branching in the higher molecular weight fractions.[12]

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Dextran-Induced Changes in Fibrin Fiber Size and Density Based on Wavelength Dependence of Gel Turbidity

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ABSTRACT: The effect of Dextran T70 on the polymerization, size, and density of fibrin fibers was evaluated both by monitoring the kinetics of the turbidity increase during the thrombin-induced gelation of fibrinogen and by measuring the wavelength dependence of the gel turbidity. The presence of Dextran T70 in the clotting solution increased the rate of fibrin polymerization and resulted in increased fiber cross-sectional size through enhancement of the side-to-side association. At a constant fibrinogen concentration of 1 mg/mL, the addition of greater than 4.00 mg/mL dextran resulted in the subsequent production of fibers whose density was decreased relative to fibers formed in the absence of dextran. Above 4.00 mg/mL dextran, measurable amounts of dextran were associated with the fibrin network. The previously proposed mechanism for dextran–fibrin interaction, steric exclusion, is not sufficient to explain the results reported here.

Introduction

Dextran has been used as a plasma expander for more than 30 years;[1,2] however, its antithrombogenic properties were not initially appreciated until 1954 when bleeding complications were first recognized.[3] The antithrombogenic effect of dextran is puzzling since dextran has been shown to accelerate thrombin-initiated fibrin clotting.[4] Dextran has subsequently been shown to interact with fibrinogen, Factor VIII, and numerous other blood proteins,[5-13] as well as platelets.[12,14-17]

Dextran interacts both with fibrinogen, reducing its solubility,[18,19] and with fibrin, accelerating its polymerization.[4] The interaction between fibrin and dextran has been studied by a variety of techniques, all of which indicate that fibrin fibers formed in the presence of dextran are coarser, producing fibrin fibers with larger cross-sectional areas than those formed in the absence of dextran.[20-25] These coarse gels also have an increased turbidity.[22-25]

While some of the above studies have utilized the measurement of gel turbidity to study the effect of dextran on fibrin gel formation, none have yielded quantitative information about the dimensions of the fibrin fibers. We recently reported a technique for calculating the mass per unit length and radius of fibrin fibers based on a measurement of the wavelength dependence of gel turbidity.[26] This technique assumes that the fibrin fiber is a long rod. This assumption is supported by numerous reports utilizing a diverse array of investigational techniques, including electron microscopy and gel perfusion. Fiber dimensions derived from the turbidity technique are in excellent agreement with values obtained from light scattering and gel perfusion procedures and furthermore allow one to calculate fiber density.[27] In this report, we show that the previously observed dextran-induced turbidity changes can be quantitatively interpreted in terms of the fiber structure. The previously proposed mechanism of dextran effect on fibrin polymerization, based on steric exclusion, is not sufficient to explain the observed changes in the fiber structure described in the report.

Materials

Human fibrinogen (Grade L, A.B., Kabi, Stockholm, Sweden) was dissolved in 0.3 M NaCl at 25 °C. The solution was cleared by centrifugation at 30000*g* for 20 min. The fibrinogen solution was dialyzed against 0.3 M NaCl for 18 h to remove any free calcium. The solution was then frozen at −70 °C. Clottability was greater than 95%. Fibrinogen concentration was determined from absorbance at 280 nm, using an extinction coefficient of 1.6 mL/(mg cm).

Thrombin solutions were prepared by dissolving Parke-Davis bovine thrombin in water to reach a concentration of 130 NIH units/mL. Free calcium was removed by dialysis against 0.3 M NaCl. The thrombin was also stored in small aliquots at −70 °C.

Dextran (T70, MW = 70 000; Pharmacia Corp.) solutions were prepared by dissolving powdered dextran in deionized distilled water to the desired concentration. The solutions were used fresh, stored at 2 °C, and never frozen.

Methods

Fibrin Gel Formation. All gels for this study were formed by the addition of thrombin in a final concentration of 1.25 NIH

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units/mL to a solution of buffered fibrinogen. The solutions contained 5 mM Ca^{2+}, 0.05 M Tris (pH 7.4), and a predetermined molarity of NaCl.

