Inhibition of Anchorage-dependent Cell Spreading Triggers Apoptosis in Cultured Human Endothelial Cells

Fabio Re,* Adriana Zanetti,* Marina Sironi,* Nadia Polentarutti,* Luisa Lanfrancone,‡ Elisabetta Dejana,§ and Francesco Colotta*

*Istituto di Ricerche Farmacologiche "Mario Negri," Centro Daniela e Catullo Borgomainerio, 20157 Milano, Italy; ‡Clinica Medica I, Policlinico Monteluce, 06100 Perugia, Italy; §CEA, Departement de Biologie Moleculaire et Structurale, Lab d'Hematologie, INSERM U217, CEN-G, Grenoble, France

Abstract. When cultivated on substrates that prevent cell adhesion (the polymer polyhydroxyethylmethacrylate, bovine serum albumin, and Teflon), human endothelial cells (EC) rapidly lost viability with a half-life of ~10 h. Dying EC showed the morphological and biochemical characteristics of apoptosis. The apoptotic process of suspended EC was delayed by the protein synthesis inhibitor cycloheximide. To obtain information as to the mechanism involved in the apoptosis of suspended EC, we investigated whether adhesion to matrix proteins or integrin occupancy in EC retaining a round shape may affect EC suicide. EC bound to low coating concentration of either fibronectin or vitronectin, retaining a round shape and failing to organize actin microfilaments, underwent to rapid cell death; by contrast, cells on high substrate concentrations became flattened, showed actin microfilament organization, and retained viability. Addition of saturating amounts of soluble vitronectin to suspended round-shaped EC did not reduce the process of apoptosis. Finally, when suspended EC bound Gly-Arg-Gly-Asp-Ser–coated microbeads (~10 microbeads/cell), yet retaining a round shape, the apoptotic process was not affected. Oncogene-transformed EC in suspension were less susceptible to cell death and apoptosis than normal EC. Overall, these data indicate that cell attachment to matrix or integrin binding per se is not sufficient for maintaining cell viability, and that cells need to undergo some minimal degree of shape change to survive. Modulation of interaction with the extracellular matrix can, therefore, be an important target for the control of angiogenesis.

THE formation and regression of new vascular structures is a regulated process that governs organ development during embryogenesis (for review see reference 11). This suggests that not only capillary proliferation, but also capillary involution, depends on physiological control mechanisms. The composition and organization of the extracellular matrix are known to markedly influence remodeling of blood vessels. Capillary basement membrane dissolution correlates with microvessel retraction, endothelial cell (EC)[1] rounding, and associated capillary regression (21, 24). This series of events are likely caused by the fact that EC must be adherent to matrix proteins to survive and proliferate. When EC are cultured under conditions that prevent adhesion and spreading, they stop growing and lose viability (20, 24, 31).

Address all correspondence to Francesco Colotta, M.D., Istituto di Richerche Farmacologiche "Mario Negri," via Eritrea 62, 20157 Milano, Italy. Phone: 39-2-3901-4530; fax: 39-2-354-6277.

1. *Abbreviations used in this paper*: CH, cycloheximide; EC, endothelial cells; fn, fibronectin; poly(HEMA), polyhydroxymethacrylate; vn, vitronectin.

Although the mechanisms by which adhesion to substrates governs EC proliferation and survival are not completely understood, integrin occupancy, cytoskeletal organization, and the consequent change in cell shape seem to play a pivotal role (20, 36; and for review see reference 24). EC proliferate more rapidly in growth factor containing medium as they become flatter, and they stop growing as they take an increasingly rounded form, suggesting that the extent to which cells spread determines their ability to enter into the cell cycle (20).

Normal or programmed cell death is an active process of self destruction, usually termed, on the basis of its morphological and biochemical characteristics, apoptosis (for review see references 25, 35). Programmed cell death is an important event involved in the regulation of cell number in several physiologic and pathologic conditions, including morphogenesis, thymic selection, and leukocyte senescence. Apoptosis is an active process of autodestruction that requires RNA and protein synthesis (37, 39, 40). The activation of the genetic program of cell death is under the control of environmental signals (35), such as growth factors (12, 27, 28, 38, 41, 43), cytokines (6, 7), glucocorticoids (39), and radiations (37).

Since EC adhesion to matrix is an absolute requirement for survival and proliferation in response to growth factors, we reasoned that failure to adhere to a substratum may represent a signal to activate a suicide process in these cells. Our results demonstrate that when adhesion is prohibited, EC rapidly undergo cell death with the morphological and biochemical characteristics of apoptosis. Occupancy and clustering of integrin receptors in conditions that do not allow cell spreading did not prevent the apoptotic process. These data suggest that round cell shape caused by an altered interaction with extracellular matrix proteins and prevention of cytoskeletal organization represent signals for cell suicide in EC.

Materials and Methods

Cell Culture Media and Reagents

The following reagents were used for culture of EC: pyrogen-free saline for clinical use (Societa per Aziani Laboratorio Farmacologico, Bergamo, Italy); pyrogen-free distilled water (Societa per Aziani Laboratorio Farmacologico); PBS (Gibco, Paisley, Scotland); 10× concentrated M199 medium (Biochrom KG, Berlin, Germany); penicillin and streptomycin for clinical use (Farmitalia, Milano, Italy); aseptically collected FCS (Hyclone Laboratories, Logan, UT); BSA (Sigma Chemical Co., St. Louis, MO). All reagents contained <0.125 endotoxin unit/ml of endotoxin as checked by the Limulus Amebocyte Lysate assay (Microbiological Associates, Walkersville, MD). Cycloheximide (CH) was from Sigma Chemical Co. Oxygen peroxide was from Merck (Darmstadt, Germany). Human plasma fibronectin (fn) was purified from freshly drawn, citrated blood plasma by affinity chromatography on gelatin-Sepharose (13). Human plasma vitronectin (vn) was purified from human plasma, as previously described (42). For binding assays (see below), plasma vn was radiolabeled with ^{125}I and the iodogen procedure (15), reaching a specific radioactivity of 0.48 μCi/μg protein.

Antibodies

The anti $\alpha_v\beta_3$ mAb LM 609 (affinity-purified mouse IgG) (4) was kindly obtained from Dr. D. A. Cheresh (The Scripps Research Institute, La Jolla, CA); an irrelevant mAb (mouse IgG) was used as a control.

Peptides

The synthetic peptides Gly-Arg-Gly-Glu-Ser (GRGES) and Gly-Arg-Gly-Asp-Ser (GRGDS) were synthesized in our laboratory as described (8). The synthetic (cyclical) peptide Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala (GPenGRGDSPCA) was from Telios Pharmaceuticals, Inc. (San Diego, CA).

Cells

Human EC were obtained from umbilical veins and cultured as described in detail in previous reports (9) on flasks coated with gelatin from Sigma Chemical Co. We used routinely confluent cells at the 2nd through 5th passages maintained in M199 medium with 20% FCS, and supplemented with 50 μg/ml endothelial cell growth supplement (Collaborative Research Inc., Lexington, MA) and 100 μg/ml heparin (Sigma Chemical Co.), hereafter defined as complete medium.

