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The Extracellular Matrix as a Cell Survival Factor

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Programmed cell death (PCD) or apoptosis is a naturally occurring cell suicide pathway induced in a variety of cell types. In many cases, PCD is induced by the withdrawal of specific hormones or growth factors that function as survival factors. In this study, we have investigated the potential role of the extracellular matrix (ECM) as a cell survival factor. Our results indicate that in the absence of any ECM interactions, human endothelial cells rapidly undergo PCD, as determined by cell morphology, nuclei fragmentation, DNA degradation, protein cross-linking, and the expression of the PCD-specific gene TRPM-2. PCD was blocked by plating cells on an immobilized integrin β_1 antibody but not by antibodies to either the class I histocompatability antigen (HLA) or vascular cell adhesion molecule-1 (VCAM-1), suggesting that integrin-mediated signals were required for maintaining cell viability. Treatment of the cells in suspension with the tyrosine phosphatase inhibitor sodium orthovanadate also blocked PCD. When other cell types were examined, some, but not all, underwent rapid cell death when deprived of adhesion to the ECM. These results suggest that in addition to regulating cell growth and differentiation, the ECM also functions as a survival factor for many cell types.

INTRODUCTION

Programmed cell death (PCD), or apoptosis, is the process whereby cells are induced to activate their own death or cell suicide. PCD occurs in a wide variety of cell types and is required during the development of many tissues (reviewed in Ellis *et al.*, 1991). The term "apoptosis" has been used historically to refer to the unique morphology of cells undergoing PCD. Apoptotic cells appear shrunken, with extensive membrane blebbing and nuclear fragmentation (reviewed in Wyllie *et al.*, 1980). Apoptotic cells ultimately fragment into membrane-bound vesicles or apoptotic bodies that contain cellular remnants of proteins and fragmented chromatin. These apoptotic bodies are eventually phagocytosed by neighboring cells and scavenging macrophages (Wyllie *et al.*, 1980). In contrast, cells undergoing pathological death or necrosis swell and then rupture, releasing their cellular contents, thereby eliciting inflammatory reactions (Wyllie *et al.*, 1980).

Concomitant with changes in morphology, cells undergoing PCD also actively degrade their DNA and form extensive protein cross-links. Studies have shown that DNA degradation requires the activation of an endogenous deoxyribonuclease (Wyllie, 1980; Compton, 1992). In some, but not all, cases of PCD (e.g., in glucocorticoid-treated thymocytes), this deoxyribonuclease is specific for internucleosomal DNA such that the degraded DNA will form a 200-bp ladder pattern when separated by gel electrophoresis (Wyllie, 1980). In thymocytes, DNA degradation appears to be a key step in committing a cell to the PCD pathway (McConkey *et al.*, 1989). PCD-induced protein cross-linking appears to be dependent on the activation of an endogenous transglutaminase(s) required for the formation of apoptotic envelopes. (Fesus *et al.*, 1987).

In many models of PCD, cells are induced to die as a result of changes in environmental stimuli. For example, hormone depletion will induce PCD in hormone-dependent tissues such as the prostate gland (Kyprianou and Isaacs, 1988), mammary gland (Strange *et al.*, 1992), uterine epithelium (Rotello *et al.*, 1989), and breast cancer cells (Bardon *et al.*, 1987). In insects, changes in steroid hormone levels control the massive cell death accompanying metamorphosis (Ellis *et al.*, 1991). Growth factors also regulate programmed cell death. Treatment of endothelial cells with serum-free medium will induce PCD (Araki *et al.*, 1990). Certain neurons deprived of growth factors, such as nerve growth factor, will also initiate PCD (Ellis *et al.*, 1991). And some mature T cells are dependent on interleukin-2 to prevent PCD (Duke and Cohen, 1986). In general, these ex-

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amples of PCD suggest that the absence of a "survival factor," such as a particular hormone or growth factor, will induce a cell to initiate its own death.

Like hormones and growth factors, the extracellular matrix (ECM) plays an important role in the regulation of cell growth, differentiation, and behavior (reviewed in Daniels and Solursh, 1991; Shimizu and Shaw, 1991). ECM-cell interactions are mediated to a large extent by the integrins, a family of more than 20 different $\alpha\beta$ heterodimers (reviewed in Hynes, 1992). Recent work has demonstrated that, in addition to their role as adhesion receptors, integrins also function as signaling receptors. Integrins have been found to regulate many intracellular signaling pathways such as tyrosine phosphorylation, cytoplasmic alkalization, intracellular Ca^{2+} fluctuations, and inositol lipid metabolism (reviewed in Juliano and Haskill, 1993; Schwartz, 1993). The recent identification of a nonreceptor focal adhesion tyrosine kinase (FAK) involved in adhesion-dependent phosphorylation suggests that the FAK kinase may be involved in integrin signaling (Guan and Shalloway, 1992; Schaller *et al.*, 1992).

