Article

Hyaluronic acid decreases the mechanical stability, but increases the lytic resistance of fibrin matrices

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Abstract

Hyaluronic acid (HA) is a large, non-sulfated glucosaminoglycan abundantly present at sites where fibrin is also formed (during wound healing, in arterial restenotic lesions and eroded atherosclerotic plaques). The aim of the present study was to characterize the structure of composite fibrin-HA clots with scanning electron microscopy (SEM), pressure-driven permeation and small-angle X-ray scattering (SAXS) and their viscoelastic properties with an oscillation rheometer. In addition the efficiency of fibrinolysis in these clots was investigated by kinetic turbidimetric and chromogenic assays for dissolution of fibrin and plasminogen activation by tissue-type plasminogen activator (tPA). Fibrin formed in the presence of native (1500 kDa) HA and its 500 kDa fragments had thicker fibers and larger pores according to the SEM and clot permeation data, whereas the 25 kDa HA fragments had only minor effects. SAXS evidenced a mild disarrangement of protofibrils. These structural alterations suggest that HA modifies the pattern of fibrin polymerization favouring lateral association of protofibrils over formation of branching points. Rheometer data showed softer fibrin structures formed with 1500 kDa and 500 kDa HA and these clots presented with lower dynamic viscosity values and lower critical stress values at gel/fluid transition. tPA-catalysed plasminogen activation was markedly inhibited by HA, both in free solution and on the surface of fibrin clots, in the presence and in the absence of 6-aminohexanoate suggesting a kringle-independent mechanism. HA of 1500 and 500 kDa size prolonged clot lysis with both plasmin and tPA and this inhibition was kringle-mediated, because it was abolished by 6-aminohexanoate and was not observed with des-(kringle1–4)-plasmin. Our data suggest that HA size-dependently modifies the pattern of fibrin polymerization with consequent inhibition of fibrinolysis. At sites of tissue injury and inflammation, HA could stabilize fibrin through modification of its structure and lysibility.
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Introduction

Thrombin, a serine-protease activated in the blood coagulation cascade converts fibrinogen, a rod-shaped soluble plasma protein of 340 kDa to insoluble fibrin polymer [1]. This 3-dimensional fibrin structure provides a solid scaffold in haemostatic and thrombotic clots, and functions as the primary matrix in the wound healing process [2]. Several factors modulate the structure and function of the fibrin network, including thrombin, chloride and calcium concentrations, as well as additional clot components and cellular interactions [3–5]. Fibrin fiber thickness, branching density and porosity of patients' plasma clots have been linked to the frequency of cardiovascular events and thrombolytic

Table 1. Structural characteristics of composite fibrin/hyaluronic acid clots.

		Additive			
		None	HA 1500 kDa	HA 500 kDa	HA 25 kDa
Clotting time (min)		25.4 ± 3.7	31.9* ± 3.3	30.9* ± 4.5	25.4 ± 2.6
Maximal clot turbidity		0.300 ± 0.018	0.372* ± 0.009	0.362* ± 0.019	0.301 ± 0.010
Fibrin fiber diameter (nm)		86.3 (70.5–104.3)	97.2* (78.0–119.4)	98.6* (80.6–120.5)	80.4* (65.8–98.1)
Fluid permeability coefficient (K_s, 10^{-9} cm^2)		0.71 ± 0.05	1.14* ± 0.09	1.07* ± 0.11	0.86* ± 0.03
Viscoelastic parameters	G' (Pa)	34.05 ± 8.31	22.21* ± 5.5	18.23* ± 4.33	24.19 ± 6.40
	G'' (Pa)	3.10 ± 0.51	2.28* ± 0.52	1.98* ± 0.39	2.48 ± 0.50
	G''/G' (–)	0.092 ± 0.009	0.104* ± 0.009	0.110* ± 0.011	0.104 ± 0.011
	τ_0 (RU)	1.00 ± 0.13	0.65* ± 0.12	0.79* ± 0.15	0.81 ± 0.15

