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Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity

(endothelium/thrombosis)

PETER P. NAWROTH*†, DEAN A. HANDLEY‡, CHARLES T. ESMON†, AND DAVID M. STERN*†

*College of Physicians and Surgeons, Columbia University, New York, NY 10006; ‡Sandoz, Inc., East Hanover, NJ 07936; and †Thrombosis/Hematology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

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ABSTRACT **Previous studies demonstrated that endothelial cells participate actively in both anticoagulant and procoagulant reactions. Although anticoagulant mechanisms predominate on the surface of quiescent endothelial cells, perturbed endothelial cells can promote coagulation through the coordinated induction of procoagulant activity and suppression of anticoagulant mechanisms. Purified recombinant interleukin 1 was infused intravenously into rabbits and coagulant properties of the native aortic endothelium were subsequently studied. Interleukin 1 infusion resulted in a time- and dose-dependent induction of the procoagulant cofactor tissue factor, while concomitantly blocking the protein C anticoagulant pathway. Tissue factor activity increased >10-fold by 3–5 hr after the infusion, while endothelial cell-dependent thrombin-mediated protein C activation decreased by 72% and assembly of functional activated protein C–protein S complex on the vessel surface was decreased by >90%. Scanning electron microscopy of major arteries demonstrated fibrin strands closely associated with the luminal endothelial cell surface with a predilection for bifurcations. Interleukin 1, a mediator of the inflammatory response, can shift the balance of procoagulant and anticoagulant reactions on the endothelium unidirectionally favoring clot formation. The surface of perturbed endothelium can thus provide a template, facilitating the development of a prethrombotic state, and provides a model for the early stages of thrombosis.**

The clinical importance of endothelial cell participation in the regulation of coagulation is emphasized by the thrombotic disorder observed in kindreds deficient in protein C or S (1–6), since function of the protein C anticoagulant pathway depends on cofactors present on the endothelial cell surface (7, 8). In addition to their multiple anticoagulant properties, endothelial cells have been shown to propagate a whole sequence of procoagulant reactions, starting with the expression of tissue factor and culminating in the formation of fibrin (9–12, 37). These considerations have led us to hypothesize that a prethrombotic state could arise in an area in which perturbed endothelium functions as a powerful focus of procoagulant reactions and where the natural anticoagulant mechanisms are suppressed. It is our hypothesis that coagulation mechanisms preventing fibrin formation predominate on the surface of quiescent endothelial cells, whereas perturbed endothelial cells can promote clot formation.

Recent studies have indicated that interleukin 1 (IL-1) can stimulate endothelial cells *in vitro* (13) and that IL-1 is also a product of cultured endothelial cells under certain conditions, including exposure to thrombin (14–17, 38). As a potent mediator of immunologic and inflammatory phenomena (18), IL-1 is released in a variety of pathologic states and thus may be a physiologic mediator of endothelial cell perturbation. This has led us to examine *in vivo* the effect of IL-1 infusion in the rabbit on procoagulant and anticoagulant properties of the native aortic endothelium.

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MATERIALS AND METHODS

Reagents and Assays. Infusion studies were carried out using recombinant murine IL-1 (15), generously provided by Peter LoMedico (Hoffmann La Roche). Heat treatment of IL-1 was carried out at 90°C for 70 min (19) and resulted in loss of IL-1 activity in the thymocyte costimulator assay (20). Control protein, a single-chain polypeptide of M_r 17,000, was also purified from *Escherichia coli* (provided by Peter LoMedico). This protein, distinct from IL-1, has no IL-1 or other known biologic activity and was also free of detectable endotoxin.

