Factor XIIIa Cross-links Lipoprotein(a) With Fibrinogen and Is Present in Human Atherosclerotic Lesions

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Abstract—During the development of atherosclerotic lesions, lipoprotein(a) [Lp(a)], a highly atherogenic lipoprotein, accumulates within fibrin clots attached to blood vessel walls. As Lp(a) accumulates within the fibrin clot with time, fatty streaks are formed that develop into occlusive atherosclerotic plaques. It is not understood, however, which mechanisms are involved in the binding of Lp(a) to fibrin and, hence, the stable incorporation of Lp(a) into the fibrin clot. The results of the present study demonstrate that factor XIIIa, a transglutaminase that catalyzes the formation of amide bonds between endo-γ-glutaminyl and endo-ε-lysyl residues of proteins, is capable of cross-linking Lp(a) to fibrinogen, the soluble precursor of fibrin. Biochemical assays were conducted to demonstrate that factor XIIIa cross-links Lp(a) with fibrinogen in a time- and concentration-dependent manner. Additionally, immunohistochemical studies revealed that factor XIII protein expression colocalizes with Lp(a) expression in human atherosclerotic plaques. It is proposed that factor XIIIa–mediated cross-linking of Lp(a) to fibrin effectively increases the local concentration of Lp(a) within a fibrin clot. The accumulation of Lp(a) within the blood vessel promotes an antifibrinolytic environment, foam cell formation, the generation of a fatty streak, and an increase in smooth muscle cell content, all of which may contribute to the pathogenesis of atherosclerosis.

Key Words: atherosclerosis ■ fibrin ■ fibrinogen ■ lipoprotein ■ lesion

lipoprotein(a) [Lp(a)] is a highly atherogenic lipoprotein complex formed of apolipoprotein(a) [apo(a)] that is disulfide-bound to the apolipoprotein B-100 moiety of LDL. It has been reported that Lp(a) binds fibrin, which becomes deposited within the blood vessel wall in fibrin clots. Furthermore, it has been suggested that Lp(a) accumulates within the vessel wall and has the potential to contribute to the development of occlusive atherosclerotic plaques with time. Individuals who develop occlusive atherosclerosis suffer from coronary heart disease, and an elevated plasma Lp(a) level has been shown to be an independent risk factor for the development of premature coronary heart disease. Other vascular diseases associated with elevated plasma Lp(a) include restenosis and stroke. The deposition of Lp(a) within the fibrin clot is believed to be a contributing factor in atherogenesis. However, the actual mechanism by which Lp(a) binds fibrin and becomes stably incorporated into a fibrin clot remains unknown.

Factor XIII (EC 2.3.2.13) is a transglutaminase that catalyzes the final step in the coagulation cascade. This enzyme exists as a zymogen and is present in 2 molecular forms. The tissue form of factor XIII, identified with the tissue form of factor XIII, at which point the tissue and plasma forms of factor XIII a are identical. Factor XIIIa, also referred to as plasma transglutaminase, fibrinoligase, and fibrin-stabilizing factor, is a calcium-dependent thiol enzyme that catalyzes the formation of amide bonds between endo-γ-glutaminyl and endo-ε-lysyl residues of proteins.

Factor XIIIa is noted primarily for its participation in the coagulation cascade, where it covalently cross-links fibrin monomers and converts soft fibrin clots into hard clots. Factor XIIIa is also capable of cross-linking α2-antiplasmin and extracellular matrix proteins such as fibronectin, vitronectin, and collagen to fibrin, thus rendering the clot more resistant to lysis and anchoring the clot to the blood vessel wall, respectively. Furthermore, factor XIIIa has been demonstrated to cross-link primary amines to the apo(a) moiety of Lp(a), suggesting a role for factor XIIIa in atherosclerosis. Also, elevated plasma levels of factor XIIIa have been observed in patients with obliterative atherosclerosis. To examine the possibility that factor XIIIa plays a role in Lp(a) deposition into fibrin clots in the development of atherosclerosis, we conducted studies to directly demonstrate that factor XIIIa is capable of cross-linking Lp(a) to fibrinogen, the soluble precursor of cross-linking Lp(a) to fibrinogen.
of fibrin. Additionally, immunohistochemistry directly demonstrated that factor XIII and Lp(a) expression was identified in human atherosclerotic lesions.

