ANCHORAGE INDEPENDENT GROWTH AND PLASMINOGEN ACTIVATOR PRODUCTION BY BOVINE ENDOTHELIAL CELLS

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ABSTRACT

Endothelial cells obtained from the aortae of 1- to 2-d-old calves were cloned at high efficiency using fibrin-coated dishes. Primary cultures as well as clones derived from them produced high fibrinolytic activity when grown on ^{125}I-fibrin-coated dishes which was 90% dependent upon the presence of plasminogen. High plasminogen-dependent proteolytic activity was also demonstrated in endothelial cell lysates and in the culture medium of the cells. The production and secretion of the plasminogen activator(s) were found to increase during the log phase of cell growth and to reach a maximum level at confluence. These endothelial cells exhibited morphological phenotypes comparable to those of transformed cells when grown in the presence of acid-treated fetal calf, dog, or human serum. Furthermore, they demonstrated anchorage independent growth, and large colonies were formed in semisolid media. Spontaneous neoplastic transformation of these cells was excluded by karyotypic analysis, lack of tumorigenicity in athymic nude mice, and limited lifespan in culture. Cell clones isolated from colonies grown in agarose demonstrated the same growth characteristics and proteolytic activity as before plating in agarose. High fibrinolytic activity, morphological changes in the appropriate serum, and growth in semisolid media may therefore be indicative of the migratory and/or invasive capacity of both nontransformed endothelial cells as well as tumor cells.

KEY WORDS endothelial cells · plasminogen activator · anchorage independent growth

Anchorage dependence and spreading on a solid substrate are thought to be the critical prerequisites for growth of nontransformed fibroblastic and epithelial cells in vitro. The loss of anchorage dependence of growth has been considered as evidence for neoplastic transformation. Growth in semisolid media has, therefore, become a widely used in vitro test for neoplastic transformation of fibroblastic and epithelial cells. Furthermore, the loss of anchorage dependence of growth has been linked to an increased production of plasminogen activator by transformed cells (20, 23, 34).

Production of plasminogen activator is not in itself a general marker for neoplastic transformation since a number of normal cells such as kidney and lung cells (23), activated macrophages (42), mouse blastocysts (40), stimulated ovarian granulosa cells (3), and granulocytes (16) also produce plasminogen activators. However, the amount of

proteases produced by most other normal mesenchymal cells is low compared to their transformed counterparts. Vascular endothelial cells have been shown by histochemical techniques (41) and by vessel perfusion (1) to produce plasminogen activator. It has also been demonstrated that during the vascularization state of tissue repair, fibrinolytic activity is high but disappears with the involution of blood vessels (21, 32). More recently, Buonassisi and Venter (7) and Loskutoff and Edgington (25) have reported that cultured rabbit endothelial cells of venous and arterial origin produce plasminogen activator in vitro, although the levels of this protease were low when compared to transformed cells.

The ability of transformed cells to grow in soft agar often correlates with the production of plasminogen activator (23, 34). Since endothelial cells produce plasminogen activator in vitro, it seemed of interest to test whether or not these cells would also grow in soft agar. With the exception of chondrocytes (17) and bone marrow cells (6, 28), no other normal cells have been reported to proliferate without anchorage to a solid substrate. Furthermore, the typical morphological growth patterns of SV-40 transformed hamster embryo fibroblasts have been correlated with high plasminogen activator-plasmin activity, and similar morphological changes were observed in normal hamster fibroblasts when cocultivated with their virally transformed counterparts (31).

This paper documents the production and secretion of plasminogen activator by bovine aortic endothelial cells, their ability to grow in soft agar, and their different morphology in various sera. The observed characteristics suggest a correlation with the migratory and/or invasive potential common to both normal endothelial cells and tumor cells.

MATERIALS AND METHODS

Culture and Cloning of Bovine Endothelial Cells

Endothelial cell suspensions, essentially free of other cell types, were obtained from thoracic aortae of 1- to 2-d-old calves by the method of Eisenstein et al. (11). These procedures gave a yield of 1.5–2.5 × 10^6 cells/aorta. The cells were plated in 75-cm^2 plastic culture flasks (Falcon, Oxnard, Calif.) in RPMI 1640 medium (GIBCO, Santa Clara, Calif.) containing 20 mM HEPES buffer and supplemented with 20% fetal calf serum (Microbiological Associates, Walkersville, Md.), 100 U/ml penicillin and streptomycin and incubated at 37°C in a humidified 5% CO_2-air atmosphere. Nonadherent cells were then removed by change of the culture medium after 30 min. Subcultures in a 1:10 dilution were obtained by trypsinization (trypsin-EDTA [1×], GIBCO) of the cells at near confluency. Population doubling levels were calculated from the number of cells harvested compared to the number of cells plated. Gross contamination by other cell types was excluded by analysis of the cells grown on cover slides for the presence of cytoplasmic Factor VIII Antigen (4). Monospecific rabbit antiserum to bovine Factor VIII, kindly provided by Dr. Edward Kirby, Temple University, Philadelphia, Penn., was used in a 1:40 to 1:80 dilution and Fluorochrome conjugated antiserum to rabbit immunoglobulins (Behring Diagnostics, Summerville, N. J.), in a 1:5 dilution.

Clones of the endothelial cell strains were obtained either by growing single cells in microwells or by plating 25–100 cells suspended in RPMI 1640 containing 20% fetal calf serum in 60-mm tissue culture dishes coated with purified fibrin (2 $\mu g/cm^2$). Cultures were fed once a week until colonies of 4- to 5-mm diameter were observed (after 2–3 wk). Single colonies were then ring isolated, trypsinized, and grown up to mass cultures. Cells were identified as of endothelial origin by morphological criteria at confluence and the assay for cytoplasmic Factor VIII Antigen.