Confluent adherent EC were detached by exposure to 0.05% trypsin–0.02% EDTA (Gibco) for 2 min at room temperature. Detached cells (routinely ≥98% viable as assessed by trypan blue assay, see below) were then centrifuged at 440 *g* and resuspended in complete medium or chemically defined medium (see below) at 3.5×10^5 cells/ml, unless otherwise stated.

Oncogene-transformed EC were used in selected experiments. LT2 and LT4 cells were obtained by introducing the large T antigen from SV-40, and have been described in detail elsewhere (17). EC-RAS and EC-RASf were obtained by infection with a retroviral vector containing a v-*ras* oncogene, whereas EC-SRC cells contained a v-*src* oncogene. Cells were detached and analyzed for survival and apoptosis in suspension, as described below, for normal EC.

In the experiments in which EC were incubated with various concentrations of vn or fn (either soluble or on plastic wells) or with RGD-coated microbeads (see below), EC from confluent adherent cultures were detached as detailed above and then resuspended in M199 medium with 4% BSA and insulin-transferrin-sodium selenite growth supplement (Sigma Chemical Co.) diluted 1:25, hereafter referred to as chemically defined medium.

EC Cultured under Nonadherent Conditions

To obtain EC cultured under nonadherent conditions, cells resuspended as detailed above were pipetted in 3.5-cm diameter plastic wells (six-well tissue culture plate, Falcon no. 3046; Becton Dickinson Labware, Lincoln Park, NJ), 3.5×10^5 cells/ml, 1.5 ml in each well, previously coated with the polymer polyhydroxyethylmethacrylate [poly(HEMA)] or with BSA, or in Teflon-bottomed dishes, and incubated for the indicated times at 37°C in 5% CO_2 in air. As control cultures, an aliquot of the same cell suspension was plated in gelatin-coated dishes, to which EC rapidly adhered and grew to confluence.

Poly(HEMA) (Aldrich-Chemie, Steinheim, Germany) was prepared and used as detailed in reference 16. 12 g poly(HEMA) was dissolved in 100 ml 95% ethanol. Then, 400 μl were pipetted into 3.5-cm diameter flat-bottomed wells and were allowed to dry at 37°C under sterile conditions for 40–48 h. After alcohol evaporation, a thin, sterile film of optically clear, nontoxic polymer remained tightly bound to the surface, as described (1, 14, 20, 36).

In some experiments, EC were not allowed to adhere by plating cells on BSA-coated plastic surfaces. 500 μl of a 1% BSA solution in PBS with Ca^{++} and Mg^{++} (Gibco) were pipetted in 3.5-cm diameter plastic wells. After 1 h incubation at 37°C under sterile conditions, wells were washed with PBS and used for EC culture.

Teflon-bottomed dishes (5.5 cm in diameter) were from Haereus (Osterode, Germany). Detached EC were resuspended at 3.5×10^5/ml in complete medium, and 2 ml of cell suspension was added in each dish.

Vn and fn Coating of Wells

Plastic wells were coated with fn or vn as described (9). To obtain wells coated with decreasing amounts of vn or fn, 500 μl of 5–0.05 μg/ml vn or fn solutions were added in 3.5-cm diameter wells and incubated for 18 h at room temperature and 4°C, respectively. Wells were washed using PBS with Ca^{++} and Mg^{++}, and then incubated with 500 μl 1% BSA in PBS for 1 h at 37°C. After washing, wells were used for cell culture. The final vn and fn concentrations in plastic wells ranged from 250 to 0.1 ng/cm^2. 1.5 ml of cells (7×10^4/ml) resuspended in chemically defined medium were added to each well. After various periods of time, wells were washed two or three times with PBS, and adherent cells were recovered by a brief exposure to trypsin-EDTA (see above), centrifuged, and examined for viability and apoptosis as detailed below.

Cell spreading was evaluated by image analysis. EC were allowed to adhere on different concentrations of fn or vn as detailed above. Images were acquired in gray color of 256 values through a FA-I CCD Grundig electronic video camera (Grundig AG, Fürth, Germany) mounted on a Zeiss Axioscope microscope using a 40× objective. Image processing and computer elaboration were done with the IBAS 2 (Kontron/Zeiss, PC 386) Image Analysis System.

Immunofluorescence Studies

Glass coverslips (13 mm in diameter) were cleaned and coated with various concentrations of fn or vn (see above), as described in detail (9). To visualize F-actin, fixed and permeabilized EC were stained with fluorescein-labeled phalloidin (Sigma Chemical Co.), and they were examined as described (9).

Incubation of Suspended EC with Soluble vn

In certain experiments, EC cultivated in poly(HEMA)-coated wells as detailed above were incubated with soluble vn. To define the concentration of soluble vn required to saturate EC receptors, cells at 10^6/ml were incubated with increasing concentrations (20–200 nM) of ^{125}I-vn. Nonspecific binding was assessed by adding a 100-fold molar excess of unlabeled vn. Cells were incubated in binding buffer (PBS diluted 1:2 in M199, 1% BSA, and 0.01% sodium azide) at 37°C for 1 h. This incubation time was selected because preliminary experiments measuring the time course of 40

nM ^{125}I-vn binding to EC indicated that equilibrium was reached within 1 h. Binding data were then examined by the LIGAND program (32). Vn binding reached saturation at 0.5 μM with a K_d of 0.24 μM and presented ~2.7 × 10^6 receptors per cell, in good agreement with previous estimates (34). Apoptosis was then evaluated at saturating vn concentrations. 1.5 ml of cells suspended in chemically defined medium (3.5 × 10^5 cells/ml) were added with 0.5 μM vn, incubated for the indicated times, and then examined for viability or estimation of apoptotic cells.

Coating of Microbeads with RGD and RGE Peptides

GRGDS and GRGES peptides linked to BSA (18) were used at a concentration of 360 μg/ml of BSA (corresponding to 19.6 μg/ml of peptide) in 0.05 M borate buffer, pH 9.5. 2.5 ml of peptide solution was mixed with ~2.5 × 10^7 magnetic beads measuring 4.5 μm in diameter (Dynal, Oslo, Norway). The mixture was incubated for 18–24 h at room temperature by slow end-over-end rotation. Then, any unreacted protein and the buffer were removed by using the Dynal magnetic device, and the coated beads were washed four times with 2.5 ml Tris-HCl, pH 9.6, which blocked any unreacted tosyl group. The peptide-coated beads were resuspended in HBSS (Gibco). Then, 7.5 × 10^6 coated beads resuspended in 100 μl HBSS were added to 1.5 ml cell suspension (5 × 10^5 cells/ml) in chemically defined medium in poly(HEMA)-coated wells measuring 3.5 cm in diameter (see above). Thus, the beads/cell ratio was 10:1. After the appropriate incubation time, cells that were bound to beads were separated from unbound cells by the Dynal magnetic device, and they were then examined for viability and apoptotic death as described below.

