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Inhibition of thrombosis and intimal thickening by *in situ* photopolymerization of thin hydrogel barriers

(polyethylene glycol/restenosis/angioplasty)

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ABSTRACT **Thin hydrogel barriers formed on the inner surface of injured arteries by interfacial photopolymerization dramatically reduced thrombosis and intimal thickening in rat and rabbit models of vascular injury. This polymerization technique allowed the synthesis of a thin hydrogel barrier that conformed to the vessel wall, directly blocking contact between blood and the damaged vessel. The illumination conditions could be varied to control the thickness of the barrier from 10 μm to >50 μm. The hydrogel was designed to degrade by nonenzymatic hydrolysis. In rats in which the carotid artery had been severely injured by crushing, treatment with the hydrogel barrier completely eliminated thrombosis ($P < 0.01$) and preserved long-term patency ($P < 0.01$). Treatment in a rabbit model of balloon injury inhibited thrombosis ($P < 0.02$) and reduced long-term intimal thickening by ≈80% ($P < 0.003$). These results suggest that blood-borne signals acting in the early phases of healing play an important role in stimulating thickening of the intima.**

Approximately 30–40% of patients treated by coronary balloon angioplasty experience clinically significant renarrowing of the vessel, or restenosis, within 6 months of initial treatment, resulting in repeat procedures and increased morbidity (1). Restenosis involves smooth muscle cell migration, proliferation, and secretion of matrix proteins, resulting in the formation of an obstructive neointimal layer (2). Thrombus formation has been implicated as a cause of restenosis (1, 3–6), as thrombus may serve as a matrix for smooth muscle cell migration and may contribute cytokines, chemoattractants, and mitogenic factors (1). Platelet-derived growth factor is both a mitogen and chemoattractant for smooth muscle cells (7–10). Thrombin also appears to be an important signal (11–14), as it stimulates smooth muscle cells to proliferate (15), express platelet-derived growth factor (16), and secrete collagen (17). Some of the signals may be intrinsic to the vessel wall. Basic fibroblast growth factor, stored in the extracellular matrix, is a potent mitogen for smooth muscle cells (18, 19) and has been shown to stimulate intimal thickening (19). Numerous pharmacological approaches have been evaluated that either directly interrupt the smooth muscle cell cycle (20) or block the various signals derived from thrombosis and growth factor release (21, 22); yet there is presently no prophylaxis for restenosis.

In the present study, we sought to investigate a nonpharmacological approach to prevent intimal thickening by inhibiting contact between blood and subendothelial tissues with a resorbable polymeric barrier. A method was developed to synthesize a nonthrombogenic, conformal hydrogel barrier with thickness on cellular dimensions upon the arterial wall by interfacial photopolymerization of a macromolecular precursor *in situ*. In two animal models, this novel barrier was found to virtually eliminate thrombosis in injured arteries. The transient presence of the barrier also preserved patency and reduced intimal thickening long after the disappearance of the barrier.

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MATERIALS AND METHODS

Precursor Synthesis. A macromolecular precursor was synthesized that consisted of a central chain of polyethylene glycol (PEG) to provide water solubility, with flanking regions of lactic acid oligomer to provide water lability and tetraacrylate termini to provide polymerizability. The precursor was synthesized by dissolving 50 g of 10,000-Da PEG (Sigma) in toluene (Mallinckrodt, ACS grade) and refluxing under argon for 1 hr; 4.5 g of DL-lactide (Aldrich) and 50 μl of 50% (vol/vol) stannous octoate (ICN) in toluene were added. The solution was refluxed under argon for 16 hr to achieve an average of five lactic acid groups per end, as estimated by proton NMR (23). The solution was cooled to ≈20°C, and the polymer was precipitated with hexane (Mallinckrodt, ACS grade), filtered, washed, and dried. This polymer was dissolved in tetrahydrofuran (Mallinckrodt, ACS grade) under argon and cooled to ≈15°C; 5.23 ml of triethylamine (Aldrich) and 3 ml of acryloyl chloride (Aldrich) were added to the mixture while bubbling argon through the solution. The mixture was then refluxed under argon for 24 hr. Triethylamine hydrochloride precipitate was removed by filtration. The macromolecular precursor was precipitated with hexane, filtered, washed, and dried. The precursor was stored at 0°C under argon until use.