Based on the importance of growth factors and hormones in maintaining cell viability and the increasing evidence that integrins function in an analogous manner to control both signaling pathways and cellular function, we have investigated the role of the ECM as a cell survival factor. In this study we report that the ECM is required for preventing PCD and that integrin-mediated events are involved.

MATERIALS AND METHODS

Chemicals

Antibody TS2/16 (IgG1) to the integrin β_1 subunit was a gift from Dr. Martin Hemler (Harvard Medical School, Boston, MA), antibody 489 to VCAM-1 (IgG1) was a gift from Dr. John Harlan, and antibody W6/32 to HLA (IgG2a) was a gift from Dr. David Cheresh (The Scripps Research Institute, La Jolla, CA). Fibronectin was prepared from human plasma by affinity chromatography on gelatin-Sepharose (Miekka *et al.*, 1982). Herbimycin A (GIBCO Laboratories, Grand Island, NY) was used at a final concentration of 1 μg/ml. All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless stated otherwise.

Cells

Human umbilical vein endothelial cells (HUVECs) were obtained from Dr. David Loskuoff (The Scripps Research Institute, La Jolla, CA). Cells were subcultured by using phosphate-buffered saline (PBS)/2 mM EDTA and were plated on tissue culture plastic in growth medium consisting of M199 medium (GIBCO Laboratories) supplemented with 20% fetal bovine serum (FBS; GIBCO Laboratories), 23 μg/ml endothelial cell growth supplement (ECGS; Upstate Biotechnology, Lake Placid, NY), 4 mM glutamine (GIBCO Laboratories), and 68 ng/ml heparin (Sigma Chemical). Cells were used between passages 2 and 10. Serum-free medium (SFM) consisted of M199 medium supplemented with 0.1% endotoxin- and protease-free bovine serum albumin (BSA) (Calbiochem, San Diego, CA) and growth media supplement G, containing insulin, selenium, and transferrin (GIBCO Laboratories). Defined medium consisted of SFM with 10 ng/ml basic fibroblast growth factor (bFGF) (Upstate Biotechnology), 90 ng/ml heparin, and 10 μg/ml high-density lipoprotein (Biomedical Technologies, Stoughton, MA). Incubation of cells in defined medium gave essentially the same results as incubation in serum-containing growth medium. For interleukin 1α (IL-1α)-treatment, cells were incubated with 50 ng/ml IL-1α (R & D Systems, Minneapolis, MN) in growth medium for 24 h before the experiment.

Human peritoneal mesothelial cells (LP-9) were a generous gift of Dr. James Rheinwald (Biosurface Technology, Cambridge, MA). Cells were maintained on tissue culture plastic in M199 medium supplemented with 20% FBS, 30 μg/ml ECGS, and 0.4 μg/ml hydrocortisone (Sigma Chemical) and were detached with PBS/2 mM EDTA. Human ureteral epithelial cells were a gift of Dr. Ada Elgavish (University of Alabama at Birmingham School of Medicine, Birmingham, AL). Cells were detached with PBS/2 mM EDTA. The human gut epithelial cell line Caco-2 was a gift of Dr. Martin Kagnoff (University of California at San Diego, San Diego, CA). Cells were maintained on tissue culture plastic in Dulbecco's modified Eagles medium (GIBCO Laboratories) with 10% FBS and were detached by trypsinization. Human cruciate ligament fibroblasts were a gift from Dr. Virgil Woods (University of California at San Diego, San Diego, CA) and were grown in Dulbecco's modified Eagle's medium with 10% FBS.

Suspension and Coated Dishes

Suspension culture dishes were made by coating tissue culture plastic with heat-denatured 10 mg/ml BSA in PBS for 5 min, followed by 5 ml (per 10-cm dish) of melted 2% agarose (BioRad Laboratories, Richmond, CA) in M199. The agarose completely prevented cells from attaching to the dish. Cells in serum-free medium were sometimes incubated in dishes blocked with denatured BSA without agarose, which also blocked cell attachment. No differences were observed between the two types of suspension dish.

Fibronectin (FN)-coated dishes were made by incubating bacteriological plastic dishes with FN (50 μg/ml in PBS) for 1–2 h. Dishes were then rinsed and blocked with denatured BSA. For antibodies, the dishes were first coated with 50 μg/ml goat anti-mouse IgG (Sigma Chemical) in PBS for 1–2 h, rinsed, blocked with denatured BSA, and then incubated with the specific antibody. Anti-β_1 (TS2/16) and anti-HLA (W6/32) were used at 1:150 dilutions of ascites in PBS. Anti-VCAM-1 (489) was used at 20 μg/ml in PBS.

Nuclear Fragmentation and DNA Degradation

Nuclear fragmentation was detected by acridine orange staining. Cells were stained with 10 μg/ml acridine orange (Sigma Chemical) in PBS for 1–2 min. Cells were then viewed using the 10× and 40× dry objectives of a diaphot microscope (Nikon, Garden City, NY) with a xenon arc light source. A total of 200 cells were scored, and the fraction with fragmented nuclei was calculated.