Culture of Bovine Smooth Muscle Cells

After removal of the endothelial cells (see above) from the aorta, small pieces of the inner layer of the vessel were plated in 60-mm plastic dishes (Falcon) in the presence of 2 ml of the same growth medium as bovine endothelial cells. 7–10 d later, outgrowing smooth muscle cells were ring isolated, trypsinized, and grown up to mass culture (36).

Culture of Human Endothelial Cells

Primary cultures of human endothelial cells were derived from the vein of the umbilical cord. HUC strain I was a kind gift of David Loskutoff, Scripps Clinic, La Jolla, Calif. HUC strain II was obtained as described by Jaffe et al. (18). The cells were grown in the same growth medium as the bovine endothelial cells except that fibroblast growth factor was added (15) at a concentration of 1 μg/ml medium. All cell cultures used were routinely checked every 6–8 wk for mycoplasma contamination by the DAPI technique (37). DAPI (4′-6-diamidino-2-phenylindole) was a kind gift of Dr. O. Dann, Friederich Alexander University, Erlangen, West Germany.

Assay for Plasminogen Activator

Crude bovine fibrinogen (Sigma Chemical Co., St. Louis, Mo.) was purified by the method of Laki (22) and depleted of plasminogen according to Mosesson (29). Fibrinogen was then iodinated with ^{125}I by the method of Frisbie and Roberts (13). ^{125}I-fibrin-coated culture dishes (Falcon) were prepared as previously described (19, 23). Briefly, ^{125}I-fibrinogen was diluted with unlabeled fibrinogen to obtain a final concentration of 2 μg fibrinogen/cm^2 with 3,000 cpm/μg for the 60-mm tissue culture dishes (for cell-associated plasminogen activator assay) and 10 μg fibrinogen/cm^2 with 1,000 cpm/μg for the 35-mm dishes (intra- and extracellular plasminogen activator assay). Dishes were then dried overnight at 45°C. Fibrinogen was converted into fibrin by incubating the dishes with RPMI 1640 containing 10% fetal calf serum (as the thrombin source) for either 2 h with 2 ml of medium (35-mm dishes) or 4 h with 5 ml of medium (60-mm dishes) just before use. Bovine thrombin (Parke-Davis, Detroit, Mich.) at a concentration of 1 NIH U/ml medium was

used instead of fetal calf serum for the plasminogen dependency assay.

Assay for Cell-associated Plasminogen Activator Activity

Cells at near confluence were trypsinized, washed once with phosphate-buffered saline (PBS), and resuspended in PBS at a concentration of 2.5×10^6 cells/ml. 0.2 ml of this cell suspension in 5 ml of RPMI 1640 containing 10% of the appropriate serum was plated in 60-mm dishes coated with ^{125}I-fibrin and incubated at 37°C in the presence of 95% air and 5% CO_2. Aliquots of the supernate were taken at the indicated time intervals, and the released radioactivity was counted in a Gammacounter 200 (Searle Analytical, Chicago, Ill.). Plasminogen activator activity was expressed as the percentage of radioactivity released into the supernate compared to the total radioactivity of ^{125}I-fibrin/dish. A blank was obtained in each experiment by incubating a dish with the corresponding growth medium alone. This fibrinolytic activity, which never exceeded 5% after 24-h incubation, was subtracted from that obtained by incubation of the cells to obtain the true fibrinolytic activity.

Secreted Plasminogen Activator Activity

Cells (5×10^4) were seeded in 60-mm culture dishes and grown for up to 2 wk after they reached confluence with medium change every second day. Growth medium was removed from cultures either in log phase of growth or at confluence, the cells washed once with sterile PBS and then incubated for an additional 24 h in the presence of 5 ml RPMI 1640 containing no fetal calf serum. The supernate was then harvested, centrifuged at 1,500 rpm for 5 min to remove cells and debris, and aliquots of the supernate were taken and assayed for the presence of fibrinolytic activity as described below.

Intracellular Plasminogen Activator Activity

Cells grown in 60-mm dishes as described above were washed twice with cold PBS, scraped off the dish with a rubber policeman in the presence of cold PBS, and centrifuged at 1,000 *g* for 5 min. The cell pellet was lysed in 15 vol of cold 0.01 M Tris-HCl, pH 8.0, containing 0.5% Triton X-100. Aliquots of the postnuclear fraction were incubated with 2 ml of 0.1 M Tris-HCl, pH 8.0, containing 1% acid-treated, pooled human serum for 2 h on ^{125}I-fibrin-coated dishes (35-mm Diam). Radioactivity of aliquots from the supernate was determined in a Gammacounter. The value obtained was expressed as the percentage of the radioactivity released by 100 Plough units of urokinase under identical conditions, after subtraction of the radioactivity released by 1% acid-treated human serum only. One unit was defined as the amount of activity which released 10% of the urokinase activity in 2 h at 37°C. Protein in the cell lysate was determined according to Lowry et al. (26), and the plasminogen activator activity was expressed in U/mg cellular protein.

Morphological Changes

Cells (5×10^5) were plated onto regular culture dishes (60 mm) or dishes coated with purified fibrin, 2 $\mu g/cm^2$, and incubated in the presence of RPMI 1640 medium containing either 10 or 20% fetal calf serum, 10% plasminogen-depleted fetal calf serum, 10% dog serum, or 10% human serum. After incubation for 24 and 48 h at 37°C in the presence of 95% air and 5% CO_2, the cultures were monitored for morphological changes using the criteria of Ossowski et al. (31).

Other Methods

Saturation density of the cells was determined by plating 5×10^4 cells in RPMI 1640 containing 20% fetal calf serum in 60-mm dishes. Cultures were fed every second day, and cell counts were determined using a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) after trypsinization of duplicate cultures.

Tumorigenicity of three different cell strains was tested by subcutaneous injection of 10^7 cells into nude athymic mice. The animals were checked twice weekly during an 18-wk period for the appearance of tumors.