Human factor X (130 units/ml) was purified to homogeneity by the method of DiScipio *et al.* (21), and purified human factor VIIa (22) was generously provided by W. Kisiel (University of New Mexico, Albuquerque, NM). These reagents were used to assay the tissue factor by incubating samples with factors VIIa (8 nM) and X (1.1 μM) for 10 min at 21°C in incubation buffer [10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$, and fatty acid-free bovine serum albumin (2 mg/ml)] at a final vol of 0.1 ml. One sample was removed from each reaction mixture, added to 0.5 ml of 50 mM Tris·HCl, pH 7.8/175 mM NaCl/10 mM EDTA/ovalbumin (0.5 mg/ml), and assayed for factor Xa by monitoring hydrolysis at 405 nM of the chromogenic substrate S2222 (Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; Helena Laboratories, Beaumont, TX) (0.1 ml; 0.5 nM) (23). Factors VIIa and X were present at saturating concentrations and factor Xa formation, which was linear, was limited only by the amount of tissue factor.

α-Thrombin (2.5 units/μg), antithrombin III (inhibitory activity of 1.0 unit of thrombin per μg), and protein C were purified to homogeneity from bovine plasma, as described (24–26). Thrombin-mediated protein C activation was studied as described (18). The amount of activated protein C formed was determined from a standard curve constructed with known amounts of enzyme. Activated protein C formation over endothelial cell monolayers was linear over the time of the assay. Where indicated, goat anti-rabbit thrombomodulin IgG (27) (generously provided by N. Esmon, Oklahoma Medical Research Foundation) or control IgG from nonimmune animals was preincubated with the aortic segments.

Human protein S and bovine factor Va were purified to homogeneity as described (21, 28). These reagents were used to study activated protein C–protein S-mediated factor Va inactivation. Activated protein C–protein S-mediated factor Va inactivation was studied as described (8) using a one-stage clotting assay (29). Under the conditions used here, activated protein C and protein S were present at saturating concen-

Abbreviation: IL-1, interleukin 1.

trations. Rates of factor Va inactivation were determined from the slope of the linear initial portion of a plot of factor Va activity versus incubation time.

Binding of protein S to aortic endothelium was studied by using ^{125}I-labeled protein S prepared by the lactoperoxidase method (30), using Enzymobeads according to the manufacturer's instructions.

IL-1 Rabbit Infusion Studies. Recombinant murine IL-1, heat-treated IL-1, or control protein was infused intravenously into rabbits (2–3 kg; Hare-Marland, Hewitt, NJ) from an ear vein in 1 ml of pyrogen-free saline. At the indicated intervals after the infusion, animals were sacrificed by humane euthanasia with an intravenous overdose of sodium pentobarbital. The chest was opened with the heart still beating and the thoracic aorta was dissected free, excised, and rapidly placed in calcium-free magnesium-free Hanks' balanced salt solution (GIBCO), containing fatty acid-free bovine serum albumin (25 mg/ml) at 21°C. After washing the vessels three times in the same buffer, adventitial tissue was removed and aortas were cut longitudinally. Aortic segments were spread flat and placed within a lucite template similar to that described in a previous study (30). After vessel segments were mounted on the lucite template, wells were washed three times with incubation buffer (200 μl per wash) and then 100 μl of incubation buffer was added to each well. Assays for endothelial cell hemostatic properties were then carried out as described.

Scanning electron microscopy, using standard methods (31), demonstrated a continuous monolayer devoid of adherent blood cells (Fig. 1). The endothelial cells exhibit a protuberant character, presumably resulting from lack of transmural pressure. Direct cell counts showed $0.88 \pm 0.09 \times 10^6$ cells (mean ± 1 SD) per cm^2 vessel surface area.

To examine arterial endothelium for evidence of fibrin deposition *in vivo*, animals were infused intravenously with IL-1 (100 ng/kg) and, after an 8-hr incubation period, anesthetized (sodium pentobarbital; 50 mg/kg, intraperitoneally) and subjected to whole body beating heart perfusion fixation using glutaraldehyde (3%) and sodium cacodylate (0.1 M, pH 7.4; 510 mOsm) at 37°C and 90–110 mm Hg. After 30 min of perfusion fixation, major arteries (carotid, aorta, femoral, renal) were excised *en toto*. Samples were rinsed in buffer, osmicated, dehydrated in an ascending ethanol series, critical point-dried, and gold-coated. Samples were viewed in an AMRAY 1600 scanning electron microscope. Control animals, treated with saline alone, were perfused with glutaraldehyde and processed as described above.