DNA degradation was detected by gel electrophoresis essentially as described (Wyllie, 1980). Cells were pelleted at 400 × *g* and washed twice with ice cold TBS (137 mM NaCl, 2.7 mM KCl, and 25 mM tris(hydroxymethyl) aminomethane [Tris], pH 7.4). Pellets were resuspended in 50 μl TE (1 mM EDTA and 10 mM Tris, pH 8.0) and lysed with 0.5 ml of extraction buffer (10 mM Tris, pH 8, 0.1 M EDTA, and 0.5% SDS) with 0.5 mg/ml proteinase K. Samples were incubated overnight at 50°C. Samples were then extracted twice with phenol: chloroform:isoamyl alcohol (25:24:1) and then precipitated with 0.2 M NaCl and 2 volumes of ethanol. DNA was recovered by centrifugation, and pellets were washed with 70% ethanol, air dried, and resuspended in 25 μl TE with 20 μg/ml RNAse A. Samples were then incubated at 37°C for 1 h. DNA was again extracted, precipitated, and washed as above, and then resuspended in 20 μl TE. Finally, DNA samples were separated on 1.2% agarose gels with 0.25 μg/ml ethidium bromide, visualized by UV fluorescence and photographed.

DNA degradation was quantitated by extraction with nonionic detergent essentially as described (Wyllie, 1980). Approximately 1–5 × 10^6 cells were harvested by centrifugation at 400 × *g* and washed twice with ice cold TBS. Cells were then lysed in 0.5 ml extraction

buffer (10 mM Tris, pH 8, 20 mM EDTA, 0.5% Triton X-100) for 30 min on ice. Lysates were spun at 14000 × *g* for 15 min to separate the intact chromatin (pellet) from the degraded DNA (supernatant). After centrifugation, the supernatants were removed and saved, and the pellets were resuspended in 0.5 ml extraction buffer. The amount of DNA in the pellet and supernatant fractions was determined by the diphenylamine reaction, as described (Burton, 1956). The extent of DNA degradation was then expressed as the percentage of total DNA found in the supernatant.

Protein Cross-linking

Cells were rinsed once in cold PBS, extracted with 10 mM Tris, pH 7, 2% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and leupeptin, and then heated to 90°C for 5 min. Protein concentration was determined by the method of Lowry using BSA as a standard. Tris (100 mM, pH 6.8), 10% glycerol, and 20 mM dithiothreitol (DTT) were added, and the samples were again heated to 90°C for 5 min. Samples normalized for protein concentration were run on a 7% SDS polyacrylamide gel and stained with Coomassie brilliant blue.

Northern Blot Analysis

Total RNA was prepared from HUVECs using RNAzol B (Biotecx Laboratories, Houston, TX). Total RNA, 15 μg per sample, was denatured with formaldehyde and blotted onto nylon membranes (Amersham, Arlington Heights, IL) according to Sambrook *et al.* (1989). Duplicate samples were stained with ethidium bromide to determine RNA integrity. Filters were hybridized using [α-^{32}P]dCTP random primed probes for the testosterone-repressed prostate message 2 (TRPM-2) (Bandyk *et al.*, 1990) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort *et al.*, 1985). TRPM-2 was a generous gift of Dr. Ralph Buttyan (Columbia University, New York) and GAPDH was a gift of Dr. Ron Bowditch (The Scripps Research Institute, La Jolla, CA).

Anti-Phosphotyrosine Immunoblots

Cells were rinsed once in cold PBS and extracted with 10 mM Tris, pH 7, 2% SDS, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 μg/ml aprotinin and leupeptin. Samples were immediately heated to 90°C for 5 min. Protein concentrations were determined by the method of Lowry using BSA as a standard. Tris (100 mM, pH 6.8) 10% glycerol, and 20 mM DTT were added, and the samples again were heated to 90°C for 5 min. Samples normalized for protein concentration were run on a 6% SDS polyacrylamide gel and electrophoretically transferred to nitrocellulose paper using the BioRad mini transblot system. Blots were blocked with 10% goat serum in pH 7.0 Tris-buffered saline with 0.5% Tween 20, incubated for 4–6 h with 1 μg/ml PY20 anti-phosphotyrosine antibody (Zymed, South San Francisco, CA), rinsed, and incubated with a 1:4000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Calbiochem). Blots were again rinsed and visualized using chemiluminescence (ECL kit, Amersham) according to the manufacturer's instructions. Note that the HRP-conjugated secondary antibody also nonspecifically recognized the prestained high molecular weight standards (GIBCO Laboratories). We found, however, that binding of the secondary antibody to phosphotyrosine-containing proteins was highly specific as it was completely dependent on PY20 and was blocked by addition of soluble phosphotyrosine.