***In Vitro* Evaluation.** Carotid arteries were explanted from male Sprague–Dawley rats (250–350 g, after euthanasia) and mechanically denuded by passage over a thin wooden rod. The denuded arteries were incubated in 1 mM eosin Y (Sigma), a nontoxic photoinitiator, in Hepes-buffered saline (10 mM, pH 7.4) for 1 min to adsorb the photoinitiator onto the surface of the vessel. The arteries were then rinsed twice in Hepes-buffered saline and infused with a 23% solution of the macromolecular precursor that also contained 100 mM triethanolamine (Aldrich) and 0.15% *N*-vinylpyrrolidone (Aldrich). The arteries were illuminated using an argon ion laser (514 nm, 70 mW/cm^2, 2-s exposure; American Laser, Salt Lake City) to convert the liquid precursor to a hydrogel on the surface of the vessel where the photoinitiating dye had been adsorbed. Heparin anticoagulated (10 units/ml, Sigma)

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human blood was used for evaluation of thromboprotection since it was readily available and reacted potently with the rat subendothelium. Treated vessel segments were perfused with this blood at a wall shear rate of 100 s^{-1} using a syringe pump. Thick sections of these vessels were examined by environmental scanning electron microscopy (ElectroScan, Wilmington, MA) in a hydrated state.

Polymerization Kinetics. Fluorescent beads, 1 μm in diameter (Polyscience), were incorporated into the precursor formulation to aid in visualization of the hydrogel barrier. Polymerization kinetics were examined in male Sprague–Dawley rats (250–350 g). The rats were euthanized by CO_2 asphyxiation, and both carotid arteries were surgically exposed. A zone was isolated by placing atraumatic arterial clamps on the common, internal, and external carotid arteries. Polyethylene tubing (PE-50, Clay Adams) was inserted into the external carotid, and blood was removed from the zone with multiple withdrawals and reinfusions of Hepes-buffered saline. The isolated zone was filled with 1 mM eosin Y in Hepes-buffered saline, which was allowed to adsorb to the vessel wall for 1 min. The eosin Y was withdrawn, and the zone was rinsed twice with saline. The zone was then filled with a 23% solution of the precursor that also contained 100 mM triethanolamine and 0.15% *N*-vinylpyrrolidone. The vessel was then externally illuminated with a 1-kW Xe arc lamp that emitted light between 400 and 600 nm (Optomed, Austin, TX) at an irradiance of 35 mW/cm^2. Illumination times were varied between 2 and 15 s, with five vessels per time point. Vessels were immediately explanted, and unpolymerized precursor was rinsed off. The vessels were cryosectioned and visualized by fluorescence microscopy (Leitz). The images were analyzed by digital video image processing (Hamamatsu Photonics, Bridgewater, CT). The thickness of the gel barrier was determined by measuring multiple chords and averaging.

Gel Retention Under Arterial Flow. Gel retention upon the arterial surface was evaluated in male New Zealand White rabbits (2.5–3.5 kg). The rabbits were anesthetized with ketamine (30 mg/kg), acepromazine (3.5 mg/kg), and xylazine (7 mg/kg) given intramuscularly. No anticoagulant was used. The left carotid artery was surgically exposed and isolated as described above. Polyethylene tubing (PE-50) was inserted into the internal carotid artery, and blood was removed from the zone as described above. The hydrogel barrier was formed as described above, using the Xe arc lamp at an irradiance of 70 mW/cm^2 with a 2-s exposure. The animals were randomly assigned to groups after application of the hydrogel, and segments of the common carotid with the hydrogel barrier were explanted (after euthanasia by pentobarbital overdose) either without exposure to blood flow or after 2-hr exposure to blood. The vessel segments were cryosectioned and analyzed as described above.

