor represented by the change in modulus.

1. Introduction

Fibronectin is a high molecular weight glycoprotein found in various tissues which interacts with other macromolecules by both noncovalent and covalent binding [1]. In particular, its interaction with fibrin has been extensively studied [1–4]. The covalent binding to fibrin consists of ϵ-(γ-glutamyl) lysine linkages to the α chains catalyzed by fibrinoligase (Factor XIIIa), with two binding sites for each fibronectin molecule. Fibronectin appears to have important roles in blood coagulation and other physiological processes [1]. The present study was undertaken to determine whether fibronectin affects the elastic modulus and other mechanical properties of fibrin clots formed in its presence.

2. Materials and methods

Human fibrinogen, Lot 57031, obtained from Kabi AB, Stockholm, was dissolved in and dialyzed against buffer in which ionic strength 0.05 was contributed by trishydroxymethylaminomethane and the remainder by sodium chloride. The clottability was 92 to 95%. Protein concentration was determined spectrophotometrically by absorption at 280 nm with correction for scattering at 320 nm. Bovine thrombin from Parke-Davis was dissolved in the same buffer. Fibronectin was prepared (D.F.M.) from human plasma protein side-fractions as previously described [5]. Fibrin stabilizing factor (Factor XIII) was prepared (L.L.) from human plasma as previously described [6]. Trasylol (proteolytic inhibitor) was obtained from FBA Pharmaceuticals. Materials for gel electrophoresis (acrylamide with cross-linking comonomer, dithiothreitol, Coomassie Brilliant Blue dye) were obtained from Biorad Laboratories.

Clotting mixtures contained approximately 7.0 (usually $\pm$ 0.1) mg/ml of fibrinogen, thrombin appropriate for a clotting time between 7 and 30 min (0.5 to 0.65 u/ml for unligated, 0.14 to 0.24 u/ml for ligated), and 2 units/ml of Trasylol (to inhibit fibrinolysin in case of possible contamination). For fine, transparant

clots (with minimal lateral aggregation of the fibrin protofibrils [7,8]) the pH was 8.5 and total ionic strength 0.45. For coarse, opaque clots (with extensive lateral aggregation to form coarse fibers) the pH was 7.5 and ionic strength 0.15. For an unligated clot, the mixture contained also 1 mM of ethylene diamine tetraacetate. For a ligated clot, the mixture also contained 15 mg/ml of fibrinoligase preactivated from fibrin stabilizing factor and 0.0032 M calcium chloride. When fibronectin was added (in trishydroxylmethylaminomethane-sodium chloride buffer), its final concentration was usually 1.2 mg/ml and the concentrations of all other components were the same as above. The fibronectin/fibrinogen w/w ratio was thus somewhat higher than under physiological conditions (0.17 versus 0.12). The ratio of contaminating fibronectin to fibrinogen in the Kabi fibrinogen, judged from gel electrophoresis, was usually much less than 0.01 but occasionally 0.02. The temperature (ambient) was between 23° and 25°C. One portion of the mixture was introduced before clotting into the Plazek apparatus [9,10] for mechanical measurements and another was reserved for gel electrophoresis after clotting.

Polyacrylamide gel electrophoresis, following conventional procedure [11] with solubilization by sodium dodecyl sulfate-urea and reduction by dithiothreitol, was performed on the fibrinogen mixture and also on the clot after incubation for about 22 h. Bands were observed, under appropriate conditions, for α, β, γ, and γ-γ chains as well as fibronectin and several representing α chains linked to fibronectin [3]. They were scanned at 550 nm in a Gilford 2400-S spectrophotometer generously made available by Professor M.T. Record Jr.. The areas of the recorder peaks were calculated by approximating the peaks with triangles.

In the Plazek apparatus, the disk-shaped clot is deformed in torsion; the maximum shear strain was less than 3%. Measurements with brief imposition of stress for 25 s and subsequent removal were made at intervals to follow the increase in shear modulus (the measure of elastic stiffness) with elapsed time after clotting. Finally, after about 22 h, a creep run was made under constant stress for 10^4 s followed by recovery for 10^4 s to determine the reversibility of the deformation.

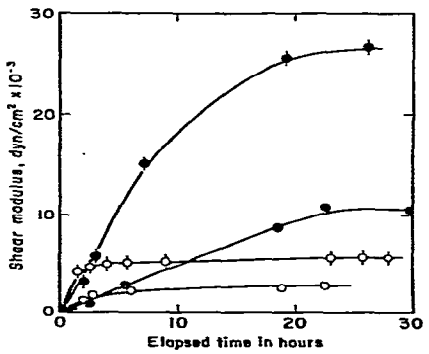

Fig. 1. Plots of initial shear modulus against time elapsed since clotting, for coarse clots. Open circles, unligated; black circles, ligated with fibrin stabilizing factor. Plain circles, without fibronectin; slotted circles, with fibronectin.

3. Results

3.1. Increase in modulus after clotting

For coarse clots, the shear modulus is plotted against time elapsed after clotting in fig. 1. Without ligation, the maximum stiffness is attained in a few hours and the final modulus is relatively small. However, the presence of fibronectin, even under these conditions where it is not attached to the fibrin by covalent bonds, causes the modulus to rise more rapidly and attain a final value twice as high.

With ligation, the modulus continues to increase for many hours and attains much higher values, associated in these coarse clots with slowly developing α-α ligation as shown by Hermans et al. [12]. Here, fibronectin has a large effect in increasing the rate of rise of modulus and increasing the final value by a factor of 2.4.