RESULTS

Endothelial Cells in Suspension Undergo Programmed Cell Death

We have investigated the effect on viability of incubating HUVECs in suspension. We have employed PCD of HUVECs during serum starvation (Araki *et al.*, 1990) as a control for PCD and as a convenient system for comparison. When cell monolayers were incubated in serum-free medium for 18 h, 20–30% of the cells detached and exhibited the typical apoptotic morphology described by Wyllie *et al.* (1980), specifically, cell shrinkage and membrane blebbing (Figure 1A). PCD was blocked in these cells by incubation in serum-free medium containing bFGF, consistent with previous studies (Araki *et al.*, 1990). When cells were incubated

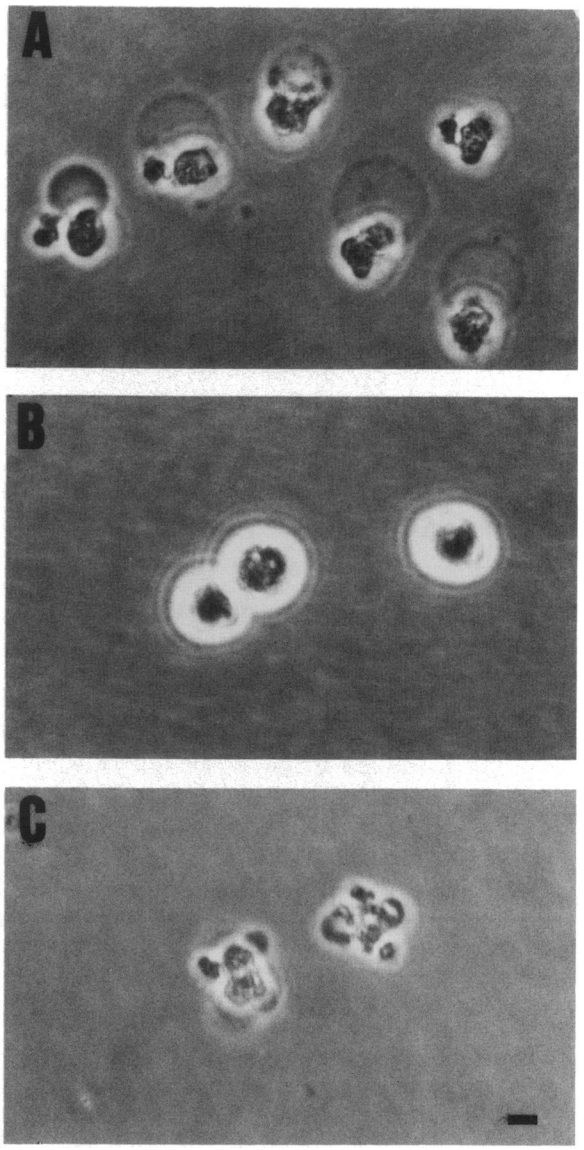

Figure 1. Morphology of HUVECs undergoing programmed cell death. (A) Adherent cells were incubated in serum-free medium for 18 h to induce PCD and the floating cells were collected and photographed. (B) Cells incubated in suspension in defined medium for 30 min. (C) Cells incubated in suspension in defined medium for 18 h. Cells were photographed using phase contrast optics. Magnification, ×400; bar, 10 μm.

