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Endothelial cell culture on fibrillar collagen: Model to study platelet adhesion and liposome targeting to intercellular collagen matrix

(vessel wall model/shape of adherent platelets/fibronectin/targeting of liposomes)

E. I. CHAZOV, A. V. ALEXEEV, A. S. ANTONOV, V. E. KOTELIANSKY, V. L. LEYTIN, E. V. LYUBIMOVA, V. S. REPIN, D. D. SVIRIDOV, V. P. TORCHILIN, AND V. N. SMIRNOV

Union of Soviet Socialist Republics Research Center of Cardiology, Academy of Medical Sciences, Petroverigsky Lane 10, Moscow 101837, Union of Soviet Socialist Republics

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ABSTRACT **Human umbilical endothelial cells (ECs) were grown on fibrillar type I collagen in 16.4-mm multiwell tissue culture plates. Human platelets were added to the wells, and platelet adhesion to collagen was examined by scanning electron microscopy and radioisotopic technique in the absence of ECs and in preconfluent and confluent EC cultures. Single adherent platelets of different shapes as well as small aggregates were seen on collagen surface. Human plasma fibronectin added to the system stimulated platelet adhesion and their spreading on collagen. ECs had no effect on the percentage of platelets adherent to collagen-coated gaps in preconfluent culture but decreased the number of spread platelets. It is demonstrated that collagen-coated gaps can bind ^{14}C-labeled liposome–antibody and ^{14}C-labeled liposome–fibronectin conjugates. ECs grown on fibrillar collagen are suggested as useful models for screening of antiplatelet drugs and for the study of drug targeting to the areas of vascular injury for prevention of thrombosis.**

At present, injury of the endothelial lining of the vessel wall is viewed as one of the key mechanisms of thrombogenesis and, possibly, of atherogenesis (for review, see refs. 1–3). The injury or loss of endothelial cells (ECs) results in the loss of athrombogenic properties by the vessel wall and this leads to adhesion of blood platelets to the injured surface. It is thought that the recognition of injury occurs through a specific interaction of platelets with subendothelium, of which several constituents, particularly collagen, have high affinity toward platelets (1, 2).

To study the mechanism of platelet adhesion to the injured vessel wall, everted vessel segments or surfaces coated with fibrillar collagen have been used (2, 4–6). In the work reported here, an experimental model is described which includes partial reconstitution of the luminal surface of "normal" and "injured" vessel wall. This surface is imitated by a bilayer structure of confluent or preconfluent EC cultures grown on fibrillar collagen. The model was used for: (*i*) quantitative studies of platelet adhesion to collagen-coated gaps in preconfluent EC cultures and to the surface of ECs; (*ii*) examination of effects produced by plasma fibronectin and ECs on the adhesion and spreading of platelets on fibrillar collagen; and (*iii*) attempts to develop an approach for specific recognition of collagen-coated gaps in preconfluent EC culture by ^{14}C-labeled liposome-antibody to type I collagen and ^{14}C-labeled liposome–fibronectin conjugates.

MATERIALS AND METHODS

Isolation of Fibronectin, Collagen, and Antibodies to Collagen. Human plasma fibronectin was isolated by affinity chromatography on gelatin-Sepharose (7) followed by chromatography on DEAE-cellulose (8). Type I collagen was prepared from bovine tendons by extractions with 0.5 M CH_3COOH, subsequent multiple reprecipitation with 0.9 M NaCl, and chromatography on DEAE-cellulose (9). Fibronectin and type I collagen preparations were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (10, 11) and were at least 95% pure. Antibodies to type I collagen were raised in rabbits by injection of 5 mg of type I collagen with complete Freund's adjuvant into foot pads and subcutaneously followed by a booster injection of 5 mg of antigen without adjuvant intraperitoneally on the 10th day (12). Antibodies to type I collagen were purified by immunoadsorption of immobilized type I collagen after by chromatography on type II and III collagen immunosorbents.