Estimation of Viable Cells

Cells were cultivated under nonadherent conditions as detailed above. After the appropriate incubation time, an aliquot of cells was cytocentrifuged and used to estimate apoptotic cells as detailed below. To assess viability, 1 vol of trypan blue (0.4%; Gibco) was added to 5 vol of cells. After incubation at room temperature for 5 min, cells were counted in a hemocytometer. All counts were done in triplicate after coding of samples. Viability of EC cultured under adherent conditions was checked by detaching cells by brief exposure to trypsin and the trypan blue assay, as described above.

Estimation of Apoptotic Cells

Cytocentrifuged cells were stained with May-Grunwald-Giemsa (Merck), and they were examined by oil immersion light microscopy at a final magnification of 400. All samples were coded, and ≥500 cells per slide were examined. Apoptotic cells were identified according to the following criteria (25): condensed and fragmented nuclei, blebbing of plasma membranes, and decrease in cell size.

DNA Fragmentation

Fragmented and intact DNA were evaluated as described (5). Suspended EC (5 × 10^5) were centrifuged in Eppendorf tubes at 13,000 *g* for 2 min, washed with cold PBS, and lysed with 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.2% Triton X-100 (Merck) (hypotonic buffer). After incubation on ice for 15 min, low and high molecular weight DNAs were separated by centrifugation at 13,000 *g* at 4°C for 20 min. Centrifugation-resistant low molecular weight DNA in the supernatant was precipitated with 12.5% TCA (Merck) for 18 h, whereas pellets were added with 300 μl of cold 12.5% TCA. Samples were then centrifuged at 13,000 *g* at 4°C for 5 min, and DNAs in the precipitates were extracted with 30 μl of 5 mM NaOH and 30 μl of 1 M perchloric acid (Merck) at 70°C for 20 min. Then 120 μl of diphenilamine reagent (3) was added, and samples were incubated at 37°C for 18 h. 120 μl from each sample was then transferred to flat-bottomed, 96-well plates (Falcon Labware), and absorbance at 600 nm was measured on an automated plate reader (Titertek Multiskan; Flow ICN, Milano, Italy). Fragmentation was calculated as percentage of total DNA (supernatant and pellet) recovered as low molecular weight DNA in the supernatant.

DNA Electrophoresis

Cells (5 × 10^5) cultivated as detailed above were centrifuged in Eppendorf tubes at 13,000 *g* for 2 min, washed in phosphate-buffered saline, and lysed in 0.5 ml hypotonic buffer (see above) for 15 min on ice. Lysates were then centrifuged at 13,000 *g* at 4°C for 20 min. Pellets of high molecular weight DNA were added with 0.4 ml of 10 mM EDTA, 50 mM Tris, 0.5% sarcosyl (Sigma Chemical Co.), and 0.5 μg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany), and incubated at 48°C for 18 h, whereas centrifugation-resistant, low molecular weight DNA was incubated with 20 μg/ml RNase A (Boehringer Mannheim GmbH) at 37°C for 1 h. Low and high molecular weight DNAs were then extracted with phenol/chloroform, precipitated with 0.5 M NaCl and 1 vol of propanol, resuspended in water, combined with loading buffer (2.5% Ficoll, 0.025% bromophenol, and 0.025% xylene cyanol; Sigma Chemical Co.), heated at 75°C for 5 min, and electrophoresed in 1% agarose containing 1 μg/ml bromide (Sigma Chemical Co.) at 50 V in 40 mM Tris-acetate. DNA was visualized by UV examination and photographed with a Polaroid camera. Size of DNA was compared with a standard ladder (Boehringer Mannheim GmbH).

Results

We examined the survival of EC cultured on plates coated with nonadhesive substrates. To this aim, EC were detached from gelatin-coated dishes, resuspended in complete medium, and plated in plastic wells previously coated with nonadhesive substrates. As a first approach, wells were coated with saturating amounts of the nonadhesive polymer poly(HEMA). This compound is nontoxic and has been previously used in a number of studies to prevent cell spreading (1, 14, 20, 36). Under these conditions, EC remained in suspension and acquired a spheroidal conformation (20, 36). Viability of EC in suspension was examined at various periods of time after plating. As shown in Fig. 1, EC in suspension rapidly died, with an estimated half-life of ~10 h (range was 9–14 h in eight different experiments). After 36–48 h, <5% cells were still viable. Comparable results were obtained when cells were cultured on plates coated with BSA or on Teflon-bottomed plates, to which cells did not adhere, thus acquiring a spherical conformation (Fig. 1). Control cultures where the same EC suspension in complete medium was plated in gelatin-coated wells rapidly adhered and grew to confluence, retaining ≥98% viability (Fig. 1).

Then we examined the mechanism of EC death. The morphological and biochemical analysis demonstrated the typical characteristics of apoptosis. As reported in Table I, a pro-

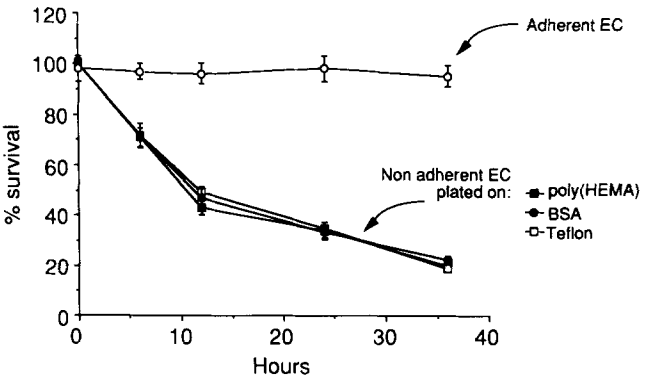

Figure 1. Viability of EC plated on surfaces that prevent cell adhesion. Growing EC were detached from gelatin-coated plates and resuspended in complete medium as detailed in Materials and Methods. Cells were then plated in 3.5-cm diameter wells previously coated with poly(HEMA) or BSA, or in Teflon-bottomed 5.5-cm diameter plates. At various time intervals, aliquots of cells were harvested and viability assessed by trypan blue dye exclusion. Data are mean ± SD of counts in triplicate from one representative experiment out of six. In control cultures, in which the same cell suspension used for viability assay was plated in gelatin-coated plates, cells rapidly attached and grew to confluence.

