Occlusive Arterial Thrombosis in Cynomolgus Monkeys With Varying Plasma Concentrations of Lipoprotein(a)

J. Koudy Williams, Dwight A. Bellinger, Timothy C. Nichols, Thomas R. Griggs, Thomas F. Bumol, Rebecca L. Fouts, and Thomas B. Clarkson

Lipoprotein(a) (Lp[a]) is a newly recognized risk factor for the development of coronary heart disease and stroke in human beings; however, the mechanisms by which Lp(a) increases the risk of coronary heart disease remain unclear. The purpose of this study was to examine the effects of Lp(a) on the occurrence of occlusive arterial thrombosis. Occlusive arterial thrombus formation was examined in 18 cynomolgus monkeys with high plasma Lp(a) concentrations (>35 mg/dL, n=6), intermediate Lp(a) concentrations (20–25 mg/dL, n=6), and low Lp(a) concentrations (<12 mg/dL, n=6). A Goldblatt clamp was positioned around the left common carotid artery to produce a stenotic segment, and the artery was pinch-injured with needle holders. A 20-MHz Doppler velocity crystal, placed distal to the stenosis/injury site, was used to detect cyclic flow reductions (indicative of transient thrombosis) or permanent cessation of flow velocity (indicative of more stable occlusive thrombosis). All monkeys with high Lp(a) concentrations developed permanent cessation of flow, whereas only one of six arteries from low-Lp(a) monkeys developed permanent cessation of flow (p<0.05). Arteries from monkeys with intermediate Lp(a) concentrations developed pronounced cyclic reductions of flow but did not progress to permanent cessation of flow. There were no differences in plasma von Willebrand factor activity among the three groups. Immunohistochemical analysis of the damaged arterial segments indicated incorporation of Lp(a) into the adventitia, media, and intima of arteries from monkeys with low and high plasma Lp(a) concentrations, as well as the presence of an occlusive thrombus in arteries that developed permanent cessation of flow. It is concluded that plasma concentrations of Lp(a) may influence the development of occlusive arterial thrombosis of stenotic or injured arteries. (Arteriosclerosis and Thrombosis 1993;13:548–554)

KEY WORDS • cynomolgus monkeys • lipoprotein(a) • platelets • thrombosis • von Willebrand factor

There is substantial evidence of a significant association between plasma concentrations of lipoprotein(a) (Lp[a]) greater than 20 mg/dL and coronary heart disease and cerebrovascular disease in human beings.1–3 The mechanisms by which Lp(a) increases the risk of coronary heart and cerebrovascular disease remain unclear. Lp(a) has a high affinity for fibrin and can compete with both plasminogen and tissue-type plasminogen activator for binding to plasmin.4–6 Lp(a) also interferes with the net fibrinolytic process.4,7 Results of in vitro studies have indicated that Lp(a) may aid in the formation and inhibit the lysis of an arterial thrombus.8–10 However, no in vivo studies have been done to determine whether relative differences in plasma Lp(a) increase the risk of occlusive arterial thrombosis. Therefore, the objective of this study was to determine in vivo whether cynomolgus monkeys with high circulating plasma concentrations of Lp(a) were more prone to formation of an occlusive thrombus in an injured artery than monkeys with intermediate or low plasma concentrations of Lp(a).

Methods

Animal Model

Eighteen male cynomolgus monkeys (Macaca fascicularis) were used in this study. Monkeys ranged in age from 3 to 12 years (estimated by dentition) and had been fed commercial laboratory chow (Purina Monkey Chow, Ralston Purina Co.) since weaning at 6 months of age. All procedures involving animals were conducted in compliance with state and federal laws, standards of the Department of Health and Human Services, and guidelines established by the Institutional Animal Care and Use Committee.

Thirty monkeys selected randomly from a colony of cynomolgus macaques were classified by plasma Lp(a) concentrations. Of these, 18 monkeys were chosen for

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High-Lp(a) group and Effects of Lp(a) on Thrombosis

Determination of von Willebrand Factor Activity

Plasma Lp(a) concentrations were determined using an enzyme-linked immunosorbent assay for Lp(a) that was developed at the Bowman Gray School of Medicine Lipoprotein Core Laboratory.