EC Cultures on Fibrillar Collagen. To prepare collagen-coated multiwell tissue culture plates, 0.3 ml of type I collagen solution (1 mg/ml in 2 mM CH_3COOH/0.165 M NaCl/0.024 M potassium phosphate, pH 7.1) was added to each 16.4-mm well of Falcon 3008 multiwell plates, incubated for 40–50 min at 37°C, and dried in air. Human umbilical ECs were isolated, cultivated, and identified according to Gimbrone *et al.* (13). Cells (6–7 × 10^4) in medium 199 were seeded into each well and grown for 3–5 days (preconfluent cultures) or 7–9 days (confluent cultures).

Preparation of Platelets and Study of Adhesion. Human platelets were obtained from platelet-rich plasma by gel filtration on Sepharose 2B (14) at 37°C in Ca^{2+}-, Mg^{2+}-free Tyrode solution containing apyrase (Sigma) at 0.2 mg/ml and bovine serum albumin (Sigma) at 3.5 mg/ml. To prepare ^{51}Cr-labeled platelets, platelet-rich plasma was incubated for 40–60 min at 37°C with $Na_2{}^{51}CrO_4$ (100–400 mCi/mg of chromium; 1 Ci = 3.7 × 10^{10} becquerels; Amersham) prior to gel filtration. The specific activity of ^{51}Cr-labeled platelets was 30,000–50,000 cpm per 10^8 platelets.

Platelet adhesion was studied in wells containing fibrillar type I collagen confluent EC culture grown on type I collagen preconfluent EC culture grown on the same support as type I collagen pretreated with fibronectin. In the last case, 50–200 μg of fibronectin in 0.15 ml of Tyrode solution containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ was added to each well and incubated for 90 min at 37°C. Unbound protein was either washed out with Tyrode solution or the adhesion was studied in the presence of free fibronectin. Platelet suspension (0.15 ml) was added to each well (1.5–2.0 × 10^7 platelets) in Tyrode solution containing Ca^{2+}, Mg^{2+}, apyrase (0.2 mg/ml), and bovine serum albumin (3.5 mg/ml). The multiwell plate was incubated at 37°C for 40 min with horizontal rotation at 36 rpm. Nonadherent platelets

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Abbreviations: EC, endothelial cell; SEM, scanning electron microscopy.

were removed, and wells were washed once with 0.2 ml of Tyrode solution. To quantitate platelet adhesion, well bottoms were cut out and ^{51}Cr radioactivity was measured. To determine the number of adherent platelets by scanning electron microscopy (SEM), 0.2 ml of Tyrode solution containing 2.5% glutaraldehyde (pH 7.3) was added to each well after the removal of nonadherent platelets. The multiwell plate was incubated for 2 hr at 37°C, and then each well washed three times with 0.2 ml of 0.15 M NaCl (pH 7.3) and dehydrated with increasing concentrations of ethanol (40% to 100%). Segments of the well bottom were cut out, dried at the critical point, and coated with platinum/palladium. Platelets were counted by using Philips PSEM at ×500.

Preparation of Liposomes, Protein–Liposome Conjugates, and Binding Studies in EC Cultures. ^{14}C-Labeled liposomes were prepared in 5 mM NaH_2PO_4/0.01 M $Na_2B_4O_7$/0.145 M NaCl, pH 8.5, with a mixture of lecithin, cholesterol, and phosphatidylethanolamine in the molar ratio 6:2:2 (15). [^{14}C]Cholesterol oleate (34 Ci/mol, Amersham) was used as a nonexchangeable membrane marker. To prepare the conjugates of ^{14}C-labeled liposome and fibronectin, ^{14}C-labeled liposome and antibody to type I collagen, and ^{14}C-labeled liposome and nonimmune rabbit IgG, ^{14}C-labeled liposomes were activated with glutaraldehyde (15) and adjusted to a lipid concentration of 1 mg/ml, and protein was added to the liposome suspension to a final concentration of 1 mg/ml. The suspension was incubated overnight at 4°C. Unbound protein was separated from a corresponding conjugate by gel filtration on Sepharose 4B. The protein-to-lipid ratio in the conjugates was 0.1–0.2 mmol of protein per mol of lipid, which corresponds to the binding of 10–20 protein molecules per liposome of average size ≈1000 Å (100,000 molecules of lipid). The specific activity of liposomes and their conjugates was 4.5 μCi/mg of lipid.