RESULTS AND DISCUSSION

Tissue factor, a cofactor for the initiation of coagulation (32), is not normally expressed at high levels on the surface of endothelial cells (33), but it can be induced in cultured endothelium in response to a variety of agents, including IL-1 or tumor necrosis factor (13, 37). Anticoagulant properties, such as endothelial cell cofactor activity for the protein C pathway (7, 8), are readily accessible. In contrast, after infusion of IL-1, there was a time-dependent increase of >10-fold in the tissue factor activity expressed on the surface of native aortic endothelium (Fig. 2*A*). Tissue factor activity was maximal by 5 hr and was reversible with a decline to pretreatment values by 24 hr. The observed factor VIIa-dependent factor Xa formation was due to endothelial cell tissue factor, since scanning electron microscopy (Fig. 1) demonstrated the presence of a continuous layer of endothelium without evidence of other adherent cells or exposure of the internal elastic lamina. Furthermore, control protein and heat-treated IL-1 did not induce tissue factor (Fig. 2*A*), indicating that functionally active IL-1 was the effective perturbant. Tissue factor activity was proportional to the dose of IL-1 infused and it appeared to be uniformly distributed throughout the proximal, mid, and distal portions of the thoracic aorta (Fig. 2*B*). Thus, infusion of IL-1 can induce procoagulant activity in endothelium *in vivo*, although the

