Elevated plasma concentrations of lipoprotein(a) are a risk factor for the development of a variety of atherosclerotic disorders. Despite intensive study, the mechanisms by which lipoprotein(a) promotes these disorders remain to be unequivocally defined. It has been demonstrated that lipoprotein(a), through its unique constituent apolipoprotein(a) (apo(a)), stimulates vascular smooth muscle cell (SMC) migration and stimulates matrix metalloproteinase (MMP-2) activity, which may play a role in the pathogenesis of atherosclerotic disease (1). However, the relationship between the expression of apo(a) and the resulting pathogenic effect remains to be defined. Here we report that apo(a) inhibits pericellular plasminogen activation and stimulates vascular smooth muscle cell (SMC) migration and proliferation. These effects arise from the ability of apo(a) to inhibit the formation of active transforming growth factor-β (TGF-β) from its latent precursor, which in turn is caused by the ability of apo(a) to decrease the formation of plasmin from its precursor plasminogen. We utilized a battery of recombinant apo(a) variants that represent systematic deletions of the various domains in the molecule to further probe the mechanism underlying the effect of apo(a) on SMC responses. All recombinant apo(a) variants that contained kringle IV type 9 were able to stimulate SMC proliferation and migration and to decrease the formation of active TGF-β; conversely all recombinant apo(a) variants lacking kringle IV type 9 had no effect on these parameters. The kringle IV type 9-dependent effects of apo(a) on SMC proliferation required the presence of plasminogen, suggesting for the first time that this kringle mediates the ability of apo(a) to inhibit pericellular plasmin formation.

Elevated plasma concentrations of lipoprotein(a) (Lp(a)) constitute an emerging risk factor for the development of a variety of atherosclerotic and thrombotic disorders (1, 2). Despite intensive investigations, the true biological role of this lipoprotein and the mechanisms by which it exerts its pathogenic effects remain to be defined.

Lp(a) consists of a lipoprotein moiety that is essentially indistinguishable from low density lipoprotein as well as the unique glycoprotein apolipoprotein(a) (apo(a)) (1, 2). Apo(a) is covalently linked, via a single disulfide bond, to apolipoprotein B in the Lp(a) particle (3, 4). Apo(a) bears a striking homology to plasminogen as it is composed of a large number of repeated motifs closely related to kringle 4 of plasminogen as well as single copy domains homologous to the kringle 5 and latent protease of plasminogen (5). Unlike plasminogen, however, apo(a) cannot be activated by plasminogen activators and lacks proteolytic activity (6).

Thus, the pathogenic mechanisms of Lp(a) are generally assumed to arise from the dual homology of Lp(a) to low density lipoprotein and plasminogen. Indeed Lp(a) has been shown to accumulate in atherosclerotic plaques, to be able to contribute to foam cell formation, and to be able to inhibit fibrinolysis and hence exacerbate intravascular thrombosis (for reviews, see Refs. 2 and 7). However, some effects of Lp(a) appear to be unique in that they do not appear to follow from the homology of this lipoprotein to low density lipoprotein or plasminogen. Examples include the stimulation of leukocyte adhesion molecule expression (8, 9), stimulation of actin cytoskeletal rearrangements in endothelial cells (10), induction of monocyte chemoattractant activity in endothelial cells (11), induction of interleukin-8 expression in THP-1 macrophages (12), potentiation of platelet aggregation (13), and inhibition of tissue factor pathway inhibitor (14). An effect of Lp(a) that may be of particular significance to atherosclerotic disorders is the ability of this lipoprotein, through its apo(a) moiety, to inhibit the formation of active transforming growth factor-β (TGF-β) (15–17). It has been demonstrated that this effect of Lp(a) and apo(a) stimulates vascular smooth muscle cell (SMC) migration (15) and proliferation (16). It has been proposed that this results from inhibition by apo(a) of pericellular plasminogen activation (15–17); plasmin cleaves latent TGF-β to release the bioactive TGF-β homodimer (18). However, inhibition of pericellular plasminogen activation by apo(a) has never been directly demonstrated. In addition, the domains in apo(a) that are responsible for its effects on SMC behavior remain to be defined.