Plasma concentrations of Lp(a) in monkeys are shown in Table 1. Monkeys designated as Hi Lp(a) (n=6) had plasma concentrations of Lp(a) ranging from 20 to 25 mg/dL. Monkeys designated as Int Lp(a) (n=6) had plasma concentrations of Lp(a) ranging from 38 to 48 mg/dL. Monkeys designated as Lo Lp(a) (n=6) had plasma concentrations of Lp(a) ranging from 1 to 11 mg/dL.

Table 1. Categorization of Monkeys by Lp(a) Concentrations and Effects of Lp(a) on Thrombosis

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Lp(a) concentration (mg/dL)</th>
<th>Nature of flow reduction</th>
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<tr>
<td></td>
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<td>CFR</td>
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<td>High-Lp(a) group</td>
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<tr>
<td>1</td>
<td>42</td>
<td>+</td>
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<tr>
<td>2</td>
<td>42</td>
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<tr>
<td>3</td>
<td>43</td>
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<tr>
<td>4</td>
<td>48</td>
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<td>5</td>
<td>38</td>
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</tr>
<tr>
<td>6</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>Intermediate-Lp(a) group</td>
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<tr>
<td>1</td>
<td>25</td>
<td>++</td>
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<tr>
<td>2</td>
<td>22</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>21</td>
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<tr>
<td>6</td>
<td>25</td>
<td>++</td>
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<tr>
<td>Low-Lp(a) group</td>
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<tr>
<td>1</td>
<td>5</td>
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<td>5</td>
<td>11</td>
<td>+</td>
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<tr>
<td>6</td>
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</table>

Lp(a), lipoprotein(a); CFR, cyclic flow reduction; PCF, permanent cessation of flow.

*Second injury.

+, Weak or irregular CFR; ++, more pronounced, regular CFR.

Further analysis. Plasma Lp(a) concentrations were determined using an enzyme-linked immunosorbent assay for Lp(a) that was developed at the Bowman Gray School of Medicine Lipoprotein Core Laboratory.

Plasma concentrations of Lp(a) in monkeys are shown in Table 1. Monkeys designated as Hi Lp(a) (n=6) had plasma concentrations of Lp(a) ranging from 38 to 48 mg/dL. Monkeys designated as Int Lp(a) (n=6) had plasma Lp(a) concentrations ranging from 20 to 25 mg/dL. Monkeys designated as Lo Lp(a) (n=6) had plasma concentrations of Lp(a) ranging from 1 to 11 mg/dL.

Determination of von Willebrand Factor Activity

Plasma samples were taken from tranquilized monkeys (ketamine hydrochloride, 15 mg/kg body wt i.m.) 2 weeks before the thrombosis experiment for determination of plasma von Willebrand factor (vWF) activity. vWF was determined by the macroscopic agglutination test with formaldehyde-fixed human platelets and botrocetin (6 units/mL) as the agglutinating agent.10 Plasma from one of the 18 monkeys chosen at random served as the reference plasma; vWF activity for all other monkeys was calculated as a percentage compared with that referent.

Induction of Occlusive Arterial Thrombosis

Monkeys were anesthetized with ketamine hydrochloride (15 mg/kg body wt) and butorphanol (0.025 mg/kg body wt) given by intramuscular injection. The right common carotid artery of each monkey was dissected free of surrounding tissue over a 3-cm segment. A 5-mm Goldblatt clamp was placed proximally on the carotid artery, and a 20-MHz Doppler ultrasonic crystal was applied distally so that flow was not impaired. The Doppler crystal was energized by a range-gated pulsed Doppler unit.12 The signal was range-gated to maximum clarity.

The protocol for the induction of arterial thrombosis has been fully described previously12 and is briefly described here. Throughout the procedure, the Doppler signal was observed for cyclic flow reductions (CFRs) or permanent cessation of flow (PCF). After a 10-minute period of stabilization, the carotid artery was stenosed by closing the Goldblatt clamp enough to block reactive hyperemia. Stenosis was continued for 30 minutes and was then removed by opening the Goldblatt clamp.

After blood flow velocity had stabilized, the artery was injured in the area where the clamp was applied. The injury was induced by three occlusions of the artery with a spring-loaded forceps (Castroviejo Needle Holders, J. Sklar, Inc., Long Island, N.Y.). After 10 minutes of observation for reductions in flow, the Goldblatt clamp again was partially closed to block reactive hyperemia. After 30 minutes of stenosis, the clamp was reopened. After stabilization of flow velocity, the pinch injury was repeated and the Goldblatt clamp tightened a third time for a final 30-minute period of observation. The end point for each animal was the observation of alterations of blood flow velocity (either CFR or PCF). Thus, not all animals received a second injury. All animals were monitored for at least 15 minutes (mean±SD, 47±19 minutes) after the initiation of altered flow.

