Urokinase treatment preserves endothelial and smooth muscle function in experimental acute arterial thrombosis

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Purpose: Pharmacologic lysis or balloon thrombectomy are options to treat acute arterial thrombosis; however, little is known about their effects on functional changes in the arterial wall. The aim of this study was to determine function of the endothelium and smooth muscle in canine arteries revascularized after acute thrombosis with balloon thrombectomy or lytic therapy.

Methods: Acute thrombosis was obtained by bilateral proximal and distal ligation of 8-cm segments of the femoral arteries in dogs. After 24 hours, the ties were removed and the arteries randomized to treatment groups: group 1, balloon thrombectomy (#4 Fogarty balloon catheter at 60 grams linear shear × 1 pass, n = 7); group 2, untreated, tie removal only (n = 6); group 3, regional intra-arterial urokinase infusion (4000 U/min × 90 min, n = 6); group 4, regional intra-arterial carrier infusion (0.43 ml/min × 90 min, n = 6); group 5, unoperated normal vessels (n = 5). After treatment, the arteries were removed and endothelial and smooth muscle responses examined in organ chambers. Endothelial loss was graded with light microscopy of vessel rings from each animal by an observer blinded to the treatment group. Findings were confirmed with scanning electron microscopy.

Results: Treatment with urokinase did not alter endothelium-dependent relaxations or smooth muscle contractions compared with carrier infusion or untreated alone. Balloon catheter thrombectomy significantly reduced endothelium-dependent relaxations compared with all other groups in response to acetylcholine, bradykinin, and thrombin ($p < 0.001$). Contractions of smooth muscle in response to potassium chloride (60 mol/L) and phenylephrine (1×10^{-6} mol/L) were also reduced ($p < 0.05$). Rings from balloon thrombectomized arteries contracted in response to calcium ionophore A23187 ($p < 0.001$); these contractions were endothelium dependent and not reduced by indomethacin or blockade of endothelin A and B receptors. No significant differences in percentage of endothelial coverage between groups were assessed by light and electron microscopy.

Conclusion: Thrombolysis with urokinase caused no or minimal abnormalities in endothelial and smooth muscle function. Endothelium present after balloon thrombectomy produces contractile factors. Although the duration and recovery of these abnormalities in function are unknown, these findings support preferential use of urokinase over balloon thrombectomy when possible in acute arterial thrombosis or embolism. (J Vasc Surg 1996;23:851-9.)

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Arterial balloon thrombectomy has been well established as the standard for treating acute arterial embolism and thrombosis.[1,2] Despite the efficacy of this technique for removing thrombus from arteries, complications have been reported after its use, including vessel rupture, perforation, and balloon fracture with distal embolism.[3-5] A potential late complication of this procedure is intimal hyperplasia and possible progression of atherosclerosis.[6-8] Experimental and

clinical experiences indicate that balloon catheters may disrupt or denude the endothelium.[9] Most experimental studies examining balloon thrombectomy have used a vigorous technique with the intent of completely denuding the endothelium, and their analysis has focused on disruption of morphologic features rather than functional changes.[10,11] Little is known about functional changes caused in arteries thrombectomized with balloon catheters with standardized low shear.

Thrombolytic therapy with direct-acting activators of plasminogen such as urokinase has become the first line of treatment for acute thrombosis. The efficacy of intra-arterial thrombolytic therapy in achieving lysis of thrombus and initial patency of recently thrombosed arteries and grafts has been well established.[12-17] Data on functional changes after urokinase treatment are not available.

The vascular endothelium plays an important role in regulation of arterial tone, in differentiation and growth of the underlying smooth muscle and in thrombogenicity through release of substances such as nitric oxide[18] and endothelins.[19,20] How lytic therapy affects the ability of endothelium to produce and release vasoactive substances that are so important to the normal function of an artery is unclear. Therefore this study was designed to determine effects of thrombolytic therapy and balloon thrombectomy on endothelial and smooth muscle function in an experimental model of acute arterial thrombosis.

MATERIAL AND METHODS

Animal model. Male mongrel dogs (weight 24 to 26 kg) were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Animal care was in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). Hydration was maintained with intravenous sodium chloride (0.9%) solution at a rate of 7 ml/kg/hr.