The plasminogen kringle 4-like domains in apo(a) are present in 10 types that differ in amino acid sequence (5). Apo(a) kringle IV (KIV) types 1 and 3–10 are present in single copies in all apo(a) isoforms (19, 20). KIV type 2 is present in varying copy numbers (from 3 to >40) (21, 22), which gives rise to the Lp(a) isoform size variation observed in the human population. We utilized a battery of recombinant apo(a) variants in which individual domains are systematically deleted to define the
sequences in apo(a) that mediate stimulation of smooth muscle cell proliferation and migration. We demonstrate a key role for apo(a) KIVn: this is the first report of a functional role for this kringle beyond mediating assembly with apolipoprotein B. The apparent requirement of this kringle for inhibition of pericellular plasminogen activation by apo(a) is in contrast to the domain requirements for inhibition by apo(a) of plasminogen activation on fibrin, suggesting different inhibitory paradigms in these two milieus.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Apo(a) Variants—The recombinant apo(a) (r-apo(a)) variants used in this study are shown schematically in Fig. 1A. Details of the construction of the expression plasmids encoding the respective variants have been described previously: 17K (23); 6K (KIV1–6), KIV5–6, KIV5–7, KIV6–7, and KIV10–16 (24); KIV10–16 (17KΔD56A) (17KΔα), and 17KΔP (25); and KIV5–9 (26). All expression plasmids were constructed in the pRK5 vector (23), which contains the cytomegalovirus promoter and SV40 transcription termination signal. An expression plasmid encoding apo(a) KIV types 1–4 (KIV1–4) was constructed as follows. The expression plasmid pRK5ha17 (encoding 17K) (23) was digested with EcoRI and SalI to excise the apo(a) cDNA. The insert was replaced by an EcoRI/SmaI fragment (encoding the apo(a) signal sequence, KIV7, eight copies of KIV, and all but the last 4 amino acids of KIV5) and an EcoRV/Sall fragment (encoding the last 4 amino acids of KIV and the entire protease domain but containing a stop codon after KIV) in a three-part ligation.

**Stable Cell Lines**—Stable cell lines expressing the respective r-apo(a) variants were generated in human embryonic kidney 293 cells by the transient transfection method previously described (23). For expression of recombinant proteins, stable lines were grown in OptiMEM (Invitrogen); conditioned medium containing apo(a) cDNA. The insert was replaced by an EcoRI/SmaI fragment (encoding the apo(a) signal sequence, KIV7, eight copies of KIV, and all but the last 4 amino acids of KIV5) and an EcoRV/Sall fragment (encoding the last 4 amino acids of KIV and the entire protease domain but containing a stop codon after KIV) in a three-part ligation. The r-apo(a) variant KIV1–4 was purified by lectin-Sepharose affinity chromatography followed by gel filtration chromatography. Briefly, conditioned medium containing r-apo(a) was collected every 48 h. All r-apo(a) variants, with the exception of KIV1–4, were purified from conditioned medium using lysine-Sepharose affinity chromatography as follows. Conditioned medium was passed over a 50-ml lysine-Sepharose column equilibrated in phosphate-buffered saline containing 0.5 mM NaCl and 0.2 mM ε-aminoacetic acid. Protein-containing fractions were pooled, diafiltered extensively against 20 mM HEPES, pH 7.4, 0.15 M NaCl at 4 °C, and concentrated with polyethylene glycol 20,000. The purified proteins were aliquoted and stored at −70 °C.