Rat Arterial Crush Model. A crush injury in the carotid artery of the rat was employed as a model of severe vascular injury. Male Sprague–Dawley rats (250–300 g) were anesthetized with pentobarbital (50 mg/kg). No anticoagulant was used. The left carotid artery was surgically exposed and isolated as described above. Polyethylene tubing (PE-50) was inserted into the external carotid, and blood was removed from the isolated zone as described above. A 4-mm length of the common carotid was crushed with hemostats by a surgeon who was blind to the treatment groupings. The hydrogel barrier was applied to animals designated for treatment through the polyethylene tubing as described above, using the argon ion laser as the external illumination source under conditions as described for the *in vitro* evaluation. The tubing was removed, the external carotid was ligated, and the clamps were removed to allow blood flow. The unpolymerized precursor was rinsed into the vasculature. The injured segment of the common carotid was explanted either 2 hr or 14 days after injury (after CO_2 asphyxiation). The segment was rinsed of blood, fixed in 2% glutaraldehyde, dehydrated in a graded ethanol series, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with Masson's trichrome. Digital video image processing was used to determine the cross-sectional areas of the thrombus and lumen at 2 hr. Patency was determined at 14 days by examining multiple sections of each vessel.

Rabbit Balloon Denudation. A balloon denudation injury in the carotid artery of the rabbit was employed as a model more closely related to balloon angioplasty. Male New Zealand White rabbits (2.5–3.5 kg) were anesthetized with ketamine (30 mg/kg), acepromazine (3.5 mg/kg), and xylazine (7 mg/kg) given intramuscularly. No anticoagulant was used. The left carotid artery was surgically exposed, and a zone was isolated as described previously. Polyethylene tubing (PE-50) was inserted into the internal carotid artery, and blood was removed from the isolated zone as described above. The tubing was removed, and a 4F arterial embolectomy catheter (Baxter Healthcare, McGaw Park, IL) was inserted through the internal carotid. The balloon on the catheter was inflated and dragged through the common carotid. This process was performed a total of three times by a surgeon who was blinded to treatment grouping. The balloon catheter was removed, and the tubing was replaced. The hydrogel barrier was applied as described above except that the external illumination was carried out with the Xe arc lamp. Each point along the vessel was illuminated at an intensity of 70 mW/cm^2 for $\approx$2 s. The tubing was removed, the internal carotid was ligated, and the clamps were removed to allow blood flow. The unpolymerized precursor was rinsed into the vasculature. The injured segment of the common carotid artery was explanted either 2 hr or 14 days after injury (after pentobarbital overdose) and processed for histological analysis as described above. Slides were stained with either Verhoeff's elastin stain or Masson's trichrome stain. Digital video image processing was used to measure the cross-sectional areas of the thrombus and lumen (2 hr) and of the intima and media (14 day).

Half of each of three vessel segments from each group, randomly selected, were reserved for scanning electron microscopy to determine the presence of endothelial cells at the 14-day time point. The tissue was cut open longitudinally, dehydrated in a graded ethanol series, and dried by critical point drying. Samples were coated with gold and imaged by scanning electron microscopy. A region in each vessel segment corresponding to the approximate center of the injured zone in the animal was examined.

RESULTS

***In Vitro* Evaluation.** Environmental scanning electron microscopy was utilized to examine the morphology of the hydrogel barrier in its hydrated state. A photomicrograph is shown in Fig. 1. The hydrogel is the bright vertical band, with the lumen to the left and the arterial wall to the right. The thickness of the barrier in this case is $\approx$15 μm. No platelet attachment was observed after exposure to heparin anticoagulated human blood flowing at a shear rate of $\approx$100 s^{-1} for 10 min.

Polymerization Kinetics and Gel Retention. Illumination conditions were investigated as a potential means for control of the thickness of the hydrogel barrier. A linear relationship between barrier thickness and illumination duration was observed, as illustrated in Fig. 2. Under the polymerization conditions used in all studies in rabbits, the barriers were $\approx$13 μm thick. The hydrogel barrier remained adherent and did not decrease in thickness during 2-hr exposure to blood flow *in vivo* under conditions of arterial flow. The preflow thickness was 13.6 $\pm$ 1.3 μm (mean $\pm$ SEM) and the postflow