In binding studies, medium 199 (0.5 ml) containing 10% fetal calf serum, ^{14}C-labeled liposome, or ^{14}C-labeled liposome–protein conjugate (approximately 200,000 dpm) was added to each well. The multiwell plate was incubated for 30 min at 37°C with horizontal rotation (36 rpm). Unbound liposomes were removed, and each well was rinsed twice with 0.5 ml of Tyrode solution. Tightly bound radioactivity was solubilized with two 0.5-ml portions of 10% (vol/vol) Triton X-100; the eluates were pooled and assayed for radioactivity. In each experiment the binding was calculated as the average of six or seven parallel determinations.

RESULTS

Modeling of the Luminal Surface of the Vessel Wall. To simulate *in vitro* the structure of the luminal surface of a large vessel, ECs were grown in 16.4-mm multiwell tissue culture plates with well bottoms coated with fibrillar type I collagen. After immobilization of the collagen, a dense layer of collagen fibrils formed on the well bottom (Fig. 1*A*). For the purpose of this study the collagen layer imitates the subendothelium of a large vessel after extensive denudation.

To simulate the noninjured luminal surface of the vessel wall, ECs were grown to confluency on type I collagen. At confluency, the collagen fibrils were completely covered with the monolayer of polygonal ECs (Fig. 1*B*). The cell density in confluent culture was about 0.8–1.0 × 10^5 cells per cm^2.

To imitate the luminal surface of the vessel wall with small focal injuries of endothelial lining, preconfluent EC cultures were used (Fig. 1*C*). On days 3–5 after the seeding, separate single cells as well as cells contacting each other were seen on the collagen surface. These cells covered 30–50% of the bottom area. Collagen-coated gaps were seen between individual cells.

Platelet Adhesion. Incubation of platelets in wells containing a layer of fibrillar collagen led to platelet adhesion to the collagen surface (Fig. 1*D*). The adhesion was dependent on time and dose (Fig. 2). Under standard experimental conditions, the adhesion reached its plateau after 40 min of incubation. The number of adherent platelets was a linear function of platelet count in the range 1.0 × 10^5 to 2.4 × 10^7 platelets per well.

