Lactadherin inhibits enzyme complexes of blood coagulation by competing for phospholipid-binding sites

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Lactadherin, a glycoprotein of the milk-fat globule membrane, contains tandem C domains with homology to discoidin-type lectins and to membrane-binding domains of blood-clotting factors V and VIII. We asked whether the structural homology confers the capacity to compete for the membrane-binding sites of factor VIII and factor V and to function as an anticoagulant. Our results indicate that lactadherin competes efficiently with factor VIII and factor V for binding sites on synthetic phosphatidylinerse-containing membranes with half-maximal displacement at lactadherin concentrations of 1 to 4 nM. Binding competition correlated to functional inhibition of factor VIII–factor IXa (factor Xase) enzyme complex. In contrast to annexin V, lactadherin was an efficient inhibitor of the prothrombinase and the factor Xase complexes regardless of the degree of membrane curvature and the phosphatidylinerse content. Lactadherin also inhibited the factor VIIa–tissue factor complex efficiently whereas annexin V was less effective. Because the inhibitory concentration of lactadherin was proportional to the phospholipid concentration, and because lactadherin was not an efficient inhibitor in the absence of phospholipid, the major inhibitory effect of lactadherin relates to blocking phospholipid sites rather than forming inhibitory protein-protein complexes. Lactadherin was also an effective inhibitor of a modified whole blood prothrombin time assay in which clotting was initiated by dilute tissue factor; 60 nM lactadherin prolonged the prothrombin time 150% versus 20% for 60 nM annexin V. These results indicate that lactadherin can function as a potent phospholipid-blocking anticoagulant. (Blood. 2003;101:2628-2636)

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Introduction

Lactadherin is a 47 000-Da molecular weight (MW) glycoprotein of milk-fat globules. It has also been known as PAS-6/7, indicating the 2 glycosylation variants,1 bovine-associated mucoprotein, BA-46, P47, and MFG-E8.2 Lactadherin has a domain structure of EGF1-EGF2-C1-C2 in which EGF indicates epidermal growth factor homology domains, and the C domains share homology with the discoidin family, including the lipid-binding C domains of blood coagulation factor VIII and factor V. The second EGF domain displays an Arg-Gly-Asp motif,3 which binds to the α3 and αvβ5 integrins.1,4-6 The second C domain binds to phospholipids.6

In milk-fat globules, lactadherin lines the surface of the phospholipid bilayer that surrounds the central triglyceride droplet, apparently stabilizing the bilayer.7 Lactadherin decreases the symptoms of rotavirus infection in infants, possibly by binding to rotavirus particles via carbohydrate moieties.8 In tissue sections, symptoms of rotavirus infection in infants, possibly by binding to a zona pellucida-binding protein on the acrosomal cap of sperm.11 These tissues remains unknown. Lactadherin has been identified as lactadherin concentrations of 1 to 4 nM. Binding competition correlated to functional inhibition of factor VIII–factor IXa (factor Xase) enzyme complex. In contrast to annexin V, lactadherin was an efficient inhibitor of the prothrombinase and the factor Xase complexes regardless of the degree of membrane curvature and the phosphatidylinerse content. Lactadherin also inhibited the factor VIIa–tissue factor complex efficiently whereas annexin V was less effective. Because the inhibitory concentration of lactadherin was proportional to the phospholipid concentration, and because lactadherin was not an efficient inhibitor in the absence of phospholipid, the major inhibitory effect of lactadherin relates to blocking phospholipid sites rather than forming inhibitory protein-protein complexes. Lactadherin was also an effective inhibitor of a modified whole blood prothrombin time assay in which clotting was initiated by dilute tissue factor; 60 nM lactadherin prolonged the prothrombin time 150% versus 20% for 60 nM annexin V. These results indicate that lactadherin can function as a potent phospholipid-blocking anticoagulant. (Blood. 2003;101:2628-2636)

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phospholipid-binding sites used by the factor VIII–factor IXa enzyme complex and the factor Xa–factor Va enzyme complex of the coagulation cascade so that it functions in vitro as a membrane-blocking anticoagulant.29 The well-defined membrane-binding and anticoagulant properties of annexin V make studies with annexin V suitable controls for studies with lactadherin.

The present study was undertaken to determine whether lactadherin could function as an anticoagulant by competing with factor VIII for membrane-binding sites. Our results indicate that lactadherin competes efficiently for membrane-binding sites of factor VIII, inhibiting the intrinsic pathway of coagulation. Lactadherin also inhibited the prothrombinase complex and the factor VIII–tissue factor complex, implying that lactadherin competes for membrane-binding sites of other coagulation proteins. Lactadherin also inhibited clotting of whole blood, suggesting that lactadherin is also able to inhibit coagulation reactions on cell membranes.