Plating efficiency of the cells in soft agar and agarose was determined by the method of MacPherson and Montagnier (27). Four dishes containing 10^4 and 10^5 cells, respectively, were set up for each cell line. In each experiment the human fibrosarcoma cell line HT 1080 (19) was plated at the same cell densities as a positive control. 24 h after seeding, the cultures were examined for the presence of single cell suspension. Cultures were fed weekly with RPMI 1640 medium supplemented with 10% fetal calf serum. After 3 wk the cultures were examined with a stereomicroscope (×40), and colonies containing more than eight cells were counted. Colonies were picked out with a Pasteur pipette and grown up in regular growth medium for further characterization of the cells growing in semisolid media. Cells were then retested for plasminogen activator activity, chromosomal pattern, and for the presence of Weibel-Palade bodies (courtesy of Dr. Weibel, University of Bern, Switzerland).

Chromosome preparations were made according to Paul et al. (33) and banded with Trypsin-Giemsa (39). A minimum of 20 metaphases were photographed and then counted for each cell strain analyzed, and at least five metaphases were karyotyped.

Serum was depleted of plasminogen as described by Deutsch and Mertz (9). The same method was used to prepare plasminogen from human serum. Acid-treated fetal calf serum was obtained by acidifying the serum with 1 N HCl to pH 3.0. The serum was kept for 2 h at room temperature and then the pH was readjusted to 7.4.

RESULTS

Characterization of Endothelial Cells

The primary cultures, subcultures, and clones isolated from mass cultures as well as from agarose grew with the typical cobblestone appearance of endothelial cells (see Fig. 7 *B*). The cells could be subcultured up to 22 times (~65–70 population doublings) before starting to senesce and to die. Overgrowth by smooth muscle cells or fibroblasts was never observed, ruling out gross contamination by these cell types. Furthermore, >99% of the cells of the primary cultures as well as of the clones isolated from agarose stained positively for Factor VIII Antigen (Fig. 1). Bovine smooth muscle cells and mouse fibroblasts, on the contrary, did not stain in the presence of monospecific rabbit antiserum to bovine Factor VIII Antigen. The chromosomal pattern of three different parental cell

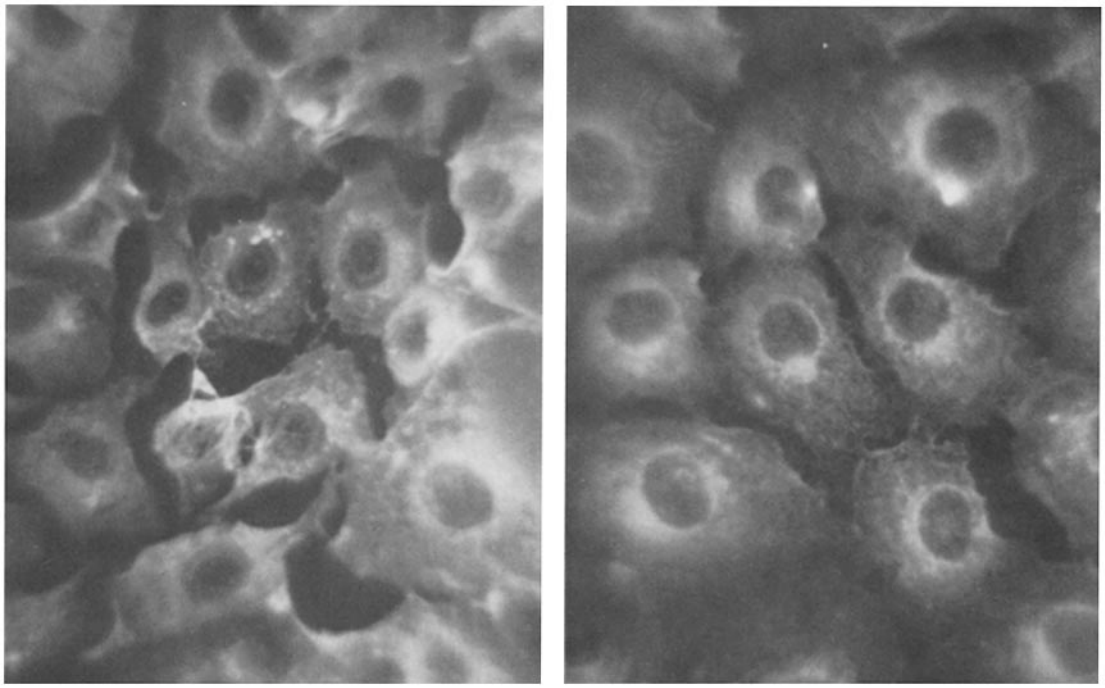

FIGURE 1 Immunofluorescence study of cultured bovine endothelial cells. Cells fixed on a coverslip were treated with specific rabbit antiserum against bovine Factor VIII Antigen and fluorescein-conjugated goat antirabbit globulin as outlined in Materials and Methods. *Left*, a primary culture (EC A), and *right*, cells derived from a colony growing in agarose (EC A p4 SA Cl_2).

strains as well as of clones isolated before and after forming colonies in agarose are shown in Table I. All metaphases were examined from photographs of well banded chromosomal spreads. The majority of metaphases had a normal diploid chromosomal complement of 60 chromosomes (24). No chromosomal aberrations were observed in any metaphase including rings, dicentrics or Robertsonian translocations. The few cells with a chromosomal number of 58–59 or 119–120 represented metaphases in which a random chromosome was lost on preparation or a normal tetraploid cell, respectively. Fig. 2 illustrates a typical normal diploid karyotype seen in these endothelial cells. The particular metaphase shown was obtained from a clone which had previously been isolated from agarose. These cell strains were also examined for tumor formation by injection of 10^7 cells/animal into nude mice. No tumors were detected after 18 wk of observation. This finding, together with the chromosomal analysis, the density-dependent growth (see Fig. 6*A*), and the limited lifespan of the cultures excludes the possibility of spontaneous transformation.