The r-apo(a) variant KIV5–9 was purified by lectin-Sepharose affinity chromatography followed by gel filtration chromatography. Briefly, conditioned medium was applied to a 50-ml lectin-Sepharose (Amersham Biosciences) column equilibrated in 20 mM HEPES, pH 7.4, 1 M NaCl. The column was washed with this buffer, and KIV5–9 was eluted with this buffer containing 0.5 M N-acetyl-d-glucosamine. Protein fractions containing r-apo(a) were pooled and concentrated with polyethylene glycol 20,000. The concentrated sample was then subjected to gel filtration chromatography on a Sepharose 4B column developed with 20 mM HEPES, pH 7.4, 0.5 M NaCl. Fractions containing KIV5–9 were pooled, dialyzed against 20 mM HEPES, pH 7.4, 0.15 M NaCl at 4 °C, and concentrated with polyethylene glycol 20,000. The purified protein was aliquoted and stored at −70 °C.

**RESULTS**

**Recombinant Apo(a) Stimulates SMC Proliferation**—Previous studies have demonstrated that Lp(a), through its apo(a) component, stimulates human vascular SMC proliferation (16). To identify the domain or domains in apo(a) that mediate these effects, we utilized a battery of r-apo(a) variants representing...
systematic deletions of key domains in the molecule (Fig. 1A). All variants were expressed in human embryonic kidney cells and were purified to homogeneity (Fig. 1B) from conditioned medium harvested from stably expressing cell lines. The 17K r-apo(a) contains all of the domains found in apo(a) and in fact represents a physiologically relevant isoform (23). The asterisk over kringle IV type 10 in 17KAsp denotes the fact that this kringle contains an Asp → Ala substitution at position 56 that abolishes its strong lysine-binding site. B, purified r-apo(a) proteins (1.0 μg of each) were subjected to SDS-PAGE on a 4–20% polyacrylamide gradient gel. Protein bands were visualized by staining with Coomassie Brilliant Blue. The positions of molecular mass standards are shown to the right of the gel.

Recombinant Apo(a) Variants Used in these Studies—A schematic diagram of the topology of the r-apo(a) variants is shown (Fig. 2). The 17K r-apo(a) represents a physiologically relevant apo(a) isoform and contains all of the kringle domains present in all apo(a) isoforms. Kringle IV type numbering is provided at the top. The bar over kringle KIV type 9 represents the unpaired cysteine residue in this kringle. The asterisk over kringle IV type 10 in 17KAsp denotes the fact that this kringle contains an Asp → Ala substitution at position 56 that abolishes its strong lysine-binding site.

Effect of Apo(a) Domains on TGF-β Activation—To demonstrate that TGF-β was capable of altering SMC migration speed under our experimental conditions, we performed digital time lapse video microscopy on SMCs that had been treated with exogenous active TGF-β or with a neutralizing monoclonal antibody against active TGF-β. As controls, cells were left untreated or were treated with mouse IgG of the same isotype as the anti-TGF-β antibody. Addition of exogenous TGF-β had no effect on SMC migration speed, while neutralizing the existing active TGF-β associated with the cells resulted in a significant increase in SMC migration speed (data not shown). These findings indicate that alteration in the concentration of TGF-β can influence the migration speed of the SMCs under these conditions.

We next assessed whether apo(a) could influence the amount of active and/or latent TGF-β produced by SMCs and what domains in apo(a) were responsible for such an effect. We treated SMCs with the different r-apo(a) variants for 96 h and tested the amount of latent and active TGF-β in the conditioned medium using a mink lung epithelial cell bioassay. Total (latent plus active) TGF-β was determined by acidification of the conditioned medium prior to bioassay. None of the r-apo(a) variants had a significant effect on total TGF-β, indicating that r-apo(a) likely does not impact TGF-β biosynthesis and secretion (Fig. 6A). However, all but three of the r-apo(a) variants did significantly decrease the amount of active TGF-β present in the conditioned medium.
in the conditioned medium (Fig. 6B), suggesting that r-apo(a) affects the activation of latent TGF-β in our SMC cultures. The three r-apo(a) variants that did not have an effect on active TGF-β levels are the same three variants lacking KIV9 that also did not stimulate SMC proliferation or migration (Figs. 3 and 4).

