Binding Of Recombinant Apolipoprotein(a) to Extracellular Matrix Proteins

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Abstract
Elevated levels of lipoprotein(a), which consists of apolipoprotein(a) [apo(a)] covalently linked to a low-density lipoprotein-like moiety, is an independent risk factor for the development of atherosclerosis. We show that a recombinant form of apo(a) [r-apo(a)] binds strongly to fibronectin and fibrinogen, weakly to laminin, and not at all to von Willebrand factor, vitronectin, or collagen type IV. In contrast to the binding of plasminogen to fibrinogen, r-apo(a) binding does not appear to be mediated by lysine-dependent interactions, based on the inability of ε-aminocaproic acid concentrations up to 0.2 mol/L to significantly decrease r-apo(a) binding to fibronectin. Plasminogen competed weakly for the binding of r-apo(a) to fibronectin, whereas r-apo(a) completely abolished plasminogen binding. The 29- and 38-kd heparin-binding thermolysin fragments of fibronectin, previously identified as the lipoprotein(a) binding domains, were digested with trypsin, and a peptide that retained the ability to bind r-apo(a) was isolated; the sequence of the peptide (AVTTIAPYDLK) corresponds to the amino terminus of the 29- and 38-kd domains. A synthetic peptide with this sequence was able to compete effectively with fibronectin for r-apo(a) binding.

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could provide a means of anchoring Lp(a) within developing atheromas, thereby potentiating the atherogenic effects of this lipoprotein.

In this study we examined the ability of recombinant apo(a) [r-apo(a)] to interact with a variety of ECM components, and we further characterized the interaction of r-apo(a) with fibroactin, including the ability of plasminogen to compete with r-apo(a) for fibroactin binding, as well as identifying the sequence in fibroactin to which r-apo(a) binds.

**Methods**

**Materials**

Gelatin-sepharose, e-aminocaproic acid (e-ACA), thermolysin, trypsin, and bovine serum albumin (BSA) were obtained from Sigma. Trifluoroacetic acid was from Pierce Chemical Co, and acetonitrile was from BDH. Purified laminin, vitronectin, and collagen type IV were obtained from Telios, and von Willebrand factor was from Calbiochem. Fibrinogen and plasminogen were the generous gifts of Dr M. Nesheim, Queen's University. r-Apo(a) was purified from the cell line 293/ apo(a).24,25 The fibroactin peptide containing an r-apo(a) binding site (see below) was synthesized on an Applied Biosystems model 431A peptide synthesizer.

**Enzyme-Linked Immunosorbent Binding Assays**

The wells of microtiteration plates (Falcon) were coated with 10 μg/mL of various ECM proteins or a synthetic fibroactin peptide (see below) in bicarbonate coating buffer (50 mmol/L NaHCO₃, pH 9.6) and incubated for 16 hours at room temperature. After this and subsequent incubations the wells were washed four times with wash buffer (10 mmol/L sodium phosphate, pH 6.8, 150 mmol/L NaCl, and 0.02% [vol/vol] Tween-20). Nonspecific binding sites were blocked with 3% BSA in wash buffer for 30 minutes at 37°C. Purified r-apo(a), in a range of concentrations in wash buffer containing 4% PEG-8000 as described in the figure legends, was added to the wells and incubated for 2 hours at room temperature. The wells were subsequently washed and incubated for 1 hour at room temperature with 1 μg/mL 2G7, a monoclonal antibody directed against apo(a).24 In wash buffer containing 4% PEG-8000. After washing, the wells were incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham; 3 μg/mL in wash buffer containing 4% PEG-8000). Binding was detected by the addition of development buffer containing the substrate O-phenylenediamine dihydrochloride (0.42 mg/mL). Upon appearance of a color gradient, the color development reaction was stopped with 2 mol/L H₂SO₄, and the absorbance at 492 nm was determined using a Titertek plate reader. PEG-8000 was included in the buffers to enhance specific antigen-antibody interactions.