TABLE I

Chromosomal Analyses of Bovine Endothelial Cells

	No. of chromosomes				
	58	59	60	119	120
EC A	1	2	16	—	1
EC I	—	3	16	—	1
EC I p4 Cl_{12}	1	1	18	—	—
EC A p4 SA Cl_2	1	1	17	—	1
EC A p4 SA Cl_3	1	1	15	1	2
EC IV	1	2	17	—	—

The chromosomes were fixed, stained, and banded with Giemsa as described in Materials and Methods. A minimum of 20 metaphases were counted. EC A, EC I, and EC IV are parental, uncloned cell strains. EC I p4 Cl_{12} was cloned at passage 4 from EC I. EC A p4 SA Cl_2 and EC A p4 SA Cl_3 are endothelial cell clones isolated from agarose.

Cloning of Endothelial Cells

Since the plating efficiency of single cells into microwells was ~5%, only one clone (EC I Cl_{12}) was isolated by this method. For the isolation of all other clones, endothelial cells were seeded at a

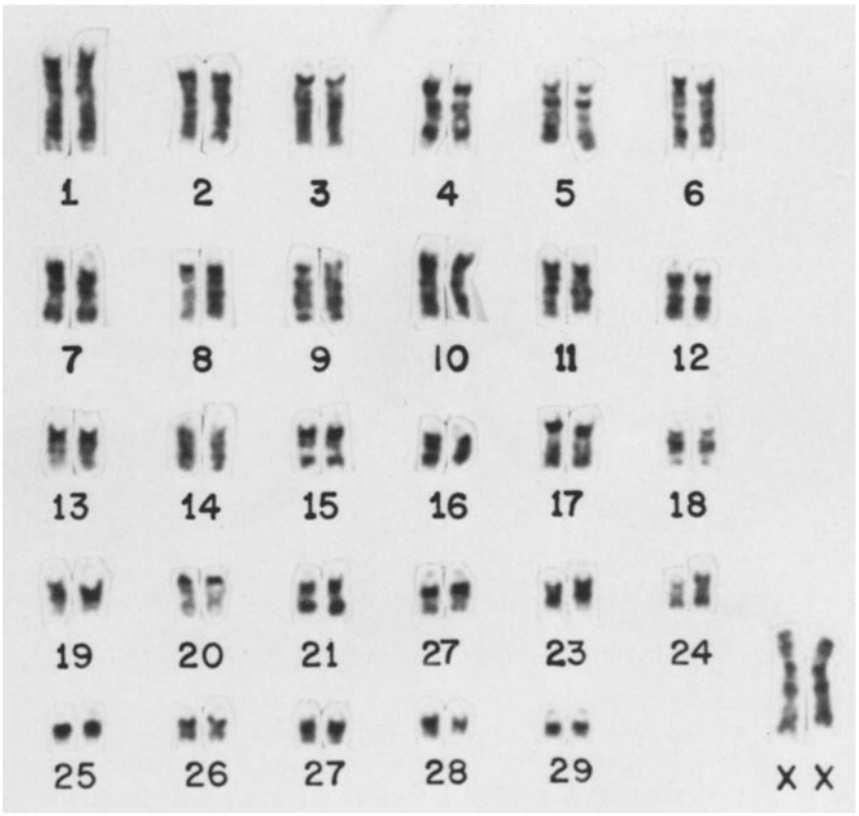

FIGURE 2 Karyotype of bovine endothelial cells. This normal diploid chromosomal pattern, representative for all cell strains tested, was obtained from a clone isolated from agarose (EC A p4 SA Cl_2).

concentration of 25–100 cells in 60-mm tissue culture dishes coated with fibrin. Plating efficiency was increased up to 60% using this method, and much larger colonies were obtained than in uncoated control dishes (Fig. 3).

Plasminogen Activator Activity

Plasminogen activator activity was determined in cultures growing on ^{125}I-fibrin coated dishes (= cell-associated activity), in whole cell lysates (= intracellular activity), and in the supernatant media of growing cultures (= secreted activity).

The cell-associated fibrinolytic activity of three different endothelial cell strains and several clones in the presence of various sera as the plasminogen source is shown in Table II. All cell strains digested a large amount of the fibrin present on the dish within 24 h, although considerable variations among different cell strains were observed. The highest proteolytic activity was consistently obtained in the presence of human and dog sera which contain high levels of plasminogen and relatively low concentrations of inhibitors of fibrinolysis (23, 35). The proteolytic activity was in general not affected by the passage number, although some variations were observed and the clones did not differ significantly from their respective parental strains. Sublines isolated from colonies which grew in soft agar exhibited high fibrinolytic activity essentially unchanged from their parent cell strains. Smooth muscle cells derived from the same vessels did not show any significant proteolytic activity except when incubated with dog serum. Primary cultures of human endothelial cells derived from the umbilical cord did not produce any significant fibrinolytic activity in either fetal calf or human serum.

The kinetics of production of cell-associated plasminogen activator by cloned endothelial cells grown on ^{125}I-labeled fibrin dishes in the presence of different sera as the plasminogen source is shown in Fig. 4. In the presence of 10% fetal calf