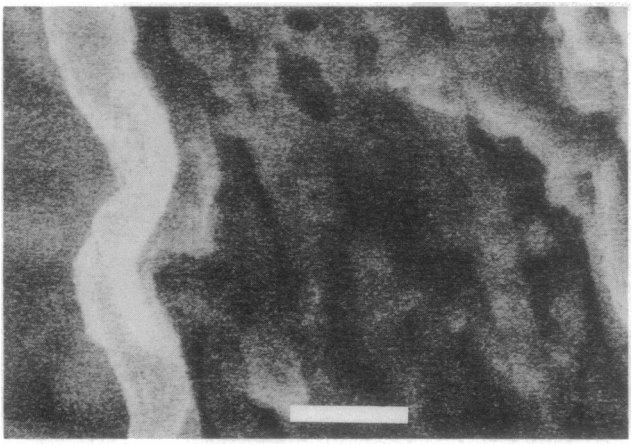

FIG. 1. Environmental scanning electron microscopy was used to observe hydrogel coatings upon arterial surfaces after 10-min *ex vivo* exposure to whole human blood anticoagulated with heparin at venous wall shear rates, without dehydration or other sample preparation. The hydrogel is the bright vertical band, with the lumen to the left and the vessel wall to the right. No mural thrombus or individual platelets were observed to be adherent to the treated arterial surface. (Bar = 10 μm.)

thickness was 13.0 ± 1.3 μm ($P > 0.7$ by Mann–Whitney U test, four vessels per group).

Rat Arterial Crush Model. A rat model of severe crushing arterial injury resulted in extensive thrombosis and consistently low long-term patency in control animals. The hydrogel barrier completely inhibited thrombosis at the 2-hr time point ($n = 4$, $P < 0.01$ by Mann–Whitney U test), while control vessels were 89% ± 5% (mean ± SEM) obstructed by thrombus after 2 hr ($n = 4$). In addition, hydrogel treatment completely preserved patency at 14 days, where no residual polymer or effect of injury was evident ($n = 7$, $P < 0.01$ by χ^2 test): all control vessels were completely occluded ($n = 7$).

Rabbit Balloon Denudation. A rabbit model of balloon denudation with stretch injury resulted in extensive thrombosis and consistent intimal thickening in control animals. The fractional occlusion by thrombus after 2 hr was reduced from 87% ± 5% (mean ± SEM) in the control group to 3% ± 2% in the treatment group ($P < 0.02$ by Mann–Whitney U test, four animals per group) (Fig. 3*A*). Intimal thickening was measured 14 days after balloon injury and treatment. The

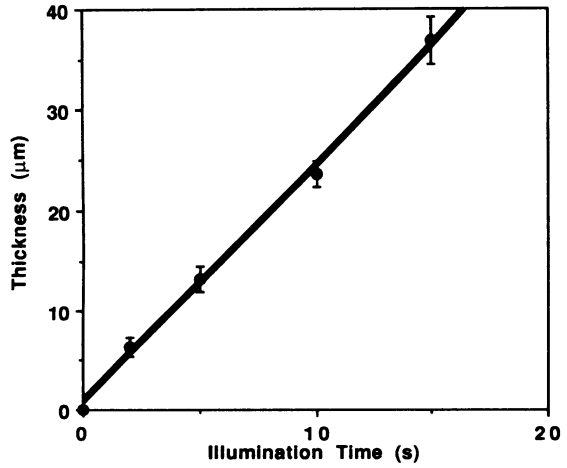

FIG. 2. The thickness of the hydrogel barrier could be controlled by the illumination conditions. External illumination was performed with a Xe arc lamp, filtered to emit between 400 and 600 nm, at an intensity of 35 mW/cm² for various durations. Means and SEM are plotted, with five samples per group.

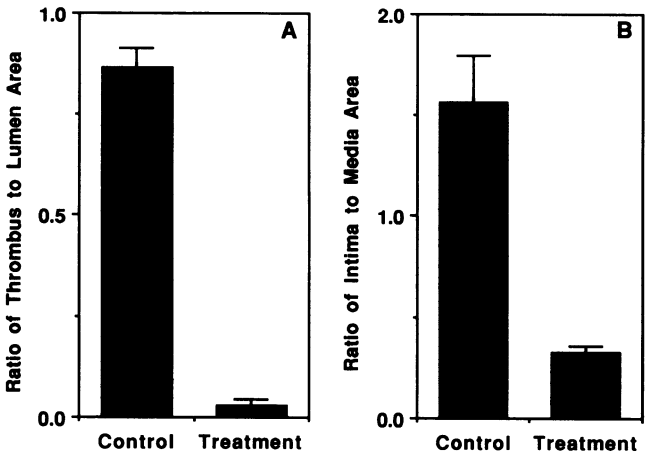

FIG. 3. (*A*) The fractional obstruction of the vessel due to thrombus formation 2 hr after balloon injury in the rabbit is represented as the ratio of the thrombus to lumen cross-sectional areas. The means and SEM are shown with four animals per group. (*B*) The ratio of the intimal to medial cross-sectional areas is shown as a normalized index of intimal thickening 14 days after balloon denudation and stretch injury in the rabbit. The means and SEM are shown with seven animals per group.