Materials and methods

Materials

Human factor X, human factor Xa, and human factor IXa were from Enzyme Research Laboratories (South Bend, IN); human factor V, human factor Va, and corn trypsin inhibitor were from Haematologic Technologies (Burlington, VT). Recombinant human factor VIII was a gift from D. Pittman of Genetics Institute (Cambridge, MA). Human factor VIIa, human prothrombin, and human α-thrombin were from Enzyme Research Laboratories. Both recombinant human tissue factor and lipidated recombinant human tissue factor were from American Diagnostica (Greenwich, CT). Lactadherin was a gift from Drs C. W. Heegaard and J. R. Rasmussen of the Department of Molecular and Structural Biology, University of Aarhus (Denmark). Annexin V was from Sigma (St Louis, MO). Bovine brain PS, egg yolk PE, and phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Chromogenic substrates S-2238 and S-2765 were from BioMerieux (Alabaster, AL). Lactadherin was supplied at a concentration of 1 mg/mL in phosphate-buffered saline. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining revealed only 2 bands corresponding to the previously described lactadherin doublet with approximate MWs of 47 kDa and 50 kDa. Lactadherin was stored at −80°C and aliquotted after thawing; then, each aliquot was subjected to fewer than 3 cycles of flash-freezing and rapid thawing. The purity and handling of other proteins were as previously described.30

Preparation of phospholipid vesicles

Phospholipid vesicles were prepared by evaporation chloroform from the desired phospholipids (PS:PE:PC percentage ratio, 4:20:76 and 15:20:65), resuspending in methylene chloride, and re-evaporating twice under argon. Desired phospholipids (PS-PE-PC percentage ratio, 4:20:76 and 15:20:65), Phospholipid vesicles were prepared by evaporating chloroform from the materials.

Relipidation of tissue factor

Recombinant human tissue factor was relipidated into phospholipid vesicles of the indicated composition by means of the octyl-β-D-glucopyranoside method.35 The nominal molar ratio of tissue factor to phospholipid monomer was 1:7500.

Fluorescein-Glu-Gly-Arg chloromethyl ketone labeling of factor IXa

Factor IXa was labeled with fluorescein-Glu-Gly-Arg chloromethyl ketone (Haematologic Technologies), essentially as described for the dansyl-Glu-Gly-Arg chloromethyl ketone.36 Free fluorescein-peptide was removed by ultrafiltration (Centricon 30) (Millipore). Labeling efficiency, as judged by the ratio of absorbance at 280 nm to absorbance at 490 nm, divided by extinction coefficients for factor IXa and fluorescein, was 0.2 fluorescein-peptide to 1 factor IXa.

Flow cytometry binding assay

Liposomes were prepared as previously described.16 Glass microspheres of 1.6-μm nominal diameter (Duke Scientific, Palo Alto, CA) were cleaned, size-restricted, incubated with sonicated vesicles of the indicated composition, and washed 3 times in 0.15 M NaCl, 0.02 M Tris-HCl, 0.1% defatted bovine albumin, and 10 μM egg PC as sonicated vesicles. Liposomes were stored at 4°C and used within 8 hours of synthesis. Recombinant human factor VIII and purified human factor V were labeled with fluorescein maleimide as described.37 Fluorescein-labeled factor VIII (4 nM) or fluorescein-labeled factor V (4 nM) was incubated with lactadherin or annexin V for 15 minutes at room temperature; the mixture was added to liposomes for an additional 10 minutes; and membrane-bound factor VIII or factor V was measured by flow cytometry. This procedure was performed on 25-μL aliquots of 125-nm samples with an approximate liposphere concentration of 1 × 10^9/mL by means of a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer. Data acquisition was triggered by forward light scatter with all photomultipliers in the log mode. Noise was reduced during analysis by eliminating events with forward and side scatter values different from those characteristic of the liposomes. Mean log fluorescence was converted to linear fluorescence for values depicted in the Figures. Only experiments in which the fluorescence histogram indicated a log-normal distribution, as judged by inspection, were analyzed quantitatively. Flow cytometry experiments were performed in 0.14 M NaCl, 0.02 M Tris-HCl, 1.5 or 5 mM CaCl_2 as indicated in Figure legends, and 0.1% bovine albumin, pH 7.5.