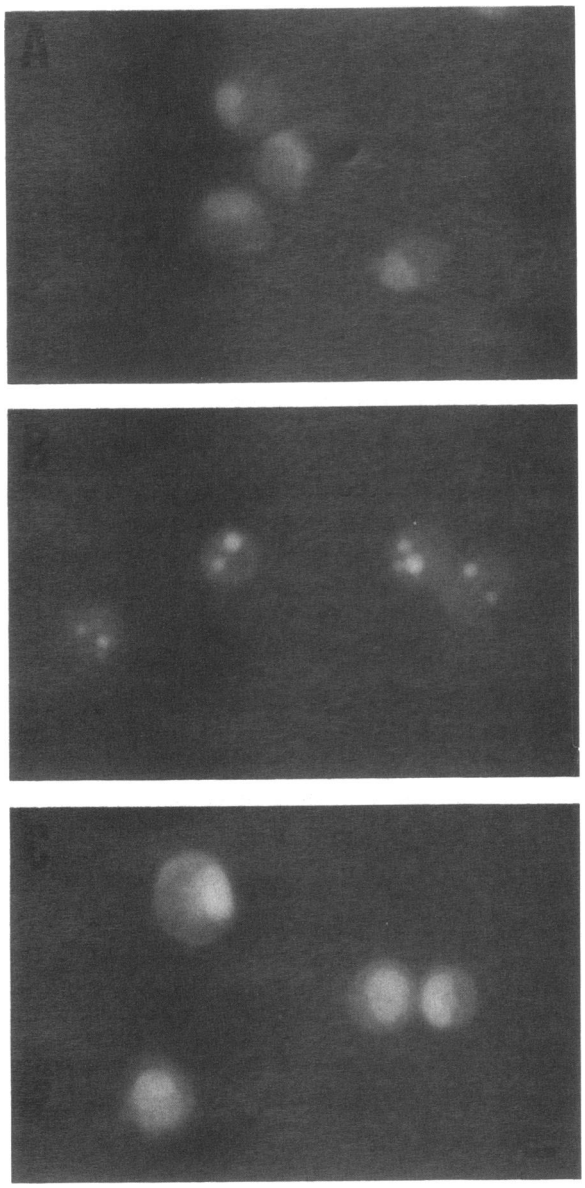

Figure 2. Nuclear fragmentation. Cells were detached and incubated in defined medium in suspension for (A) 30 min, (B) 18 h, and (C) 18 h with 100 μM sodium orthovanadate. Cell nuclei were stained with the cell permeable DNA-binding dye acridine orange and viewed using fluorescence microscopy. Magnification, ×400; bar, 10 μm.

in suspension for 18 h, they exhibited the same shrinkage and membrane blebbing as serum-starved cells, consistent with PCD (Figure 1C). Approximately 20–30% of the cells looked apoptotic after 18 h, and nearly all cells appeared apoptotic after 24–30 h.

We next asked whether the cells in suspension exhibited any of the other features common to apoptosis. One such feature is nuclear fragmentation (Wyllie *et al.*, 1980), which can be visualized with the cell permeable, DNA-binding dye acridine orange. As shown in Figure 2A, cells in suspension for 30 min contained intact nuclei. This nuclear staining was identical to that in attached cells. Cells in suspension for 18 h, however, showed extensive nuclear fragmentation (Figure 2B). In many cases these fragments were found associated with apoptotic bodies, the small membrane-bound vesicles released by apoptotic cells (Wyllie *et al.*, 1980). Similar patterns of nuclear fragmentation were also detected in cells undergoing PCD during serum starvation. Neither fragmented nuclei nor the apoptotic morphology was observe in cells killed by exposure to parachloromercuribenzoic acid (PCMB), a common model for necrotic cell death (Sahaphong and Trump, 1971).

Many investigators have reported that endogenous deoxyribonucleases are activated in cells undergoing PCD (Compton, 1992). To assay for degradation, DNA was analyzed by agarose gel electrophoresis. As shown in Figure 3, DNA isolated from attached cells in growth medium migrated as a single, high molecular weight band (lane 1, Figure 3). In contrast, DNA isolated from cells in suspension for 25 h exhibited considerable degradation (lane 4, Figure 3). An identical pattern of degradation was detected in cells incubated in serum-free medium for 25 h (lanes 2 and 3, Figure 3). In many reported cases of PCD, DNA is degraded to form 200-bp ladders (e.g., Wyllie, 1980). We did not consistently observe a DNA ladder from either the cells in suspension or the control cells under serum starvation, although in most experiments faint banding was detectable. The absence of a clearly defined banding pattern may be due to nonspecific DNA degradation. DNA degradation was, however, always detected in both the cells in suspension and in the cells under serum starvation, and the DNA pattern was identical between the two.

Another commonly used assay for measuring DNA degradation is detergent extraction (Wyllie, 1980). In attached cells in growth medium, a low percentage of DNA was extractable (Figure 4). This degradation could potentially be due to normal cell turnover, because we have observed small numbers of floating, apoptotic cells in well-fed, confluent monolayers. In contrast, DNA extraction measured in cells in suspension for 18 h was

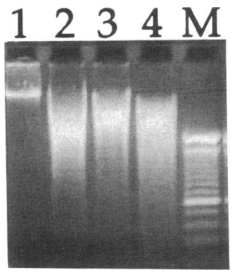

Figure 3. DNA degradation. Cells were lysed and cellular DNA isolated and subjected to gel electrophoresis on a 1.2% agarose gel. Lane 1, DNA isolated from adherent cells in growth medium. Lane 2, DNA isolated from adherent cells starved for 25 h in serum-free medium. Lane 3, DNA isolated from cells in suspension for 25 h in serum-free medium. Lane 4, DNA isolated from cells in suspension for 25 h in growth medium. A 100-bp DNA ladder is shown in lane M. The gel contained 0.25 μg/ml ethidium bromide and was photographed using UV fluorescence. Similar results were obtained in four separate experiments.