By using an aseptic surgical technique, the femoral arteries were exposed. The adventitia and vasa vasorum were disturbed only in the areas where ties were to be placed. Both femoral arteries were ligated with a 2-0 silk suture proximally below the inguinal ligament and 8 cm distally above the femoral bifurcation. Side branches of the femoral arteries were double ligated. Lack of blood flow was confirmed with an ultrasonic flow probe (Transonics Systems Inc., Ithaca, N.Y.). The groin wounds were closed in layers. Twenty-four hours later the femoral vessels were exposed again, ties were removed, and each artery was assigned to one of four treatment groups. The iliac arteries were cannulated with an 18-gauge Teflon catheter for infusions and arteriograms. Angiograms were performed in all limbs with 50% diatrizoate meglumine after treatment but before harvesting the vessels. The animals were sacrificed after the arteries were harvested.

Thirty arteries were randomized before the start of the study into four groups. Group 1 underwent balloon thrombectomy (n = 7). A #4 Fogarty balloon catheter (Baxter Healthcare, Santa Ana, Calif.) connected to a force transducer (Model FT03, Grass Instrument Co., Quincy, Mass.) was inserted through an arteriotomy at the level of the femoral bifurcation. The balloon was positioned in the iliac artery and inflated with fluid while withdrawing the catheter under arterial pressure. While withdrawing the balloon through the femoral artery, inflation was controlled to keep the linear shear stress as close to 60 gm as possible (Fig. 1). The balloon was removed after one withdrawal in all limbs. Group 2 was untreated; the ties were removed from the ligated artery after 24 hours, and the vessel was harvested (n = 6). Group 3 received urokinase (n = 6). A Harvard pump (Harvard Apparatus, Millis, Mass.) was used for continuous intra-arterial infusion of urokinase. Two hundred fifty thousand units of urokinase as lyophilized powder were reconstituted with 5 ml of sterile water and brought to a volume of 27 ml with sterile saline for a final concentration of 9260 U/ml. The lytic agent was then infused at 0.43 ml/min (4000 U/min) for 90 minutes. Group 4 received the carrier solution (0.5% mannitol, 5% human albumin, and 1% sodium chloride in sterile water), which was intra-arterially infused at 0.43 ml/min for 90 minutes (n = 6). Group 5 consisted of unoperated normal vessels (n = 5). All arteries were removed and cut into sections for histologic examination and organ chamber experiments.

In vitro studies. Femoral arteries were removed from each animal and sectioned into four rings (5 mm in length). The rings were immersed in a chilled, modified Krebs-Ringer bicarbonate solution (control solution) of the following millimolar concentration: sodium chloride 118.3, potassium chloride 4.7, calcium dichloride 2.5, magnesium sulfate 1.2, potassium phosphate 1.22, sodium bicarbonate 25.0, glucose 11.1, edetate calcium disodium 0.026. The endothelium was removed from two of the four rings by gentle intraluminal rubbing with a fine forceps tip.

Rings were suspended between a fixed point and a force transducer (Gould UC2, Cleveland, Ohio) to measure isometric force. The rings were placed in organ baths filled with 25 ml control solution at 37° C and gassed with 95% oxygen and 5% carbon dioxide. The rings were allowed to equilibrate for 30 minutes. Rings were then gradually stretched in stepwise fashion to the optimal point of their length-tension relation as determined by tension developed to potassium chloride (20 mmol/L). Contraction to potassium chloride (60 mmol/L) was measured. One group of rings with and without endothelium from each treatment group was incubated with the arginine analog N^8-monomethyl-L-arginine (L-NMMA) (10^{-4} mol/L) for 30 minutes before the first concentration response curve. L-NMMA was readded to these chambers before each concentration response curves. The rings were then contracted with prostaglandin F_{2a} (2×10^{-5} mol/L). Cumulative concentration-response curves to the following agents were then recorded after submaximal contraction with phenylephrine (1×10^{-6} mol/L): acetylcholine (10^{-9} to 10^{-6} mol/L), bradykinin (10^{-9} to 10^{-6} mol/L), and thrombin (10^{-2} to 1.0 U/ml). Contractions were measured from baseline tension in response to endothelin-1 (10^{-11} to 10^{-7} mol/L). Relaxations to nitric oxide (3×10^{-8} to 10^{-5} mol/L) in rings without endothelium and calcium ionophore A23187 (10^{-9} to 10^{-6} mol/L) in rings with endothelium were measured from the maximal contraction obtained with endothelin-1. In parallel experiments, rings from balloon thrombectomized arteries were incubated with indomethacin (1×10^{-5} mol/L) and SB-209670 (1×10^{-5} mol/L) (nonselective antagonist of endothelin A and B receptors) before the calcium ionophore A23187 dose response curves (n = 2).