Turbidity measurements were made with a Cary 118C spectrophotometer. Fibrin polymerization kinetics were studied at 632.8 nm against a blank composed of the buffer lacking fibrinogen. Wavelength-dependence measurements were made 60 min after the addition of thrombin. The gels were scanned from 800 to 350 nm.

The amount of dextran associated with the fibrin network was obtained from the differential refractive index of the supernatant of clots formed in the presence of dextran relative to the supernatant of a clot formed in the absence of dextran. This value was then compared to a standard curve of dextran concentation vs. refractive index to yield the dextran concentration in the supernatant.

Theory

The turbidity, τ, of a solution is a measure of how much light is scattered as it passes through a solution. Therefore, we may define τ as the integral over the scattering angle θ for all the scattered light

$$\tau = \int_0^\pi 2\pi d^2(i_\theta/I_0) \sin\theta \, d\theta \quad (1)$$

where

$$i_\theta/I_0 = R_\theta(1 + \cos^2\theta)/d^2 \quad (2)$$

i_θ is the scattered intensity at an angle θ per unit of volume, I_0 is the intensity of the primary beam, and d is the distance between the scattering volume and the detector. R_θ is the Rayleigh ratio and depends on the size and shape of the scattering particles. The angular dependence of the scattered intensity for a sufficiently large particle is quite dependent on the shape of the particle, provided polydispersity and the excluded-volume effect are not excessive.[28,29] For very long, thin rod-shaped particles[30-32]

$$R_\theta = ck\lambda\mu/4n \sin(\theta/2) \quad (3)$$

where

$$k = 2\pi^2n^2(dn/dc)^2/N\lambda^4 \quad (4)$$

c is the concentration in grams per cubic centimeter, λ is the wavelength of the incident light in vacuo, μ is the mass/length ratio of the fibers in daltons per centimeter, n is the refractive index of the solution, dn/dc is the refractive index increment of the solute, and N is Avogadro's number. That eq 3 is appropriate for the study of fibrin gels has been previously demonstrated.[27,32] Substituting for i_θ/I_0 and R_θ eq 1 becomes

$$\tau = (\pi ck\lambda\mu/2n)\int_0^\pi (1 + \cos^2\theta)(\sin\theta)/\sin(\theta/2)\, d\theta \quad (5)$$

Integration yields

$$\tau = (44/15)\pi kc\lambda\mu/n \quad (6)$$

Substituting for k

$$\tau = [(88/15)\pi^3n(dn/dc)^2c/N]\mu\lambda^{-3} \quad (7)$$

This implies that a plot of τ vs. λ^{-3} should be a straight line whose slope is proportional to the mass/length ratio. A similar treatment indicates a λ^{-2} dependence for spheres. For very turbid gels, eq 5 may be replaced by

$$(44/15)\pi kc\lambda/n\tau = \mu^{-1}(1 + 92\pi^2r^2n^2/77\lambda^2 + \ldots) \quad (8)$$

where r is the radius of the rods in the solution. Substituting for k gives

$$[(88/15)\pi^3n(dn/dc)^2/N](c/\tau\lambda^3) = \mu^{-1} + (92\pi^2n^2/77)(r^2/\mu)\lambda^{-2} \quad (9)$$

or more simply

$$(c/\tau\lambda^3) = A\mu^{-1} + B(r^2/\mu)\lambda^{-2} \quad (10)$$

where

$$A = [(88/15)\pi^3n(dn/dc)^2/N]^{-1} \quad (11)$$

and

$$B = (92\pi^2n^2/77)A \quad (12)$$

Equation 10 implies that a plot of $c/\tau\lambda^3$ vs. λ^{-2} should give a straight line whose intercept is proportional to the reciprocal of the mass/length ratio. In addition, the ratio of slope to intercept is proportional to the square of the radius of the rods. The values of the constants A and B in this study were

$$A = 6.76 \times 10^{22} \quad (13)$$

and

$$B = 1.41 \times 10^{24} \quad (14)$$

where the values for n of water and dn/dc of fibrinogen at 633 nm have been used. A complete derivation of eq 8 may be found in ref 26.