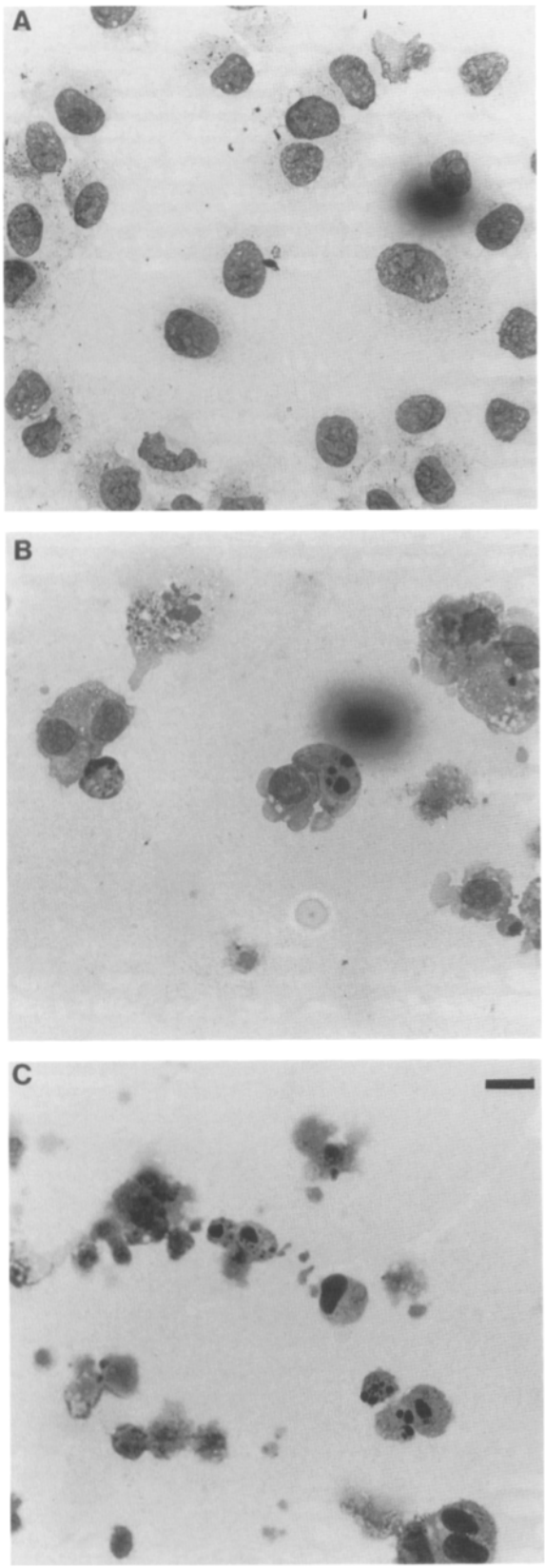

Figure 2. Morphological characteristics of EC cultured under nonadherent conditions. Cells were cytocentrifuged after 12 (*B*) or 18 h (*C*) of culture in poly(HEMA) coated wells, stained with May-Grunwald-Giesmsa, and then examined at a final magnification of 400. Cells showed the typical characteristics of apoptosis (nuclear condensation and fragmentation, surface blebbing and reduction in cell size). For comparison, an aliquot of the same cell suspension was plated in gelatin-coated plates, cultivated for 18 h, and then detached and cytocentrifuged (*A*). Identical results were obtained in three different experiments. Bar, 40 μm.

Table I. EC Showing Morphological Characteristics of Apoptosis and Percent DNA Fragmentation after Plating of Cells on Substrates That Prevent Cell Adhesion

Time	Percent of apoptotic cells			Percent of fragmented DNA
	Poly(HEMA)	Teflon	BSA	
0	≤2	≤2	≤2	9.1 ± 3.8
6	10.5 ± 2.7	12.4 ± 4.9	11.9 ± 3.8	20.6 ± 5.1
12	25.8 ± 5.8	21.9 ± 4.1	22.7 ± 7.2	34.5 ± 2.1
24	49.8 ± 4.4	47.8 ± 7.1	45.6 ± 5.6	57.0 ± 18.1
36	85 ± 9.8	88.5 ± 10.2	84 ± 5.7	70.6 ± 8.8

Growing adherent EC were detached by brief exposure to 0.05% trypsin–0.02% EDTA, resuspended in complete medium, and either examined immediately (t = 0) or plated on 3.5-cm diameter wells previously coated with poly(HEMA) or BSA, or in Teflon-bottomed plates, as detailed in Materials and Methods. At various intervals of time, cells were harvested and evaluated for morphological characteristics of apoptosis in cytocentrifuged preparations. At least 200 cells were counted for each cytocentrifuged preparation. Data of DNA fragmentation are from cells cultivated on poly(HEMA)coated wells. All data are mean ± SD from one representative experiment counted in triplicate. Similar results were obtained in three different experiments. Control cultures, in which the same cell suspension used for these experiments was plated on gelatin-coated plates, rapidly attached to the substrate and grew to confluence.

gressively increasing percentage of cells showed highly condensed and fragmented nuclei, blebbing of plasma membrane, and decrease in cell size (Fig. 2). The biochemical hallmark of apoptosis is the fragmentation of DNA in multiples of ~200 bp. As reported in Table I, a consistent proportion of DNA from EC in suspension was recovered as low molecular weight DNA in a time-dependent fashion. The electrophoresis analysis (Fig. 3) clearly demonstrated the characteristic pattern of fragmentation of DNA in multiples of 200 bp, once again in a time-dependent fashion. The specificity of the apoptotic process in nonadherent EC was determined by examining EC death induced by H_2O_2 (5 mM, 4 h), ethanol (1.5% vol/vol, 4 h), and heat shock (45°C, 4 h). All these treatments induced 70–80% EC death, as assessed by trypan blue dye exclusion, but neither morphological evidence of apoptosis nor DNA fragmentation were evident under these experimental conditions (two experiments, not shown).

Apoptosis is an active process that requires macromolecular synthesis (37, 39, 40). When EC in suspension were incubated in the presence of the protein synthesis inhibitor CH, the process of apoptosis was considerably delayed, suggesting that the process was, at least in part, actively conducted by cells. As reported in Table II, CH (0.1 μg/ml) augmented cell viability at all time points examined, reduced the number of cells showing the morphological characteristics of apoptosis (Table II), and reduced DNA fragmentation as assessed both by quantitative determination of low molecular weight DNA (Table II) and gel electrophoresis (Fig. 2). Thus, when EC are prevented from adhering to a substrate, they rapidly undergo an active process of cell death with the morphological and biochemical characteristics of apoptosis.