ratio of the intimal area to the medial area, a normalized measure of intimal thickening, was reduced by ≈80%, from a value of 1.56 ± 0.24 (mean ± SEM) in control animals to a value of 0.32 ± 0.04 in treated animals ($P < 0.003$ by Mann–Whitney U test, seven animals per group) (Fig. 3*B*). Representative histological sections of both time points are shown in Fig. 4.

Scanning electron microscopy was employed to indirectly demonstrate the absence of the hydrogel barrier at the 14th day and to qualitatively determine whether the application of the barrier interfered with reendothelialization after degradation. Both control and treated vessel segments were essentially reendothelialized at the 14th day, as shown in Fig. 5. Since the barrier is very nonadhesive to cells, the observation of reendothelialization would indirectly indicate the absence of the barrier.

DISCUSSION

Many of the signalling molecules in the complex process of intimal thickening are derived from blood. Thick polyether gel coatings have been shown to be capable of blocking thrombosis on denuded arteries *ex vivo* (24). With this in view, we have developed a technique for synthesizing a resorbable, conformal hydrogel barrier upon arterial surfaces *in situ*. The barrier was designed to block contact of blood with the vessel wall and thus block platelet deposition and thrombosis upon subendothelial tissues. This should substantially reduce exposure to substances derived from platelets—e.g., thrombin and platelet-derived growth factor. Moreover, the hydrogel barrier provides resistance to protein permeation (25) and may limit exposure to substances derived from plasma.

The design and properties of the barrier material and its precursor are critical in this investigation. We have utilized a nonionic, highly hydrated material, so that the resulting barrier will be essentially nonthrombogenic (26). In addition to providing a barrier for the adhesion of cells, the hydrogel also presents a barrier for the permeation of cells and proteins. The hydrogel is a three-dimensional network that is much too tight to permit cell permeation. The resistance to diffusion of small molecular weight species, such as oxygen, glucose, and amino acids, is essentially nil, being the same as through a stagnant layer of water, while the permeability of