It has previously been reported that apo(a) results in a decrease in cell-associated plasmin activity presumably via inhibition of plasminogen activation, which could explain the ability of apo(a) to result in a decrease in TGF-β activation. In contrast to published reports (15, 16), we were unable to reliably quantify the amount of cell-associated plasmin activity in our cell cultures. As an alternative strategy, we depleted fetal calf serum of plasminogen by chromatography over lysine-Sepharose and then tested the ability of r-apo(a) variants to stimulate SMC proliferation in the absence of plasminogen. The SMCs were exposed to different r-apo(a) variants at a concentration of 50 nM for 96 h in the presence of complete or plasminogen-deficient serum. The results (Fig. 7) indicate that SMC proliferation is generally decreased in the absence of plasminogen in the serum and that no apo(a) species was able to stimulate SMC proliferation under these conditions. Consistent with the results presented above (Fig. 3), apo(a) species containing KIV9 did significantly stimulate SMC proliferation in the presence of normal serum.

While strong evidence continues to emerge from retrospective case-control and prospective studies that elevated plasma concentrations of Lp(a) are an important risk factor for coronary artery disease and other atherosclerotic disorders (1), the mechanisms underlying these observations remain elusive. It is critical to elucidate the pathogenic mechanisms of Lp(a) to identify potential targets for therapeutic intervention. Since plasma Lp(a) levels are relatively resistant to lowering by lifestyle changes or pharmacological treatment (1, 31), this strategy would constitute an important alternative to therapies aimed at lowering plasma levels of Lp(a). A particularly promising avenue of research is to identify domains in apo(a) that mediate the unique pathogenic effects of this lipoprotein; the role of these domains in atherosclerosis can then be directly tested in animal models expressing recombinant human apo(a) species containing mutations in these domains or lacking these domains altogether. A potentially important pathological effect of Lp(a) is its ability to inhibit plasmin-dependent TGF-β activation and thus to stimulate vascular smooth muscle cell proliferation and migration (15–17). We demonstrate here that these effects are dependent on the presence of apo(a) kringle IV type 9.
Our findings both confirm and extend the reports of Kojima et al. (15) and Grainger et al. (16). Kojima et al. (15) reported that addition of Lp(a) to cocultures of bovine aortic endothelial and smooth muscle cells resulted in a decreased activation of latent TGF-β, and as a result conditioned medium harvested from Lp(a)-treated cocultures was capable of stimulating endothelial or smooth muscle cell migration in in vitro wound assays. Grainger and co-workers (16) reported that Lp(a) and the
apo(a) component of this lipoprotein increased human and rat vascular smooth muscle cell proliferation in vitro and that Lp(a) and apo(a) decreased the amount of active TGF-β in the medium of these cells without affecting the amount of latent TGF-β. Our findings serve to unify these observations in that we showed that stimulation of smooth muscle cell migration and proliferation as well as inhibition of TGF-β activation are all uniformly dependent specifically on the presence of apo(a) kringle IV type 9. Using digital time lapse video microscopy we showed for the first time that apo(a) directly increases the speed of smooth muscle cell migration. Kojima and co-workers (15) reported that when Lp(a) was added directly to smooth muscle cell cultures, the lipoprotein had no effect on the basal migration of the cells. However, since these incubations were performed in the absence of serum, the lack of effect of Lp(a) can be attributed to the absence of plasminogen on which latent TGF-β activation is dependent under these conditions.

It has recently been reported that apo(a) kringle IV type 10, expressed as a glutathione S-transferase fusion protein in Escherichia coli, induces smooth muscle cell growth, while a mutant version of the kringle in which the lysine-binding site has been abolished has no effect (32). Moreover the wild-type kringle decreased the amount of active TGF-β in the cell medium without affecting the concentration of latent TGF-β; the mutant kringle had no effect on active TGF-β levels (32). These results are surprising in light of the fact that the incubations of the kringles with the smooth muscle cells were done in the absence of serum. As such, an apo(a)-dependent inhibition of plasmin-mediated TGF-β activation would not be possible under these conditions. It is possible that in the absence of serum, the apo(a) kringle is able to affect TGF-β activation by a different mechanism. The apo(a) kringle IV type 9-dependent effects on smooth muscle cell proliferation documented in our study require the presence of plasminogen (Fig. 7). It is possible that the effects