Fibrin clots containing various HA size-variants at 0.2 g/l were prepared from 2.5 g/l fibrinogen for turbidimetry, scanning electronmicroscopy, fluid permeability and oscillation rheometry experiments to evaluate key structural and physical determinants of the composite fibrin networks (maximal clot turbidity and clotting time, fibrin fiber diameter, fluid permeability coefficient reflecting porosity and viscoelastic parameters reflecting mechanical strength as described in Experimental procedures). Plateau values of storage modulus (*G*'), loss modulus (*G*'') and loss tangent (G''/G') at complete clotting (Fig. 3A) are presented. The critical shear stress for the gel/fluid transition in the fibrin structure (τ_0) illustrated in Fig. 3B is expressed in relative units (RU) compared to pure fibrin. Asterisks indicate $p < 0.05$ according to Kolmogorov-Smirnov test in comparison to pure fibrin, n = 4–12, values are reported as mean ± standard deviation or median and bottom-top quartile range in parenthesis.

resistance [6]. The lytic susceptibility of blood clots may also be related to microembolization, a common event after acute myocardial infarction [7]. Recently we have described that certain extracellular matrix components of the atherosclerotic plaque (chondroitin-sulfate, dermatan-sulfate and decorin) decrease the mechanical and fibrinolytic resilience of the fibrin structure [8]. Hyaluronic acid (HA), a large, non-sulfated glycosaminoglycan is ubiquitously present in extracellular matrices, and appreciated as an important player in wound healing, vascular disease and cancer, where fibrin deposition also occurs [9,10]. HA is found at high concentrations in articular joint synovial fluid (2–3 g/l), human umbilical cord, and the vitrous body of the eye, but its circulating plasma concentration is very low (0.01–0.1 mg/l) [11]. Among human solid tissues, articular cartilage contains 0.5–2.5 g/l, and skin up to 0.5 g/l HA, other organs have less. Plasma HA concentration rises in certain diseases (and may be utilized in diagnostic tests), but the reported values vary over a broad range up to 0.2 g/l in rheumatoid arthritis. Microenvironments of many solid tumours have been found to be rich in HA, and HA content has been linked to poor outcome in cancer patients [9,11]. In healthy arterial wall it is localized in the endothelial glycocalix, the intima and the adventitia, while in atherosclerotic vessels restenotic lesions and eroded plaques have been found rich in HA [11–13]. Vascular HA has been suggested to play a dual role: in the endothelial glycocalix it may protect endothelial integrity, whereas HA in plaques could promote cell proliferation and neointimal expansion [14,15]. In advanced atherosclerotic lesions, hyaluronic acid co-localizes with versican, a prothrombotic vascular proteoglycan, and platelets can adhere to HA with specific CD44 receptors, hence versican-HA complexes could serve as platelet adhesion targets at the plaque/thrombus interface [13]. Together with fibrin, HA is also a major component of the primary matrix formed following tissue injury [10]. High-molecular weight HA (over 10^6 Da, "native" HA) released from cells becomes fragmented by hyaluronidase enzymes, as well as by reactive oxygen species at sites of tissue injury and inflammation, leading to a set of HA fragments of highly variable chain length. HA size-variants have been shown to display varying, sometimes even opposing effects on the immune system, angiogenesis and tumour biology. Thus, native HA is anti-inflammatory and anti-angiogenic, while smaller HA fragments promote inflammatory reactions, and angiogenesis, which are crucial events in wound healing and tissue remodelling, as well as in cancer progression [9,10,16]. Despite some early data on the specific binding of human fibrinogen to HA, little is known on the potential role of HA in modulating the formation and behaviour of the fibrin matrix, and no data are available on the size-dependency of these effects [17,18].

We aimed to characterize the HA effects on fibrin structure, and also describe the composite HA/fibrin matrices as a cofactor and substrate for the fibrinolytic system. Fibrinolysis is largely based on a serine-protease, plasmin, formed from its zymogen, plasminogen by plasminogen activators [19,20]. Fibrin has been shown to accelerate tissue plasminogen activator (tPA) mediated plasminogen activation, a phenomenon dependent on fibrin network characteristics [21,22]. Once formed on fibrin, plasmin would proteolytically dissect fibrin fibers leading to the formation of water-soluble fibrin

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