Drugs. The following drugs were used: acetylcholine, bradykinin, calcium ionophore A23187, endothelin-1, L-NMMA, thrombin, indomethacin, and SB209670. Drugs were prepared daily with distilled water and kept refrigerated. The calcium ionophore was dissolved in dimethyl sulfoxide and diluted in distilled water (final bath concentration of dimethyl sulfoxide was 8.2×10^{-3} mol/L). Nitric oxide was prepared according to the method as previously described.[21] All concentrations refer to final molar (mol/L) concentrations of drugs in the organ bath or superfusion solution.

Calculations and statistical analysis. All data are expressed as mean ± SEM. The n value represents the number of arteries. Relaxations are expressed as a percentage of the contraction to phenylephrine or endothelin-1. Contractions are expressed as increases in grams of tension or percentage of the contraction to potassium chloride (60 mmol/L). Differences in contractions among groups were determined by the use of a one-way analysis of variance of the area under the curves. The Student's-Newman-Keuls post hoc analysis was used to identify differences between groups. Statistical significance was accepted when $p < 0.05$.

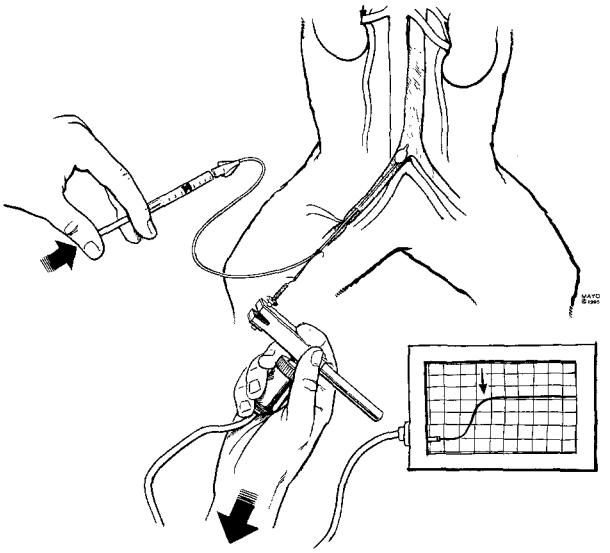

Fig. 1. Diagram demonstrating method of controlled balloon catheter thrombectomy. Linear shear is measured with force transducer. Linear shear is kept close to 60 grams by controlling balloon inflation during catheter withdrawal.

Histologic analysis. Endothelial damage of rings fixed immediately after the intervention was assessed by light microscopy of sections (stained with hematoxylin and eosin) and electron microscopy. Damage was scored by an observer blinded to the treatment groups. Rings were divided into quadrants and percentage of endothelial denudation assessed. This percentage was converted to a score for each ring (0% to 10% = 1, 11% to 25% = 2, 26% to 50% = 3, 51% to 75% = 4, and 76% to 100% = 5). The results were expressed as a mean ± SEM. Analysis of variance was used to identify differences between treatment groups.

RESULTS

All femoral arteries were filled with soft thrombus 24 hours after ligation. Removal of the proximal and distal ligatures resulted in most but not all of the thrombus being swept away. Balloon thrombectomy resulted in linear shear measurements of 60 ± 10 mm Hg. All vessels were patent after treatment as demonstrated by arteriography.

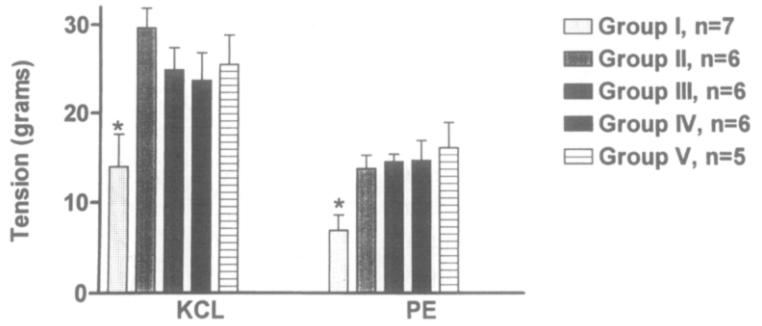

Fig. 2. Contraction-response curves to phenylephrine (PE) (1×10^{-6}) and potassium chloride (KCl) (60 mmol/L) in femoral arterial rings with intact endothelium. Data are mean ± SEM of change in tension (grams). Responses of rings without endothelium (not shown) were not different from those with endothelium. Contraction of balloon-thrombectomized rings was significantly decreased ($p < 0.05$) compared with other groups.