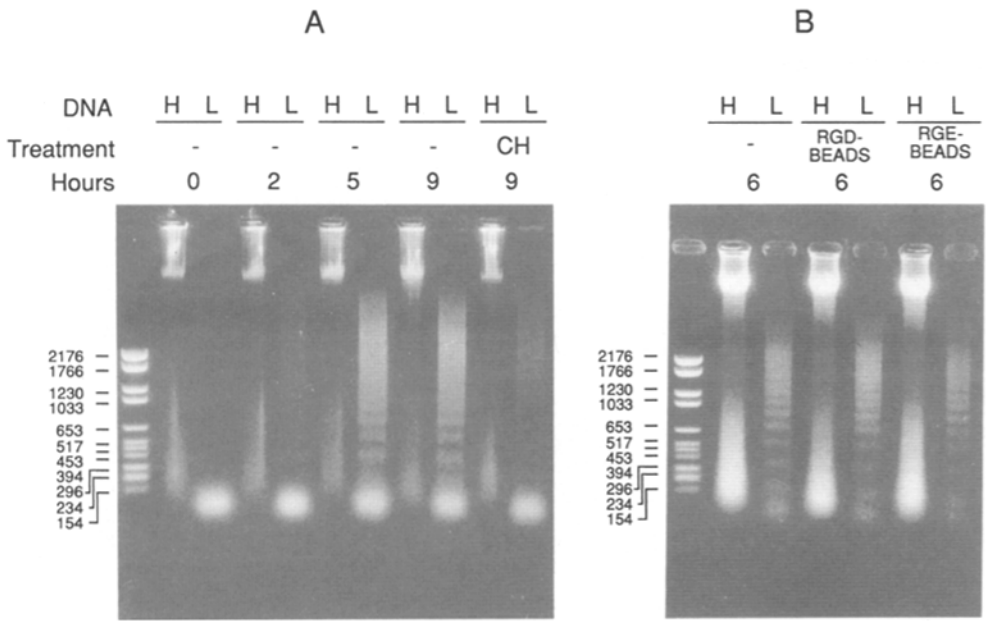

Figure 3. Gel electrophoresis analysis of high (*H*) and low (*L*) molecular weight DNAs isolated from EC cultured in poly(HEMA)-coated wells. (*A*) Kinetics of DNA fragmentation and effect of cycloheximide. Growing adherent EC were detached and resuspended in complete medium. An aliquot of the cell suspension was immediately used for extraction of high (*H*) and low (*L*) molecular weight DNAs (time = 0), whereas the remaining cells were plated in poly(HEMA)-coated wells, and DNAs were extracted after 2–9 h of cultivation. The last two lanes show the DNAs extracted from EC cultivated for 9 h in poly(HEMA)-coated wells in the presence of 0.1 μg/ml CH. After extraction, DNAs were analyzed by gel electrophoresis through 1% agarose gel. (*B*) Effects of RGD- and RGE-coated microbeads on DNA fragmentation. EC resuspended in chemically defined medium were plated in poly(HEMA) wells in the presence or absence of RGD- or RGE-coated microbeads. After 6 h incubation, high and low molecular weight DNAs were extracted and examined by 1% agarose gel electrophoresis.

Nonadherent EC acquired a spherical conformation, as opposed to the flattening shape of adherent cells. Cell flattening is essentially mediated by cell adhesion to extracellular matrix proteins through integrin receptors. Integrins, once bound to extracellular matrix components, cluster and promote cytoskeletal organization that, in turn, is responsible for cell spreading (for review see references 2, 19). To obtain information about the mechanisms that trigger the apoptotic process in nonadherent EC, we investigated whether cell adhesion to matrix proteins and integrin occupancy in conditions that prevent cell spreading could inhibit EC suicide. Since FCS, which is present in complete medium, contains significant amounts of adhesive substrate molecules, in these experiments, EC were resuspended in chemically defined medium supplemented with growth factors. Control cultures, in which the same EC suspension in chemically defined medium was plated in gelatin-coated wells, were fully adherent and retained ≥98% viability.

Table II. Effects of Cycloheximide on the Apoptotic Process of Suspended EC

Time	CH	Percent of viable cells	Percent of apoptotic cells	Percent of DNA fragmentation
0	−	95.1 ± 2	≤2	8.0
	+	94.9 ± 3.2	≤2	ND
24	−	30 ± 5.7	55 ± 8.5	54.5
	+	67.5 ± 4.9	15.4 ± 8.7	19.4
36	−	9.2 ± 6.1	76.0 ± 10.2	69
	+	38.6 ± 4.9	20.1 ± 8.9	20.2
50	−	ND	95 ± 4.8	82.4
	+	ND	29.2 ± 6.5	30.4

EC were treated and plated as detailed in Materials and Methods and Table I. Data are from one representative experiment out of three in which we obtained similar results. CH was used at 0.1 μg/ml. ND, not done.

First, the cells were plated on increasing amounts of matrix proteins such as vn and fn, as detailed in Materials and Methods. As expected, EC remained in suspension and round shaped when plated in BSA-coated wells. Increasing vn or fn density resulted in progressive flattening of cells and actin microfilament organization (Fig. 4, *A* and *B*). After 12 h of culture, viability of human EC on BSA-coated plates was 50 ± 5 and 45 ± 4% in *A* and *B*, respectively (mean ± SD of counts in triplicate), whereas fully flattened cells in plates coated with 250 ng/cm² vn or fn retained 98 ± 8 and 95 ± 6% viability, respectively. At 50, 10, 2.5, 0.5, and 0.1 ng/cm² fn or vn, cells acquired a progressively more spherical form and viability was 90 ± 6, 70 ± 5, and 58 ± 3%; 54 ± 4 and 51 ± 6% for fn; and 88 ± 7, 65 ± 8, 48 ± 4, 47 ± 2, and 43 ± 5% for vn (mean ± SD). (Fig. 4). Similar results were obtained after 24 and 36 h of culture (Fig. 4, *A* and *B*). These findings were confirmed in three different experiments. EC attached to these concentrations of vn or fn showed a different degree of spreading: the examination of cell size by image analysis revealed that the area occupied by EC was 2,240 ± 523, 1,790 ± 106, 641 ± 152, 550 ± 102, 400 ± 93, and 300 ± 100 μm² for EC plated on 250 through 0.1 ng/cm² fn, and 1,885 ± 552, 1,133 ± 432, 679 ± 135, 443 ± 177, 80 ± 16, and 80 ± 1 μm² for EC on 250–0.1 ng/cm² (mean ±SD of four to eight determinations for each substrate). Nevertheless, EC plated on 50 and 250 ng/cm² vn or fn survived equally well, suggesting that a low to moderate spreading is sufficient to maintain cell viability. These results were confirmed and extended by examining the percentage of cells showing the morphological characteristics of apoptosis (Table III).