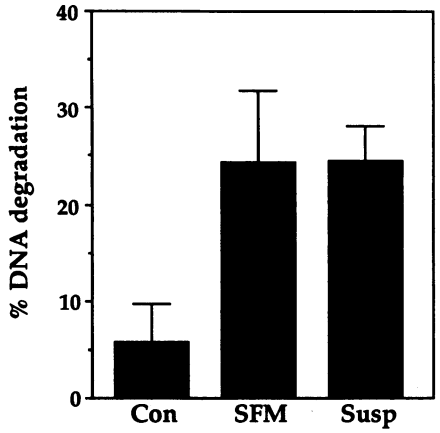

Figure 4. Detergent solubility of degraded DNA. Cells were lysed iyn nonionic detergent and the intact chromatin was separated from soluble DNA fragments by centrifugation. DNA was measured using the diphenylamine reaction and the percent of the DNA found in the supernatant calculated. Con, adherent cells in defined medium; SFM, adherent cells starved for 18 h in serum-free medium; Susp, cells in suspension in defined medium for 18 h. Bars represent the average of six experiments (±SD).

~fourfold higher (Figure 4). A similar level of degradation was measured in cells incubated in serum-free medium for 18 h (Figure 4). Note that percent DNA degradation as determined by this assay is not equivalent to cell death, because a significant fraction of the total DNA was resistant to extraction even in cells that were 100% apoptotic by morphology. The levels of DNA degradation were, however, highly reproducible and so enabled us to compare relative levels of PCD in cells under various conditions.

In addition to changes in cell morphology and DNA integrity, apoptotic cells are also subject to extensive protein cross-linking due to the activation of intracellular transglutaminases (Fesus *et al.*, 1987). Transglutaminases are Ca^{2+}-dependent enzymes that catalyze the formation of glutamyl-lysine bonds. To determine the presence of protein cross-linking in our system, cell extracts were subjected to SDS-polyacrilymide gel electrophoresis (PAGE). We rationalized that protein cross-linking activity should be detectable as the presence of very high molecular weight protein complexes. As shown in Figure 5, very high molecular weight protein complexes (arrow) that were unable to penetrate the stacking gel were detected in both the serum-starved cells (lane 1) and the cells in suspension for 18 h (lane 3). These high molecular weight complexes were not detected in attached cells in growth medium (lane 2) or from cells in suspension incubated in the presence of the Ca^{2+} chelator EGTA (lane 4). Incubation with 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid EGTA) had no effect on morphological apoptosis or DNA fragmentation of these cells, suggesting that the EGTA was inhibiting a Ca^{2+}-dependent transglutaminase.

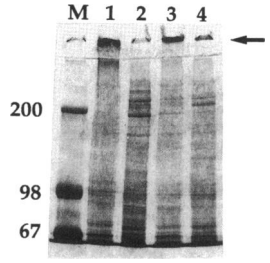

Figure 5. Protein cross-linking. Cell extracts were subjected to SDS-PAGE in the presence of DTT and proteins stained with Coomassie blue. High molecular weight protein complexes that did not enter the stacking gel are indicated by the arrow. Lane 1, adherent cells starved for 18 h in serum-free medium. Lane 2, adherent cells in defined medium. Lane 3, cells in suspension for 18 h in defined medium. Lane 4, cells in suspension for 18 h in defined medium in the presence of 5 mM EGTA. Lane M contains molecular weight markers. Similar results were obtained in three separate experiments.

Finally, we examined the expression of the apoptosis-specific gene TRPM-2. TRPM-2 (also known as SGP-2 and clusterin) was first identified in regressing rat ventral prostate tissue during hormone withdrawal–induced apoptosis (Leger *et al.*, 1987; Bettuzzi *et al.*, 1989). Subsequently, TRPM-2 protein and mRNA expression were shown to be induced in other models of PCD (Buttyan *et al.*, 1989; Jenne and Tschopp, 1992). Northern blot analysis was carried out on total RNA isolated from attached cells in serum-free medium for 23 h and cells in suspension for 0, 7, 12, and 23 h. As shown in Figure 6, low levels of TRPM-2 mRNA were present initially (lane 2). Expression of TRPM-2 increased after 7, 12, and 23 h of incubation in suspension (lanes 3–5, Figure 6). The highest level of TRPM-2 expression was detected in cells in suspension for 23 h (lane 5, Figure 6) and was similar to the level of expression observed in cells serum starved for 23 h (lane 1, Figure 6).

A Role for Integrins

We next investigated whether integrins were involved in regulating this process. To analyze the involvement of integrins, cells were plated onto bacterial plastic coated with various antibodies, including the integrin β_1 chain–specific monoclonal antibody TS 2/16. To assess the extent of PCD, cells were stained with acridine orange and scored for fragmented nuclei.

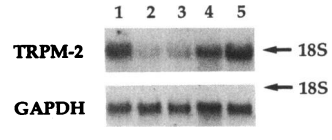

Figure 6. Expression of TRPM-2. RNA aliquots (15 μg) were separated by denaturing agarose gel electrophoresis, transferred to nylon membranes, and hybridized to a ^{32}P-labeled probe for the apoptosis specific gene testosterone-repressed prostate message 2 (TRPM-2). Lane 1, RNA isolated from attached cells serum starved for 23 h. Lanes 2–5, RNA isolated from cells in suspension in growth medium for 0, 7, 12, and 23 h, respectively. Blots were stripped and rehybridized to a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to determine RNA integrity. Arrows indicate the position of the 18S rRNA. Similar results were obtained in four separate experiments.