A qualitative assessment was made of the magnitude of CFR. A weak or irregular CFR was given a + score and a more pronounced, regular CFR was given a ++ score. The number of CFR episodes was not counted because CFR in some arteries was very weak.

Evaluation of Carotid Artery Injury

At the end of the thrombosis protocol, the Goldblatt clamp and Doppler flow probe were removed and the carotid artery segment was ligated and removed. The incisions were closed, and the monkeys were returned to their pens.

The artery was divided lengthwise into one flat artery segment. This segment was divided lengthwise once again into two separate pieces, each containing sections of upstream, damaged, and downstream artery. One piece of the artery was placed in 4% buffered formalin. This segment was dehydrated in alcohol, embedded in paraffin, and stained with hematoxylin and eosin. Sections of artery were cross sectioned and damaged segments examined with light microscopy for the presence or absence of smooth muscle damage, medial hemorrhage, disruption of the internal elastic laminae, mural thrombi, and endothelial denudation. Smooth muscle damage was defined as pale-staining cytoplasm and contracted and pyknotic nuclei.

Incorporation of Lp(a) Into the Damaged Carotid Artery

The other half of the artery segment (from the Hi-Lp[a] and Lo-Lp[a] monkeys only) was placed im-
Immediately in OCT compound (Lab-Tek, Naperville, Ill.) and frozen in liquid nitrogen. Segments of artery were cut with a cryostat and examined for incorporation of Lp(a) into the damaged segments of artery. Six-micron frozen sections were cut and fixed in acetone and stained with a monoclonal mouse anti-Lp(a) antibody using a biotin–streptavidin–horseradish peroxidase system. Sections were first treated with 3% goat serum in phosphate-buffered saline (PBS), pH 7.4, to block any nonspecific binding sites. Mouse anti-human Lp(a) (Cappel catalog No. 59407) diluted to 10 μg/mL or nonspecific antibody (monoclonal mouse immunoglobulin M [IgM]) was allowed to react with the tissue; after the tissue was washed in PBS, biotin-conjugated goat anti-mouse IgG (Tago catalog No. 6653, 1:500 dilution) was applied to the sections and incubated. After further washing, streptavidin–horseradish peroxidase (KPL No. 143000, 1:200 dilution) was added and allowed to incubate, and the slides were washed thoroughly in PBS. Enzyme activity was visualized by treatment with 0.5 mg/mL diaminobenzidine (Sigma No. D5905) in tris (hydroxymethyl)aminomethane buffer, pH 7.4, plus 0.015% H₂O₂; the reaction was terminated by a water wash. Slides were counterstained with hematoxylin, dehydrated, and coverslipped in a xylene-based mounting medium. Substitution of the primary antibody with PBS or a nonspecific mouse monoclonal antibody was used as a control for nonspecific binding.

**Statistical Analyses**

Logistic regression analysis was used to determine the effect of Lp(a) on categorical outcomes such as PCF (yes, no), CFR (yes, no), and the degrees of arterial injury caused by stenosis and pinch injury. The effect of Lp(a) on vWF activity was determined by the Wilcoxon rank sum test. The association of measured variables with one another was evaluated with linear regression analyses.

**Results**

**Flow Velocity Changes After Stenosis and Injury**

Stenosis alone of carotid arteries did not result in CFR or PCF in any of the carotid arteries. CFRs occurred in the carotid arteries of all monkeys (except No. 1 in the Lo-Lp[a] group) in all three experimental groups after the first injury \( (p>0.05) \); Table 1). PCF occurred in all arteries from the Hi-Lp(a) monkeys (Table 1; \( p<0.05 \) versus other groups) after the first injury and stenosis. PCF occurred in only one monkey in the Lo-Lp(a) group (Table 1). PCF did not occur in any
FIGURE 2. Photomicrograph showing immunohistochemical analysis of an injured arterial segment in a cynomolgus monkey. Lipoprotein(a) is stained brown. Brown staining is seen in the adventitia (AD), media (MED), intima (INT), and the thrombus (TH) in the lumen. Counterstained with hematoxylin; magnified ×23.

of the arteries from Int-Lp(a) monkeys. However, the CFR in the Int-Lp(a) arteries were more pronounced than in Lo-Lp(a) arteries (Figure 1). Furthermore, CFRs from Int-Lp(a) monkeys did not diminish over time as they did in Lo-Lp(a) monkeys.

vWF Activity

Plasma vWF activity was similar in all three groups (p > 0.05). Activity was 102±10% in Hi-Lp(a), 94±6% in Int-Lp(a), and 103±6% in Lo-Lp(a) monkeys.

