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Evidence that Vascular Endothelial Cells Can Induce the Retraction of Fibrin Clots (41260)

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Abstract. Cultured endothelial cells (EC) from bovine aorta and umbilical vein induced retraction of a fibrin clot formed by addition of thrombin to cell-free plasma. Fibrin clot retractile (FCR) activity increased with time (1–24 hr) and with the number of cells in the system (1–4 × 10^6/ml, final concentration), and was inhibited at 22° or in the presence of Na_2–EDTA; moreover, no retraction occurred when batroxobin was used as a clotting agent instead of thrombin. FCR of EC thus showed many characteristics in common with platelet- and fibroblast-induced clot retraction. FCR activity of bovine EC increased with the number of subcultures, being very low in cells harvested from primary cultures. In contrast, human EC had high activity in primary cultures. Like fibroblasts, EC with a higher density in culture showed lower FCR, suggesting that confluency inhibits the cell contractile capacity. FCR could thus represent a simple *in vitro* test to further characterize the biology of EC and to evaluate their role in the development of fibrin thrombi.

It has been suggested that vascular endothelium plays a role in maintaining the patency of the circulatory system when intravascular fibrin deposition occurs (1). However, the interaction between fibrin and endothelial cells has not yet been studied thoroughly. The experiments reported below offer evidence that cultured bovine and human endothelial cells can indeed interact with fibrin and are able to induce the retraction of a fibrin clot.

Materials and Methods. *Primary cultures.* Bovine endothelial cells (EC) were harvested from freshly excised aortas by means of a perfusion technique using collagenase (Worthington CLS II) as described by Booyse *et al.* (2). Human endothelial cells were obtained from umbilical veins according to Gimbrone *et al.* (3).

Bovine smooth muscle cells (SMC) were derived from aortas pretreated with collagenase to remove endothelial cells. After discarding the adventitia and the outer part of the media, smooth muscle segments were dissected, cut into 1- to 2-mm^2 pieces, and incubated for 2 hr in serum-free medium containing 0.1% collagenase plus 0.5% elastase. Tissue segments treated in this way were then suspended in culture medium and placed in plastic flasks.

Culture media. Bovine and human EC were grown in Eagle's minimum essential medium on Hanks' balanced salt solution supplemented with HEPES (20 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), and either 10% adult bovine serum for bovine EC or 20% heat-inactivated fetal calf serum for human EC. The medium was replaced every second day. Bovine SMC were grown in the same culture medium supplemented with 5% fetal calf serum and 5% newborn calf serum. These cells were fed twice a week.

Subcultures. EC and SMC were subcultured by brief exposure to a 0.05% trypsin–0.02% EDTA solution. The subculture ratio was 1:2. EC were characterized by positive immunofluorescent staining with a specific anti-factor VIII antibody (Behringwerke, Scoppito, Italy).

Fibrin clot retraction (FCR). For FCR assays cells were grown in plastic flasks (25 cm^2) containing 5 ml of culture medium. Unless otherwise stated, confluent cultures were used. No obvious contamination with SMC was detected in EC cultures used for FCR tests. FCR was assayed on single-cell suspensions according to a previously described technique (4).

Cell number and viability were deter-

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mined by the trypan blue exclusion technique. Experiments were carried out in an "optically corrected" water bath, with perfectly parallel glass walls, where test tubes could be placed at a fixed distance from the walls to avoid aberrations due to light diffraction. Glass test tubes (5 × 70 mm) were incubated at 37° for 24 hr. Then 200 μl of cell suspension, 100 μl of human platelet-free citrated plasma, and 50 μl of thrombin (20 NIH U/ml, topostasine, Roche, Milano, Italy) or batroxobin (100 μg/ml reptilase, Boehringer-Biochemia, Milano, Italy) were added in rapid succession.

After addition of the clotting enzyme, the contents of the tube were rapidly mixed using a 2-mm-thick glass rod with a flattened base which was left in the tube. The diameter of the clot around the glass rod was measured at intervals with microcalipers applied to the outer surface of the bath. Retraction values were expressed as percentage of complete clot retraction according to a procedure previously described (4). The presence of plasma antiplasmin(s) in the system prevented the occurrence of any clot lysis during the 24-hr clot retraction period; this was also confirmed by undetectable (<1 ng/ml) levels in serum of fibrinogen-related material, measured by the tanned red cell hemagglutination inhibition test (5). The data presented in the figures are means of three to five replicate experiments for the same cell preparation. Standard deviations, omitted in the graphs, never exceeded 10% of the means. Each figure reports the data obtained from one cell culture. Similar results have been obtained from at least three different cell cultures.