A second approach consisted in cultivating cells on poly(HEMA)-coated plates in the presence of soluble vn. To

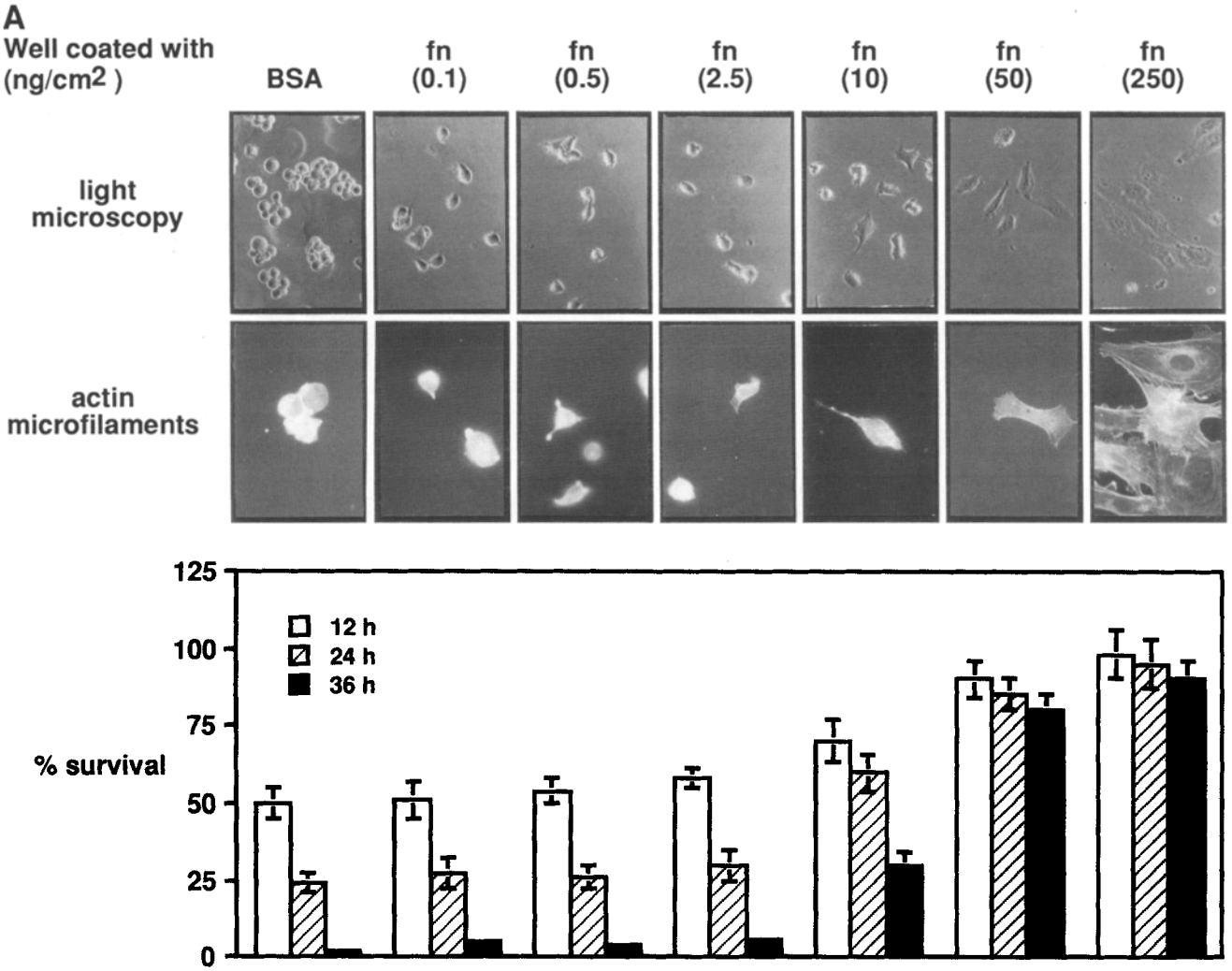

Figure 4. Effects of increasing amounts of matrix proteins on EC spreading and survival. EC from adherent cultures were detached and resuspended in chemically defined medium and then plated in BSA-coated wells (nonadherent condition) or in plates coated with increasing amounts of fn (*A*) or vn (*B*). After 12 h cultivation, cells were examined for cell spreading by light microscopy and actin microfilament organization, whereas after 12, 24, and 36 h cells were examined for survival (as assessed by trypan blue dye exclusion) as shown in the lower part of each panel. Similar results were obtained in four different experiments.

establish the vn concentration able to saturate EC receptors, binding assays were performed. Soluble vn bound to EC in a concentration- and time-dependent manner, reaching the equilibrium at 1 h and saturation at 0.5 μM. The binding presented a K_d of 0.24 μM and a maximal number of receptors at saturation of 2.7×10^6 receptors per cell. These data are in agreement with previously reported studies on soluble vn binding to EC (34). The anti-$\alpha_v\beta_3$ mAb LM609 used at 25 μg/ml inhibited by 45–67% the specific vn binding to EC (range of two experiments performed at 40 nM vn added to cells for 1 h at 37°C). The cyclic RGD peptide (GPenGRGDSPCA) inhibited by 72–78% (at 100 μM, range of two experiments performed at 40 nM vn added to cells for 1 h at 37°C) vn-specific binding, while GRGES at 100 μM was ineffective (not shown). These concentrations of mAbs and RGD peptide gave comparable inhibition of EC adhesion to substrate-linked vn (7 μg/ml in coating, 2 h adhesion).

When EC were then incubated with a saturating vn concentration (0.5 μM), we found that survival of EC cultivated in the absence or presence of soluble vn was unchanged (60% and 59% at 8 h, 47% and 50% at 12 h, and 31% and 30% at 24 h, respectively).

We studied the effect of the binding of RGD-coated beads to suspended EC cultivated in chemically defined medium. Almost all of EC in suspension bound ~10 RGD-coated beads, yet cells retained a round shape (Fig. 5). Under identical conditions, ≤1% cells bound beads coated with the control peptide RGE (Fig. 4). It was found that binding of RGD-coated beads to EC in suspension did not alter the process of cell death. After 8 h of culture, control cells (no beads added) showed 70.5 ± 4% survival. EC incubated with RGD- or RGE-coated microbeads had a survival of 71 ± 3% and 70.4 ± 7%, respectively. Similar results were obtained after 24 h of culture (not shown). In addition, binding of RGD-coated microbeads to round-shaped EC in suspension did not influence the number of cells showing the morpho-