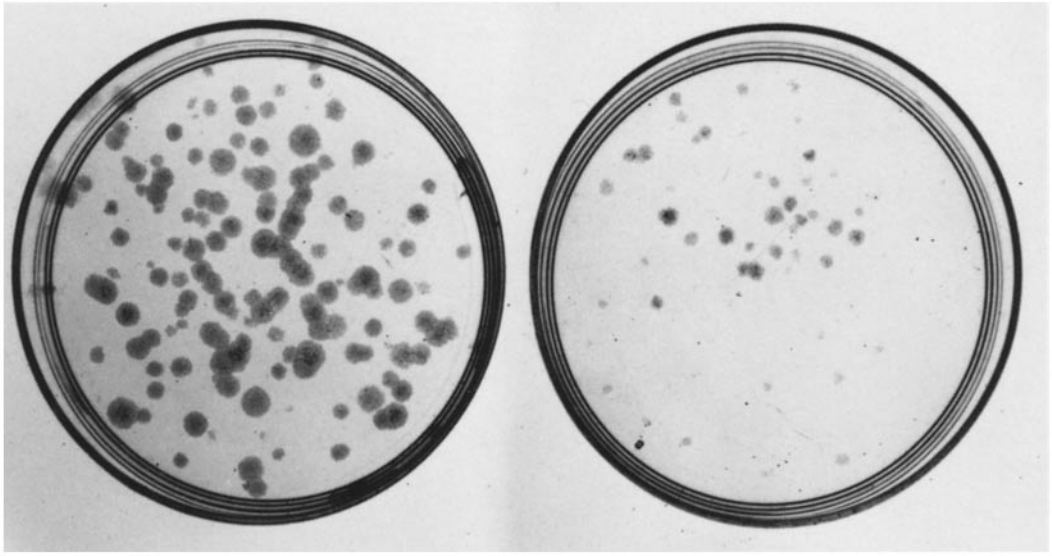

FIGURE 3 Clonal growth of endothelial cells. 50 cells suspended in 5 ml RPMI 1640 containing 20% fetal calf serum were plated into each culture dish. *Left*, a dish coated with fibrin; *right*, an uncoated dish. Cultures were fed once a week and the colonies were stained with Giemsa after 2 wk.

serum, which has a low concentration of plasminogen and high levels of inhibitors of fibrinolysis (23, 35), delayed fibrinolysis was observed. After acid treatment of fetal calf serum, which removes the inhibitors of fibrinolysis (31), fibrin digestion was as rapid as in the presence of human or dog serum. Almost no radioactivity was released when the cells were grown for 24 h in the presence of plasminogen-depleted fetal calf serum or in the absence of any serum. Fibrinolytic activity was restored when plasminogen was added to the culture medium (Table III).

The cell lysates demonstrated high fibrinolytic activities which were >90% dependent upon the presence of plasminogen (data not shown). The enzyme assay for plasminogen activator was linear during 2 h of incubation in the presence of 1% acid-treated human serum as long as <25% of the radioactivity was released (Fig. 5). Dilution and mixing experiments with urokinase proved the absence of large amounts of inhibitors in the cell lysates.

High fibrinolytic activity which was >95% dependent upon the presence of plasminogen (data not shown) was also detected in the serum-free supernatant growth medium from endothelial cell cultures. Production and secretion of plasminogen activator was highest in resting, confluent endothelial cell cultures (Fig. 6). Secreted protease activity, expressed in U/10^6 cells per 24 h, and intracellular protease activity, expressed in U/mg cellular protein increased steadily with cell density and reached a maximum in confluent cultures at 12–14 d after seeding. During the following 14 d with the cultures at confluence, the enzyme activities released per 24 h remained constantly high, indicating that maximum enzyme production occurs while the cells are arrested in the G_1 phase of the cell cycle.

Morphological Changes

Endothelial cells grown for 24 or 48 h on fibrin-coated culture dishes in the presence of 10 or 20% fetal calf serum showed a typical cobblestone appearance (Fig. 7). In the presence of plasminogen-depleted fetal calf serum, the cells demonstrated a fibroblast-like appearance while the presence of 10% acid-treated fetal calf serum or 10% human serum caused morphological changes comparable to class 1+ according to the criteria of Ossowski et al. (31). The most striking morphological changes were observed in the presence of 10% dog serum where multilayered cell aggregates, which remained attached to the surface of the culture dish, were observed (class ++). These morphological changes were more prominent after 48 h incuba-

TABLE II

Cell-associated Plasminogen Activator Activity of Different Endothelial and Smooth Muscle Cells

Cell strain	Origin	Passage No.	Population doublings	Fibrinolytic activity			
				FCS	at FCS	DS	HS
				%	%	%	%
EC A	Newborn calf	2	7	12		47	46
		4	13	35		52	51
EC A p4 Cl_1	Clone of EC A isolated before growth in agarose	4	37	6	21	39	33
EC A p2 SA Cl_1	Clones of EC A isolated after growth in agarose	14	71	45		50	68
EC A p4 SA Cl_2		2	38	29	26	54	53
EC A p4 SA Cl_3		2	38	21		50	
EC I	Newborn calf	3	10	45		60	71
		5	16	34		66	74
		7	22	54	53	80	64
		13	40	56	63	54	84
		16	49	10		52	38
		17	52	19		56	40
		22	67	41		60	45
EC I p4 Cl_{12}	Clone of EC I	6	40	46		58	
		9	49	44	52	65	60
EC IV	Newborn calf	3	10	38		34	58
SM A Cl_2	Smooth muscle clone of newborn calf	4	19	1	0	21	4
HUC I	Human umbilical cord (vein)	1	4	0			1
HUC II		1	4–5	0			0

5×10^5 cells suspended in 5 ml RPMI 1640 containing 10% of the serum indicated were seeded onto ^{125}I-fibrin-coated dishes and incubated at 37°C. for 24 h. Aliquots of the supernate were then taken and the released radioactivity was counted. Fibrinolytic activity is expressed as the percentage of the total radioactivity present on the dish released into the supernate. FCS = fetal calf serum, at FCS = acid-treated fetal calf serum, DS = dog serum, HS = human serum, p = passage number when cells were cloned, and Cl = clone, EC = bovine endothelial cells, and HUC = human endothelial cells.

tion and less striking in the absence of fibrin on the dish.