Mathematical model

Competition of lactadherin for phospholipid-binding sites of factor VIII was compared with the following model, fVIIIB/fVIIIB(max) = [fVIII]/([K_d × (1 + [lactadherin][K_d]) + [fVIII]]), where fVIIIB is membrane-bound factor VIII, fVIIIB(max) is maximum bound factor VIII when the concentration of factor VIII is saturating, K_d is the dissociation constant of factor VIII with phospholipid-binding sites, and K_d is the dissociation constant of lactadherin with the binding sites recognized by factor VIII. The model assumes that phospholipid-binding sites are limiting. For the curves depicted in Figures 1 through 3, all data were normalized to the calculated value of fVIIIB/fVIIIB(max) at the indicated concentrations of factor VIII without lactadherin. For factor Xase complex activity, fVIIIB/fVIIIB(max) was assumed to be proportional to residual activity. Curve fitting was by eye while K_d was varied, as the concentrations of factor VIII and lactadherin were known and the K_d value for factor VIII had been determined experimentally (Figure 1). The same model, substituting IV for VIII, was used for comparison with competition binding experiments using factor V and with inhibition of the prothrombinase complex.

Factor Xase assay

The activation of factor X by factor IXa in the presence of lactadherin or annexin V was measured with a 2-step amidolytic substrate assay38 with the following modifications. Factor IXa, 0.1 nM, was incubated with the
specified concentrations of factor X and varied concentrations of lactadherin and annexin V in 150 mM NaCl, 50 mM Tris (pH 7.4), 1.5 mM CaCl₂ (for 4% PS-solubilized vesicles), or 5 mM CaCl₂ (for the other vesicles), 1 nM factor VIII, and 0.1 nM thrombin for 5 minutes at 25°C; final reaction volume was 40 μL. The reaction was then stopped by the addition of EDTA (ethylenediaminetetraacetic acid) to 7 mM final concentration. The amount of factor Xa generated was determined immediately with the use of the chromogenic substrate S-2765 (0.31 mg/mL) on a Molecular Devices (Sunnyvale, CA) enzyme-linked immunosorbent assay (ELISA) plate reader in kinetic mode. The results displayed in the Figures are the means of duplicates from a representative experiment. For studies without phospholipid, 40 nM factor IXa was mixed with 40 nM factor VIII and 250 nM factor X in the reaction buffer. The reaction was started by the addition of 2 nM thrombin and 1.5 mM Ca²⁺. The reaction was allowed to proceed for 30 minutes prior to quenching with EDTA and reading, as above.

**Factor VIII–tissue factor assay**

Relipidated tissue factor, at the indicated concentration, was mixed with 100 nM factor X, 0.1 nM factor VIIa, and varied concentrations of lactadherin or annexin V. The reaction was started by addition of 1.5 mM CaCl₂ and allowed to proceed for 5 minutes at 25°C. The reaction was stopped with EDTA, and the quantity of factor Xa formed was determined as described above for the factor Xase assay.

**Prothrombinase assay**

Cleavage of prothrombin to thrombin was measured in a 2-step amidolytic assay analogous to that for factor X activation as previously described. First, 1 nM factor Va, 6.2 pM factor Xa, and lactadherin or annexin V at specified concentrations were incubated for 5 minutes at 25°C in a solution containing 150 mM NaCl, 50 mM Tris, 1.5 mM (for 4% PS, sonicated vesicles) or 5 mM (for the other PS content and/or vesicle size) CaCl₂, and 0.05% wt/vol ovalbumin, pH 7.8, prior to the addition of 1 μM prothrombin. After 5 minutes at 25°C, the reaction was stopped by the addition of EDTA to a final concentration of 7 mM. Thrombin formation was assessed in a kinetic microplate reader immediately after addition of 0.1 mM chromogenic substrate S-2238. The results displayed in the Figures are the means of triplicates from a representative experiment.

**Activated partial thromboplastin time and prothrombin time assays**

Pooled normal plasma, anticoagulated with 1:9 dilution of 3.8% citrate, was stored at −80°C until use. All reagents were prewarmed to 37°C, and assays were performed in triplicate. For the activated partial thromboplastin time (aPTT) assay, 76 μL plasma was mixed with 76 μL aPTT reagent (aPTT-SA) (Helena Laboratories, Beaumont TX) and 76 μL lactadherin diluted into Tris-buffered saline. After 10 minutes, 76 μL 25 mM Ca²⁺ was added to start the clotting reaction. Time to fibrin-strand formation was measured with a fibrometer. The aPTT reagent was either used at full strength or diluted 1:16 in 100 μM ellagic acid, as indicated (to maintain a constant ellagic acid concentration as the phospholipid was diluted).

