Antithrombogenic Effect of Urokinase Bound to Collagen Substrate with Bifunctional Antibodies

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In vitro experiments simulating collagen-induced thrombogenesis in arteries damaged during angioplasty demonstrate a marked inhibitory effect of surface-bound urokinase. In contrast to free urokinase, bound enzyme is more effective in preventing platelet aggregation on the collagen surface.

Key Words: *transcutaneous transluminal coronary angioplasty; collagen; urokinase; platelet aggregation*

Transcutaneous transluminal coronary angioplasty is a surgical method of treatment of ischemic heart disease (IHD) for a broad spectrum of indications: unstable stenocardia, acute myocardial infarction, and the state after aorto-coronary bypass grafting [3]. This technique is effective in restoring vascular permeability, but in the course of the operation extensive traumatic lesions occur in the layers of the vascular wall. Collagen exposed to the vascular lumen due to this damage is a powerful inducer of platelet activation and aggregation, leading to the formation of a parietal clot [2,5].

Clot formation is usually prevented by systemic administration of fibrinolytics, anticoagulants, and vasodilators [4]. For this purpose we proposed that during vascular angioplasty urokinase in combination with bifunctional antibodies be intracoronarily injected with a catheter. Bifunctional antibodies exhibiting affinity to urokinase and to collagen of the vascular wall localize urokinase at the site of collagen exposure, protecting the lesioned vascular wall against the formation of a platelet clot. In the present study we investigated *in vitro* the fundamental possibility of an antithrombogenic effect of urokinase immobilized on a collagen substrate with bifunctional antibodies.

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MATERIALS AND METHODS

Thrombogenesis was simulated in a special system comprising microchambers (wells of Falcon multiwell culture plates), magnet stirrers made of a hard magnetic material (Be-Fe-B), and a magnetic field generator, as described previously [1]. The stirrers agitate the fluid in the wells at a rate of 1 rev/sec, which corresponds to a 500-sec^{-1} shear rate at the bottom of the wells, as in elastic arteries. Whole blood was drawn from the ulnar vein of donors and centrifuged at 1000 g for 12 min not later than 15 min after being drawn. Sodium citrate (130 mM) was used as an anticoagulant. The cell count was determined with an automatic platelet counter. It ranged from 4.5 to 5×10^8 cells/ml. Rat collagen in a concentration of 10 μg/ml was preliminarily absorbed in the wells of the culture plates. Platelet-rich plasma (PRP) was placed in the wells, and agitated at a rate of 1 rev/sec for 0-60 min. Rat collagen, type I-III, was extracted from rat tail tendon with 2% pepsin in 500 mM acetic acid, pH 2.5, purified by redissolving in acetic acid, and sedimented in acid and neutral NaCl solutions (1 M). Rabbit polyclonal anticollagen antibodies were isolated from the serum of

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immunized rabbits by elution from immunosorbent (Sepharose-2B-CL-rat collagen) with a 40-mM sodium citrate buffer in 50% ethylene glycol (pH 2.2). Anticollagen antibodies were modified with MBS (maleimidobenzoyl-N-hydroxysuccinimide, Sigma) using 10 MBS molecules per antibody molecule, i.e., 90 nmol MBS (20 mM; 4.5 μl) in dimethylsulfoxide were added to 9 nmol antibodies (1.5 μg/ml; 1 ml) in borate buffer (pH 8.2) and incubated at room temperature for one hour. Monoclonal antibodies to urokinase were prepared by ion-exchange chromatography on DEAE-Toyopearl from the culture medium of UNG-5 hybridomas. Antibodies were modified with SATA (N-succinyl-S-acetyl-thioacetate, Calbiochem) using 5 mol SATA per mol antibody, i.e., 45 nmol SATA (10 mM; 4.5 μl) in dimethylsulfoxide were added to 9 nmol antibodies (3 mg/ml; 0.05 ml) in borate buffer (pH 8.2) and incubated at room temperature for one hour. Both preparations were purified from excess reagents on a G-25f microcolumn, transferring them to a 50 mM sodium-sulfate buffer (pH 7.0). Aliquots of both samples were taken to test the amount of succinimide and maleimide groups that entered into reaction (reaction with dithio-bis(2-nitrobenzoic acid) in a sodium-borate buffer, pH 8.7, containing 2 mM ethylenediamine tetraacetate; calibration performed with β-mercaptoethanol, 0-50 nmol; optical density measured on a spectrophotometer at 412 nm).