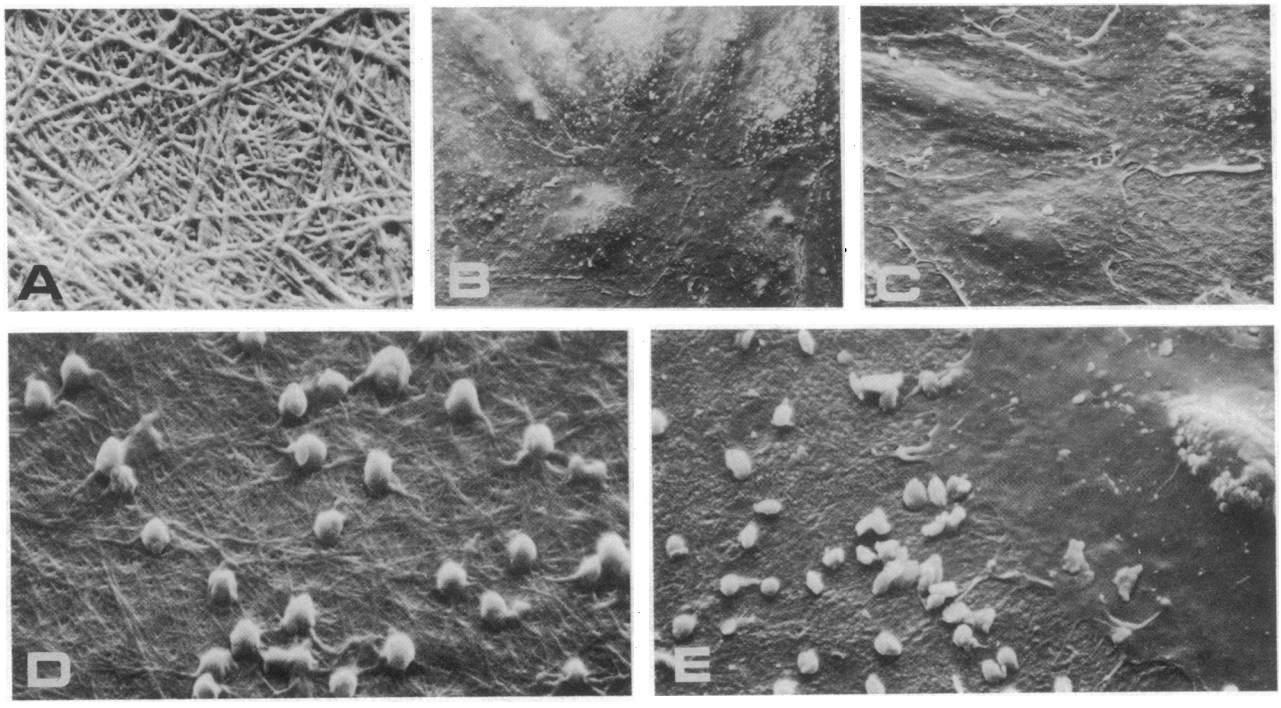

FIG. 1. Imitation of the luminal surface of the vessel wall and platelet adhesion. (*A*) Well bottom coated with fibrillar type I collagen. (×6140.) (*B*) Confluent EC culture on type I collagen, day 8. (×770.) (*C*) Preconfluent EC culture on type I collagen, day 3. (×850.) (*D*) Platelet adhesion to fibrillar type I collagen. (×2350.) (*E*) Platelet adhesion to type I collagen-coated gaps in preconfluent EC culture. (×1170.)

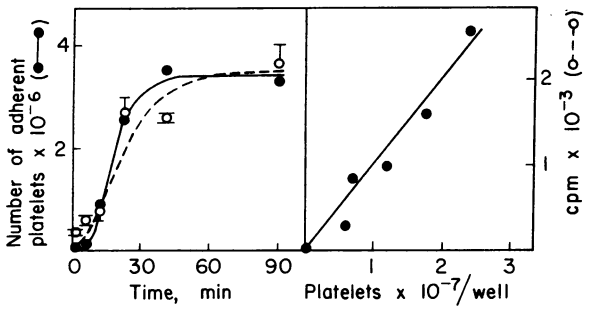

FIG. 2. Platelet adhesion to fibrillar type I collagen. (*Left*) As a function of time. (*Right*) As a function of dose. ^{51}Cr-Labeled platelets (1.5×10^7) were added to each well. Adhesion was measured by SEM and radioisotopic techniques. The values are expressed as mean ± standard error of the mean ($n = 4$).

Similar dependences were observed by SEM and radioisotopic techniques with ^{51}Cr-labeled platelets.

When platelets were incubated with the preconfluent EC culture grown on type I collagen (Fig. 1*E*), platelet adhesion to the surface of ECs was low ($0.9 \pm 0.3 \times 10^3/mm^2$, mean ± SEM; $n = 4$). This value is approximately 10% of the adhesion to fibrillar collagen in the absence of ECs ($9.5 \pm 1.6 \times 10^3/mm^2$; $n = 7$). In the preconfluent cultures the platelets adhered to collagen-coated gaps between cells. The adhesion density in these areas ($8.9 \pm 2.1 \times 10^3/mm^2$; $n = 4$) did not differ from the adhesion to fibrillar collagen in the absence of ECs. The adhesion of platelets to the surface of ECs in confluent and preconfluent endothelial cultures was similar ($0.9 \pm 0.3 \times 10^3/mm^2$; $n = 3$).