For the prothrombin time (PT) assay, 100 μL plasma was mixed with 100 μL lactadherin in Tris-buffered saline and 100 μL PT reagent (Thromboplatin-C Plus) (Baxter, Miami, FL). Because package instructions call for use of the PT reagent at a 2:1 ratio with plasma, the PT reagent was supplemented with 16 mM Ca²⁺ to achieve the manufacturer’s intended final Ca²⁺ concentration. The PT reagent was also diluted 1:24 in 16 mM Ca²⁺, then used in the same manner as full-strength PT reagent.

**Whole-blood prothrombin time assay**

Blood was drawn from healthy, nonsmoking, non-aspirin-using volunteers by means of 19-gauge butterfly needles. The first 3 mL blood was discarded. Subsequently, 20 mL blood was gently drawn. The blood was rapidly discharged into a polypropylene tube containing sodium citrate, final concentration 10 mM. To suppress contact activation, corn trypsin inhibitor was added to the tube (final concentration, 25 μg/mL). The blood was kept at room temperature, gently mixed by inversion approximately every 10 minutes, and used within 3 hours of collection. For the clotting reaction, 150-μL aliquots of blood were prewarmed to 37°C for 10 minutes, then diluted 1:1 with 10 mM CaCl₂, the indicated concentration of lactadherin or annexin V, and 50 μL relipidated tissue factor (0.38 μM phospholipid). The time to fibrin-strand formation was monitored with a fibrometer. Experiments were performed in triplicate.

**Results**

We hypothesized that the tandem C domains of lactadherin confer phospholipid-binding properties that enable it to compete with factor VIII and/or factor V for membrane-binding sites and function as an anticoagulant. To test this hypothesis, we performed a competition membrane-binding experiment in which lactadherin competed with fluorescein-labeled factor VIII for membrane binding sites (Figure 1A). The membranes had a composition of 4% PS, 20% PE, and the balance as PC. Bilayers were supported on 2-μm-diameter glass microspheres (lipospheres), and binding of factor VIII was evaluated by flow cytometry. Lactadherin effectively competed for all factor VIII-binding sites, with half-maximal displacement occurring at approximately 1.5 nM lactadherin. The competition predicted by a mathematical model approximated the data when lactadherin was assigned a Kᵣ of 0.5 nM.

![Figure 1. Competition for factor VIII- and factor V-binding sites by lactadherin or annexin V.](image-url)

(A) 4 nM fluorescein-labeled factor VIII was mixed with the indicated concentration of competitor, lactadherin (●) or annexin V (○), in the presence of 1.5 mM Ca²⁺. Lipospheres were added, and bound factor VIII was evaluated by flow cytometry after 10 minutes. Liposphere membrane composition of PS-PE-PC was 4:20:76. The inhibition curve was modeled under the assumption that the Kᵣ of factor VIII with lipospheres was 4.8 mM; the Kᵣ (×) for lactadherin, 0.5 mM. (B) First, 4 nM fluorescein-labeled factor V was mixed with lactadherin (●) or annexin V (○), and bound factor V was evaluated under the same conditions as those in panel A. The inhibition curve was modeled under the assumption that the Kᵣ of factor V with lipospheres was 4.3 mM; the Kᵣ (×) for lactadherin, 1.0 mM. (C) Lactadherin (●) or annexin V (○) was mixed with 0.1 nM factor IXa, 1 nM factor VIII, and 100 nM factor X, prior to the addition of 1 μM sonicated vesicles with 1.5 mM Ca²⁺ and thrombin. The reaction was stopped after 5 minutes, and factor Xa was measured with chromogenic substrate S-2765 in a kinetic microplate reader. Lactadherin was an effective competitor for binding sites of factor VIII and factor V and an inhibitor of the factor Xase complex. Results displayed are from a single experiment representative of at least 2 experiments for all conditions.
For comparison with lactadherin, we asked if annexin V would compete for factor VIII–binding sites in the same assay (Figure 1A). Annexin V competed for approximately 20% of factor VIII–binding sites at a concentration of 32 nM. These results indicate that lactadherin is a more potent competitor for the phospholipid-binding sites of factor VIII on membranes with 4% PS.

To determine whether lactadherin is also able to recognize the phospholipid-binding sites of factor V, we performed similar competition experiments with fluorescein-labeled factor V (Figure 1B). Lactadherin competed efficiently for the binding sites recognized by factor V with half-maximal inhibition at an approximately 2-fold higher concentration. Annexin V competed for only 20% of the factor V–binding sites at concentrations up to 32 nM. The corresponding $K_i$ for a curve approximating the data was 1.0 nM.