**Competition Experiments**

Microtiteration plates were coated with fibroactin or the fibroactin peptide as described above. A range of concentrations of either plasminogen or r-apo(a) or both plasminogen and r-apo(a) in equimolar amounts was subsequently bound to the wells as described above. In some experiments r-apo(a) binding to fibroactin was assayed in the presence of a range of concentrations of the synthetic fibroactin peptide, up to 20 μg/mL, or e-ACA, up to 0.2 mol/L. Binding of r-apo(a) to the peptide was also assayed in the presence of a similar range of concentrations of e-ACA. Bound apo(a) or plasminogen was detected using either the anti-apo(a) 2G7 monoclonal antibody or a monoclonal antibody specific for plasminogen (the kind gift of Dr Frank Castellino, University of Notre Dame). The secondary antibody and color development reaction were as described above.

**Isolation of the 29- and 38-kd Heparin-Binding Domains of Fibroactin**

Human fibroactin was isolated from plasma by the method of Vuento and Vaheri.25 The purified protein (25 mg) was digested with 0.25 mg thermolysin in 50 mL buffer (20 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, and 2.5 mmol/L CaCl₂) for 5 hours at room temperature. The reaction was terminated by the addition of EDTA to 5 mmol/L, and the digest was dialyzed against 5 mmol/L sodium phosphate, pH 6.8, at 4°C.

The digestion products were loaded on a 10 × 1.5-cm column of hydroxyapatite (BioRad) that had been equilibrated at 4°C with 0.5 mmol/L sodium phosphate, pH 6.8. The column was washed with 50 mL of the same buffer, and the fibroactin fragments were eluted with a linear gradient of sodium phosphate, pH 6.8 (0.5 to 190 mmol/L; total volume, 200 mL), at a flow rate of 15 mL/h. Fractions of 5 mL were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) by using a 10% gel.26 Fibroactin fragments were visualized by silver staining.27 Fractions that contained a mixture of both the 29- and 38-kd thermolysin fragments were pooled, dialyzed against 100 mmol/L sodium phosphate, pH 6.8, at 4°C, and reapplied to a hydroxyapatite column (10 × 1.5 cm) equilibrated with this buffer. The column was eluted by using a linear gradient of sodium phosphate, pH 6.8 (0 to 0.2 mol/L; total volume, 200 mL), with a flow rate of 15 mL/h, and 5-mL fractions were collected. Separation of the 29- and 38-kd fragments was assessed by SDS-PAGE as described above. Samples of each fraction (20 μL) from the hydroxyapatite columns were tested for their ability to bind r-apo(a) by using an enzyme-linked immunosorbent assay (ELISA) as described above.

**High-Performance Liquid Chromatography Separation of Tryptic Digests of the 29- and 38-kd Fragments**

The isolated 29- and 38-kd fragments as well as fractions containing a mixture of the two fragments were dialyzed against 200 mmol/L NH₄HCO₃, pH 8.0, and digested by addition of 1:100 (wt/wt) ratio of trypsin for 72 hours at room temperature. The tryptic digests were lyophilized, dissolved in 1 mL solvent A (0.1% trifluoroacetic acid), and filtered through a 0.22-μm Millipore filter (Millipore Corp). The digests were applied to a Superpac Pep S (Pharmacia) high-performance liquid chromatography (HPLC) reversed-phase column equilibrated with solvent A and eluted with a linear gradient from 100% solvent A to 70% solvent B (trifluoroacetic acid/H₂O/acetoniitrile, 0.1:9:90 [vol/vol/vol]) over 90 minutes at a flow rate of 0.8 mL/min. Peptides were detected by monitoring the absorbance at 218 nm, and fractions of 1 mL were collected. Each fraction was lyophilized and resuspended in 100 μL distilled water, and a sample of 20 μL was tested for its ability to bind r-apo(a) by using ELISA as described above. Fractions containing the highest r-apo(a) binding activity were subjected to automated Edman sequence analysis on an Applied Biosystems 470A gas phase sequenator.