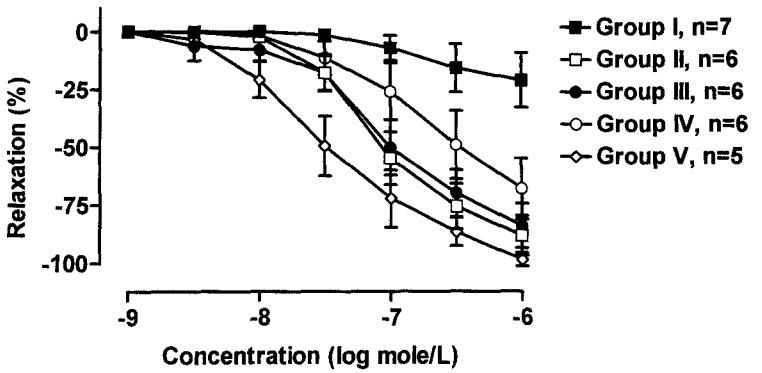

Fig. 3. Contraction-response curves to acetylcholine in femoral arterial rings with intact endothelium. Data are mean ± SEM of percent change in tension from contraction to phenylephrine (1×10^{-6} mol/L). Relaxation of balloon-thrombectomized rings was significantly decreased ($p < 0.001$) compared with groups 2, 3, and 4.

Organ chamber studies

All rings contracted to potassium chloride (60 mmol/L) and phenylephrine (1×10^{-6} mol/L). There were no differences in contractions between urokinase and carrier-infused arteries. Contraction of rings from balloon thrombectomized arteries was significantly less in response to potassium chloride and phenylephrine compared with all other groups ($p < 0.05$; Fig. 2).

Acetylcholine. In rings with endothelium, acetylcholine caused concentration-dependent relaxations in urokinase, carrier-infused, and untreated arterial rings without significant differences among these groups. Relaxations of rings from balloon thrombectomized arteries were significantly less than groups 2, 3, and 4 ($p < 0.001$; Fig. 3). In this group, 5 of 7 rings relaxed to acetylcholine while 2 of 7 had no response. Rings from all groups with endothelium mechanically removed contracted without significant differences.

Bradykinin. In rings with endothelium, bradykinin caused concentration-dependent relaxations in urokinase, carrier-infused, and untreated arterial rings without significant differences among these groups. Rings from balloon-thrombectomized arteries contracted significantly in response to bradykinin ($p < 0.001$ for group 1 vs all other groups; Fig. 4) with only 1 of 7 rings relaxing below initial tension developed to phenylephrine. Rings from all groups with endothelium mechanically removed contracted without significant differences among groups.

Thrombin. In rings with endothelium, thrombin caused concentration-dependent relaxations in uroki-

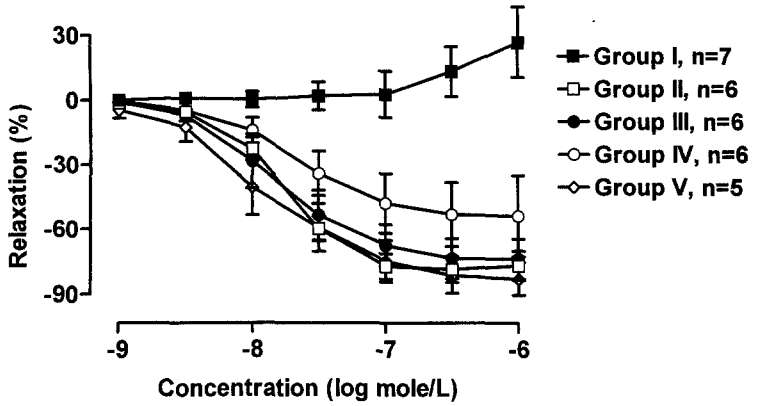

Fig. 4. Contraction-response curves to bradykinin in femoral arterial rings with intact endothelium. Data are mean ± SEM of percent change in tension from contraction to phenylephrine (1×10^{-6} mol/L). Relaxation of balloon thrombectomized rings was significantly decreased ($p < 0.001$ for group 1 vs all other groups) compared with all other groups.