We performed factor Xase assays in the presence of increasing concentrations of lactadherin to determine whether the competition for factor VIII–binding sites would translate into inhibition of the factor Xase complex (Figure 1C). The phospholipid composition of the sonicated vesicles used was the same as for the binding experiments depicted in Figure 1A. Lactadherin was a potent inhibitor of the factor Xase complex, with half-maximal inhibition at approximately 6 nM and greater than 98% inhibition at 32 nM. Annexin V was an ineffective inhibitor of the factor Xase complex with lower than 10% inhibition at 64 nM annexin V. These results confirm that lactadherin is able to inhibit the factor Xase complex, probably by competing with factor VIIIa and/or factor IXa and factor X for phospholipid-binding sites. They do not explain why annexin V, which also binds to phospholipid membranes with high affinity, is an ineffective inhibitor under these conditions.

We asked whether lactadherin is capable of inhibiting the prothrombinase complex in a manner similar to the factor Xase complex (Figure 2). Prior reports indicate that membrane binding and anticoagulant efficacy of annexin V is related to the PS content of membranes and inversely related to the curvature of phospholipid membranes. Thus, we evaluated vesicles with maximal curvature (sonicated, nominal diameter of 20 nm), intermediate curvature (extruded, nominal diameter 73 ± 25 nm), and minimal curvature (large multilamellar vesicles, diameters greater than 400

![Figure 2](image-url)

**Figure 2. Relationship of vesicle curvature and PS content to inhibition of the prothrombinase complex by lactadherin or annexin V.** Sonicated vesicles (△ and ○), extruded vesicles (● and □), and LMVs (■ and △) contained 4% PS (top row) or 15% PS (bottom row). Effective concentrations of the vesicles for supporting the prothrombinase complex were identified in phospholipid titration experiments (A,D). Subsaturating phospholipid concentrations were selected for inhibition experiments (open symbols; A,D). The indicated phospholipid composition/concentration was added to factor Xa, factor Va, prothrombin, and either lactadherin or annexin V. After 5 minutes, the reaction was quenched with EDTA, and thrombin was measured with chromogenic substrate S-2238 in a kinetic microplate reader. Lactadherin was an effective inhibitor of the prothrombinase complex regardless of membrane curvature or PS content (B,E). In panel B, $K_i$ indicates $K_i = 1$ nM. In contrast, inhibition by annexin V was inversely related to curvature and directly related to PS content (C,F). The Ca$^{2+}$ concentration was 1.5 mM for sonicated vesicles of 4% PS (△; A-C) and 5 mM for all other conditions. Results displayed are from a single experiment representative of either 2 or 3 experiments for all conditions.
inhibited approximately 90% of prothrombinase activity at 128 nM concentration, similar to results in a prior report.\textsuperscript{39,41} Annexin V was not as effective as lactadherin under any conditions evaluated. These results indicate that lactadherin efficiently inhibits the prothrombinase complex and that, in contrast to annexin V, inhibition is not closely tied to PS content or to vesicle curvature.

We asked whether inhibition of the factor Xase complex by annexin V and lactadherin may also be related to PS content of membranes and membrane curvature (Figure 3). Our results indicate that, like the prothrombinase complex, the factor Xase complex is supported at lower concentrations of sonicated vesicles than extruded vesicles or LMVs (Figure 3A,D). Also, the inhibition of the Xase activity on sonicated vesicles was approximated by a model in which lactadherin is assigned a Kd of 0.5 nM (Figure 3B), similar to the value correlating to direct competition for factor VIII-binding sites (Figure 1A). Lactadherin inhibited factor Xase complex more than 95% on all vesicle types, similar to inhibition of the prothrombinase complex (Figure 3B,E). However, the difference between the lactadherin concentrations required for inhibition of the factor Xase complex on sonicated versus extruded vesicles and LMVs was not as large as for the prothrombinase complex (Figure 2B,E). Annexin V was a more effective inhibitor of the factor Xase complex than the prothrombinase complex, with inhibition reaching 80% for LMVs of 4% PS (Figure 3C) and 95% for LMVs of 15% PS (Figure 3F). However, annexin V remained a poor inhibitor of the factor Xase complex on sonicated vesicles, with lower than 20% inhibition for 4% PS and lower than 50% for 15% PS. Together, these results indicate that lactadherin is a potent, near-complete inhibitor of the prothrombinase and factor Xase complexes on synthetic membranes regardless of membrane curvature and over a wide range of PS content.