Growth in Soft Agar and Agarose

All bovine cell strains and clones tested grew in soft agar (data not shown) and in agarose (Table IV), although the plating efficiency varied from 0.1 to 11.3% after 21 d of incubation. The colony sizes also varied considerably (8 to >256 cells/colony) in the different cell strains, and larger colonies were observed with higher plating efficiency. A typical small colony is shown in Fig. 8. Table IV also demonstrates that the cloning efficiency declined with increasing passage number and, with the exception of the cell strain EC 1 which still grew in agarose at the 15th passage (46th population doubling), all other cell strains as well as the clones tested did not grow in agarose after the 8–10th passage (~25–30th population doubling). Clones of endothelial cells derived from the aortae of fetal calves also grew in agarose although at a low plating efficiency. The human fibrosarcoma cells, which were plated as a positive control, grew at a higher plating efficiency (30–40%) than endothelial cells. The colonies grew faster than those derived from bovine endothelial cells, and after 3 wk growth >80% of the colonies consisted of 250 or more cells. Bovine smooth muscle cells and human endothelial cells derived from the vein of the umbilical cord did not form any colonies in either agarose or agar.

Colonies of bovine endothelial cells growing in agarose were isolated, cultured as monolayers, and tested again for various parameters. Examination of the cells by electron microscopy proved the presence of Weibel-Palade bodies. The morphology at confluence showed the typical cobblestone appearance of endothelial cells, and all cells stained positively for Factor VIII Antigens (Fig. 1). Cell-associated, intracellular and extracellular

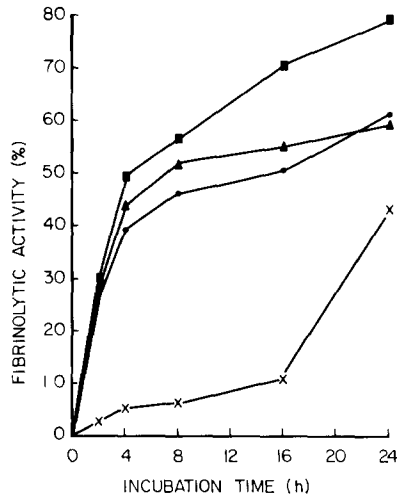

FIGURE 4 Cell-associated plasminogen activator activity. 5×10^5 cloned endothelial cells (EC I Cl_{12}) suspended in 5 ml RPMI 1640 containing 10% of the indicated serum were seeded on dishes coated with ^{125}I-fibrin and incubated at 37°C. Samples of the supernate were taken at the times indicated and the released radioactivity determined. Plasminogen activator activity is expressed as the percentage of radioactivity released into the supernate compared to the total radioactivity of ^{125}I-fibrin/dish. The value was corrected by subtracting the fibrinolytic activity developed by corresponding serum. (×) fetal calf serum; (▲) acid treated fetal calf serum; (●) dog serum; and (■) human serum.

fibrinolytic activity was essentially the same as in the parental cell strains (Table II). Cytogenetic analysis also showed the same normal diploid chromosomal pattern as in the original cell strains (Table I and Fig. 2). No colony formation was observed when the cells were replated into agarose, probably due to aging of the cells.

DISCUSSION

Primary and cloned bovine endothelial cultures obtained from the aortae of 1- to 2-d-old calves exhibited high fibrinolytic activity in vitro which was dependent upon the presence of plasminogen. These results confirm the findings of Buonassisi and Venter (7) and Loskutoff and Edgington (25) who also detected plasminogen-dependent proteolytic activity in endothelial cells derived from rabbit aorta and vena cava, respectively. The proteolytic activities in our cell strains and clones were much higher than those reported by these authors and were comparable to those detected in transformed cells (23). Smooth muscle cells, the most likely contaminants of our primary cultures, did not produce significant levels of plasminogen activator. High levels of plasminogen activator were also found in the cell lysates but, in contrast to Loskutoff and Edgington (25), we did not find inhibitors of fibrinolysis.

TABLE III

Plasminogen Dependency of Expression of Fibrinolytic Activity

Serum	Fibrinolytic activity after 24 h incubation
	%
10% Fetal calf serum	23.5
10% Acid-treated fetal calf serum	40.0
None	1.1
10% Fetal calf serum plasminogen depleted	3.5
10% Fetal calf serum plasminogen reconstituted	27.7

^{125}I-fibrinogen-coated dishes were converted to ^{125}I-fibrin by incubation for 4 h with thrombin (1 NIH U/ml RPMI). 5×10^5 cells (EC I Cl_{12}) were seeded on the dish in the presence of the growth medium containing the indicated serum. Percent fibrinolytic activity was determined as described for Table II.

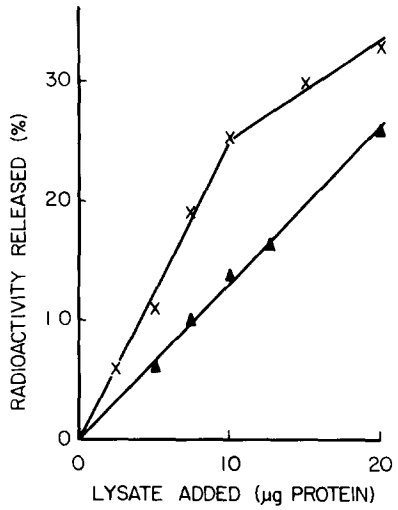

FIGURE 5 Assay of plasminogen activator in lysates of endothelial cells. Lysates of endothelial cells were incubated with 1% acid-treated human serum in 0.1 M Tris-HCl, pH 8.0, on ^{125}I-fibrin dishes (35 mm). The radioactivity solubilized in 2 h at 37°C was expressed as a percentage of the radioactivity released by 100 U of urokinase under identical conditions. The lysates used were obtained from the cell strain EC I (▲) and its clone EC I Cl_{12} (×) while the cells were at confluence.