For fine clots, the increase in modulus with elapsed time is not shown because it is accomplished in a much shorter time [13,14]. There is little or no α-α ligation; only the γ chains are ligated [15]. Only the final value of the modulus is reported.

3.2. Magnitude of final modulus

The final values of shear modulus, averaged from two or three replicate experiments which in most cases agreed within 8%, are listed in the last column of table 1.

Table 1
Final shear modulus (after 22 h) and ligation data

Clot type	Ligation	Fibronectin	γ-γ Ligation %	Unligated α %	Ligated fibronectin %	Shear modulus dyn/cm^2 × 10^{-3}
coarse	unlig.	absent	a)	b)	–	2.74
		present	a)	b)	–	5.45
coarse	lig.	absent	77–96	60–67	–	10.6
		present	98, 100	0, 15	81, 87	25.6
fine	unlig.	absent	a)	b)	–	0.88
		present	a)	b)	–	0.72
fine	lig.	absent	96, 99	b)	–	3.40
		present c)	94, 97	82, 84	35, 61	1.62

a) Less than 5% γ-γ ligation in the original fibrinogen, unchanged after clotting. b) Essentially 100%.
c) In these experiments, the concentration of fibronectin was somewhat higher (1.6, 2.0 mg/ml).

In coarse clots, the fibronectin increased the modulus whether ligated or not, as stated previously. In fine clots, on the contrary, the fibronectin scarcely affected the modulus when unligated and it *decreased* the modulus by a factor of 0.48 when ligated.

3.3. Ligation: γ-γ, α-α and α-fibronectin

In unligated clots there was of course no ligation of either γ or α chains or covalent binding of fibronectin. In fine ligated clots formed without fibronectin, γ-γ ligation was essentially complete and α-α ligation was negligible. In fine ligated clots formed with fibronectin, γ-γ ligation was essentially complete, portions of the α chains and of the fibronectin disappeared after clotting, and α-fibronectin bands appeared. On a molar basis, the loss of α chains was about 1.8 times the loss of fibronectin subunits; a linkage of one α chain per fibronectin subunit, plus involvement of about 8% of the α chains in α-α ligation would account for this ratio.

In coarse ligated clots, the γ-γ ligation was also essentially complete. Without fibronectin, the α-α ligation was less than half complete under the conditions of our experiments. With fibronectin, the disappearance of α chains was essentially complete and most of the fibronectin was bound to α chains.

3.4. Creep and creep recovery

In a creep experiment, the creep compliance, or time-dependent strain per unit stress, is plotted logarithmically against time of duration of the applied stress. These plots are shown in fig. 2 for fine clots with and without ligation and with and without fibronectin. The ligated clots have smaller compliance (corresponding to higher modulus) and they creep very little; the unligated clots undergo substantial creep at long times. The presence of fibronectin has no effect in either case except to change the scale factor corresponding to the change in modulus already noted. After recovery for 4×10^3 s, the deformation is almost completely recovered for the ligated clots but for the unligated clots there is a substantial permanent deformation (21%). The relation of such unrecoverable strain to structural rearrangement in fine unligated clots will be reported elsewhere [16]; it is unaffected by the fibronectin. Creep and creep recovery of coarse clots with and without fibronectin were very similar except for the differences in scale corresponding to higher moduli and somewhat higher recovery in the

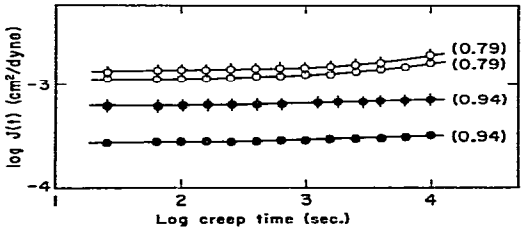

Fig. 2. Logarithmic plots of shear creep compliance against creep time, for fine clots. Key to symbols same as in fig. 1. Numbers in parentheses denote fractional recovery at 4000 s after removal of stress.

unligated case; again, the presence of fibronectin had little effect on the time dependence even when bound by ligation.

4. Discussion

The ligated coarse clots are formed under conditions most nearly approximating physiological, although many components of plasma are absent. The substantial enhancement of elastic modulus by fibronectin is presumably due to stiffening of the bundles of protofibrils in the thick clot fibers by additional linkage of α chains, through the difunctional fibronectin molecules, binding the protofibrils together. The models of protofibril packing proposed by Hermans [17] could probably accommodate interspersed fibronectin molecules. The difference in modulus may be partially due to the enhancement of α-α ligation by fibronectin under our experimental conditions, but the fact that fibronectin also increases the modulus of unligated clots indicates that it has a specific effect. It is difficult to speculate about the mechanism until it is known whether the elastic energy storage is associated with bending or stretching the fibers or both.

Without ligation, the modulus of coarse clots is much lower, but it is still increased by fibronectin, which presumably interacts by noncovalent binding, as has been observed from retention in clots at lower temperatures [2].

The reduction of modulus by fibronectin in fine ligated clots is puzzling. It has been suggested [13] that the rigidity of these clots is primarily due to the steric immobilization of the long stiff interpenetrating protofibrils. Perhaps the attachment of fibronectin molecules dangling from pendant α chains separates the protofibrils enough to lessen the immobilization. However, better understanding of the relation of macroscopic rigidity to the mechanical properties of the fibrils themselves is necessary to interpret these observations.

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