We asked whether lactadherin might also have the capacity to inhibit the factor VIIa–tissue factor complex (Figure 4A). Recombinant tissue factor was reconstituted into vesicles containing 4% PS and 20% PE by dialyzing octylthioglucoside away from the tissue factor-phospholipid mixture. Vesicles prepared in this way have curvature comparable to extruded vesicles.\textsuperscript{42} Lactadherin inhibited the factor VIIa–tissue factor complex more than 90%. The quantity of lactadherin required for 50% inhibition varied with the tissue factor concentration and corresponding phospholipid concentration. Annexin V was a weaker inhibitor of the factor VIIa–tissue factor complex (Figure 4B), with lower than 50% inhibition at an annexin V concentration of 64 nM. These results suggest that lactadherin has the capacity to compete for membrane-binding sites of blood coagulation proteins other than factor V and factor VIII.

To directly probe the possibility that lactadherin can compete with vitamin K-dependent blood coagulation proteins for membrane binding, we asked whether lactadherin is able to compete with fluorescein-labeled factor IXa (Figure 5). Fluorescein-labeled factor IXa bound to lipospheres with a dissociation constant of approximately 0.5 μM (data not shown), consistent with prior reports of the membrane-binding affinity of factor IX/factor IXa. Unlabeled factor IXa competed with fluorescein-labeled factor IXa, indicating that membrane binding was not enhanced by derivatization by fluorescein-Glu-Gly-Arg chloromethyl ketone at the active site. Lactadherin competed with fluorescein-labeled factor IXa for membrane-binding sites to the same extent as unlabeled factor IXa. However, half-maximal inhibition occurred at approximately 4 nM lactadherin versus 300 nM factor IXa.

To confirm that the mechanism through which lactadherin inhibits membrane-bound blood coagulation complexes is via competition for membrane-binding sites, we performed experiments with varying phospholipid concentrations (Figure 6). When the phospholipid vesicle concentration was limiting, lactadherin inhibited 50% activity of the factor Xase complex (Figure 6A) and the prothrombinase complex (Figure 6B) at a concentration of approximately 2 nM. The concentration required increased for each increment in the phospholipid concentration, indicating that the required lactadherin is related to the phospholipid concentration rather than the concentrations of blood coagulation proteins. Furthermore, when the phospholipid concentration was 2.5 μM,
the factor Xase complex and prothrombinase complex maintained more than 90% activity at 8 nM lactadherin, a concentration that inhibits activity more than 90% when the phospholipid concentration is limiting. We also evaluated the effect of lactadherin on the factor VIII–factor Xa complex (Figure 6C). The concentrations of factor VIIIa, factor IXa, and factor X were chosen to be at or below their apparent K<sub>i</sub>’s or K<sub>i'</sub>’s (Michaelis constants), respectively, thus optimizing the sensitivity of the reaction to inhibitory action of lactadherin. Lactadherin caused lower than 50% inhibition at concentrations up to 512 nM. Thus, lactadherin is at least a 1000 times better inhibitor in the presence of phospholipid membranes. These results suggest that the only mechanism through which lactadherin inhibits the factor Xase and prothrombinase complexes at the concentrations employed is by competitive occupation of phospholipid-binding sites and that protein-protein complexes between lactadherin and factor VIIIa, factor Va, factor IXa, or factor X do not occur or do not significantly inhibit function of these enzyme complexes.

To further investigate the dependence of lactadherin’s inhibitory properties on phospholipid concentration, we evaluated inhibition of plasma clotting in aPTT and PT assays. Commercial aPTT and PT reagents contain high concentrations of phospholipids of unknown or unspecified composition. Lactadherin inhibited the aPTT assay by approximately 10% at a concentration of 1000 nM and had no effect at concentrations of 100 nM or lower. However, when the aPTT reagent was diluted to 6% of the original concentration, the aPTT was prolonged 5%, 20%, and 1000% at concentrations of 10, 100, and 1000 nM, respectively. Similarly, 1000 nM lactadherin inhibited the prothrombin time by less than 20% when the PT reagent was present at 50% of manufacturer’s suggested usage. However, when the PT reagent was diluted to 2% of the stock concentration, the PT was prolonged 5%, 30%, and 500% by lactadherin concentrations of 10, 100, and 1000 nM, respectively. These results are consistent with the model in which the major mechanism through which lactadherin inhibits blood coagulation enzyme complexes is through competition for phospholipid-binding sites. Further studies, with defined phospholipid membranes, will be required to determine whether the apparent K<sub>i'</sub>’s for inhibition of the isolated prothrombinase and Xase complexes correlate with inhibitory concentrations for plasma.