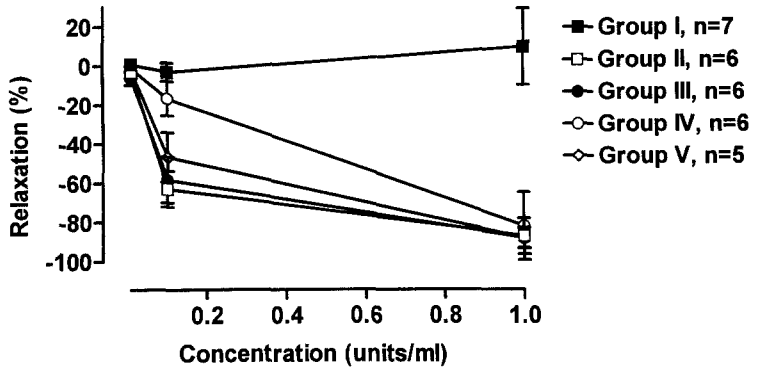

Fig. 5. Contraction-response curves to thrombin in femoral arterial rings with intact endothelium. Data are mean ± SEM of percent change in tension from contraction to phenylephrine (1×10^{-6} mol/L). Relaxation of balloon-thrombectomized rings was significantly decreased ($p < 0.001$) compared with all other groups.

nase, carrier-infused, and untreated arterial rings. There were no significant differences among these groups. Rings from balloon-thrombectomized arteries contracted significantly compared with all other groups ($p < 0.001$; Fig. 5). Rings from all groups with endothelium mechanically removed contracted without significant differences among groups.

Endothelin-1. Concentration-dependent increases in tension were observed to endothelin-1. The contractions were not significantly different in rings with or without endothelium.

Calcium ionophore A23187. In rings with endothelium contracted with endothelin-1, calcium ionophore A23187 caused concentration-dependent relaxations in rings from urokinase, carrier-infused, and untreated arteries. There were no significant differences among these groups. Rings from balloon-thrombectomized arteries contracted significantly in response to calcium ionophore A23187 compared with all other groups ($p < 0.001$; Fig. 6). The contractions to calcium ionophore A23187 in the balloon thrombectomy group seen at higher concentrations was endothelium dependent and not abolished by incubation with indomethacin or SB209670 as demonstrated in parallel experiments (n = 2). L-NMMA did not alter the contractions seen in the balloon thrombectomy group.

Nitric oxide. Rings from all groups relaxed in a

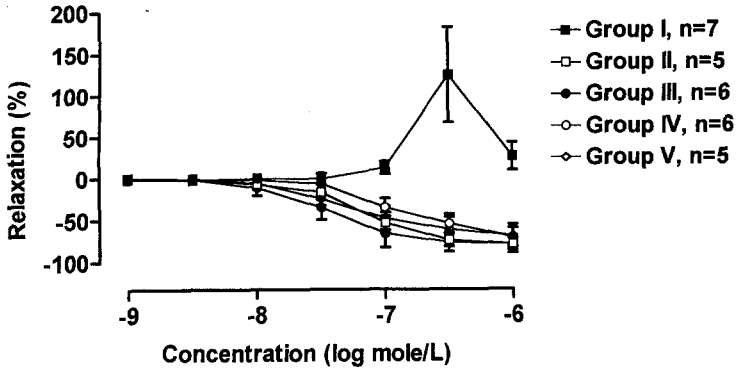

Fig. 6. Contraction-response curves to calcium ionophore A23187 in femoral arterial rings with intact endothelium. Data are mean ± SEM of percent change in tension from contraction to endothelin-1 (1×10^{-7} mol/L). Balloon-thrombectomized rings paradoxically contracted ($p < 0.001$) compared with all other groups.

Table I. Degree of endothelial loss in treated arteries

Treatment group	*n*	*Mean ± SEM*
Urokinase infusion	6	1.5 ± 0.34
Carrier infusion	4	2.25 ± 0.75
Balloon thrombectomy	7	3.0 ± 0.57
Untreated	5	1.8 ± 0.8

Percentage of endothelial loss for rings from each vessel was determined by an observer blinded to treatment groups converted to numeric value according to scale: 0% to 10% = 1, 11% to 25% = 2, 26% to 50% = 3, 51% to 75% = 4, 76% to 100% denudation = 5. Differences were not significant ($p > 0.05$).

concentration-dependent fashion to nitric oxide. Relaxations were not significantly different among groups.