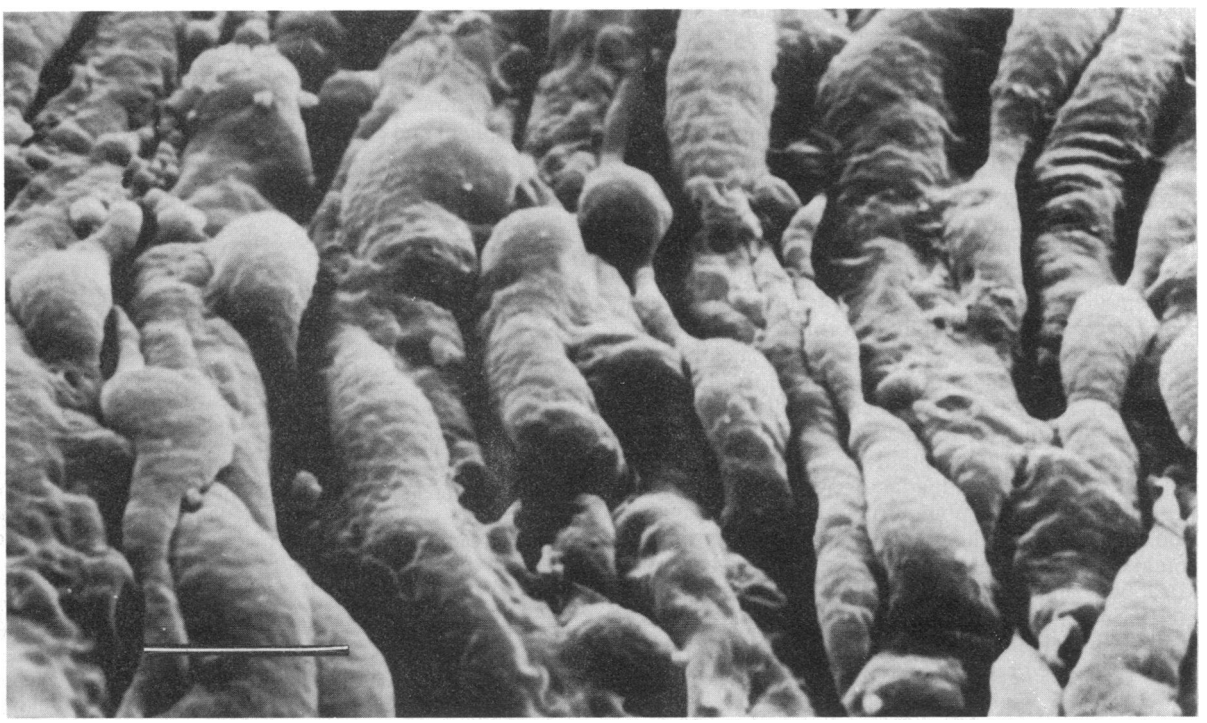

FIG. 1. Scanning electron micrograph of native rabbit aortic endothelium after being subjected to the experimental conditions described in *Methods*. A rabbit was infused with IL-1 (100 ng/kg) and was sacrificed 8 hr later. The endothelium is observed as an intact monolayer. (Bar = 10 μm.)

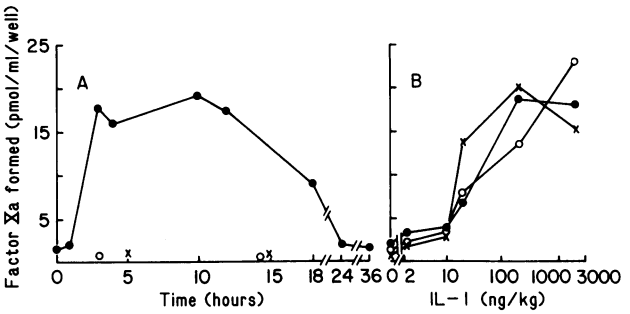

FIG. 2. Induction of tissue factor activity in rabbit aortic endothelium after IL-1 infusion. (*A*) Time course of tissue factor induction. Rabbits (2–3.5 kg; New Zealand White) were infused with recombinant murine IL-1 (6×10^3 units/mg) (0.5 μg per kg of body weight, ●), heat-treated IL-1 (0.9 μg per kg of body weight, ○), or control protein (lot 11319-180-42) (0.8 μg per kg of body weight, ×). Animals were sacrificed at the indicated times and the tissue factor activity of the native aortic endothelium was assayed by monitoring factor VIIa-dependent factor X activation as described in *Methods*. In the absence of factor VIIa, no factor Xa was detectable. (*B*) Dependence of tissue factor induction on the dose of IL-1. Eight hr after infusion of the indicated dose of IL-1, rabbits were sacrificed, aortas were isolated, and segments from proximal (○), mid (×), and distal (●) portions were assayed as described above. Factor Xa formed over 10 min is shown.