**Results**

**Binding of r-Apo(a) to ECM Proteins**

The binding of purified r-apo(a) to various ECM proteins was measured by using an ELISA in which the ECM proteins were attached to the wells of plastic microtiteration plates as described in “Methods” (Fig 1). r-Apo(a) bound so tightly to fibrinogen and fibroactin that the binding sites on these ECM proteins appeared to be nearly saturated even at the lowest concentration of r-apo(a) that was tested (1 mmol/L). r-Apo(a) bound only weakly to laminin, and the binding was not saturated even at the highest concentration of r-apo(a) tested (3 μmol/L). The amount of r-apo(a) that bound
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nMr-apo(a)

FIG 1. Line graph showing the binding of recombinant apolipoprotein(a) [r-apo(a)] to extracellular matrix components. Purified fibrinogen, fibronectin, laminin, collagen type IV, vitronectin, and bovine serum albumin (BSA) were immobilized to the wells of microtiteration plates. A range of concentrations of purified r-apo(a) (1 nmol/L to 3 pmol/L) was added to the wells and incubated for 2 hours at room temperature. The wells were subsequently washed, and binding was detected by the addition of a monoclonal antibody specific for apo(a) as described in "Methods." The results indicate that r-apo(a) binds strongly to fibronectin and fibrinogen, more weakly to laminin, and not at all to collagen type IV or vitronectin.

Competition of Plasminogen and r-Apo(a) for Fibronectin Binding

Owing to the high degree of sequence homology that exists between apo(a) and plasminogen, it is possible that apo(a) and plasminogen bind to fibronectin in a similar manner. Experiments were therefore performed in which the binding of r-apo(a) to fibronectin was challenged with equimolar amounts of plasminogen. Control experiments (Fig 2A and 2B) demonstrated that the monoclonal antibody against apo(a) showed little cross-reactivity with plasminogen and, conversely, that the monoclonal antibody directed against plasminogen showed little cross-reactivity with r-apo(a). The epitopes recognized by these antibodies did not appear to be masked by the binding of plasminogen and apo(a) to immobilized fibronectin. When plasminogen and r-apo(a) were both present in the binding assay, r-apo(a) was found to compete very effectively for plasminogen binding to fibronectin, but plasminogen competed very poorly, if at all, for r-apo(a) binding (Fig 2C).

Effect of e-ACA on the Binding of r-Apo(a) to Fibronectin

The binding of apo(a) and plasminogen to substrates such as fibrinogen is dependent on lysine residues exposed on the substrate and can be quantitatively inhibited by lysine analogues. In keeping with previous studies, the lysine analogue e-ACA inhibited the binding of plasminogen to fibronectin (data not shown). However, increasing concentrations of e-ACA (up to 0.2 nmol/L) did not inhibit the binding of r-apo(a) to fibronectin (data not shown).

Fig 2. Line graphs showing the competition of recombinant apolipoprotein(a) [r-apo(a)] and plasminogen (pmgn) for binding to fibronectin. A, A range of concentrations of r-apo(a) (1 nmol/L to 3 pmol/L) was added to duplicate wells of microtiteration plates containing immobilized fibronectin and allowed to bind for 2 hours at room temperature. The wells were subsequently washed, and binding was detected using monoclonal antibodies (Ab) specific for either apo(a) or plasminogen as described in "Methods." Results indicate no significant cross-reactivity to apo(a) of the anti-plasminogen antibody. B, The experiment was performed as described above except that purified plasminogen was used as antigen. Results indicate no significant cross-reactivity to plasminogen of the anti-apo(a) antibody. C, The experiment was performed as described above except that equimolar amounts of both plasminogen and r-apo(a) (1 nmol/L to 3 pmol/L of each) were added to the wells. Results indicate that r-apo(a) can essentially abolish plasminogen binding to fibronectin but that plasminogen can only modestly interfere with r-apo(a) binding (compare anti-apo(a) antibody curves in A and C).
Identification of a Tryptic Peptide From the Heparin-Binding Domain of Fibronectin That Binds to r-Apo(a)