By using SEM, one can follow the changes in the shape of platelets during their interaction with collagen-coated surface (Fig. 3). The initial suspension of platelets used in the adhesion experiments contained discs (34 ± 5%), discs with pseudopods (27 ± 5%), spheres (23 ± 5%), and spheres with pseudopods (16 ± 4%) (means ± SEM; $n = 8$). After 40 min of incubation with the layer of fibrillar collagen, this ratio was drastically changed. The majority of adherent platelets were spheres and discs with pseudopods seen as singles or small aggregates. Discs were found only rarely, some platelets were spread, and thrombi were absent.

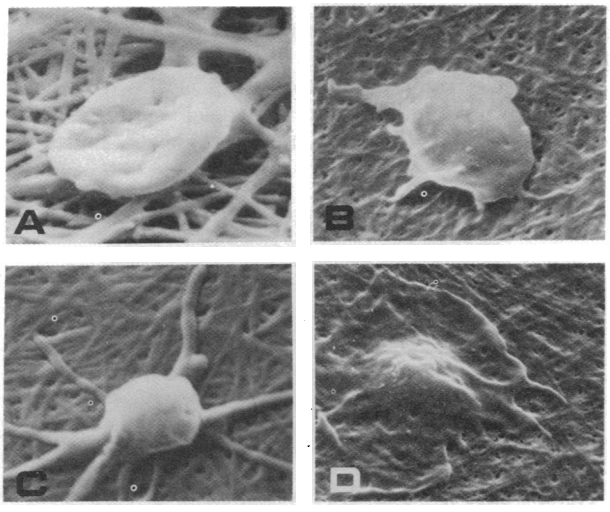

FIG. 3. Shape of platelets adherent to fibrillar type I collagen. (*A*) Disc. (×9000.) (*B*) Disc with protuberances and pseudopods. (×5760.) (*C*) Sphere with pseudopods. (×6750.) (*D*) Spread platelet. (×4420.)

Effects of Fibronectin and ECs on Adhesion and Spreading of Platelets. The addition of human plasma fibronectin to the system containing ^{51}Cr-labeled platelets and the layer of fibrillar collagen stimulated total adhesion of platelets to collagen (Table 1). When 50–200 μg of fibronectin was added to the incubation mixture, 2- to 3-fold stimulation of adhesion was observed. In the absence of fibronectin, the ratio between spread and nonspread platelets was 1:4. In the presence of this protein, it was 2:3 (Fig. 4; Table 1). Thus, fibronectin stimulates not only the adhesion of platelets to collagen-coated surface but also their spreading.

The shape of platelets adherent to collagen-coated gaps in preconfluent EC culture was altered compared to that observed after adhesion in the absence of ECs (Table 1): the number of spread platelets was decreased and no effect of ECs on the total adhesion was seen. It is likely that ECs do not affect primary contacts of platelets with collagen but rather inhibit the process of platelet spreading.

Binding of ^{14}C-labeled Liposome–Carrier Conjugates to Collagen Matrix in EC Cultures. The EC cultures grown on fibrillar type I collagen and imitating various degrees of vessel wall injury can be used as a convenient model to locate "denuded" areas in the vascular wall. The conjugates between liposome and a carrier were used for this purpose, the carrier molecule being capable of locating the injured surface and securing the conjugate in this area and the liposome serving as a container for a potential drug. Because in our model the layer of fibrillar type I collagen imitates exposed subendothelium of the injured vessel, fibronectin and antibodies to type I collagen were tested as carriers. Both proteins have high affinity toward type I collagen and can be conjugated with liposomes.

The results of experiments on binding of ^{14}C-labeled liposomes and liposome–protein conjugates by fibrillar type I collagen-coated surfaces and by preconfluent and confluent EC cultures grown on fibrillar type I collagen are presented in Fig. 5. The presence of the molecule with high affinity to type I collagen in a conjugate increased the binding of the conjugate by collagen-coated surface 3.5- to 4-fold compared to the binding of ^{14}C-labeled liposome–nonimmune IgG complex. The monolayer of ECs grown on type I collagen weakly bound all types of conjugates and ^{14}C-labeled liposomes. Preconfluent EC culture with collagen-coated gaps between ECs was characterized by values of binding for liposome–antibody to type I collagen and liposome–fibronectin intermediate between fibrillar type I collagen surface and confluent EC culture.