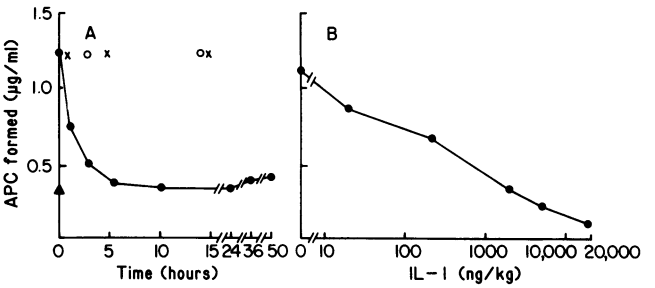

FIG. 3. Effect of IL-1 on endothelial cell-dependent thrombin-mediated protein C activation. (*A*) Time course. Rabbits were infused with IL-1 (1 μg per kg of body weight, ●), heat-treated IL-1 (1.2 μg per kg of body weight, ○), or control protein (1.3 μg per kg of body weight, ×) and sacrificed at the indicated times. Rabbit aortic segments were then incubated with thrombin and protein C, as described in *Methods*, to assess their ability to promote thrombin-mediated protein C activation. Where indicated, goat anti-rabbit thrombomodulin IgG (100 μg/ml) was incubated with the aortic segment for 45 min (▲). Control IgG from nonimmune animals had no effect on the assay. Results are expressed as activated protein C (APC) formed per 40 min (mean of duplicate determinations). Under these conditions, protein C activation was negligible in the absence of endothelium and little or no activated protein C formed in the presence of endothelium if either exogenous protein C or thrombin was omitted. (*B*) Dependence on the dose of IL-1. Eight hr after infusion of the indicated dose of IL-1, animals were sacrificed, and the aortas were assayed for endothelial cell-dependent thrombin-mediated protein C activation as described above. Activated protein C formed per 40 min (mean of duplicate determinations), is shown, and the experiments were repeated three times.

physiological significance of this tissue factor remains to be clarified.

Since endothelium has potent anticoagulant mechanisms, induction of procoagulant activity may not be sufficient for the vessel surface to promote development of a prethrombotic state. This led us to examine the effect of IL-1 infusion on anticoagulant properties of endothelium. We chose to monitor the protein C pathway, since endothelium supplies both a cell-surface protein promoting thrombin-mediated protein C activation, thrombomodulin (7), and cofactor activity, allowing formation of functional activated protein C–protein S complex (8). IL-1 infusion resulted in a time-dependent decrease in thrombin-mediated protein C activation (Fig. 3*A*). Formation of activated protein C was due to interaction with endothelial cell thrombomodulin, as indicated by the 75% inhibition of activated protein C formation in the presence of anti-thrombomodulin IgG (Fig. 3*A*). By 5 hr after IL-1 infusion, there was a 72% decrease in the rate protein C activation compared with controls, and there was only minimal recovery up to 50 hr. Decreased formation of activated protein C was also dependent on the dose of IL-1 infused (Fig. 3*B*), falling to <12% of the controls at an IL-1 dose of 20 μg/kg. Control experiments indicated that decreased protein C activation was not due to inactivation of added thrombin or activated protein C formed.

Activated protein C requires the presence of membrane surfaces and another plasma protein, protein S, for optimal anticoagulant activity (26, 34, 35). Recent studies from our laboratory have demonstrated effective assembly of functional activated protein C–protein S complex on the endothelial cell surface promoting factor Va inactivation (8) and thereby regulating thrombin formation. As expected, inactivation of factor Va in the presence of activated protein C and protein S was observed with aortic endothelium from untreated rabbits, animals infused with control protein, or heat-treated preparations of IL-1 (Fig. 4*A*). In contrast, aortic segments from animals infused 3–5 hr previously with IL-1 showed very attenuated rates of factor Va inactivation (Fig. 4*A*). After 24 hr, only minimal recovery was observed. The loss of endothelial cell-dependent factor Va inactivation by activated protein C and protein S was dependent on the dose of IL-1 infused (Fig. 4*B*) and occurred at concentrations of IL-1 that depressed protein C activation (Fig. 3*B*) and induced tissue factor (Fig. 2*B*) on the endothelial cell surface. In a limited number of studies, it was apparent that aortic endothelium from IL-1-treated animals bound considerably less radioiodinated protein S than control aortic segments [when incubated with ^{125}I-labeled protein S (12 nM), control animals bound 3.2 ± 0.6 fmol per well, whereas IL-1-treated animals (100 ng per kg of IL-1) bound 0.4 ± 0.3 fmol per well]. In contrast, factor X binding to endothelium (36) was not decreased. Therefore, loss of cell-surface protein S binding sites from the aortic endothelium of IL-1-treated animals is probably responsible for the defective factor Va inactivation.