Tissue Concentrations of Lp(a)

Tissue Lp(a) level was evaluated in the arteries of Lo-Lp(a) and Hi-Lp(a) monkeys. Lp(a) was incorporated into the artery at the site of injury of both groups. Anti-Lp(a) was located in the intima, media, and adventitia of the arteries (Figure 2). The thrombi in the lumen of arteries were darkly stained with anti-Lp(a). On light microscopic examination, there appeared to be no difference between groups in the amount or distribution of anti-Lp(a) in the injured arteries. We did not measure tissue Lp(a) in the Int-Lp(a) group because no differences were seen in the initial comparisons between the Lo-Lp(a) and Hi-Lp(a) groups. The Lp(a) antibody was specific for tissue Lp(a), as shown in Figure 3. The nonspecific antibody did not stain with horseradish peroxidase (left) but did with anti-Lp(a) (right).

Histological Examination of Arterial Injury

The luminal surfaces of carotid arteries in the area of stenosis and injury from monkeys in all three experimental groups were characterized by platelet accumulation and formation of microthrombi. However, occlusive thrombi were seen only in the carotid arteries of Hi-Lp(a) monkeys (Figure 2).

The degrees of arterial injury at the Goldblatt clamp site are listed in Table 2. Medial hemorrhage was present in all carotid arteries (p > 0.05; Figure 4). Damaged smooth muscle cells were present in all arteries from the Hi-Lp(a) monkeys and in five of six arteries from both the Int-Lp(a) monkeys and the Lo-Lp(a) monkeys (p > 0.05; Figure 4). Disruption of the internal elastic laminae was seen in three of six arteries from both the Hi-Lp(a) monkeys and Int-Lp(a) monkeys and in two of six arteries from the Lo-Lp(a) monkeys (p > 0.05). There was endothelial cell denudation in two of six arteries from the Hi-Lp(a) monkeys and in three of six arteries from both the Int-Lp(a) and the Lo-Lp(a) monkeys (p > 0.05).

Discussion

The three major findings of this study are the following: 1) carotid arteries from monkeys with high plasma concentrations of Lp(a) (>35 mg/dL) developed PCF in response to arterial stenosis and injury, which was associated with development of occlusive thrombosis; 2) arteries from the Int-Lp(a) and Lo-Lp(a) monkeys developed transient CFRs that, although associated with the development of microthrombi and transient thrombosis, did not progress to PCF; and 3) increased risk of permanent arterial thrombosis in Hi-Lp(a) mon-
keys occurred despite similar vWF activity among experimental groups. It has not been shown previously that high in vivo concentrations of plasma Lp(a) are associated with increased risk for the development of permanent occlusive arterial thrombosis.

Studies in human beings indicate that 20 mg/dL is the critical plasma concentration of Lp(a), with higher amounts associated with increased risk of coronary heart disease and cerebrovascular disease.1-3 In the present experiment, occlusive thrombosis in monkeys was associated with plasma Lp(a) concentrations greater than 35 mg/dL. The carotid arteries from Int-Lp(a) monkeys (with Lp[a] concentrations of 20–25 mg/dL) responded to stenosing injury in a more pronounced manner than those from the Lo-Lp(a) monkeys, but the arteries did not develop PCF or occlusive thrombosis. It is unknown whether monkeys and human beings differ in the critical Lp(a) concentration required for increased heart disease risk, or whether the apparently higher critical values of Lp(a) in monkeys reflect the sensitivity of the methods used in this experiment.

There is debate whether concentrations of plasma Lp(a), tissue Lp(a), or both influence the risk of coronary heart disease. In the current study, Lp(a) was incorporated into the injury site of arteries in both Hi- and Lo-Lp(a) monkeys. There was no apparent difference in amount or distribution of anti-Lp(a) in the arteries between these two groups, and there was no staining for anti-Lp(a) in undamaged artery sections. This finding could be interpreted to mean that tissue Lp(a) concentrations are not as critical as plasma Lp(a) concentrations in thrombosis risk. However, tissue Lp(a) was not quantified, and arteries in the present experiment were not atherosclerotic.