**Fig. 5.** Effect of r-apo(a) on SMC migration speed as assessed by digital time lapse video microscopy. **A**, human aortic SMCs were incubated for 96 h in the presence of a 50 nM concentration of the indicated r-apo(a) variants or in the absence of r-apo(a). The cells were then seeded in 35-mm plates for observation of their migration. Depicted for each condition are the hourly migration paths of 10 cells over a period of 8 h. Distances from the origin are in μm. **B**, the migration speeds of the cells (10 cells/condition) depicted in **A** were determined by dividing the sum of the distances traveled each hour by the total time elapsed and are displayed on the graph as the means and S.D. The asterisks represent migration speeds that are significantly different (p < 0.05) from the migration speed in the absence of r-apo(a).
of apo(a) kringle IV type 10 are masked when this kringle is part of a larger apo(a) molecule or in the presence of serum.

Morishita and co-workers (33) reported that expression of recombinant apo(a) directly in vascular smooth muscle cells had no effect on the proliferation of these cells. However, these proliferation assays were performed at an extremely low concentration of fetal calf serum (0.05%). Indeed these investigators could not detect active TGF-β in conditioned medium from these cells, while latent TGF-β was present in abundance (33). On the other hand, conditioned serum-free medium harvested from HepG2 (human hepatoma) cells transfected with an apo(a) expression plasmid was able to stimulate smooth muscle cell proliferation (33). This was taken as evidence that apo(a) could stimulate smooth muscle cell proliferation in a manner independent of an effect on plasmin-mediated TGF-β activation. However, the concentration of active TGF-β in this HepG2 cell conditioned medium was not measured. Moreover, it is possible that expression of endogenous plasminogen by the hepatoma cells would allow for the formation of plasmin and thus the activation of TGF-β.

**FIG. 6.** Apo(a) decreases TGF-β activation, and kringle IV type 9 is required for this effect. Human aortic SMCs were incubated for 96 h in the presence of a 50 nM concentration of the indicated r-apo(a) variants or in the absence of r-apo(a). Conditioned medium was harvested from the cells, and the concentrations of total (latent plus active) TGF-β (A) or active TGF-β (B) were determined using a mink lung epithelial cell bioassay. For determination of total TGF-β, the conditioned medium was acidified prior to TGF-β bioassay. The data correspond to the mean and S.D. of TGF-β determinations of conditioned medium from triplicate wells and are representative of at least two independent experiments. The asterisks represent active TGF-β concentrations that are significantly different (p < 0.05) from those in the absence of r-apo(a).
Apo(a) Kringle IV Type 9 and Smooth Muscle Cell Behavior

The cell surface provides a means to exert local control over the elaboration of plasmin activity. Cells promote plasmin formation both by stimulating the rate of plasminogen activation and by binding the plasmin that is formed thus protecting it from consumption by its cognate inhibitors (for a review, see Ref. 34). It is well established that Lp(a), through its apo(a) moiety, inhibits the binding of plasminogen to cell surfaces (35, 36). On the basis of this, it has been speculated that Lp(a)/apo(a) could be capable of inhibiting cell surface-associated plasminogen activation, although such an effect has yet to be directly demonstrated. Although we were unable to reproduce previous findings that Lp(a)/apo(a) caused a decrease in the amount of cell-surface associated plasmin activity (15, 16), the apo(a) kring IV type 9-dependent effects on smooth muscle cell proliferation described in this report are clearly dependent on the presence of plasminogen (Fig. 7). As such, it is clear that this kring plays a key role in the ability of apo(a) to modulate the rate of plasmin formation. The molecular details of this effect remain to be elucidated.