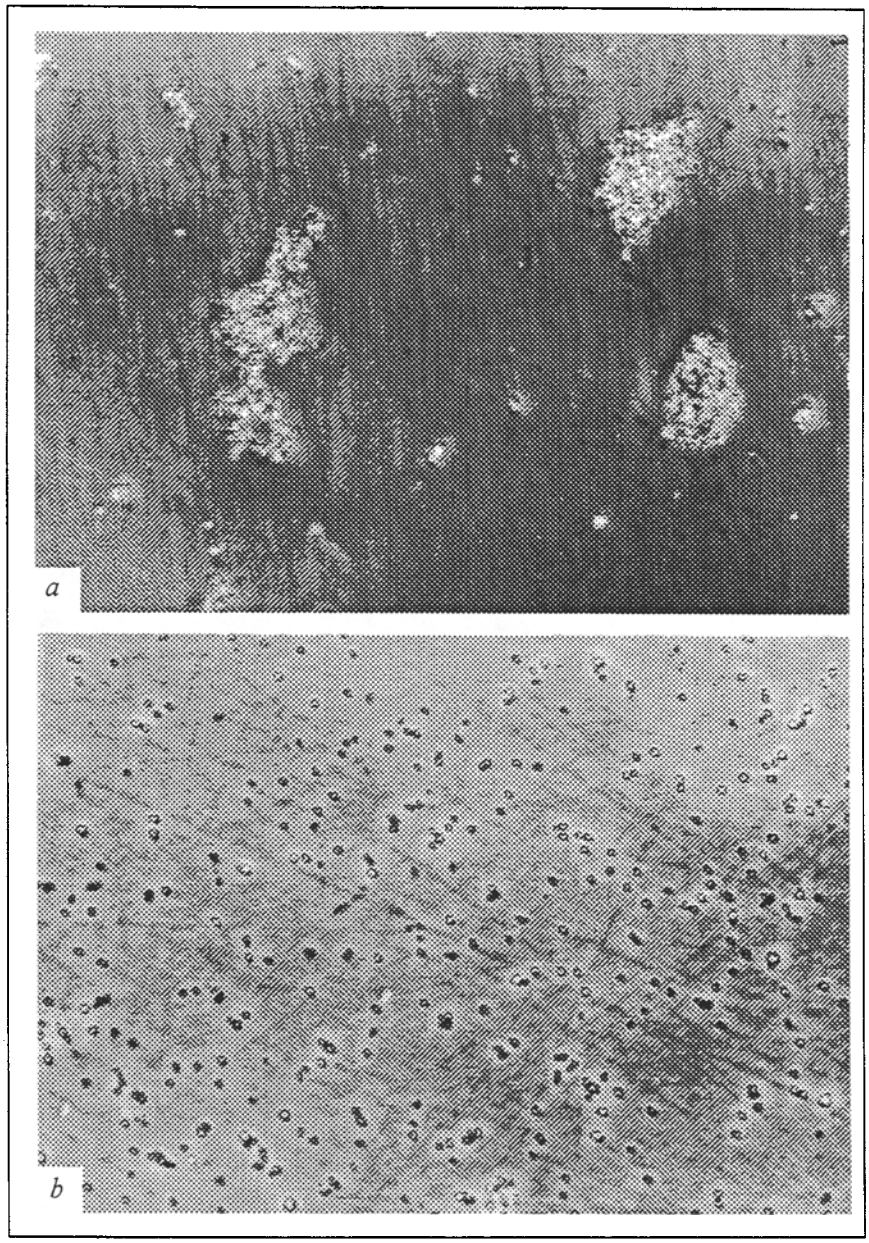

Fig. 1. *a*) platelet aggregation on collagen substrate; *b*) urokinase, immobilized on collagen substrate with bifunctional antibodies, inhibits platelet aggregation. Phase contrast, ×600.