As shown in Figure 7, the level of PCD for cells in suspension for 18 h was ~17-fold higher than for cells on fibronectin (Figure 7A). Cells plated on the integrin β_1–specific antibody also exhibited low levels of PCD, only slightly higher than for cells on fibronectin (Figure 7). Cells also adhered to plastic coated with monoclonal antibodies to either the class I histocompatability antigen HLA, or to VCAM-1. In both cases, however, the level of PCD was not significantly inhibited relative to cells in suspension.

Role of Tyrosine Phosphorylation

Because cell attachment and spreading on fibronectin or anti-integrin IgG have been shown to induce tyrosine phosphorylation of a complex of proteins in fibroblasts (Guan *et al.*, 1991), we speculated that these integrin-mediated phosphorylation events might regulate PCD. To address this question, cells in suspension were incubated in the presence or absence of the tyrosine phosphatase inhibitor sodium orthovanadate.

We found that 100 μM orthovanadate completely inhibited DNA degradation, as measured by detergent extraction (Figure 8). This suppression of PCD by vanadate appeared to be dependent on tyrosine phosphorylation, because the addition of the tyrosine kinase inhibitor herbimycin A completely reversed the effect (Figure 8). Orthovanadate also inhibited nuclear fragmentation (Figure 2C). In addition, we found that the vanadate-treated cells showed none of the morphological signs of apoptosis and were viable when replated.

To verify that the vanadate was inhibiting tyrosine phosphatase activity, cell extracts were Western blotted and probed with an antibody to phosphotyrosine. The film in Figure 9 was overexposed to allow detection of minor bands. As shown in Figure 9, cells plated on FN had numerous phosphotyrosine-containing proteins, including a major band of ~120 kDa (arrow). These phosphotyrosine-containing bands were absent in cells incubated in suspension (Figure 9). This observation is consistent with published reports indicating that cell spreading or clustering of β_1 integrins with antibodies induces tyrosine phosphorylation of predominately 120- to 125-kDa proteins (Guan *et al.*, 1991; Kornberg *et al.*, 1991; Burridge *et al.*, 1992).

Treatment of cells in suspension with 50 or 100 μM orthovanadate also increased tyrosine phosphorylation of a number of bands (Figure 9). The pattern of bands induced by adhesion and orthovanadate were distinct but overlapping. For example, a predominate band of ~130 kDa (arrowhead) was induced by vanadate but was absent in the cells spread on FN, while the major 120-kDa band in the cells spread on FN was not increased in the vanadate treated cells (arrow). Several bands at 145–170 and 200–210 kDa (bracket), however, were induced by both FN and vanadate. The identity of these proteins is at present unknown. Note that 50 μM orthovanadate inhibited PCD about one-half as well as 100 μM, suggesting a correspondence between tyrosine phosphorylation and PCD.

Other Cell Types

To determine if the ECM functions as a survival factor for other cell types, we assayed four other primary cell

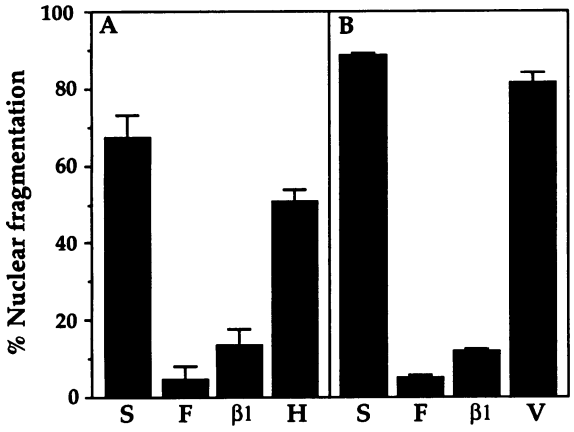

Figure 7. Nuclear fragmentation in HUVECs plated on antibodies. HUVECSs were plated in defined medium on bacterial plastic coated with either BSA (S), 50 μg/ml fibronectin (F), monoclonal anti-β_1 integrin IgG (β_1), monoclonal anti-HLA IgG (H), or monoclonal anti-VCAM-1 IgG (V). Cells were allowed to attach for 2–3 h before plates were washed to remove unattached cells. Cells were incubated for 18 h before staining cell nuclei with acridine orange and scoring by fluorescence microscopy. At least 200 nuclei were scored per experiment. Cells in A were in defined medium. The cells in B were pretreated with 50 ng/ml IL-1α for 24 h before the experiment and subsequently incubated in defined medium containing 50 ng/ml IL-1α to induce VCAM-1 expression. Bars represent the average of at least two experiments (±SD).