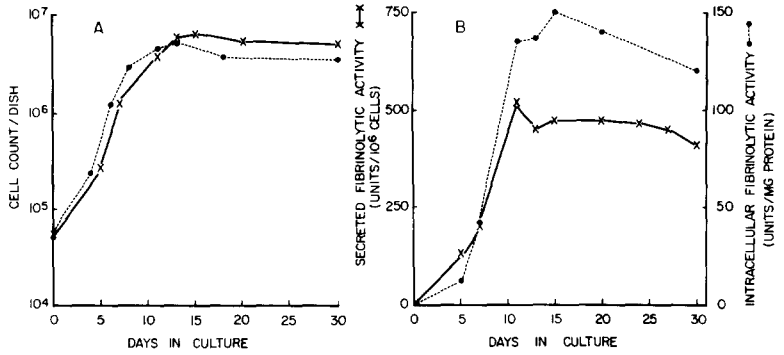

FIGURE 6 Growth curve (*A*) and intracellular and extracellular plasminogen activator production of endothelial cells (*B*). 5×10^4 cells (EC I) were seeded in 60-mm culture dishes and fed every second day. 24 h before the times indicated, quadruplicate cultures were washed with PBS and kept for a further 24 h in medium without serum. Duplicate cultures of two experiments (*A*) were trypsinized, and the cells were counted in a Coulter counter. Aliquots from the supernate of quadruplicate cultures were taken at the same time for determination of secreted plasminogen activator activity per 24 h, and lysates of duplicate cell cultures were made for the determination of intracellular protease activity as described in Materials and Methods. The secreted plasminogen activator activity is expressed in units released/10^6 cells per 24 h (×—×) and the intracellular activity in U/mg protein (●– – –●). Results are means of two different experiments.

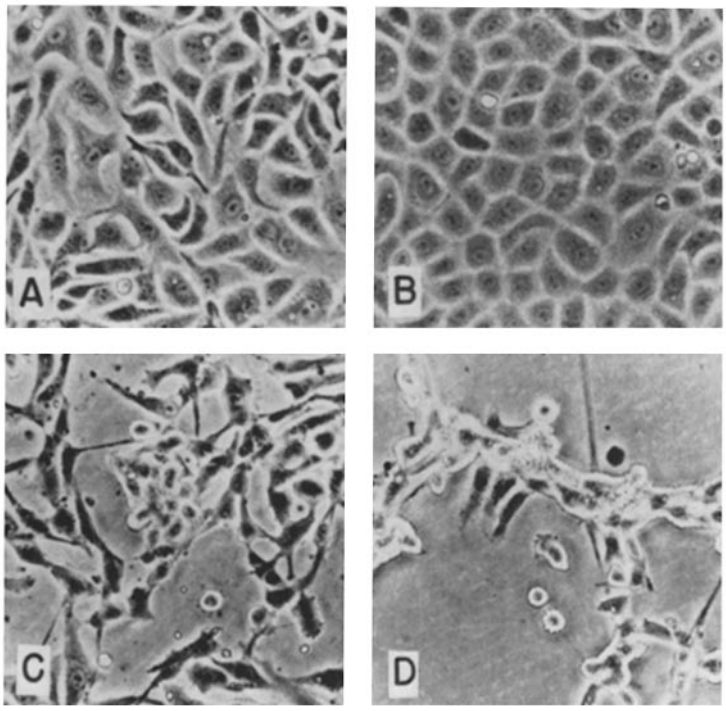

FIGURE 7 Morphological growth patterns of endothelial cells in different sera. 5×10^5 cells (EC I Cl_{12}) suspended in 5 ml RPMI 1640 containing the appropriate serum were seeded onto 60-mm culture dishes coated with fibrin (2 $\mu g/cm^2$) and incubated for 48 h at 37°C. Cultures were then examined for morphological changes and photographed by phase contrast microscopy. Culture medium contained 10% (*A*) or 20% fetal calf serum (*B*), 10% acid-treated fetal calf serum (*C*) or 10% dog serum (*D*).

TABLE IV

Plating Efficiency of Different Endothelial Cells in Agarose

Cell strain	Origin	Passage No.	Generations	Plating Efficiency
				%
EC A	Newborn calf	1	3	11.3
		2	7	4.5
		4	13	5.3
EC I	Newborn calf	6	19	1.9
		10	31	2.4
		12	37	0.9
		15	46	0.6
EC I p4 Cl_{12}	Newborn calf	3	30	0.1
EC II	Newborn calf	6	19	1.3
A14 C_1	Fetal calf	5	29	0.1*
A14 Cl_2	Fetal calf	5	29	0.1*
SM_2 Cl A	Fetal calf	5	21	0*
SM Cl_3	Newborn calf	6	23	0*
HUC I	Human umbilical	1	4	0
HUC II	cord	1	4–5	0

10^4 and 10^5 cells were seeded in agarose-containing RPMI 1640 medium supplemented with 10% fetal calf serum and fed weekly. Colonies containing >8 cells after 3 wk were counted using ×40 magnification under a stereomicroscope. Results are means of duplicate experiments. EC A, EC I, and EC II are primary endothelial cultures; EC I Cl_{12} is a clone of EC I; A14 Cl_1 and A14 Cl_2 are clones of fetal aortic endothelial cells; SM_2 Cl A is a cloned smooth muscle cell strain obtained from the fetal calf aorta, and SM Cl_3 is a cloned smooth muscle cell strain isolated from the aorta of a newborn calf. HUC I and II are primary cultures of human endothelial cells derived from the vein of the umbilical cord.

* Determined after 4 wk incubation.