Histologic study

Light microscopy of sections prepared from arteries immediately after intervention revealed small areas of denudation in all groups. The differences in amount of lumen covered with endothelium among groups were not significant ($p > 0.05$; Table I). There was no correlation in degree of endothelial denudation and relaxation or contractile responses. These findings were confirmed with scanning electron microscopy of representative arteries from each group (urokinase infusion, n = 4; carrier infusion, n = 3; balloon thrombectomy, n = 4; untreated, n = 3).

DISCUSSION

In this study, organ chamber experiments and histologic examination demonstrated that regional infusions of urokinase did not inhibit endothelium-dependent relaxations or smooth muscle contractions or visibly alter endothelial architecture. However, gentle balloon catheter thrombectomy significantly inhibited endothelium-dependent relaxations and smooth muscle contractions without significantly altering endothelial histologic features. The endothelium remaining after balloon catheter thrombectomy produced contractile factors. Thrombosis alone did not significantly alter endothelial or smooth muscle function.

Thrombolytic agents are used in patients because of their ability to alter the fibrinolytic cascade. The biology of these agents is quite diverse. The activation of plasminogen to the protease, plasmin is a mechanism for producing extracellular proteolysis[22] and may have roles in a variety of biologic processes, including fibrinolysis, placental implantation, migration of normal and malignant cells, and tissue remodeling.[23,24] Endothelial cells express receptors for plasminogen and urokinase-like plasminogen activator. These receptors may have a role in cell-cell interaction, permeability, and migration.[25-27] Given the broad spectrum of activity of plasmin and receptors for plasminogen activators such as urokinase, it was not clear if their use would result in immediate effects on the vessel wall. Results of this study demonstrate that intra-arterial infusions of urokinase in doses similar to those used in patients resulted in minimal or no alterations in endothelial-dependent relaxation or smooth muscle contraction compared with infusions of carrier solution in stark contrast to balloon thrombectomy.

Studies have used balloon catheters to completely denude the endothelium as a model for vascular injury,[10,11] whereas others have controlled the amount of force applied to the vessel wall to recreate as nearly as possible a normal operative thrombectomy.[9] Denudation of the endothelium has been associated with accelerated atherosclerosis,[28] and even mild injury may be associated with intimal hyperplasia.[4,6,29] Most studies with balloon catheter thrombectomy have described the degree of vessel injury in histologic terms. Very little is known about the functional effect of a "surgical" balloon thrombectomy in an occluded artery. It appears that endothelial injury depends on balloon inflation and the number of catheter withdrawals. "Gentle" controlled balloon thrombectomies were performed by carefully regulating dynamic shear forces during one catheter withdrawal as described by Jorgensen and Dobrin.[9] A controlled balloon thrombectomy caused insignificant denudation of endothelium compared with other groups; however, vessel rings from this group demonstrated profoundly decreased ability to generate dose-dependent endothelium-dependent relaxations and smooth muscle contractions. The arteries relaxed only slightly to the highest concentrations of acetylcholine compared with all other groups, whereas there were no appreciable relaxations to any other agent that generally elicits an endothelium-dependent relaxation (bradykinin and thrombin). The histologic presence of endothelium does not necessarily translate to normal functioning endothelium. It was apparent that the endothelium remaining within the vessel sustained a physiologic insult by the balloon. Nitric oxide causes a endothelium-independent smooth muscle relaxation.[21] All vessel rings relaxed in a similar fashion when exposed to nitric oxide. This finding proves that the smooth muscle in balloon-thrombectomized arteries is able to relax in response to endothelium-derived relaxing factors; however, the endothelium did not appear to release them in the expected fashion. The internal elastic lamina and smooth muscle appeared intact in the balloon thrombectomy group; however, smooth muscle contractions were significantly decreased. The ability of the endothelium to influence the underlying smooth muscle is responsible for the ability of the vessel to alter its tone and to undergo vessel wall remodeling.[30] Surprisingly, calcium ionophore A23187 elicited a significant endothelium-dependent contraction in the balloon-thrombectomized arteries. The mechanism of this contraction is not yet clearly understood; however, it has been seen in other tissues and may be caused by superoxide anions and alterations in cyclooxygenase pathways.[31-33] Production of endothelial contractile factors and diminished smooth muscle function may ultimately contribute to the eventual pathologic sequela of vessel stenosis or occlusion.