Cell viability assay after FCR. After the last measurement of FCR (at 24 hr) clots were placed in Rose chambers (6), filled with medium, and incubated at 37°. The chambers were inspected daily by light microscopy. The cells began to migrate from the clot after 3–5 days and reached confluence within 10 days.

Results and Discussion. Figure 1 shows a typical experiment to test the kinetics of FCR induced by bovine EC; retraction appears to increase with time (up to a maximum value) and with the number of cells in the system.

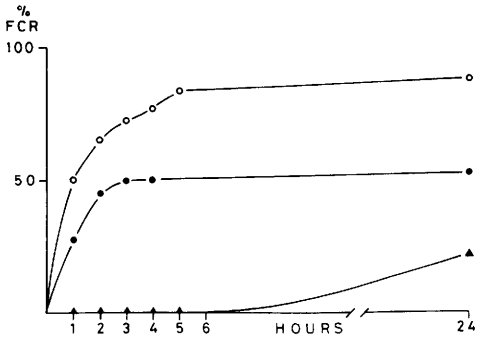

FIG. 1. Course of FCR activity of bovine EC (at third passage) harvested from a culture with 89,000 cells/cm². ○——○, 4 × 10⁶; ●——●, 2 × 10⁶; and ▲——▲, 1 × 10⁶ cells/ml (final concentration).

The retractile activity could not be detected in either mechanically disrupted cells or viable cells incubated at 22° or in the presence of Na_2–EDTA (10^{-2} *M*). This suggests that cellular integrity, the presence of adequate amounts of divalent cations, and optimal conditions of enzymatic activity (at 37°) are all factors required for FCR to occur.

Treatment of cells with aspirin (500 μ*M*, a dose sufficient to completely inhibit prostaglandin I_2 (PGI_2) production as measured by radioimmunoassay of 6-keto $PGF_{1\alpha}$) did not modify clot retraction. These experiments will be described in detail elsewhere.

When batroxobin was used as a clotting enzyme instead of thrombin, no retraction (<1%) occurred. There could be at least two reasons: on the one hand, the structure of fibrin formed by reptilase differs from that formed by thrombin (7); on the other, it is possible that only thrombin, not batroxobin, is able to activate the EC contractile system.

The different cellular effects of these enzymes have already been implicated to explain the failure of platelets to retract batroxobin clots (8). At present, we have no data to support either of the above hypotheses with regard to EC.

The retractile activity of bovine EC was virtually absent in cells harvested from primary cultures, increasing to 60–70% in

the subsequent passages. It is possible that cells in primary culture recover only slowly from the trauma of tissue disaggregation or that adhesion of cells in culture will by itself promote development of cell contractile capacities.

Within the same culture, FCR activity was lower in cells having a higher density in culture (Fig. 2), as if confluency inhibited cell retractile capacity. Figure 3 shows that, in comparison with EC from the same vascular specimen, SMC had similar or even weaker FCR activity. This rules out the possibility that contamination with SMC, even if undetectable on microscopic inspection, could have greatly influenced the retractile capacity of the EC suspensions tested.

EC from primary cultures of human umbilical veins showed greater FCR activity than bovine EC in comparable conditions (Fig. 4). This could be because bovine cells are more susceptible to damage due to enzymatic treatment for tissue disaggregation or because of faster synthesis in human EC of cytoskeleton proteins useful for clot retraction.

Data presented here thus indicate that EC of bovine and human origin, as well as bovine vascular SMC, are able to induce the retraction of a fibrin clot formed on addition of thrombin to platelet-free plasma.

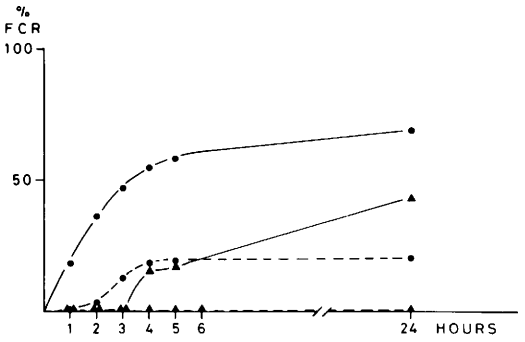

FIG. 2. Course of FCR activity of subconfluent (——) and confluent (– – –) bovine EC at fifth passage. Cells were obtained from the same culture either 3 days (cell density = 39,000 cells/cm²) or 5 days (cell density = 124,000 cells/cm²) after seeding (one of three similar experiments. ●——●, 2×10^6; and ●– – –●, 2×10^6 cells/ml final concentration; ▲——▲, 1×10^6; and ▲– – –▲, 1×10^6 cells/ml final concentration.