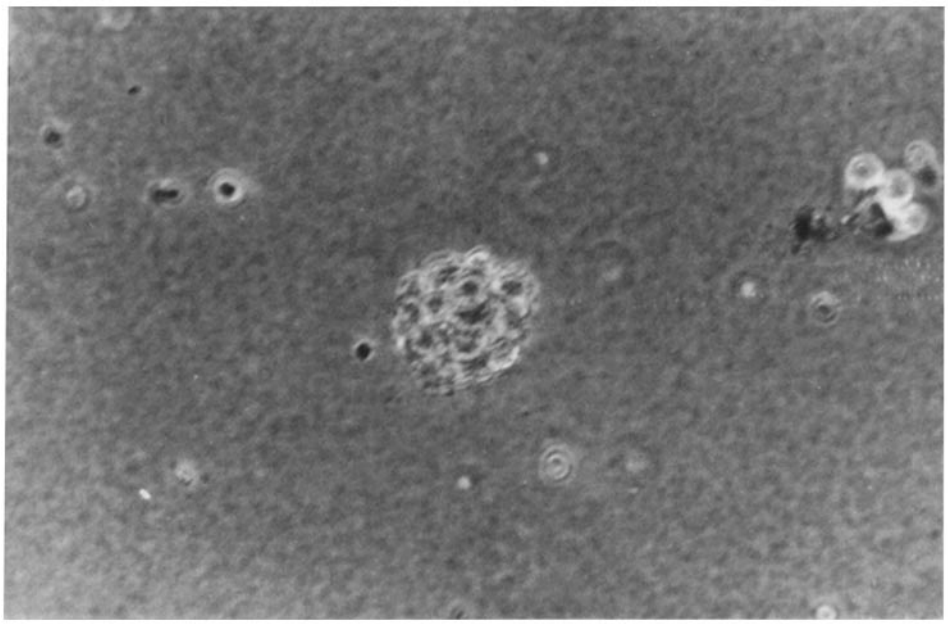

FIGURE 8 Colony of endothelial cells in agarose. 10^5 endothelial cells (EC A) were seeded in agarose as described in Materials and Methods. The picture of the colony was taken 3 wk after seeding. × 80.

Our studies show that endothelial cells secrete large amounts of plasminogen activator. The secretion of this protease increases at higher cell densities until a maximum is reached upon confluence, and thereafter it remains constantly high. The intracellular protease activity showed the same behavior. In contrast to this secretion pattern, tumor cells produce and secrete this serine protease mainly during the logarithmic phase of growth (8). Furthermore, Bosmann (5) demonstrated that leukemic L5178Y cells produce proteases mainly during the M phase of the cell cycle, and we obtained similar results using synchronized hamster fibrosarcoma cells (Laug and Jones, unpublished observations). Since endothelial cells produce and secrete plasminogen activator mainly at confluence (G_1 phase of the cell cycle), a significant difference of regulation of this protease may exist between endothelial cells and tumor cells.

Typical morphological changes as observed in SV-40 transformed hamster embryo fibroblasts (31) were found when the endothelial cells were grown for 24–48 h in the presence of dog, human, or acid-treated fetal calf serum, with the most striking changes occurring in dog serum. Although we could not detect this morphological pattern in normal kidney and lung cells with high fibrinolytic activity (23), we do not believe that this phenomenon is strictly related to neoplastic transformation. More likely, this behavior might be correlated with the migratory capability of these cell types as has been observed by Zetter et al. (43) in chick embryo fibroblasts after treatment with proteases.

Finally, we have found that calf endothelial cells of aortic origin grow in semisolid media, although plating efficiency as well as colony sizes varied considerably among the various cell strains. The plating efficiency in agarose was highest in primary cultures and declined with increasing passage number. Plating efficiency was also lower in the clones than in their parental cell strains, probably due to the many cell divisions necessary for the cloning process. This finding could also indicate that primary bovine endothelial cell cultures contain subpopulations of cells which grow in semisolid media and which are lost during subsequent subcultivations. Since the cells grown in agarose retain the characteristics of endothelial cells, it is unlikely that they represent other contaminating cell types although our cloning technique does not rule out this possibility with absolute certainty. Furthermore, cloned bovine smooth muscle cells, the most likely contaminating cell type in our system, failed to grow under the same conditions. The highly tumorigenic human fibrosarcoma cell line HT 1080, on the other hand, grew at high cloning efficiency independent of passage number and the colonies tended to be larger than the bovine endothelial cells. We are not aware of any other normal cell types that form colonies in semisolid media, aside from chondrocytes (17) and cells derived from the bone marrow (6, 28).

We have also demonstrated that, in contrast to bovine aortic endothelial cells, untransformed human endothelial cells derived from the umbilical vein do not grow in semisolid media, confirming the findings of Gimbrone and Fareed (14). Furthermore, like Dosne et al. (10), we were unable to detect any plasminogen activator production by human endothelial cells isolated from the umbilical vein. These discrepancies are most likely because endothelial cells of the umbilical vein are different insofar as they have to fulfill only a temporary function.

High plasminogen activator production, together with anchorage independent growth, is normally associated with oncogenic transformation (20, 23, 34), and endothelial cells of bovine aortic origin share these two characteristics with tumor cells. Both vascular endothelial and tumor cells also have the capability to invade surrounding tissues; but, in contrast to transformed cells, endothelial cells demonstrate a "controlled" invasiveness since they invade and vascularize tissues only when appropriate stimuli are present (12). It has been demonstrated that during the process of vascularization blood vessel growth is achieved by proliferation and forward migration of endothelial cells (38). It is attractive to hypothesize that endothelial cell migration is facilitated by the plasminogen activator-plasminogen-plasmin system, as has been shown for tumor cells in vitro (30). This hypothesis is supported by high plasminogen activator production found in other normal cells known to have migratory capability such as activated macrophages (42), granulocytes (16), mouse blastocysts (40), and stimulated ovarian granulosa cells (3). Granulocytes and stimulated macrophages also grow in semisolid media (6, 28), while the other cell types outlined above have not yet been tested for anchorage independent growth. These data suggest that growth in semisolid media and high plasminogen activator production are correlated with cell migration and/or invasion, characteristics shared by certain normal and trans-

formed cells, and that growth in soft agar is not a unique feature of tumor cells.

The continuous production and secretion of plasminogen activator by resting endothelial cells is another important observation. It has been speculated for years that blood vessels release this protease continuously to prevent intravascular coagulation in the organism (2), and our results support this hypothesis. Further studies are under way to determine whether venous endothelial cells also produce and secrete this protease and whether they produce an inhibitor as described by Loskutoff and Edgington (25) for endothelial cells from the vena cava of rabbits.

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