In addition to monitoring specific endothelial cell anticoagulant and procoagulant properties, the rabbit arterial

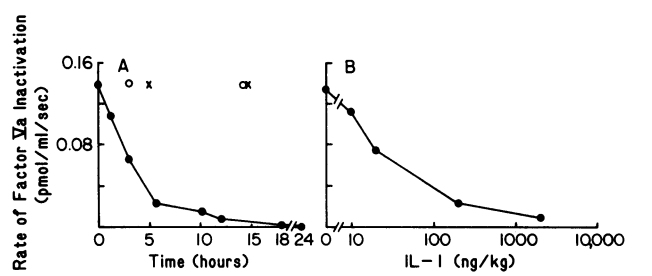

FIG. 4. Effect of IL-1 on endothelial cell-dependent activated protein C–protein S-mediated factor Va inactivation. (*A*) Time course. Rabbits were infused with IL-1 (0.3 μg per kg of body weight, ●), heat-treated IL-1 (0.5 μg per kg of body weight, ○), or control protein (0.5 μg per kg of body weight, ×) and sacrificed at the indicated times. Rabbit aortic segments were assayed for the ability to promote activated protein C–protein S-mediated factor Va inactivation as described in *Methods*. No significant factor Va inactivation occurred in the absence of endothelium. (*B*) Dependence on the dose of IL-1. Eight hr after infusion of the indicated dose of IL-1, animals were sacrificed and aortas were isolated and assayed for endothelial cell-dependent activated protein C–protein S-mediated factor Va inactivation as described above. The initial rate of factor Va inactivation is plotted versus the concentration of infused IL-1. All experiments in *A* and *B* above were repeated three times.

bed was examined for evidence of fibrin deposition. Fibrin strands closely apposed to the endothelial cell surface (Fig. 5*A*), often with associated platelets (Fig. 5*B*), were observed in the vasculature with a predilection for arterial bifurcations. Control animals, treated with buffer alone, did not demonstrate fibrin formation.

These studies demonstrate that *in vivo* infusion of IL-1 can modulate endothelial cell hemostatic properties and result in fibrin formation. Although the ultimate physiologic effect of these changes is not clear, they could potentially form the basis for a prothrombotic state on the luminal surface of the vessel wall. Quiescent endothelial cells from untreated animals had very little tissue factor activity and promoted both the formation and anticoagulant function of activated protein C. Perturbed endothelial cells from IL-1-treated animals, in contrast, had considerably enhanced tissue factor activity and did not supply cofactor activity for the protein C pathway effectively. These results indicate that the balance of anticoagulant and procoagulant activities on the endothelial cell surface can be shifted from the quiescent state, in which inhibitory mechanisms predominate, to a perturbed state, in which procoagulant properties predominate and fibrin formation is promoted. The unidirectional shift in the balance of endothelial cell anticoagulant and procoagulant properties

