Binding Of Recombinant Apolipoprotein(a) to Extracellular Matrix Proteins

Ytje Y. van der Hoo, Waheed Sangrar, Graham P. Côte, John J.P. Kastelein, Marlys L. Koschinsky

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 fibrinogen, 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 (AVTTFAPTDLK) corresponds to the amino terminus of the 29- and 38-kd domains. A synthetic peptide with this sequence was able to compete effectively with fibrinogen for r-apo(a) binding.

Key Words • apolipoprotein(a) • lipoprotein(a) • fibrinogen • extracellular matrix • atherosclerosis

lipoprotein(a) [Lp(a)] has been identified as an independent risk factor for the development of coronary heart disease (for review, see References 1 and 2). Marked inherited variability has been observed with respect to plasma Lp(a) levels, which vary from less than 1 to more than 100 mg/dL. Roughly 25% of the human population possesses Lp(a) levels above an apparent coronary risk threshold of 30 mg/dL, which more than doubles the risk of developing coronary heart disease.3-6

With respect to both lipid composition and the presence of apolipoprotein (apo) B-100, Lp(a) closely resembles low-density lipoprotein (LDL). Lp(a) is clearly distinguishable from LDL, however, by the presence of apo(a), which likely confers the unique structural and functional properties attributed to Lp(a). Human apo(a) consists of multiple tandem repeats of a sequence closely resembling plasminogen kringle IV, followed by sequences exhibiting ≈90% identity to the kringle V and protease regions of plasminogen.7 Apo(a) is covalently linked to LDL extracellularly in plasma by a single disulfide bridge mediated in apo(a) by an unpaired cysteine present in the penultimate kringle IV repeat.8 Given the high degree of similarity between Lp(a) and both plasminogen and LDL, it has been hypothesized that Lp(a) contributes to the development of atherosclerosis by both prothrombotic and proatherogenic mechanisms.9

The extracellular matrix (ECM) is composed of a complex array of glycoproteins and glycosaminoglycans. The major adhesive macromolecules include fibronectin, vitronectin, laminin, and multiple types of collagen, of which collagen type IV is the major species present in basal laminae. These components interact with a variety of integrins, in most cases through the adhesive recognition sequence RGD,10 with heparin-sulfate proteoglycans, and with other cell surface receptors. Other ECM proteins such as von Willebrand factor and fibrinogen also contain the RGD recognition sequence.

The high-molecular-weight glycoprotein fibronectin, present in an insoluble form in the ECM and in a soluble form in plasma, is involved in a wide variety of physiological functions, including the maintenance of normal cellular morphology, cell adhesion and migration, and wound healing.11 Fibronectin is composed of two highly similar polypeptides connected at their C-termini by a pair of disulfide bridges.12 Alternative splicing of the primary fibronectin mRNA transcript can give rise to as many as 10 distinct polypeptides; insoluble (cellular) fibronectin contains all the sequences present in soluble (plasma) fibronectin as well as one or two additional type III repeats.13 Fibronectin can bind to multiple substrates including fibrin, heparin, gelatin, collagen, and cell surfaces.14 Each of the binding activities of fibronectin are localized to discrete domains or regions of the molecule that can be readily isolated by limited proteolytic digestion.15,16

Fibronectin is an early marker of connective tissue formation, and elevated levels of fibronectin (both from plasma and cellular sources), fibrinogen, and fibrin are colocalized with Lp(a) in early atherosclerotic lesions and in atherosclerotic plaques.17-19 Lp(a) can bind to fibronectin20 and fibrin21,22; binding to these substrates

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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 fibronectin, including the ability of plasminogen to compete with r-apo(a) for fibronectin binding, as well as identifying the sequence in fibronectin to which r-apo(a) binds.

Methods

Materials

Gelatin-sephrose, 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 Teliios, 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 fibronectin 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 microtitration plates (Falcon) were coated with 10μg/mL of various ECM proteins or a synthetic fibronectin 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 0.5 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

Microtitration plates were coated with fibronectin or the fibronectin 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 fibronectin was assayed in the presence of a range of concentrations of the synthetic fibronectin 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 Fibronectin

Human fibronectin was isolated from plasma by the method of Vuento and Vaheiri.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 fibronectin 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 Fibronectin 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 (0.5 to 0.2 mol/L; total volume, 200 mL), at 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 a 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 Millex-GV filter (Millipore Corp). The digests were applied to a Superpach Pep S (Pharmacia) high-performance liquid chromatography (HPLC) reversed-phase column equilibrated in solvent A and eluted with a linear gradient from 100% solvent A to 70% solvent B (trifluoroacetic acid/25% acetonitrile, 0.1:9.9:90 [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 redissolved 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 microtitration plates as described in “Methods” (Fig 1). r-Apo(a) bound so tightly to fibrinogen and fibronectin 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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1 10 100 1,000 10,000
nMr-apo(a)

FIG 1. Line graph showing the binding of recombinant apo-
lipoprotein(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 microtitration 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.

to wells coated with vitronectin or collagen type IV was
no greater than the amount that bound to the negative
control (BSA). Additional experiments demonstrated
that r-apo(a) also did not bind to purified von Wille-
brand factor (data not shown).

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 anti-
body against apo(a) showed little cross-reactivity with
plasminogen and, conversely, that the monoclonal anti-
body 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.

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 pre-
vious 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

FIG 2. Line graphs showing the competition of recombinant
apolipoprotein(a) [r-apo(a)] and plasminogen (pmgn) for bind-
ing to fibronectin. A, A range of concentrations of r-apo(a) (1
nmol/L to 3 pmol/L) was added to duplicate wells of microtitra-
tion plates containing immobilized fibronectin and allowed to
bind for 2 hours at room temperature. The wells were subse-
quently 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 substituted for r-apo(a). Results Indi-
cate no significant cross-reactivity to plasminogen of the antl-
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 plasmino-
gen binding to fibronectin but that plasminogen can only mod-
estly interfere with r-apo(a) binding [compare anti-apo(a) anti-
body 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).20 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 assayed 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 when equivalent concentrations of NaCl were used (data not shown).

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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.9 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,33 and it facilitates the binding of Lp(a) to substrates abundant in developing atherosomas such as glycosaminoglycans and proteoglycans.34

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,20 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.20 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 ε-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, 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. As with the intact fibronectin molecule, binding of r-apo(a) to the peptide is modestly inhibited by ε-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
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.²³ 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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