Materials and Methods

Factor XIIIa–Mediated Cross-linking of Lp(a) With Fibrinogen: Determination by Immunoprecipitation With Anti-Fibrinogen Antibody Followed by Western Blotting With Lp(a) Antibody

Factor XIIIa was incubated with Lp(a) and fibrinogen in solution and then analyzed for Lp(a)-fibrinogen cross-linking over a time course ranging from 1 to 6 hours (n=5 per time point). In brief, purified human fibrinogen (Calbiochem) at a final concentration of 100 μg/mL was incubated with purified human Lp(a) (Sigma Chemical Co) at a final concentration of 500 μg/mL (based on the protein content of Lp(a)) in the presence of purified human factor XIIIa (Enzyme Research Laboratories) at 30 °C. The approximate molecular weight of Lp(a) was reported by the manufacturer to be 1 000,000, in agreement with a previous report, and was purified to homogeneity from fresh, nonfrozen human plasma pooled from several hundred donors. Since the Lp(a) was purified from plasma obtained from multiple donors, it can be assumed that the Lp(a) used for these studies is a mixture containing the various apo(a) isoforms and is representative of the general population. The chemical composition of the Lp(a) was reported to be ~31% protein and 69% lipid, based on earlier studies. It should be noted that the concentrations of Lp(a) and factor XIIIa used for these studies are within the physiological range detected in human plasma. Also, since fibrin will spontaneously form a soft clot and potentially trap Lp(a) within it to yield a false-positive result, fibrinogen, the soluble precursor of fibrin, was used for these studies. The reactions were conducted at 37°C in a buffer consisting of 40 mmol/L Tris, 0.15 mol/L NaCl, 2-mercaptoethanol, and 0.025% bromophenol blue and then heated at 100°C for 3 minutes. To identify the presence of Lp(a) within the immunoprecipitated complexes, samples were analyzed by Western blotting with the use of an antibody to Lp(a). In brief, the samples (30 μL each, ~60% of the pelleted material) were subjected to electrophoresis through a 4% to 20% polyacrylamide gradient gel (Bio-Rad) and then transferred to a nitrocellulose membrane by using a Bio-Rad semidyestra apparatus according to the manufacturer’s instructions. Unoccupied binding sites were blocked overnight at 4°C with 5% nonfat powdered milk in a 0.1 mol/L Tris-HCl buffer, pH 8.0, containing 1.5 mol/L NaCl and 0.5% Triton X-100 (TBST buffer). A polyclonal sheep anti-human Lp(a) primary antibody that recognizes the apo(a) component of Lp(a) (Enzyme Research Laboratories), diluted in TBST to 10 μg/mL, was then added to the membrane and allowed to incubate for 1 hour at 25°C. The membrane was washed 3 times for 20 minutes each with TBST and then incubated for 30 minutes with a donkey anti-sheep IgG secondary antibody conjugated to horseradish peroxidase (Sigma) diluted 1:5000 in TBST. The membrane was washed as described above, and the blot was developed with the enhanced chemiluminescence method (Amer sham) according to the manufacturer’s instructions. The intensity levels of each band relative to background were determined and quantified with a Molecular Dynamics densitometer.

Factor XIIIa–Mediated Cross-linking of Lp(a) With Fibrinogen: Determination by ELISA-Based Assay

Microwell ELISA plates (No. 25801, Corning) were coated with purified human fibrinogen (American Diagnostica) at a concentration of 80 μg/mL, 100 μg/mL per well, for 40 minutes at room temperature. Unoccupied binding sites were then blocked for 1 hour with 1% BSA in buffer A (40 mmol/L Tris and 0.15 mol/L NaCl, pH 8.3). Lp(a) (Sigma) was added to the wells to a final concentration of 250, 375, or 500 μg/mL (based on the protein content of Lp(a)) in buffer A containing 10 mmol/L CaCl2, and 5 mmol/L DTT (n=6). Factor XIIIa (Enzyme Research Laboratories), preactivated with thrombin as described above, was then added to the wells for a final activity of 15 U/mL. The reactions were allowed to proceed for 2 hours at room temperature and then stopped by the addition of EDTA to a final concentration of 15 mmol/L. The wells were then washed 4 times with ELISA wash solution consisting of 0.002 mol/L imidazole-buffered saline (pH 7.4) and 0.02% Tween 20 (Kirkegaard and Perry).