Results

The above derivation neglects the possible additional contributions of the network branch points. The error introduced by this simplification is apparently small, based on the agreement one obtains when comparing the results obtained from this technique with results obtained by other techniques. In addition, previous studies have indicated that branch points in the gel occur as a result of the continuation of the basic lateral monomer association with parallel alignment of the major axis of the monomer.[33,34] The fiber diameters must vary, and certainly the branch points contribute to this variability. However, we obtain and report only an average value for the fiber diameters.

The polymerization (followed by increasing turbidity) during fibrin gel assembly is plotted for fibrin gels formed in 0.10, 0.20, and 0.30 M NaCl in Figure 1. Dextran is shown to produce a more rapid rise and a higher turbidity end point for the fibrin gels formed in 0.10 M NaCl (Figure 1A), reflecting more rapid side-to-side aggregation and thicker fibers. The end-point turbidities of gels in 0.2 M NaCl are never as high as those in 0.10 M NaCl.

The turbidity changes seen for fibrin gels formed in 0.30 M NaCl demonstrate a critical dextran concentration (Figure 1C). For dextran concentrations up to 4.0 mg/mL the changes in initial turbidity and turbidity end point are small. However, at 8.0 mg/mL dextran the rate of turbidity rise and turbidity end point are dramatically increased.

The turbidity of fibrin gels formed in 0.10 M NaCl steadily increases as the initial fibrin concentration is increased. In Figure 2 it is seen that gels containing 8.00 mg/mL dextran are 2.0 times more turbid than their dextran-free counterparts.

The turbidity of fibrin gels formed at lower thrombin concentrations (0.125 NIH units/mL) is slower to develop but eventually reaches a higher end point (Figure 3). The effect of dextran on the kinetics of polymerization is marked under these conditions. In the presence of dextran the end point is higher and the slope sharper. It is apparent that the effect of reduced thrombin and increased dextran concentration may be additive because these conditions yield the highest end-point values. It has been shown previously that this effect is not the result of di-

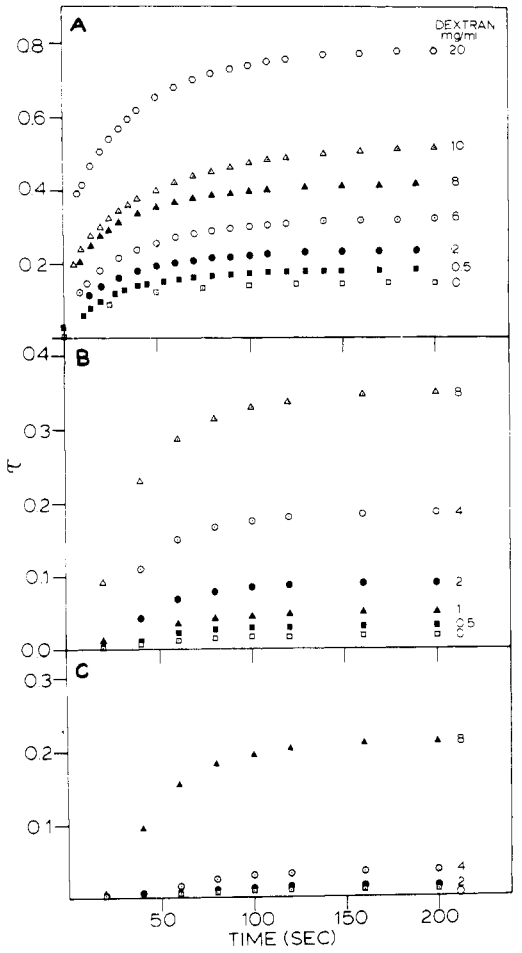

Figure 1. Kinetics of turbidity increase in fibrin gels formed in the presence of the indicated amounts of dextran. All gels contained 1 mg/mL fibrinogen, 5 mM Ca^{2+}, and 1.25 NIH units/mL thrombin and were buffered at pH 7.4 with 0.05 M Tris at 22 °C. NaCl concentrations (M): (A) 0.10; (B) 0.20; (C) 0.30. Thrombin was added at zero time.