Seminal recent studies by Gong et al. (37) indicate that the predominant effect of cell surfaces on plasminogen activation is through promotion of the rate of conversion of Glu-plasminogen to Lys-plasminogen; the latter is a much more efficient substrate for plasminogen activators. It appears that there is only a minor role for the ability of cell surfaces (i) to bind Glu-plasminogen and promote a conformational change that accelerates its activation to plasmin and (ii) to increase the local concentrations of plasminogen and its activator (34). The simplest explanation for the effect of apo(a) on plasminogen activation is that it competes with Glu-plasminogen for binding to cell surface receptors; binding to these receptors presumably induces a conformational change in Glu-plasminogen that accelerates the rate at which it is converted to Lys-plasminogen by plasmin (37). However, it cannot be excluded at this time that competition between Lp(a)/apo(a) and plasmin for cell binding increases consumption of plasmin.

Interestingly it is clear that the domains in apo(a) responsible for inhibiting plasminogen activation in the fibrin clot milieu are very different from those responsible for inhibiting plasminogen activation in the pericellular milieu. In a recent study we used our battery of recombinant apo(a) variants to identify the domain(s) in the molecule responsible for inhibiting tissue plasminogen activator-mediated plasminogen activation in the context of fibrin clot (38). In the context of a fibrin clot, apo(a) KIV and kring IV types 1–4 are absolutely required for inhibition of plasminogen activation, while the lysine-binding site in kring IV type 10 also plays a significant role. Variants such as KIV5–P, KIV9–P, KIV9–10, and KIV5–9, which are fully capable of decreasing TGF-β activation and SMC responses, are significantly or completely impaired in their ability to inhibit plasminogen activation in the context of a fibrin clot. We used a kinetic approach to gain insight into the mechanism by which apo(a) inhibits tissue plasminogen activator-mediated plasminogen activation in the presence of fibrin (38). Our data are consistent with a model in which apo(a) binds to the ternary catalytic complex of tissue plasminogen activator, plasminogen, and fibrin cofactor that is required for maximally efficient plasminogen activation; the resultant quaternary complex has a reduced turnover number compared with the ternary complex. Clearly, the different domain requirements for inhibition of plasminogen activation in the fibrin clot and pericellular milieu reflect the distinct roles for fibrin and the cell surface as cofactors for tissue plasminogen activator-mediated plasminogen cleavage and plasmin-mediated Glu- to Lys-plasminogen conversion, respectively.

Apo(a) kring IV type 9 contains the cysteine residue involved in covalent bond formation with apoB in the Lp(a) particle (3, 4). This is the first report of a function for kring IV type 9 beyond its role in Lp(a) assembly. Previous studies by our group have revealed the presence of a functional lysine-binding site in this kring (39). This lysine-binding site is on a different face of the molecule than the site for apoB binding and thus may maintain its lysine binding function even in the context of the Lp(a) particle (39). While apo(a) kring IV types 5–8 and type 10 also bind lysine (40), it is possible that kring IV type 9 has the unique ability to mediate cell surface binding of Lp(a)/apo(a).
Smooth muscle cell migration and proliferation play key roles in the development of atherosclerosis and in arterial remodeling after interventions such as balloon angioplasty and stenting. However, while the preponderance of the evidence indicates that elevated concentrations of Lp(a) is a significant risk factor for coronary artery disease events and ischemic stroke (1), the association of Lp(a) levels with restenosis remains controversial (Ref. 41 and references therein). It is important to note that inhibition of TGF-β activation could result in numerous proatherosclerotic effects beyond stimulation of smooth muscle cell responses. TGF-β is a potent anti-inflammatory and profibrotic factor (42, 43). Thus, inhibition of TGF-β activation would result in increased invasion of the developing atheroma by inflammatory cells and therefore (i) a decrease in plaque stability due to degradation of the extracellular matrix and promotion of smooth muscle cell death and (ii) an increase in plaque thrombogenicity following rupture due to the presence of apoptotic cells and microparticles. Decreased TGF-β activation could also destabilize the plaque by inhibiting extracellular matrix synthesis by plaque-resident smooth muscle cells. Our identification of kringle IV type 9 as the domain in apo(a) that mediates inhibition of TGF-β activation provides the opportunity to directly test the significance of this function of apo(a) in animal models of atherosclerosis by deletion or mutagenesis of this domain.

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