The two modified antibodies were mixed in an equimolar ratio and incubated for 1 h in a buffer containing 50 mM hydroxyamine, 2 mM ethylenediamine tetraacetate, and 50 mM sodium phosphate, pH 7.0. After the buffer was replaced with a borate buffer by means of gel-filtration (Sepharose G-25f), the reaction mixture was dialysed against borate buffer, pH 8.2. The reaction was stopped by the addition of 2 mM iodacetamide and, 20 min later, of 4 mM β-mercaptoethanol. Conjugated antibodies were purified by gel-filtration on AcA-44 Ultragel. The bifunctional properties of the conjugate were determined by enzyme immunoassay, and its ability to deliver urokinase to plastic was assessed as the amidolytic activity of urokinase reacting with the synthetic substrate (Glp-Gly-Arg-pNA, Serva). The heteroconjugate formed by polyclonal antibody to collagen and monoclonal antibody to urokinase was absorbed by the collagen substrate from a concentration of 10 μg/ml during 1 h under stirring; 5 μg/ml uric urokinase (160,000 Plough units) were then added, and incubation was performed under stirring for 0.5 h. The plates with collagen substrate, as well as the plates with collagen and bifunctional antibodies and urokinase adsorbed, were used for

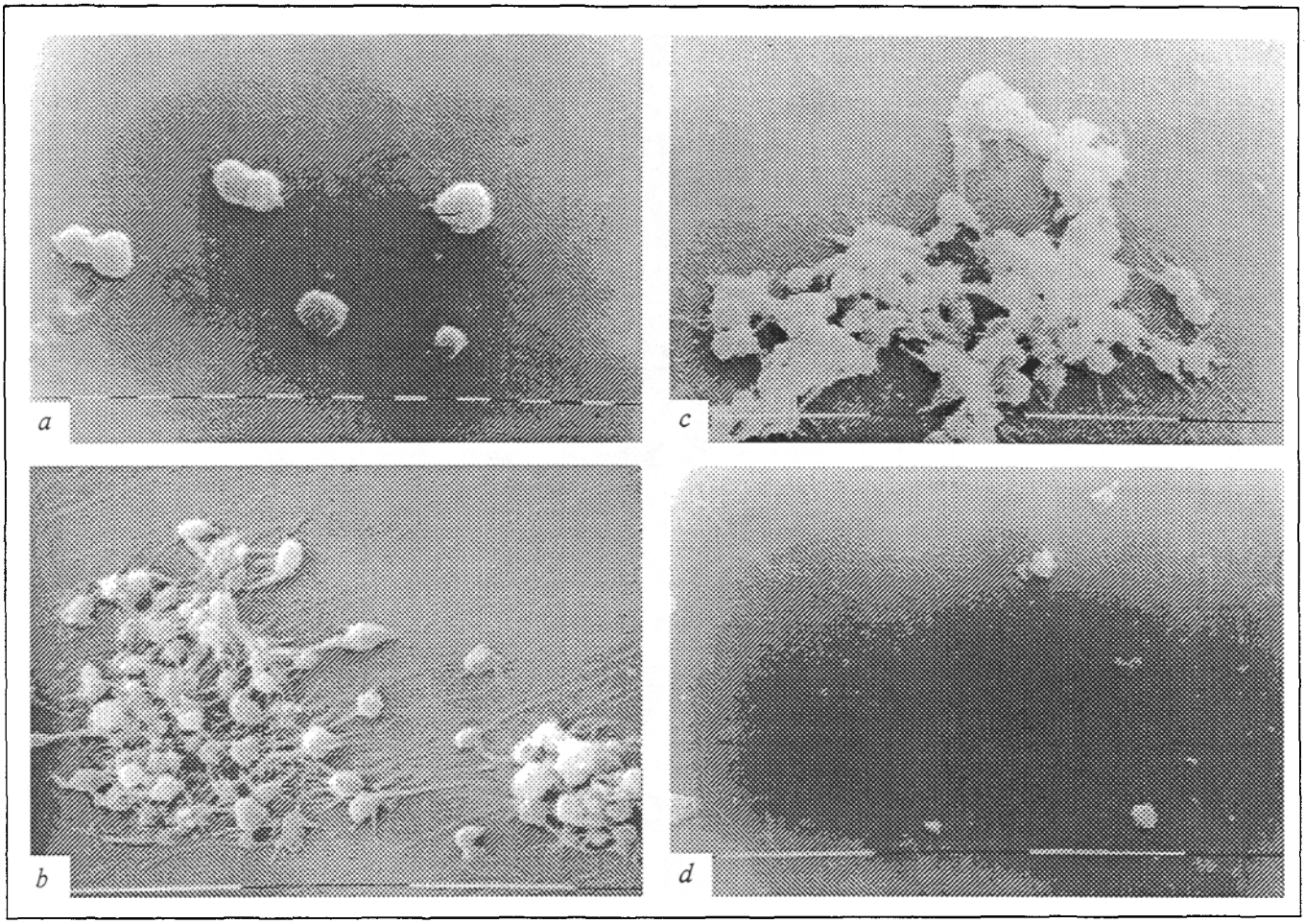

Fig. 2. *a*) interaction of platelets with plastic surface; *b* and *c*) platelet activation and aggregation on collagen substrate; *d*) absence of platelet activation in the presence of urokinase immobilized on collagen substrate. Electron microscopy, ×2500.

thrombogenesis simulation *in vitro*. PRP (1 ml) was added to the wells. The stirrers, which were also the covers of microchambers, agitated PRP at a rate of 1 rev/sec (60 rpm) for 0-60 min, so that the shear rate at the bottom of the wells was 500 sec^{-1}. Platelet aggregation on the collagen substrate was assessed using a phase-contrast and then an electron microscope.

After the termination of stirring, PRP was removed from the wells, and the cell count was determined; the bottom of the wells was rinsed with Tyrode solution (NaCl 137 mM, KCl 2.7 mM, sodium phosphate 0.36 mM, glucose 0.1%, $MgCl_2$ 1 mM, pH 6.5), in which the number of platelets was counted. A 2.5% glutaraldehyde solution in Tyrode buffer (0.5 ml) was then added to each well and incubated at room temperature for 2 hours. The bottoms of the wells were rinsed with physiological saline and cut off. The samples obtained were dehydrated in ascending grades of alcohol (30-100°), shadowed with silver, and examined under an electron microscope (×2500-10,000).

TABLE 1. Interaction of Platelets with Collagen Substrate