The 29- and 38-kd thermolysin fragments of fibronectin, which encompass the heparin-binding domain of the molecule, contain a binding site for Lp(a). The 29- and 38-kd fragments were isolated from a thermolysin digest of fibronectin by two rounds of chromatography over a hydroxyapatite column. This procedure yielded several fractions that contained exclusively either the 29- or the 38-kd fragment as well as a number of fractions that contained a mixture of the two fragments (Fig 4). The isolated 29- and 38-kd fragments as well as a mixture of the two fragments in approximately equal amounts were digested extensively with trypsin. The resulting digests were then subjected to reversed-phase HPLC, and each fraction that was collected was assessed for its ability to bind to r-apo(a) by ELISA (Fig 5). In all three digests the strongest binding of r-apo(a) was observed to correspond to a tryptic peptide that eluted from the HPLC column in fraction 42 (Fig 5). Samples of fraction 42 from all three digests were subjected to amino acid sequence analysis; in all cases, the same major amino acid sequence was obtained: AVTTIPAPTDLK. Comparison of this sequence with the complete sequence of fibronectin shows that the apo(a)-binding peptide represents the amino-terminus of both the 29- and 38-kd fragments.

To substantiate that the tryptic peptide identified above represents a true apo(a) binding site on fibronectin, the ability of a synthetic version of this peptide to

complement with fibronectin for binding to r-apo(a) was tested. The results showed a dose-dependent inhibitory effect of the synthetic peptide on the binding of r-apo(a) to immobilized fibronectin (Fig 6A). The addition of increasing amounts of e-ACA did not significantly inhibit r-apo(a) binding to the immobilized peptide (Fig 6B). The slight inhibition observed was comparable to that obtained when equivalent concentrations of NaCl were added to the assay (data not shown).

Discussion

While the mechanisms by which Lp(a) exerts its pathogenic effects are at present not well understood, it has been hypothesized that Lp(a) has both prothrombotic properties, by virtue of the similarity of apo(a) with plasminogen, and atherogenic properties, as a consequence of its similarity to LDL. The apo(a) component of Lp(a) can compete with plasminogen for lysine binding sites present on such substrates as fibrin/ fibrinogen and cell surfaces, and this may result in a hypercoagulable state in vivo. The LDL component of Lp(a) confers upon it the ability to be bound, internalized, and degraded in a similar fashion to LDL, and it facilitates the binding of Lp(a) to substrates abundant in developing atherosomas such as glycosaminoglycans and proteoglycans.

We studied the binding of r-apo(a) to various protein components of the ECM. Using r-apo(a) we confirmed that Lp(a) binds tightly to fibronectin, and we demonstrated that r-apo(a) binds weakly to laminin and not at all to von Willebrand factor, vitronectin, or collagen type IV. The binding of r-apo(a) to fibronectin is of a similar magnitude to that observed to fibrinogen; significant binding of Lp(a) to the latter substrate has been previously demonstrated. The ability of Lp(a) to bind to fibronectin, an early marker of atherogenesis, and thus to become anchored within developing lesions, could facilitate both the prothrombotic and atherogenic potential of Lp(a). The nature and significance of the weak interaction between r-apo(a) and laminin remains
to be characterized. Laminin is highly abundant in the basal laminae that underlie the vascular endothelium, suggesting that the ability of Lp(a) to bind this macromolecule could serve as a means of retaining Lp(a) within developing atheromas and enhancing its atherogenic effects.

In contrast to the binding of apo(a) to substrates such as fibrinogen and fibrin, apo(a) binding to fibronectin does not appear to be mediated to any significant degree by lysine affinity sites; although the lysine analogue e-ACA was able to slightly decrease r-apo(a) binding to fibronectin (Fig 3), a similar effect was achieved using an equivalent amount of NaCl. These results agree with those of Salonen et al,20 who report that the binding of Lp(a) to fibronectin is not lysine dependent. As such, this is one of the few reports of an apo(a)-substrate interaction that is not lysine dependent. Elucidation of the mechanism of apo(a) binding to fibronectin awaits the definition, using truncated r-apo(a) species, of the sequences in apo(a) that mediate this interaction.