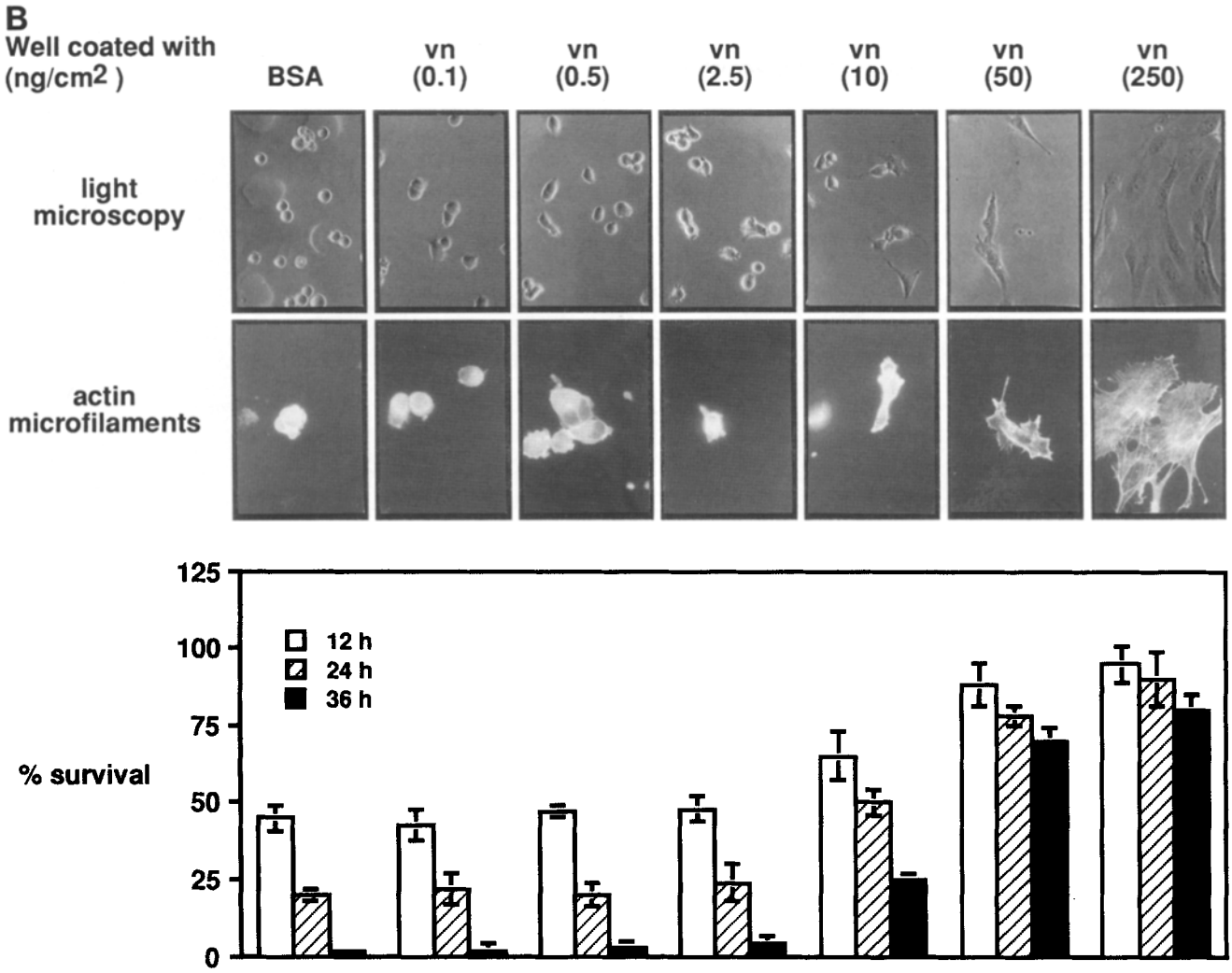

Figure 4.

Table III. EC Showing Morphological Characteristics of Apoptosis after Cultivation on Different ECM Densities

ECM	Density	Percent of apoptotic cells* 12 h	24 h	36 h
	ng/cm²			
‡	–	25 ± 5	48 ± 8	86 ± 9
FN	0.1	30 ± 5	52 ± 6	84 ± 3
	0.5	28 ± 4	48 ± 5	79 ± 4
	2.5	29 ± 7	46 ± 6	70 ± 5
	10	15 ± 3	28 ± 7	41 ± 9
	50	≤2	≤2	4 ± 2
	250	≤2	≤2	≤2
VN	0.1	28 ± 3	43 ± 5	82 ± 3
	0.5	29 ± 6	50 ± 7	80 ± 7
	2.5	21 ± 5	47 ± 9	72 ± 9
	10	11 ± 3	26 ± 3	39 ± 5
	50	≤2	≤2	5 ± 2
	250	≤2	≤2	≤2

* Cells cultivated on different ECM densities for 12–36 h were detached and examined for cells showing morphological characteristics of apoptosis on cytospin preparations.
‡ As a control, nonadherent EC were cultivated on BSA-coated dishes as described in Materials and Methods.

logical characteristics of apoptosis (23.4%, 25.6%, and 22.7% in untreated EC and in EC incubated with RGD- and RGE-coated beads, respectively, 12 h of culture) or the DNA fragmentation, as assessed by agarose gel electrophoresis (Fig. 2, 6 h of incubation). These findings were confirmed in four different experiments. Overall, these results suggest that the occupancy of RGD-dependent integrin receptors in absence of cell spreading does not prevent EC apoptosis.

Having found that normal EC undergo cell death by apoptosis when in suspension, we investigated whether this phenomenon was operative in transformed EC. The data shown in Table IV indicate that transformed EC are less susceptible to cell death when cultivated in suspension. Viable transformed EC fully retained their proliferative potential when recovered and plated under standard adherent conditions. Transformed EC in suspension were also evaluated for morphological characteristics of apoptosis. The percentage of transformed EC undergoing cell death by apoptosis was much lower than that exhibited by normal EC (Table IV).

Discussion

The major finding of this work is that prevention of EC

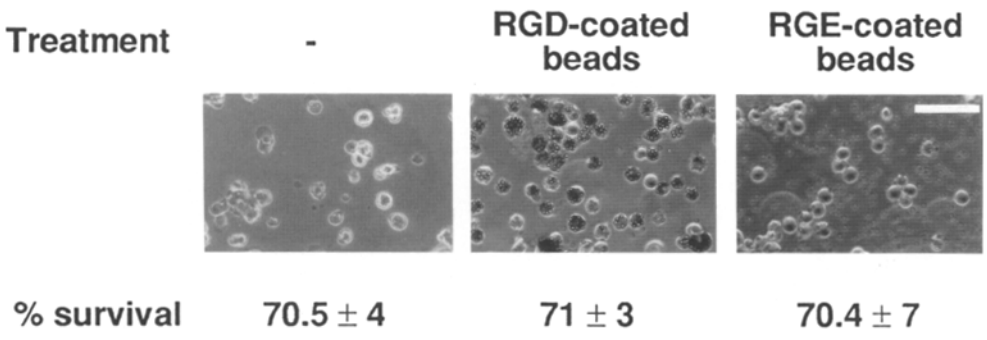

Figure 5. Survival of EC treated with RGD- or RGE-coated microbeads in poly-(HEMA)-coated wells. EC were plated on poly(HEMA)-coated wells and were either untreated (*left panel*), or added with RGE-(*middle panel*) or RGD-(*right panel*) coated microbeads (Bar, 45 μm). The beads/cell ratio was 10:1. HEC bound RGD- but not RGE-coated beads. More than 90% of cells bound ~10 RGD beads. The figure refers to 8 h of culture. These results were confirmed in four different experiments.

spreading on matrix proteins causes cell death with the morphological and biochemical characteristics of apoptosis. Adhesion of EC to matrix components such as vn and fn is mostly mediated by integrin receptors (19). These molecules, after binding to their specific ligands, cluster and promote the assembly of cytoskeletal proteins and actin microfilament polymerization that, in turn, cause cell flattening on the substratum (2, 19, 21). For induction of cell spreading, therefore, integrins need to bind to a ligand immobilized on a solid surface so that it can resist the cell tension. While this paper was in preparation, another report demonstrated that attachment to the extracellular matrix prevents apoptosis in human EC (31). These authors suggested that integrin-mediated signals can be critical for cell survival. In this paper, we bring evidence that a minimal degree of cell deformation is also necessary for a complete inhibition of apoptosis.