These results, showing inhibition of the factor Xase, the prothrombinase, and the factor VIIa–tissue factor complexes, support the hypothesis that lactadherin would inhibit the rate at which whole blood clots. To test this prediction, we used a modified whole blood prothrombin time (Figure 7). In this assay, fresh blood was anticoagulated with citrate and corn trypsin inhibitor (to minimize activation of the intrinsic pathway prior to the prothrombin time assay) in a polypropylene tube. Blood coagulation was initiated by simultaneous addition of calcium and 50 μM tissue factor prepared as in Figure 4. In the absence of lactadherin or annexin V, the time to clotting varied between 1200 and 2000 seconds for different donors. Lactadherin and annexin V led to prolongation of the clotting time, and the prolongation was similar over a concentration range of 0 to 20 nM. However, at concentrations of 40 nM and higher, lactadherin led to progressively longer inhibition of blood clotting. The clotting time was prolonged approximately 3-fold at 100 nM lactadherin, but only about 1.5-fold by annexin V. These results suggest that lactadherin is able to compete for binding sites on cell membranes to inhibit blood coagulation in a manner similar to inhibition of isolated blood coagulation complexes on phospholipid vesicles.

Discussion

Our results indicate that lactadherin binds to PS-containing membranes with sufficient affinity to compete with blood coagulation proteins. The phospholipid-binding competition makes lactadherin
a potent inhibitor of the prothrombinase, the factor Xase, and the factor VIIa–tissue factor complexes of blood coagulation. Because the quantity of lactadherin necessary to inhibit these enzyme complexes is proportional to the phospholipid concentration used, and because lactadherin does not efficiently inhibit the phospholipid-free factor VIIa–factor IXa complex, the major mechanism of inhibition involves blocking of the phospholipid surface rather than formation of inhibitory protein-protein complexes. Inhibition of whole blood prothrombin time suggests that lactadherin is able to bind to platelet membranes to inhibit blood coagulation by a mechanism similar to inhibition of reconstituted enzyme complexes on phospholipid vesicles.

The enzyme complexes of blood coagulation assemble and function efficiently only on a membrane surface. PS-containing membranes serve to increase the apparent affinity of the cofactors factor VIIa and factor Va for the enzymes factor IXa and factor X, respectively, and of the cofactor-enzyme complexes for the substrates factors X and prothrombin. The membranes also serve as allosteric activators of the enzyme-cofactor complex. PS-containing membranes also support anticoagulant activity that modulates the procoagulant activity. For example, protein C is activated by the thrombin-thrombomodulin complex on PS-containing membranes, and activated protein C inactivates factor Va and factor VIIa on PS-containing membranes. The membranes of quiescent blood cells do not display the PS necessary to enable assembly and function of the procoagulant enzyme complexes. Rather, PS is exposed only after cells are stimulated or undergo apoptosis.

In the setting of a tissue injury, the procoagulant membranes are probably on the surface of platelets that have adhered to the damaged tissues. The absence of a PS-containing phospholipid membrane effectively prevents function of the complexes. Thus, blocking the PS-containing phospholipid-binding sites on platelets appears to be a potential mechanism for preventing blood coagulation or altering the procoagulant/anticoagulant balance. Additional investigation will be necessary to determine whether lactadherin has this physiologic function.

Several proteins have been identified that can influence blood coagulation via interaction with phospholipid membranes. The hypothesis that lactadherin might function in this manner was based on the homology between the discoidin-type domains of lactadherin and those of factors VIII and V, together with the previously defined membrane-binding properties of lactadherin. Annexin V, the most thoroughly studied of these proteins, binds to PS-containing membranes with high affinity. However, annexin V binds poorly to curved membranes, requires supraphysiologic Ca\(^{2+}\) concentrations for optimal binding, and inhibits less than 80% of procoagulant function on endothelial cell membranes unless the concentration exceeds 200 nM. Likewise, annexin V is an incomplete inhibitor of the factor Xase complex on platelet membranes. Our results, indicating that inhibition of the prothrombinase complex exceeds 80% at 60 nM annexin V only when the curvature of the membrane is minimal and the PS content is 15%, are in agreement with these prior studies. The \(\beta_2\)-glycoprotein I binds to PS-containing membranes and other negatively charged lipid-containing particles such as lipoproteins. Purified \(\beta_2\)-glycoprotein I may be a more efficient inhibitor of the anticoagulant reaction in which activated protein C cleaves factor V or factor Va on a phospholipid membrane. The \(\beta_2\)-glycoprotein I bound to phospholipid is the primary antigen of lupus-type anticoagulants. When an antibody links 2 \(\beta_2\)-glycoprotein I molecules, the membrane-binding affinity is increased, and \(\beta_2\)-glycoprotein becomes a more potent in vitro anticoagulant. Whether \(\beta_2\)-glycoprotein I has a physiologic function influencing procoagulant or anticoagulant membrane interactions remains unknown. The physiologic relationship of these proteins and lactadherin to blood coagulation is a likely field for speculation and further investigation.