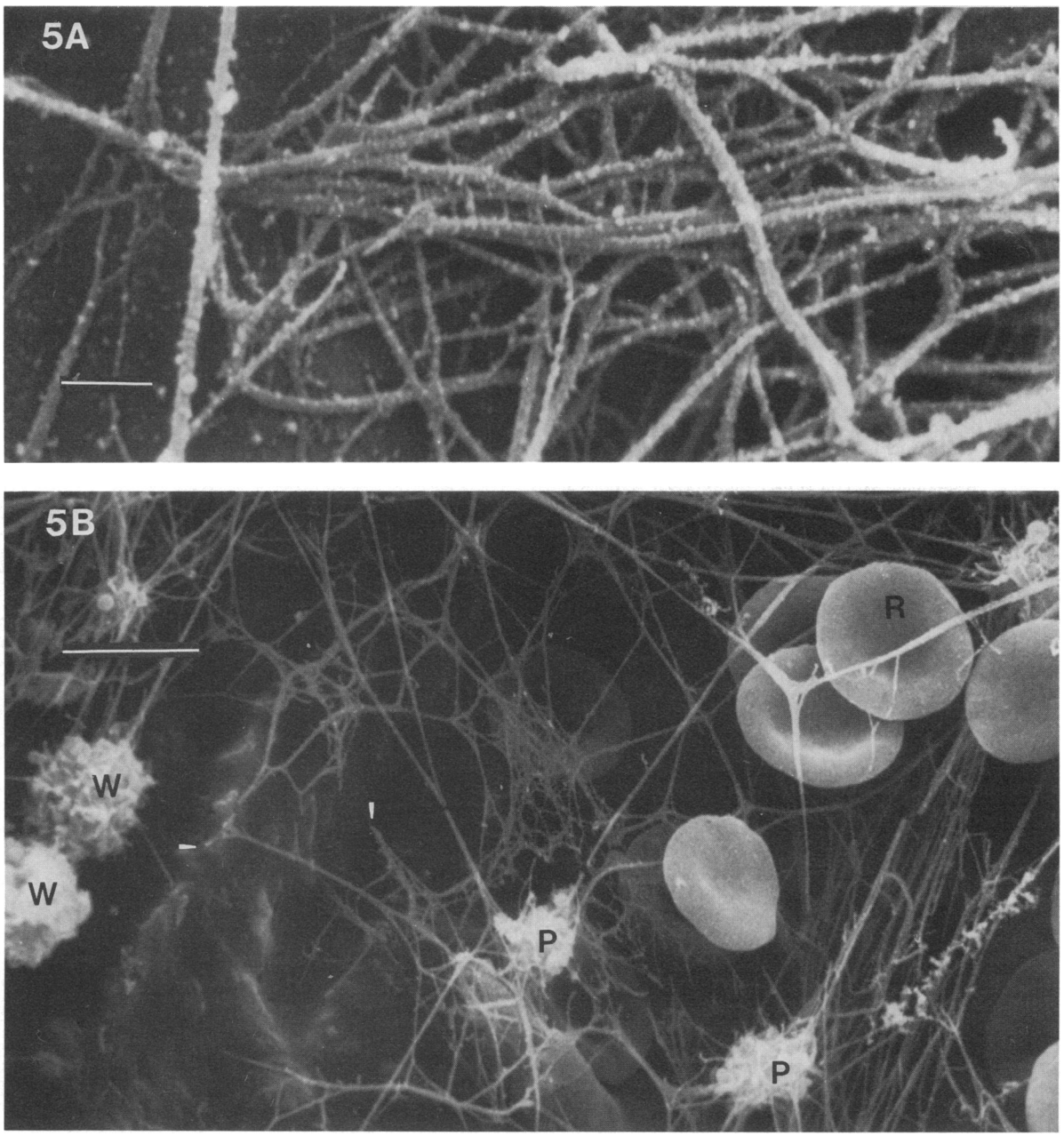

FIG. 5. The association of fibrin with the endothelium of rabbits treated with IL-1. Rabbits were infused with IL-1 (100 ng per kg of body weight), 8 hr later subjected to whole body beating heart perfusion fixation, and major arteries were examined by scanning electron microscopy as described in *Methods*. (*A*) Scanning electron micrograph of carotid artery in which fibrin is seen as an interlocking strand network in direct apposition to the luminal surface of the vascular endothelium. (Bar = 1 μm.) (*B*) Scanning electron micrograph of femoral artery demonstrating fibrin strand formation and platelet deposition. Platelets (P) exhibit an activated state as indicated by pseudopodial projections, which are in direct association with the fibrin strands. Also evident are leukocytes (W) and associated erythrocytes (R). Fibrin strands have an apparent direct association with the endothelial cell surface (marker points). (Bar = 5 μm.) In both cases, the endothelial cells demonstrate a confluent morphology and no evidence of exposure of the internal elastic lamina was observed. The experimental procedure is described in *Methods*.

suggests an explanation for the enhanced thrombotic tendency in inflammatory disease and provides a model of thrombus formation in which endothelial cells are active participants.

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