Previous reports on EC or other anchorage-dependent cell types showed that some intracellular events, such as activation of the antiporter, induction of inositol lipid turnover, activation of tyrosine kinases, and induction of early immediate growth response genes, can be induced by integrin occupancy before or in absence of cell spreading (26, 30, 33, 36). In contrast, cell growth was promoted by adhesion to extracellular matrix proteins only when this event was followed by cell spreading (20). The data reported here strongly suggest that some degree of deformation or shape change, and not the simple occupancy of integrin receptors, is required to prevent apoptosis. When cells had a round shape, yet were attached to matrix proteins such as vn, fn, and RGD peptides, they equally activated a suicide program. Occupancy of integrin receptors by soluble ligands or RGD-coated microbeads in conditions that do not allow cell spreading failed to prevent cell death by apoptosis. In contrast, when cell spreading was induced by the binding of different types of integrins (9), either $\alpha_v\beta_3$ (when the cells were seeded on vn) or $\alpha_5\beta_1$ (when the cells were seeded on fn) apoptosis was always prevented, thus suggesting that the phenomenon was not caused by the occupancy of a specific integrin, but rather by the subsequent organization of the cytoskeleton and cell spreading. Overall, these results and data reported previously (20) can be interpreted as follows: early chemical signaling by integrins is not sufficient for complex and long-lasting responses such as cell growth or survival. Cytoskeletal organization, which follows integrin engagement, and the subsequent mechanical and structural changes associated with cell spreading seem to be required for the complete cellular response to matrix interaction.

Table IV. Survival and Apoptosis of Normal and Oncogene-transformed EC Cultivated in Suspension

	Percent of survival				Percent of apoptotic cells		
Cells	12	24	48	72	24	48	72
EC	58.3	19	<2	<2	90	>95	>95
EC-SN	60	25	4	<2	87	93	>95
LT2	96	83	70	52	7	12	37
LT4	83	57	12	4	31	80	>95
EC-RAS	87	73	41	10	40	55	75
EC-RASf	98	95	57	40	<2	40	58
EC-SRC	98	98	69	63	<2	30	38

Cells were as follows: *EC*, normal endothelial cells; *EC-NS*, normal endothelial cells transfected with an empty vector; *LT2* and *LT4*, two different cell lines transfected with the large T antigen from SV-40; *EC-RAS* and *EC-RASf*, transfected with a retroviral vector containing a v-*ras* oncogene; *EC-SRC*, transfected with a retroviral vector containing a v-*src* oncogene. Cells were cultivated on poly(HEMA)-coated wells for the indicated periods of time and then examined for viability by the trypan blue dye exclusion test, and for cells showing morphological characteristics of apoptosis on May-Grunwald-stained cytospin preparations. Data represent percent of starting population.

The difference of the effect of soluble or surface-bound vn or RGD peptides on apoptosis could be related to the engagement of a different number or type of receptors on EC. However, no change in apoptosis was observed when receptor-saturating concentrations of soluble vn were used. In addition, EC attachment to vn-coated substrata or soluble vn binding to EC were inhibited by comparable concentrations of RGD peptides and $\alpha_v\beta_3$ mAbs, suggesting that similar recognition mechanisms are playing a role in both conditions.

The mechanism by which cell shape may prevent apoptosis is unknown. Alterations of cytoskeleton organization may affect cell metabolism, including DNA synthesis and transcription, and stretch-activated ion channels in the plasma membrane (10, 23). The data presented in this paper demonstrate that in addition to control DNA proliferation and cell

metabolism, cell shape may also represent a signal to make a decision as to whether or not to activate a process of self destruction in EC.

Cell death by apoptosis is a highly complex phenomenon subjected to a strict environmental control (25, 35). It is well established that growth factor deprivation triggers apoptosis in a number of different cell types (12, 27, 28, 38, 41, 43). Our data show that in EC, the availability of growth factors is not sufficient to sustain cell survival, but also attachment to a substrate is required to prevent cells from entry into the apoptotic process. Competition for adhesive interactions may represent a simple mechanism to control cell number, as suggested for cells competing for limiting amounts of growth factors (35).

The concept that abnormal location is a simple mechanism for eliminating cells has been proposed to explain cell death of misplaced cells as, for example, during neuronal or embryo development. Activation of cell death in round-shaped EC may represent a mechanism to eliminate misplaced, i.e., nonadherent, cells. If this mechanism is operative in normal anchorage-dependent cells, it is tempting to speculate that transformed cells, which are anchorage-independent, have escaped the restriction imposed on cell survival by cell shape. According to this conclusion, in this paper, we show that a number of oncogene-transformed EC lines are more resistant to cell death and apoptosis induced by the loss of adherence to a substrate.

The results reported herein indicate that after 24–36 h of cultivation under nonadherent conditions, 18–30% of the starting EC population is still alive as judged by the trypan blue dye exclusion. This arises the possibility that survived cells may represent a selected, highly functional subpopulation. To address this issue, EC were cultivated on poly-(HEMA)-coated plates for 24 h, and then recovered and plated on gelatin-coated wells. The examination of these survived cells revealed that they were essentially identical to control cultures in terms of attachment to the gelatin coating (90% of recovered cells adhered compared to 92% of control, freshly isolated detached cells) and growth rate (duplication time of 46 h compared to 48 h of control cells), as assessed by crystal violet staining. Thus, no apparent selection of a subpopulation of functionally stronger EC could be demonstrated.

Not all cell types are equally sensitive to apoptosis by detachment. While this paper was under revision, Frisch and Francis (16) reported that epithelial cells, but not fibroblasts, undergo apoptosis after disruption of matrix interactions. The present paper extends this observation to endothelial cells which, even if they share morphological and structural properties with epithelial cells, have a different embryonic origin and specific functions. Similarly to what is reported here for *ras*- and *src*-infected EC lines, v-Ha-*ras*– and v-*src*–transformed epithelial cells were more resistant to cell death after disruption of matrix interactions.

In summary, our data suggest that in addition to growth factor deprivation, a round conformation can also trigger cell death by apoptosis in EC. Induction of apoptosis in EC by loss of attachment and acquisition of a round shape may be a relevant phenomenon by which EC involution can be controlled locally by modifying interactions between cells and substrate. Capillary involution has been described in the resolution of inflammatory reactions and during embryo development (11). Moreover, it has been shown that combination of angiostatic steroids and heparin induce basement membrane breakdown, capillary retraction, and endothelial rounding in vivo (21). The data reported here may explain the antiangiogenic properties of compounds that interfere with extracellular matrix integrity, and they point to extracellular matrix metabolism as an important target for the control of angiogenesis (22, 24, 29).

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