**Studies on Plasma Lp(a) in Macaques**

Virtually all of the reported data about Lp(a) in nonhuman primates are based on studies of baboons.
and macaques. Lp(a) was first recognized in rhesus monkeys by Fless et al.\textsuperscript{13} Later, Nevan et al\textsuperscript{14} reported a relation between plasma Lp(a) levels, apolipoprotein (a) (apo(a)) isoforms, and low density lipoprotein receptor function. Azrolan et al\textsuperscript{15} reported that Lp(a) from cynomolgus monkeys is immunochemically and quantitatively similar to human Lp(a). In part, plasma Lp(a) concentrations in these macaques depended on the number of kringle IV encoding domains found in the genome and were not correlated with hepatic apo(a) mRNA abundance. Nachman et al\textsuperscript{16} reported that Lp(a) accumulates in the atherosclerotic plaques of macaques with diet-induced atherosclerosis. In the same study, no Lp(a) was found in nonatherosclerotic arteries. Results of the present study confirm that no tissue Lp(a) was found in nonatherosclerotic arteries but that Lp(a) accumulated at the site of arterial injury. Results of the present study expand on previous work by suggesting a functional relation between plasma Lp(a) concentrations in monkeys and increased risk of thrombosis.

**Potential Mechanisms**

In the present study, arteries from Lo- and Int-Lp(a) monkeys had CFRs, platelet adherence, and development of microthrombi, but PCF and occlusive thrombosis did not occur. Although the present study was not designed to examine how Lp(a) affects the formation of an occlusive thrombus, our results are consistent with those of previous studies that suggest a role of Lp(a) in fibrinolysis.\textsuperscript{7,17}

When platelets aggregate at an injury site, the stability of the aggregate is favored by fibrin deposition, which promotes conversion of the reversible platelet clumps to irreversible aggregates.\textsuperscript{17,18} Thus, factors that tend to diminish fibrin accumulation should protect against development of irreversible aggregates. The fibrinolytic system plays a major role in regulating fibrin stability at the lesion site.\textsuperscript{4,10,17} Impaired fibrinolysis should, therefore, potentiate the development of a permanent thrombus by decreasing the response to fibrin formation. Fibrinolysis may be regulated by the control of the generation or activity of plasmin\textsuperscript{17}; in turn, regulation of plasminogen activation and plasmin activity may depend on the local concentration of plasminogen and its activators and inhibitors.\textsuperscript{4,5,10,17} Results of several studies indicate that Lp(a) may regulate fibrinolysis by competing with plasminogen for binding sites on fibrin and fibrinogen.\textsuperscript{7,17} These data indicate that Lp(a) inhibits both plasminogen activation and fibrinolysis. It is hypothesized from the results of the present experiment that injury resulted in the same initial events at the artery wall in all three groups of monkeys. However, initial changes at the artery wall proceeded to permanent occlusive thrombosis only in monkeys with high plasma concentrations of Lp(a).

**Potential Limitations of the Study**

This study would have been enhanced by measurement of plasma fibrinolytic activity. Plasma fibrinolytic...
activity was not measured for two reasons: 1) this was a preliminary experiment, and 2) we were unsure of the results until all monkeys were studied and the data analyzed. However, we believe that the results of the current experiment merit publication as they stand.

There was a considerable amount of brown staining in the adventitia of the damaged arteries. The control sections done with nonspecific antibody indicated that the Lp(a) antibody was specific for tissue Lp(a). However, it is possible that external maceration of the tissue could have resulted in some nonspecific staining of the adventitia. In addition, carotid arteries contain vasa vasorum, which may have contributed to the accumulation of Lp(a).

Conclusions

In conclusion, monkeys with relatively low, intermediate, and high plasma concentrations of Lp(a) were studied to determine whether high plasma Lp(a) concentrations were associated with an increased risk of occlusive thrombosis. Results of the experiment indicate that after injury, arteries from monkeys with high plasma concentrations of Lp(a) developed a PCF that was associated with the development of occlusive thrombosis. The results of this study are the first to show an association between plasma Lp(a) concentrations and increased risk of experimentally produced thrombosis measured in vivo.

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References

15. Azrolan N, Gavish D, Breslow J: Plasma lipoprotein (a) concentration is controlled by apolipoprotein (a) size and the abundance of hepatic apo(a) mRNA in a cynomolgus monkey model. J Biol Chem 1991;266:13866–13872
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