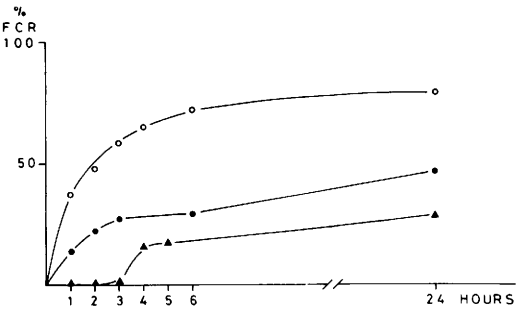

FIG. 3. Course of FCR activity of bovine SMC at third passage, harvested from a culture with 42,500 cells/ml final concentration (one of three similar experiments). ○——○, 4×10^6; ●——●, 2×10^6; and ▲——▲, 1×10^6 cells/ml final concentration.

This phenomenon has many characteristics in common with clot retraction induced by blood platelets. The latter is known to require binding of thrombin to platelets with subsequent activation of the platelet contractile protein(s) and binding of platelets to fibrin through specific receptor sites (9–11).

Endothelial cells have long been observed to contract in response to histamine-like mediators (12) and to possess a contractile protein, very similar to muscle actomyosin and platelet thrombosthenin (13): it was suggested that this contractility played a major biologic role in normal hemostasis (13).

It has more recently been shown that thrombin can bind to EC (14) and cause the cells to contract with subsequent formation

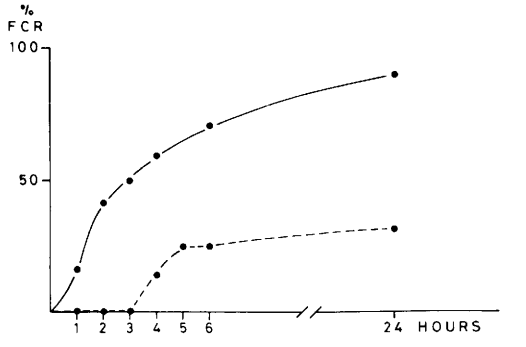

FIG. 4. Course of FCR activity of human (●——●) and bovine (●– – –●) EC (2×10^6/ml final concentration) harvested from primary cultures (one of three similar experiments).

of intercellular gaps and exposure of non-thromboresistant subendothelial layers (15). Moreover, thrombin is able to stimulate EC membrane activities such as phospholipase(s) with generation of products of the arachidonic acid cascade (mainly PGI_2) (16). On the other hand, EC have been reported to respond with migratory behavior to contact with fibrin (1). It is conceivable that both activation by thrombin and binding to fibrin are required for EC to retract.

Beside blood platelets, human and animal fibroblasts have been reported to induce the retraction of a fibrin clot in the same experimental system used in the present study for EC (4). In fibroblasts, FCR activity correlated well with the functional conditions of the cells in culture, varied with the number of subcultures, was lower with higher cell density, and was reduced in aged and absent in transformed or tumor-derived cells (4, 17, 18).

Human EC and human fibroblasts in culture displayed approximately the same degree of FCR activity and similar time kinetics (4). It is worth considering therefore that not only cells typically located in the subendothelial layers, such as fibroblasts and SMC, but also the cells of the endothelial layers, the elements that come in closest contact with intravascularly deposited fibrin, are able to interact actively with this crucial biological substrate. The FCR test could thus represent a simple *in vitro* tool to evaluate the interactions between vascular cells and developing fibrin thrombi.

Although in the FCR system the cells are in suspension and may present surface characteristics different from those found *in vivo*, this test has the advantage of measuring quantitatively a phenomenon that could otherwise be evaluated only by morphological means (1). Thus, using the same experimental system with human skin fibroblasts, it has already been shown that defective FCR activity *in vitro* did indeed correspond to *in vivo* abnormalities of the interactions between fibrin and connective tissue cells (18).

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