Thrombolysis with urokinase has gained widespread use among vascular surgeons and appears to be a safe alternative to balloon catheter thrombectomy.[16] In experiments, urokinase preserved endothelial and smooth muscle function in an experimental model of arterial thrombosis of 24-hour duration. However, gentle balloon catheter thrombectomy similar to that in use by most vascular surgeons seriously altered arterial physiologic features and the vessels' ability to normally adapt to a changing milieu. Although the significance, duration, and recovery of these abnormalities in function are unknown, these findings support preferential use of urokinase over balloon thrombectomy when possible in acute thrombosis or embolism.

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DISCUSSION

Dr. Dolores F. Cikrit (Indianapolis, Ind.). Even though you controlled for linear sheer stress, did you try using a smaller Fogarty catheter to assess its effect on smooth muscle cells? Because balloon thrombectomy caused the vessels to lose contractility, should we be using a calcium-channel blocker at the completion of our arterial thrombectomies? In your study, the clots were present for only 24 hours, and the majority of this was swept away as soon as blood flow was re-established to the vessel. This does not happen in the clinical setting. Urokinase will sit in the occluded vessel for a period of time before the clot is lysed. Did you do any studies with the distal tie on to assess the long-term effect of urokinase on the endothelial lining? It was surprising that endothelial denudation was similar in all groups. Did you use a factor VIII stain to quantify endothelium coverage? Did you do any studies with pressure perfusion to maintain endothelial contact?

Dr. David Whitley. In response to your first question, no, we did not use a smaller catheter. We had a limited amount of time and animals to do this, and we decided on one size.

As far as your second question regarding use of a calcium-channel blocker, I think this tells us that if we see spasm in an artery after a balloon thrombectomy, we would need to use something that is going to act directly on the smooth muscle if we are going to try to get rid of the spasm. Agents that normally would cause relaxation with an intact endothelium may actually end up causing a contraction—

such as acetylcholine, bradykinin, and thrombin, all of which actually cause significant contraction. We need to keep that in mind.

As far as your question about tie removal, it is true that the clot formed up to 24 hours was a soft clot, and some of that actually did sweep away when the ties were taken off. We might have seen higher concentrations of urokinase within that artery had we left the distal tie on for a period during the infusion. However, we did do a series of experiments in which we added purified urokinase directly to the organ chambers and actually saw no effect on endothelial function.

Lastly, we did not stain for factor VIII. Since this was an acute study, we had good histology and scanning electromicroscopy, so anything we saw lining the vessel was going to be endothelium. We did not do any perfusion studies. Perfusing these vessels would have been nice; however, if we had done that, we would not have been able to carry out the functional studies.

Dr. John D. Corson (Iowa City, Iowa). The pharmaceutical companies, our interventional colleagues, and others are exhorting us to try to lyse things that have been thrombosed for significant periods of time. What do we really know about the effect of the thrombus itself on endothelial function vis-à-vis the time the thrombus is there? Have you looked at that as a factor in the longer term?

Dr. Whitley. Not in the longer term. I actually have looked at the literature, and there is very little known about how it affects the function of the endothelium. In the beginning we were not quite sure if our endothelium was going to be affected by the clot sitting within the artery for a period of time. However, as you can see from the controls, the endothelium, at least by our study, seems to fare very well and had normal responses to all the agents that we looked at. I cannot give you any information on the long-term effect.

Dr. Thomas W. Wakefield (Ann Arbor, Mich.). Because you had loss of endothelial cells in the balloon group, are you sure that you just did not have enough endothelial cells remaining to respond appropriately rather than concluding that these endothelial cells did not work because of the balloon injury?

Dr. Whitley. We had endothelium present in all the vessel rings that we examined. We studied adjacent vessel rings before hanging them in the organ chamber. We also stained and looked at the vessel rings after they came out of the chamber. All vessel rings had endothelium present. There was obviously more endothelium denuded in the balloon thrombectomy group, and we tried to correlate that with the function. There was not a positive correlation between the degree of denudation and the function. Usually in these vessel rings if you have any endothelium present, you will have almost near-normal endothelium-dependent relaxation.