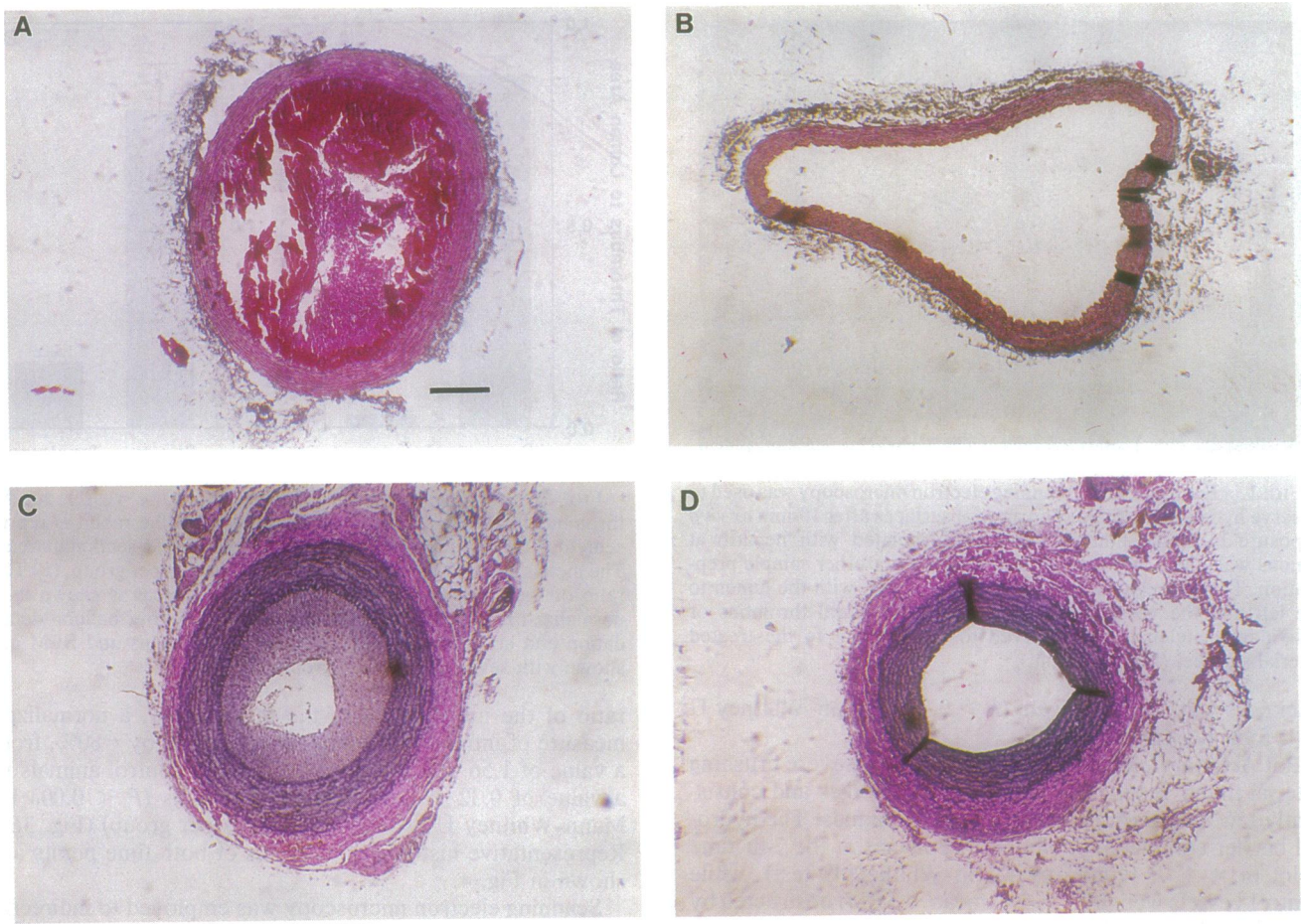

FIG. 4. Representative histological sections of arterial segments are shown. (*A* and *B*) Control rabbit carotid 2 hr following injury (*A*) and hydrogel-treated rabbit carotid 2 hr following injury (*B*), both stained with Masson's trichrome. The thrombus and the vessel wall are readily distinguishable. (*C* and *D*) Control rabbit carotid 14 days following injury (*C*) and hydrogel-treated rabbit carotid 14 days following injury (*B*), both stained with Verhoeff's method to accentuate the elastin bands. The neointima is the lighter region internal to the most internal elastin band. (Bar = 200 μm.)

the barrier to proteins depends upon their molecular weight. Since the barrier is impermeable to macromolecules, it may be used for the controlled release of macromolecular drugs. The drug may be entrapped in the three-dimensional meshwork of the gel and as the meshwork loosens by degradation, the drug is slowly released. This may allow the use of both a physical barrier and a pharmacological approach in the prevention of thrombosis and intimal thickening.

The hydrogel barrier is degraded nonenzymatically by hydrolysis of the lactic acid regions, thus resulting in ultimate degradation products of PEG, lactic acid, and oligomers of acrylic acid (23). The degradation period of the barrier material used in this study is at most a few days (27). This duration may be widely shortened or lengthened by altering the length or composition of the α-hydroxy acid oligomer (23).

The properties of the hydrogel barrier material and of the process of its application are important in this investigation. Since diseased vessels may be far from cylindrical, we sought to utilize a method of application that would not depend upon a mold to determine the shape. This was accomplished by interfacial photopolymerization, by staining the vessel surface with one component of the photoinitiation system, and then exposing the entire segment to light in the presence of the other components. This ensured that the shape of the barrier conformed precisely to that of the vessel being treated. This approach also provided for precise control of barrier thickness. In addition, the process of barrier application also provided for barrier adhesion. When the precursor solution is applied as a liquid, it can flow into crevices on the tissue, and gelation results in adhesion via mechanical interlocking. If applied as a pregelled film, the hydrogel demonstrates no intrinsic adhesiveness to tissues. In the present models, the unpolymerized precursor was flushed into the vasculature. The volume of the unpolymerized precursor solution that was rinsed into the vasculature was $\approx$100 μl, for a precursor dose of $\approx$9.2 mg/kg. As a reference, the LD_{50} of PEG is in excess of 6.7 g/kg (28).