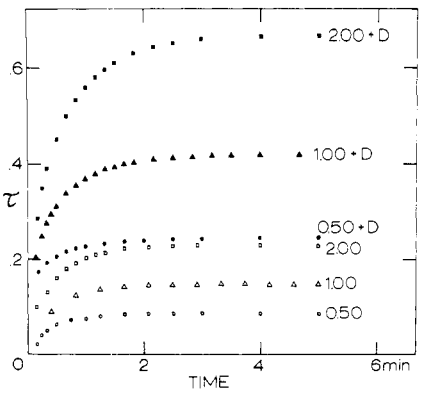

Figure 2. Effect of initial fibrinogen concentration on the kinetics of turbidity increase in fibrin gels in the presence (closed symbols) and absence (open symbols) of 8.00 mg/mL dextran. Fibrinogen concentrations (mg/mL): (○) 0.50; (△) 1.00; (□) 2.00. All other conditions are the same as for Figure 1.

luting out plasmin, a known trace contaminant of topical thrombin.[35]

The dependence of gel turbidity is plotted as $(1/\tau\lambda^3)$ vs. λ^{-2} in accordance with eq 10 and is shown in Figure 4. The intercepts of these plots are proportional to the reciprocal of the fiber mass/length ratio, and the ratio of slope and intercept is proportional to the square of the fiber radius.

For very clear gels with low turbidities the data were plotted as τ vs. λ^{-3} (Figure 5). The slopes of these lines

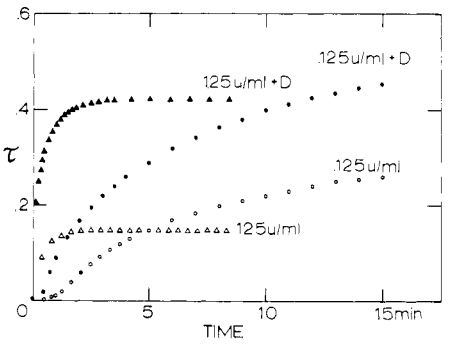

Figure 3. Effect of varying thrombin concentration on the kinetics of turbidity increase in fibrin gels in the presence (closed symbols) and absence (open symbols) of 8.00 mg/mL dextran. Thrombin concentrations (U/mL): (△) 1.25; (○) 0.125. All other conditions are the same as for Figure 1.

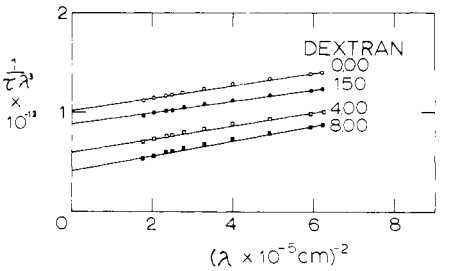

Figure 4. Wavelength dependence of the turbidity of several fibrin gels formed in the presence of the indicated amounts of dextran. The data have been fitted with straight lines. The intercept of these lines is proportional to the reciprocal of the mass/length ratio, and the ratio of slope and intercept is proportional to the square of the radius of the fibers. All other conditions are the same as for Figure 1.

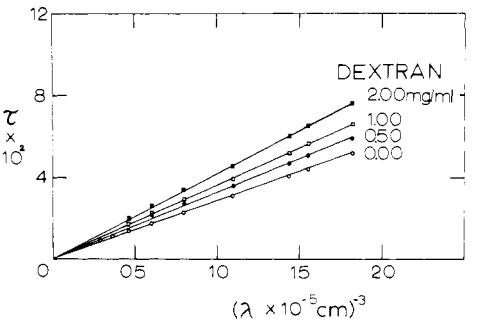

Figure 5. Wavelength dependence of the turbidity of several fibrin gels of very low turbidity. The slope of this plot is proportional to the mass/length ratio, but no information regarding the fiber radius is obtained from this plot. This proportionality between turbidity and λ^{-3} breaks down for gels with very large mass/length ratios.

are proportional to the mass/length ratio.

Mass/length ratios are calculated for gels formed as a function of NaCl molarity and dextran using eq 10 and are plotted in Figure 6. Mass/length ratios for all three NaCl concentrations increase with increasing dextran concentrations. The increases for 0.10 and 0.20 M NaCl gels are linear with increasing dextran over the range tested. Gels formed in 0.30 M NaCl maintain a low value of mass/length ratio until the dextran concentration reaches 4.0 mg/mL, but above 4.0 mg/mL dextran, show rapid increases in their mass/length ratios. This parallels the effect seen on the polymerization kinetics.