Factor VIII binds to sites on phospholipid membranes with remarkable specificity. The specificity is best illustrated by the failure of other lipid-binding proteins to compete with factor VIII for these sites. Even factor V, with structural homology and equivalent affinity for phospholipid membranes, competes for only a fraction of the sites recognized by factor VIII. The capacity of lactadherin to compete for membrane-binding sites of factor VIII, as well as to inhibit both the factor Xase complex and the prothrombinase complex, implies that lactadherin is more promiscuous than factor VIII with regard to phospholipid-binding sites. Inhibition of the factor VIIa–tissue factor complex indicates that lactadherin has the capacity to compete for the vitamin K–dependent proteins, factor VIIa, and/or factor X. The contrast between lactadherin and annexin V with regard to competing for membrane sites of both high- and low-PS content and varying membrane curvature indicate that lactadherin is also more promiscuous in its membrane requirements than annexin V. To facilitate understanding of these properties, we have initiated studies to characterize the membrane-binding properties of lactadherin versus those of factor VIII and factor V. The results indicate that lactadherin resembles factor VIII and factor V in specific binding to PS and curvature-dependent membrane binding. However, lactadherin differs in having a lower PS requirement and no apparent requirement for PE on membranes with low PS content. The displacement of membrane-binding properties of lactadherin versus those of factor VIII and factor V by lactadherin with the competition predicted from the simplest mathematical model (Figures 1-3) supports 2 conclusions. First, lactadherin is a better competitor for factor VIII–binding sites than for factor V–binding sites, with a \(K_i\) that is 2-fold lower. More potent competition for factor VIII–binding sites correlated with a 2-fold lower \(K_i\) for the factor Xase complex versus the prothrombinase complex, not only under conditions in which phospholipid was limiting but also when phospholipid was not limiting (Figure 6). The lower \(K_i\) implies that lactadherin binds with higher affinity.
to phospholipid-binding sites of factor VIII versus those of factor V. The second conclusion is that the experimental data did not precisely conform to the curves predicted by the mathematical model. The assumption of the model was that lactadherin competed for a single class of phospholipid-binding sites with factor VIII or factor V. In separate studies, we have further characterized the interaction of lactadherin with phospholipid-binding sites. Our results indicate that lactadherin recognizes at least 2 classes of phospholipid-binding sites, so both association and dissociation are kinetically complex events (J.S. E.W. Heegaard, J.T. Rasmussen, and G.E.G., manuscript in preparation). The ability to interact with multiple classes of phospholipid-binding sites probably explains part of the variation from the simple model as well as the mechanism underlying the capacity to compete with factor VIII and factor V with different apparent $K'_i$'s.

The concentrations of lactadherin required to inhibit the whole blood prothrombin time were somewhat higher than the concentrations necessary to inhibit isolated enzyme complexes (compare Figures 2–4 versus Figure 6). Our data do not indicate whether these apparent discrepancies in concentration reflect lactadherin binding to a plasma protein that partially competes with binding sites on the membranes of platelets or other cells or whether lactadherin may have a lower affinity for cell membranes versus phospholipid vesicles.

The results in this report suggest that lactadherin could serve as a physiologic or pharmacologic anticoagulant. In newborn calves, the plasma concentration rises from 0.07 $\mu$g/mL before feeding to 1.2 $\mu$g/mL after feeding, suggesting that intact lactadherin is absorbed across the gastrointestinal tract and that sufficient lactadherin circulates in the blood under these conditions to have a measurable in vitro anticoagulant effect. Lower concentrations of lactadherin have been measured in the serum of women with metastatic breast carcinoma, but not in the serum of healthy controls. The plasma levels in pregnant or lactating mammals have not been reported. However, it is plausible that sufficient lactadherin may be secreted into the blood or may “leak” from mammary glands to provide an anticoagulant effect during pregnancy or lactation. The relatively small size of lactadherin suggests that it freely traverses the placental barrier and could affect the procoagulant/anticoagulant balance of a developing fetus. The presence of lactadherin on the apical surfaces of secretory epithelia other than breast tissue suggests that lactadherin may circulate in blood even during the nonpregnant state. Thus, it is plausible that lactadherin could provide a physiologic anticoagulant function under a variety of circumstances. Our data suggest that lactadherin may be a candidate for development as a pharmacologic anticoagulant. Lactadherin functions at steps that are early in the coagulation pathway and is apparently more potent than annexin V, the only other tested agent that functions by a similar mechanism.

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