Stage of platelet activation	Plastic, PRP	Collagen, PRP	Collagen, PRP, urokinase	Collagen, PRP, uroinase, antibodies
Formation of pseudopodia	–	+	+	–
Plating	–	+	+	–
Adhesion	–	+	+	–
Aggregation	–	+	+	–

RESULTS

The results of phase-contrast microscopy showing interaction between platelets from PRP and collagen substrate, as well as collagen substrate protected with urokinase immobilized with antibodies, are presented in Fig. 1, *a, b*.

These preliminary conclusions are confirmed by the results of determination of the number of platelets remaining in the PRP after interaction with the collagen substrate and immobilized urokinase, as well as in the rinsing Tyrode solution.

The initial platelet count in PRP was 5×10^8 cells/ml. The smallest amount of cells (2.1×10^8 cells/ml) remained in PRP after interaction with the collagen substrate, and the largest amount (3.8×10^8 cells/ml) after interaction of PRP with the collagen substrate protected with immobilized urokinase.

In the first case 2.8×10^8 cells/ml remained attached to the collagen surface in the form of aggregates, and in the second case 1×10^8 cells/ml remained in the form of solitary cells. The absence of platelet aggregation after the addition of PRP to the plastic surface without collagen cover attests to the collagen-dependent aggregation of platelets. In this case, after the completion of the experiment, the number of cells in PRP was maximal (4.4×10^8 cells/ml). After interaction of PRP with collagen coating in the presence of urokinase (5 μg/ml) the number of cells constituted 3×10^8 cells/ml; 2×10^8 cells/ml remained in the form of aggregates at the bottom of the well. The interaction between platelets and collagen and the same interaction in the presence of free urokinase in the plasma, as well as in the presence of urokinase immobilized on the collagen substrate with chimeric antibodies, was studied in more detail in the series of experiments using the electron microscope. The results are presented in Table 1.

As follows from the table, platelet aggregation is collagen-dependent, evidence of which is the absence of signs of platelet activation during interaction of PRP with the plastic surface (Fig. 2, *a*). Monomolecular collagen under these conditions causes contact activation and aggregation of platelets (Fig. 2, *b*, *c*). Dissolved urokinase in a concentration of 5 μg/ml does not inhibit collagen-dependent activation and platelet aggregation; urokinase (5 μg/ml) immobilized on the collagen substrate with bifunctional antibodies (10 μg/ml) blocks contact activation and aggregation of platelets (Fig. 2, *d*).

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