The fiber density, in mass/unit volume, was calculated by dividing the mass/length ratio by the radius squared times π. Values for 0.1 M NaCl are plotted in Figure 7. Gels formed in 0.10 M NaCl in the presence of dextran

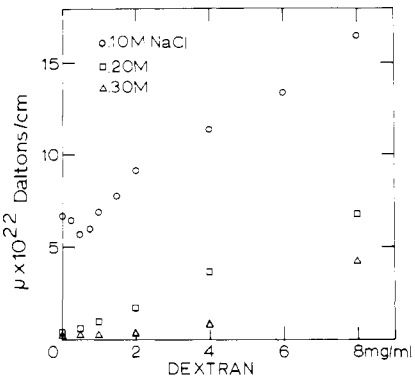

Figure 6. Changes in mass/length ratios (daltons/cm) of fibrin gels formed at various concentrations of NaCl and dextran. NaCl concentrations (M): (○) 0.10; (□) 0.20; (△) 0.30. All other conditions are the same as for Figure 1.

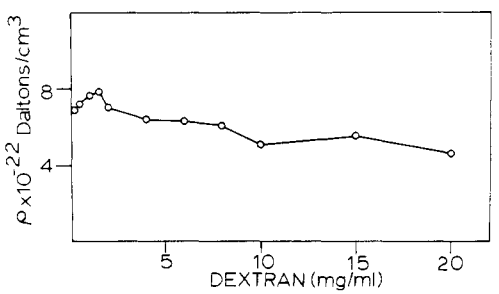

Figure 7. Density of fibrin fibers in gels formed in 0.10 M NaCl with varying amounts of dextran. All other conditions are the same as for Figure 1.

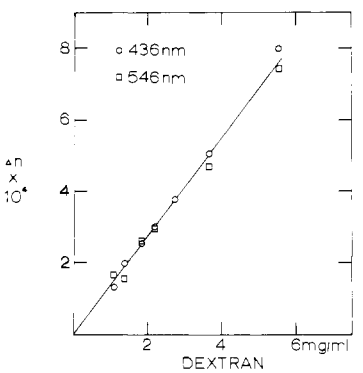

Figure 8. Refractive index changes with increasing amounts of dextran in 0.10 M NaCl. The slope of this plot allows one to calculate the concentration of dextran in an unknown sample given the value of the relative refractive index of that sample.

show an initial sharp increase in fiber density followed by a gradual decline as the dextran concentration increases.

The amount of dextran incorporated into the forming fibrin network was calculated by measuring the refractive index changes of the supernatant from synergized clots. The amount of dextran in the supernatant was calculated based on Figure 8. This value was then subtracted from the original amount of dextran present in the clotting solution to yield the amount of dextran associated with the fibrin pellet. It can be seen that, at least initially, more dextran is bound to fibrin gels formed at 0.10 M NaCl than at 0.30 M NaCl (Figure 9). The limitation of this technique is the large value of the refractive index of the blank.

Discussion

The results of this study illustrate that the main effect of dextran on thrombin-generated fibrin gels is an enhancement of side-to-side aggregation. This is reflected

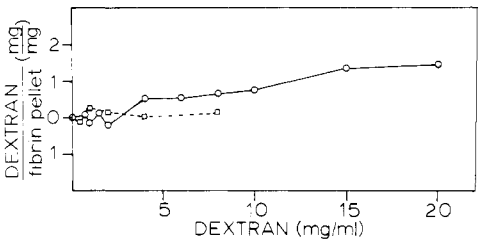

Figure 9. Amount of dextran associated with the fibrin network, in mg of dextran per mg of fibrin, as a function of increasing initial amounts of dextran (mg/mL). All gels contained 1 mg/mL fibrinogen and were clotted in 0.10 M NaCl (○) or 0.30 M NaCl (□). All other conditions were the same as for Figure 1.

by an earlier more rapid rise in turbidity and by the increased final level of gel turbidity.