The application of the hydrogel barrier very effectively prevented thrombosis on injured arterial surfaces. In the rat model of crush injury, thrombosis was completely inhibited, and in the rabbit model of balloon-induced injury, thrombosis was inhibited in excess of 95%. In the rat model, long-term patency was preserved in seven of seven crushed and treated arteries, whereas seven of seven untreated arteries were occluded at 14 days. Since this model of severe injury resulted in occlusion in control animals, the less severe balloon injury was employed. In thrombocytopenic models, where thrombosis was essentially completely inhibited by platelet depletion, intimal thickening was substantially reduced (5, 6). In the present study, this was also observed. The ratio of intimal to medial areas was reduced by $\approx$80% by application of the hydrogel barrier. It should be understood that this result represents a long-term outcome, measured after the complete degradation of the barrier. The absence of the barrier was demonstrated, albeit indirectly, by examining the artery surface for reendothelialization, which could only occur if the hydrogel barrier had degraded.

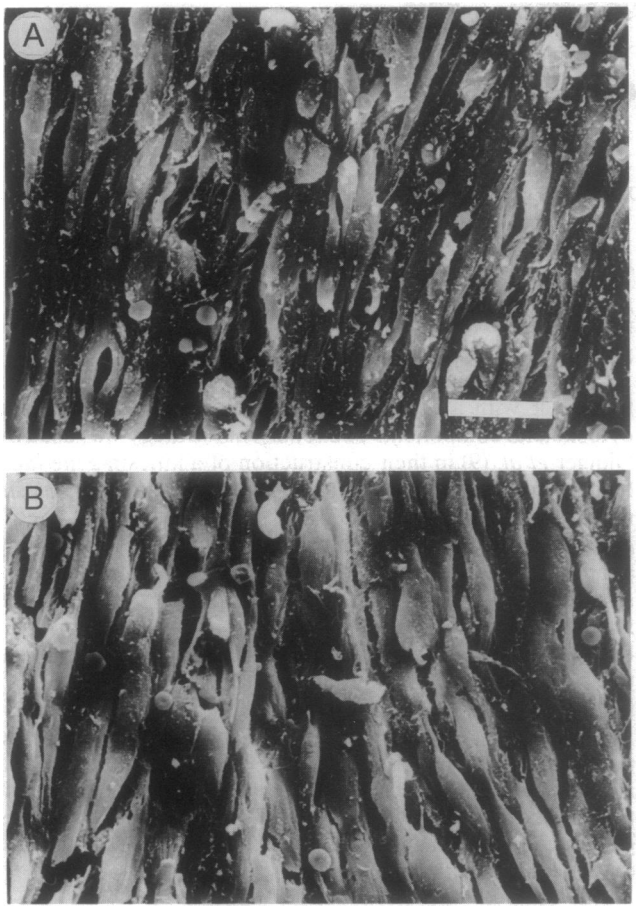

FIG. 5. Reendothelialization was examined in balloon-injured rabbit carotid arteries 14 days after injury by scanning electron microscopy. Control carotid, balloon injured but not treated (*A*), and hydrogel-treated carotid (*B*), are both shown 14 days after injury. Both control and treated groups displayed qualitatively similar degrees of reendothelialization at day 14, demonstrating that the presence of the hydrogel barrier during the first few days after injury did not inhibit the process of reendothelialization and indirectly demonstrating the absence of the barrier at the 14th day. (Bar = 25 μm.)

Based on the results of our study, it would appear that signals derived from contact with blood during the early stages of injury are responsible for the intimal thickening that occurs at later stages. Further experimentation in atherosclerotic vessels will be necessary to determine whether similar mechanisms prevail under conditions of disease. Moreover, additional research will be needed to determine the optimum duration of blockade of blood–vessel wall interactions (29).

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