SEM of ^{14}C-labeled liposome–antibody to type I collagen binding in the preconfluent EC culture is shown in Fig. 6. The binding of the conjugates occurred only to collagen-coated gaps between ECs; the conjugates were not bound by the surface of ECs.

Table 1. Effects of fibronectin and ECs on platelet adhesion to and spreading on fibrillar collagen

Surface	Adhesion*	Spreading†
Fibrillar type I collagen	1.0	20.9 ± 4.6
Fibrillar type I collagen pretreated with fibronectin	2.5 ± 0.4 $P < 0.01$	42.2 ± 3.1 $P < 0.01$
Collagen-coated gaps in preconfluent EC culture	1.1 ± 0.3 (NS)	7.0 ± 3.0 $P < 0.05$

All values are mean ± SEM. P was calculated by using Student's t test. NS, not significant.

* Adhesion is expressed in arbitrary units, 1 unit being equal to 5.9×10^3 platelets per mm^2; $n = 6$–9.

† Spreading is expressed as percentage of total adherent platelets; $n = 6$–9.

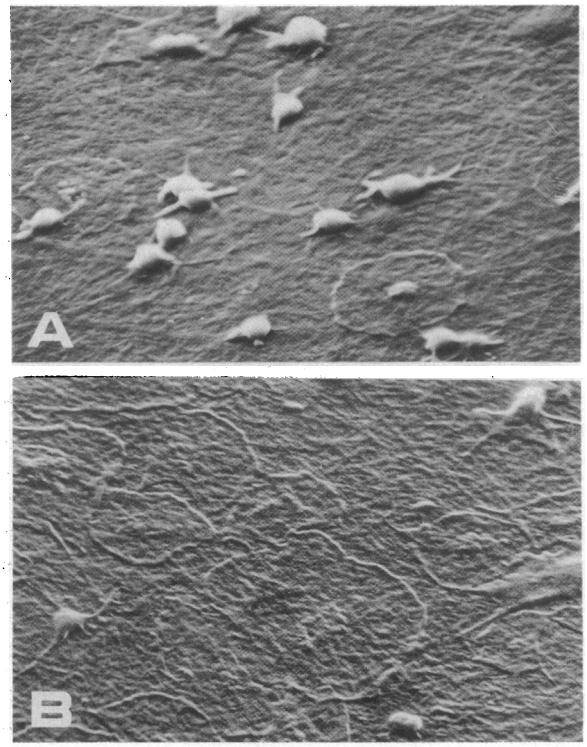

FIG. 4. Effect of fibronectin on platelet adhesion and spreading. (*A*) Platelets on type I collagen. (×2150.) (*B*) Platelets on fibronectin-treated type I collagen (100 μg of fibronectin per well). (×2600.)

DISCUSSION

In this study the two-component bilayer model consisting of human umbilical ECs grown on fibrillar type I collagen is proposed to imitate *in vitro* the vessel wall luminal surface. This model was used to study platelet adhesion and to develop an approach for liposome targeting to injured vessel areas.

Intact vessel wall as well as various degrees of injury may be imitated by using this model beginning from the loss of 1–10 ECs up to large denudation. Compared to other models used to study platelet adhesion (2, 4–6, 16, 17) our system has a number of specific features including quantitative estimation of adhesion by two independent techniques, the possibility to study platelet adhesion to the cell surfaces, and the possibility to estimate the effect of cells and various other factors on platelet adhesion and platelet shape.