It has been previously demonstrated that the enhanced polymerization rate is not due to dextran–thrombin interaction.[36] The increased speed of polymerization is thought by some authors to result from steric exclusion of fibrin by dextran.[37,39] This explanation, based on Ogston's theory of steric exclusion by long polymers, implies that the fibrin concentration is increased by a corresponding decrease in the effective aqueous volume available for the fibrin molecules.[43] Since the rate of polymerization is dependent on the fibrin concentration, the assembly process proceeds more rapidly. While this will explain the increased rate of polymerization, it does not explain the increased size of fibrin fibers.

The increase in fiber size is reflected by the increased turbidity end point and confirmed by electron microscopic studies.[22,39,40] Increased fiber diameter must result from an increased tendency toward side-to-side association rather than end-to-end aggregation. This may be demonstrated by forming gels of varying initial fibrin concentration in 0.30 M NaCl. The fiber diameters do not double in size when the initial fibrin concentration is doubled; one simply produces gels containing more of the same size fiber.[27] The reaction environment must be altered to favor side-to-side aggregation to increase fiber diameter.

It has been previously shown that one may shift from thin to thick fibrin fibers by reducing the ionic strength of the clotting solution.[23,44-46] Gels formed in 0.1 M NaCl contain much thicker fibers. As seen in this study, the addition of dextran to fibrin solutions in 0.3 M NaCl can also cause the transition from thin to thick fibers. The ability of dextran to cause this transition cannot be explained by simple steric exclusion, since this effect alone should not favor side-to-side over end-to-end polymerization. It is possible that the side-to-side interaction is promoted by a dextran-mediated fibrin monomer conformational change. However, side-to-side polymerization is salt and pH,[45,46] but not temperature, dependent,[48] which implies hydrogen bonding is potentially important for stabilization of side-to-side interaction.

It is possible that dextran may be incorporated into the fibrin fibers. We have been able to demonstrate limited association of dextran with the fibrin network. The amounts associated have been small, especially at low dextran concentrations. However, the ratio of dextran to fibrin in the network may be greater at high dextran concentrations. Previous evidence that the fibers may contain dextran is derived mainly from electron microscopic studies. All studies have shown increased fiber diameters and at least one has revealed fading of the cross striation in the presence of a large concentration of dextran.[22] In addition, previous studies on the structure of fibrin fibers in the absence of dextran have indicated the

possibility of channels within the fibers.[26,33] These spaces may be sufficient to accommodate dextran incorporation. Incorporation of dextran into the network could provide an explanation for the observed decrease in fiber density as the dextran concentration is increased. It should be emphasized that the density calculations are based on the assumption that no dextran was incorporated into the fibers. This assumption simplifies the mathematical analysis, which becomes quite complex if one must deal with a two-component fiber.

Although this study demonstrates that small amounts of dextran are associated with the fibrin network, the obvious question of whether dextran is located on the surface of the fiber or distributed throughout the fiber cannot be addressed. In principle, plots of the amount of dextran incorporated into the fibrin network against the fiber radius and against the fiber radius squared should give this information. If the dextran is contained only on the outside of the fiber, the dextran incorporated should be proportional to the surface area or the radius. If the dextran is found throughout the fiber, the amount of dextran incorporated will vary with the cross-sectional area or radius squared.

The possible consequences of the dextran-induced structural changes in the fibrin network are apparent in at least two areas. First, the mechanical strength of the gel may be reduced, and, second, the dextran-modified gels may have altered substrate properties for enzymes such as Factor $XIII_a$ and plasmin. In terms of the mechanical properties the tensile strength of fibrin gels has been shown to decrease in the presence of dextran.[21] Elasticity studies in our laboratory demonstrate a dramatic reduction in the elastic modulus of dextran-modified plasma clots.[47] The significance of dextran modification of fibrin clots on the enzymatic specificity of both Factor $XIII_a$ and plasmin is controversial. Previous studies on the plasmin digestion of fibrin have produced conflicting results. Kopec et al. found that dextran inhibited plasmin digestion.[41] In contrast, Tangen has repeatedly demonstrated an enhancement of plasmin activity in the presence of dextran.[39,40,42] Possible effects of dextran on Factor $XIII_a$ cross-linking of fibrin may be significant but have not been investigated.

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