To determine whether factor XIIIa had cross-linked Lp(a) to the immobilized fibrinogen, a sheep anti-Lp(a) antibody (Enzyme Research Laboratories), diluted to 10 μg/mL in ELISA wash solution, was added to the wells and allowed to incubate for 18 hours at 4°C. The wells were washed as described above, and then a biotinylated anti-sheep IgG antibody (Vector Laboratories), diluted to 10 μg/mL in ELISA wash solution, was added and allowed to incubate for 1 hour at room temperature. The wells were washed as described above, after which time 150 μL of streptavidin–β-galactosidase (GIBCO/BRL), diluted to 0.35 μg/mL in ELISA wash solution, was added to room temperature. The wells were washed once with ELISA wash solution, and then 150 μL of p-nitrophenyl-β-d-galactopyranoside (Sigma) at 1 mg/mL was added. After 30 minutes of incubation at room temperature, the reaction was stopped, and the resulting color product was enhanced by adding 20 μL of 1N NaOH to each well. Absorbance was then read at a wavelength of 405 nm on a SPECTRAMax 250 microplate spectrophotometer (Molecular Devices).

Immunohistochemistry

Sections of human coronary arteries with moderate to advanced atherosclerotic lesions were obtained from the cardiovascular tissue bank at the University of Washington, Seattle. The tissue samples (n=3) were obtained from diseased hearts of cardiac transplant recipients. Tissues were collected within 30 to 60 minutes after cardiac excision and placed into neutral buffered formalin. After overnight fixation, the samples were routinely processed and paraffin-embedded for histology. Hematoxylin and eosin–stained
sections were reviewed and used to select samples for immunohistochemistry. The ages of the donors, all male, were 51, 54, and 63 years. Sections were prepared for immunoperoxidase staining by using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer’s instructions. In brief, endogenous peroxidase was quenched with 0.3% H2O2 in methanol for 30 minutes. Nonspecific immunoglobulin binding sites were blocked with normal rabbit serum for 1 hour, and then the sections were incubated with a sheep anti–factor XIII primary antibody (2.5 μg/mL, Enzyme Research Laboratories) or a sheep anti-Lp(a) primary antibody generated against the apo(a) moiety of Lp(a) (2.5 μg/mL, Enzyme Research Laboratories) for 1 hour at room temperature. As a control, serial sections were incubated with sheep IgG (2.5 μg/mL, Sigma) instead of the primary antibody. The sections were then incubated for 30 minutes with a biotinylated rabbit anti-sheep IgG secondary antibody (7.5 μg/mL, Vector Laboratories) followed by 30 minutes of incubation with the Vectastain Elite ABC reagent solution. Immunoglobulin complexes were visualized on incubation with 3,3′-diaminobenzidine (Vector Laboratories) at 0.5 mg/mL in 50 mmol/L Tris-HCl, pH 7.4, and 3% H2O2. Sections were washed, counterstained with Gill’s hematoxylin, cleared, mounted with Aquamount (Polysciences), and then examined by light microscopy.

Statistics
Data were expressed as mean±SEM. For statistical analysis of factor XIIIa–mediated cross-linking of Lp(a) with fibrinogen as determined by the ELISA-based method, the t test for unpaired data was used. Statistical significance was accepted when P<0.05.