In the model described, adhesion of platelets occurs under conditions of nonlaminar flow. The study of platelet behavior in this situation is of particular interest because it is in the vascular regions with disturbed laminar flow that mural thrombi are formed more actively (1). In our model the kinetic curve of platelet adhesion to fibrillar type I collagen reaches saturation at 40 min of incubation—i.e., later than in a perfusion chamber (2, 18) or in the system with rotating probe (5). The slowing down of adhesion and the absence of thrombi formation made it possible to visualize various shapes of platelets adherent to the collagen: nonactivated platelet discs, and activated non-spread platelets including discs and spheres with pseudopods and spread platelets. Similar forms of adherent platelets were described in studies of platelet interaction with other substrates (19–21). It is likely that contact activation of platelets includes two steps: (*i*) adherent discs are transformed into discs and spheres with protuberances and pseudopods, and (*ii*) the spreading of platelets occurs. The shape changes of platelets during adhesion may reflect general principles involved in the interaction of eukaryotic cells with various substrates described by Vasiliev and Gelfand (22, 23).

Earlier, Hynes *et al.* (24) demonstrated that plasma fibronectin stimulates platelet adhesion to surfaces coated with collagen. At the same time it has been found that fibronectin inhibits platelet interaction with collagen fibrils in suspension (25, 26). We have demonstrated that the pretreatment of collagen-coated surface with plasma fibronectin stimulates adhesion as well as spreading of platelets. On the other hand, ECs have no effect on the level of platelet adhesion to collagen but inhibit platelet spreading. Because spread platelets are often found at the base of mural thrombi (2), our data may serve as evidence of a possible regulatory function of ECs and plasma fibronectin in the formation of thrombi over injured areas of vascular wall.

The model was used to explore the possibility of the liposome targeting to collagen matrix between ECs grown on fibrillar type I collagen. It has been found that liposome–antibody to type I collagen and liposome–fibronectin conjugates are selectively bound by collagen matrix between ECs. It is thought that the liposome–carrier conjugates bound by denuded areas of endothelial layer *in vivo* can function as the microreservoir for a diffusible drug or for enzyme(s) capable of transforming locally nonactive substrates into pharmacologically active drugs.

In liposome–antibody to type I collagen conjugate the antibody molecule and the surface of the liposome are spatially separated and antigen–antibody interaction occurs outside the liposome surface. Covalent binding of an antibody to the liposome surface (15) or partial embedding of an antibody into the lipid bilayer (27, 28) may result *in vivo* in the formation of an antigen–antibody–liposome complex which would activate the complement system and result in breakage of the liposome and release of a drug.

The use of liposome–carrier conjugates for directed transport of a drug to the injured area of the vessel wall *in vivo* might require the use of a mixture of antibodies to several constituents

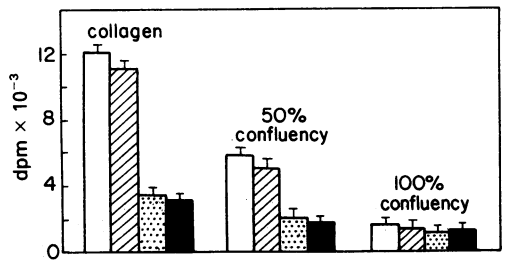

FIG. 5. Binding of ^{14}C-labeled liposome–carrier conjugates by fibrillar type I collagen in EC cultures. □, Liposome–antibody to type I collagen conjugate; ▨, liposome–fibronectin conjugate; ▩, liposome-nonimmune IgG conjugate; ■, liposomes.

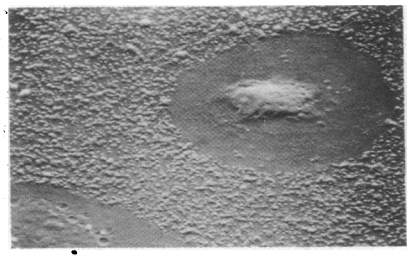

FIG. 6. SEM of ^{14}C-labeled liposome-antibody to type I collagen conjugate binding by collagen-coated gaps in preconfluent EC culture. In this experiment, after dehydration with ethanol the preparation was dried in the air to destroy the blebs and microvilli on the upper surface of ECs for better visualization of liposomes. (×1090.)

of the subendothelium and intimal basal membrane. Perhaps the mixture of Fab fragments of antibodies, to avoid the reaction of a conjugate with corresponding auto-anti-IgG-antibodies, would be the most suitable carrier.

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