With respect to the sequences in fibronectin that mediate binding to apo(a), we identified a peptide, present in the amino-terminus of the 29- and 38-kd heparin-binding thermolysin fragments of fibronectin, that is capable of binding to apo(a) (Fig 5). This peptide, of the sequence AVTTIPAPTDLK, is also capable of competing for fibronectin binding to r-apo(a) (Fig 6A). This sequence is present in both the soluble and insoluble forms of fibronectin as well as in all alternatively spliced isoforms of the protein.32 As with the intact fibronectin molecule, binding of r-apo(a) to the peptide is modestly inhibited by e-ACA (Fig 6B), although to a similar extent as by equivalent concentrations of NaCl. These findings suggest that r-apo(a) binding to the peptide is through a non-lysine-dependent mechanism, despite the presence of a lysine residue in the peptide sequence. The similarities in the r-apo(a)-binding properties of the peptide and intact fibronectin as well as the peptide competition studies further suggest that the peptide we have identified represents the major apo(a) binding site in fibronectin. In the ELISAs measuring r-apo(a) binding to the HPLC-separated tryptic fibronectin fragments, we observed several additional peaks (Fig 5). This indicates

Fig 5. Representative reversed-phase high-performance liquid chromatography (HPLC) profiles of the separation of tryptic peptides derived from the 29- and 38-kd fibronectin fragments and analysis of their binding to recombinant apolipoprotein(a) [r-apo(a)]. Fractions from the second hydroxyapatite column that contained (A) only the 29-kd thermolysin fragment of fibronectin, (B) only the 38-kd fragment, or (C) an approximately equal amount of each fragment were pooled, digested with trypsin, and subjected to reversed-phase HPLC as described in "Methods." Samples of each fraction were immobilized to the wells of microtitration plates, and the ability of r-apo(a) to bind to the wells was assessed using a monoclonal antibody to apo(a) as described in "Methods." A strong r-apo(a) binding peak eluting in fraction 42 resulted from all three digests. The peptide in each of these three fractions was subjected to amino acid sequence analysis and found to be identical. Solid line in panel A indicates the absorbance at 218 nm; dashed lines in each panel, enzyme-linked immunosorbent assay results.
Interestingly, while plasminogen was able to inhibit r-apo(a) binding to fibronectin only to a relatively minor extent, r-apo(a) was able to completely abolish plasminogen binding (Fig 2C). This observation may reflect steric interference of plasminogen accessibility to fibronectin owing to the large size (=500 kd) of the r-apo(a) molecule. Such interference may be a general phenomenon of the competition of Lp(a) with plasminogen for binding to various substrates in vivo; as such, Lp(a) could ablate plasminogen function to a disproportionate extent relative to their respective concentrations in plasma, hence magnifying the prothrombotic nature of Lp(a).

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References

Fig 6. Line graphs showing analysis of a fibronectin peptide representing a recombinant apolipoprotein(a) [r-apo(a)] binding site. A, Competition of the peptide for binding of fibronectin to r-apo(a). The wells of microtitration plates were coated with fibronectin, and a range of concentrations of purified r-apo(a) (60 pmol/L to 120 pmol/L) was added in the presence of a range of concentrations of the synthetic fibronectin peptide (0 to 20 muM/mL). After a 2-hour incubation at room temperature, the wells were washed, and r-apo(a) binding was detected with the r-apo(a)-specific monoclonal antibody 2G7. B, Effect of the lysine analogue e-aminocaproic acid (ACA) on binding of r-apo(a) to the immobilized fibronectin peptide. The wells of microtitration plates were coated with the synthetic fibronectin peptide (20 muM/mL), and a range of concentrations of r-apo(a) (6.8 to 68 pmol/L) was added in the presence of a range of concentrations of e-ACA (0 to 184 mmol/L). After a 2-hour incubation at room temperature the wells were washed, and r-apo(a) binding was detected with the anti-apo(a) monoclonal antibody 2G7.

that there may be other minor apo(a) binding sites in fibronectin. However, since none of these minor peaks were common to the three tryptic digests, they may represent incompletely digested forms of the peptide we identified in fraction 42. Additionally, since plasma-derived fibronectin was used in the studies reported here, we cannot exclude the possibility that additional apo(a) binding sites may be present within the additional type III repeats that are found in the insoluble forms of fibronectin.


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