Results
Factor XIIIa Is Capable of Cross-linking Lp(a) With Fibrinogen
To determine whether factor XIIIa cross-links Lp(a) with fibrinogen, Lp(a) and fibrinogen were incubated in solution with or without factor XIIIa (n=5). Complexes that formed between Lp(a) and fibrinogen were then immunoprecipitated with an antibody directed against fibrinogen. Those complexes were subjected to electrophoresis and then analyzed for the incorporation of Lp(a) by Western blotting with an antibody directed against Lp(a). The results demonstrated that in the presence of factor XIIIa, increasing amounts of Lp(a) became cross-linked with fibrinogen over a time course ranging from 1 to 6 hours (Figure 1). After 6 hours, the degree of cross-linking between Lp(a) and fibrinogen when factor XIIIa was present had increased 10.82±2.33-fold (Figure 1, bottom). When factor XIIIa was omitted from the reaction, only a negligible amount of Lp(a) was detected in the immunoprecipitated material, which was comparable to the amounts detected at the zero time points (Figure 1, top and bottom). Also, when unactivated factor XIII was added to the reaction (n=3), only a small amount of Lp(a) was detected in the immunoprecipitated material, indicative of a nonspecific factor XIIIa–independent interaction between Lp(a) and fibrinogen (Figure 1, top). Similar results were generated when EDTA was added to the reaction to inhibit factor XIIIa (data not shown, n=5) and when thrombin plus hirudin was added (data not shown, n=2). In additional experiments, Lp(a) was incubated at concentrations ranging from 200 to 800 μg/mL [based on the protein content of Lp(a)] with fibrinogen and factor XIIIa for 6 hours (n=3). The results showed that Lp(a) became cross-linked to fibrinogen in a concentration-dependent fashion (Figure 2). When factor XIIIa was not included in the reaction, Lp(a) was essentially undetected (Figure 2). These results indicate that factor XIIIa mediates cross-linking between Lp(a) and fibrinogen. Also, with increasing amounts of Lp(a) added to the reaction, there appeared to be complexes formed of varying molecular weight. This was evident as a “tail” that existed beneath each band that had been detected by Western blotting (Figure 2, top). With increasing amounts of Lp(a) present, the complexes appeared to be larger; hence, the “tails” became shorter. These results suggest that there are multiple Lp(a) binding sites on each fibrinogen molecule.

Factor XIIIa–mediated cross-linking of Lp(a) to fibrinogen was also analyzed by an ELISA-based assay (n=6). The results demonstrated that during a 2-hour incubation period in the presence of factor XIIIa, Lp(a) became cross-linked to the fibrinogen immobilized on the plate in a concentration-dependent manner (Figure 3). It should be noted, though, that in the absence of factor XIIIa, a measurable amount of Lp(a) nonspecifically adhered to the fibrinogen-coated plate (Figure 3), and this background signal modestly increased with time.
Figure 2. Factor XIIIa (FXIIIa)–mediated cross-linking of increasing amounts of Lp(a) with fibrinogen. Top, Complexes formed between Lp(a) and fibrinogen in the presence of FXIIIa were retrieved by immunoprecipitation with an anti-fibrinogen antibody and then evaluated for incorporation of Lp(a) by Western blotting using an antibody directed against Lp(a). Fibrinogen (100 μg/mL) was incubated with increasing amounts of Lp(a) [200 to 800 μg/mL, based on the protein content of Lp(a)] for 6 hours at 37°C in the presence (n = 3 per concentration) or absence (n = 3 per concentration) of FXIIIa (30 U/mL). In the presence of FXIIIa, Lp(a) became cross-linked with fibrinogen in a concentration-dependent manner. When FXIIIa was not included in the reaction, Lp(a) did not form a complex with fibrinogen. Arrow indicates Lp(a) immunoreactivity in complexes immunoprecipitated with anti-fibrinogen antibody. Results shown are representative of all assays conducted (n = 6). In the presence of factor XIIIa and with increasing amounts of Lp(a) included in the reaction, more Lp(a) became cross-linked with fibrinogen. Results are representative of all assays conducted (n = 6) and are presented as mean and SEM. Solid bars represent factor XIIIa omitted from reaction; hatched bars, factor XIIIa included in reaction. O.D. indicates optical density. *P < 0.05.

Figure 3. Cross-linking of Lp(a) with fibrinogen: determination by ELISA-based assay. Factor XIIIa–mediated cross-linking of Lp(a) to fibrinogen immobilized on a 96-well plate was evaluated by ELISA-based assay (n = 6). In the presence of factor XIIIa and with increasing amounts of Lp(a) included in the reaction, more Lp(a) became cross-linked with fibrinogen. Results are representative of all assays conducted (n = 6) and are presented as mean and SEM. Solid bars represent factor XIIIa omitted from reaction; hatched bars, factor XIIIa included in reaction. O.D. indicates optical density. *P < 0.05.

Factor XIII and Lp(a) Expression Is Detected in Human Atherosclerotic Lesions

To determine whether factor XIII and Lp(a) were expressed within an atherosclerotic lesion, immunohistochemistry was performed. The expression of factor XIII and Lp(a) was investigated in serial sections prepared from human coronary arteries containing atherosclerotic lesions (n = 3). The results showed that factor XIII and Lp(a) were expressed in a similar region within the atherosclerotic lesions (Figure 4), predominantly in the core of the lesions. Notably, the intensity of the immunoreactive product was greater for the Lp(a)-immunoreactive product than for the factor XIII–immunostained samples (Figure 4A and 4B, respectively). Also, additional Lp(a) staining was detected in some regions devoid of factor XIII immunoreactivity and vice versa. A serial section that was incubated with sheep IgG instead of factor XIII or Lp(a) primary antibodies was negative (Figure 4C).

Discussion

During the development of atherosclerotic lesions, Lp(a) becomes deposited into fibrin clots within the blood vessel wall and has the potential to contribute to atherogenesis. Factor XIIIa, a transglutaminase that catalyzes the formation of amide bonds between the endo-γ-glutaminy1 and endo-ε-lysinyl residues of proteins, plays a major role in the cross-linking of fibrin monomers for the generation of fibrin clots and arterial thrombi. Since the mechanism by which Lp(a) actually becomes incorporated into the fibrin clot is poorly understood, we propose the hypothesis that factor XIIIa is capable of catalyzing covalent γ-glutamyl-ε-lysine bonds between Lp(a) and fibrin and contributes to the development of atherosclerotic lesions. Our results have demonstrated for the first time that in vitro, factor XIIIa is indeed capable of catalyzing covalent γ-glutamyl-ε-lysine bonds between Lp(a) and fibrin and contributes to the development of atherosclerotic lesions. Furthermore, we have demonstrated that factor XIII and Lp(a) expression is present in human atherosclerotic lesions.

The pathophysiological role of Lp(a) in the development of vascular diseases such as atherosclerosis, myocardial ischemia, restenosis, and stroke may lie in the ability of Lp(a) to (1) become deposited within fibrin clots on vessel walls, (2) interfere with fibrinolysis, (3) enhance cholesterol accumulation in macrophages to result in foam cell formation, and (4) promote smooth muscle cell proliferation and migration. For example, the incorporation of Lp(a) onto the vessel wall potentiates the thrombotic events that occur in the development of atherosclerotic lesions. The apo(a) moiety of Lp(a) has been shown to have high homology with plasminogen and the zymogen precursor of the fibrinolytic enzyme plasmin. Owing to the high homology between the apo(a) component of Lp(a)
and plasminogen, it has been demonstrated that Lp(a) competes with plasminogen for binding to endothelial cells and monocytes. These studies suggest that in pathological conditions such as atherosclerosis, in which there is an increased amount of circulating Lp(a), Lp(a) prevents plasmin(ogen) from binding to the vessel wall and interferes with fibrinolysis of the thrombotic lesion. In addition to promoting foam cell formation and the generation of fatty streaks, Lp(a) has been demonstrated to induce smooth muscle cell proliferation and migration. Increased smooth muscle cell content is characteristic of atherosclerotic as well as restenotic lesions. Investigators have also shown that the effects of Lp(a) on smooth muscle cells are due to the fact that Lp(a) inhibits plasmin-mediated activation of transforming growth factor-β, a potent inhibitor of smooth muscle cell growth and migration.

In conclusion, we propose that factor XIIIa–mediated cross-linking of Lp(a) to fibrin effectively increases the local concentration of Lp(a) within a fibrin clot that is sequestered within the vessel wall. The accumulation of Lp(a) within the vessel wall promotes an antifibrinolytic environment, foam cell formation, and an increase in smooth muscle cell content, all of which contribute to the pathogenesis of atherosclerosis.

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References


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