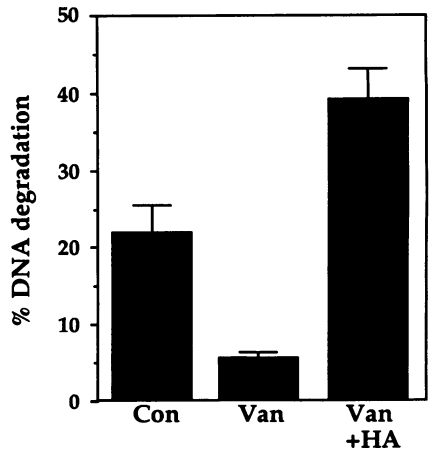

Figure 8. Effect of sodium orthovanadate on cell death. Cells were incubated in suspension in defined medium for 18 h either alone (Con), in the presence of 100 μM sodium orthovanadate (Van), or in the presence of 100 μM sodium orthovanadate plus 1 μg/ml herbimycin A (Van + HA). After 18 h, DNA degradation was analyzed as in Figure 4. Bars represent the average of three experiments (±SD).

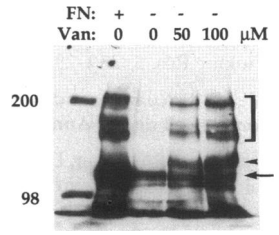

Figure 9. Effect of sodium orthovanadate on tyrosine phosphorylation. Cells in defined medium were either plated on tissue culture plastic coated with 50 μg/ml fibronectin (FN) or were incubated in suspension in the presence of 0, 50, or 100 μM sodium orthovanadate (Van). After 4 h of incubation cells were extracted and proteins were separated by SDS-PAGE and immunoblotted using antiphosphotyrosine antibodies. The arrow indicates the position of the 120-kDa protein band and the arrowhead, the 130-kDa protein band. The bracket indicates the bands common to both the cells plated on FN and the cells treated with vanadate. Similar results were obtained in three separate experiments.

types and one cell line (Caco-2 gut epithelial cells). Cells were incubated in suspension in growth medium for up to 50 h and at various times were scored for apoptosis by morphology and nuclear fragmentation. As summarized in Table 1, we found that the gut epithelial cells appeared to undergo PCD. Ureteral epithelial cells in suspension died, but exhibited an unusual morphology that was distinct from apoptotic HUVECs. Some, but not all, of these cells also contained fragmented nuclei; however, in many cases cells were devoid of any acridine staining material. Identifying the mechanism of cell death in this cell type will therefore require further analysis. Both mesothelial cells and fibroblasts were comparatively resistant to suspension-induced cell death. These cells appeared normal morphologically and contained intact nuclei after 50 h of incubation in suspension.

DISCUSSION

Our results indicate that the ECM is required for endothelial cell survival. We found that cells incubated in suspension were induced to undergo PCD, based on both morphological and biochemical criteria. Cells held in suspension became shrunken, developed extensive membrane blebbing, and contained fragmented nuclei, all characteristic of apoptosis (Wyllie *et al.*, 1980). Cells in suspension also contained degraded DNA and accumulated very high molecular weight protein complexes, both hallmarks of apoptosis (Fesus *et al.*, 1991; Compton, 1992). Finally, we found that placing cells in suspension induced the apoptosis-specific gene TRPM-2. Importantly, we found that cells incubated in suspension were identical both morphologically and biochemically to cells induced to undergo PCD as a result of serum starvation.

What factors regulate this suspension-induced PCD? Our results indicate first that integrin-mediated events are involved. We found that PCD was suppressed when cells were plated on an integrin β_1 monoclonal antibody but not when cells were plated on either HLA or VCAM-1 antibodies. Our results at this point do not allow us to determine to what extent integrin clustering alone or integrin-mediated spreading are required for this effect.

Secondly, our results with vanadate demonstrate that entry of HUVECs into PCD can be regulated by protein tyrosine phosphorylation. We found that PCD was blocked by treating cells in suspension with the tyrosine phosphatase inhibitor sodium orthovanadate, and this effect was reversed by the tyrosine kinase inhibitor herbimycin A. Interestingly, previous studies have shown that cell spreading and integrin clustering induces increased tyrosine phosphorylation of a number of different proteins (Guan *et al.*, 1991; Kornberg *et al.*, 1991; Burridge *et al.*, 1992). The data therefore suggest that integrin-mediated tyrosine phosphorylation may be involved. Proof of this hypothesis, however, must await the development of more specific probes.

Finally, we found that the ECM was required for the survival of some other cell types, such as gut epithelial and possibly ureteral epithelial cells, but not all, because both mesothelial cells and fibroblasts were able to survive in suspension for ≥50 h. In addition, neurons are also dependent on the ECM for survival (Kalcheim *et al.*, 1987; Ernsberger *et al.*, 1989). These results imply that, while the dependence of cell survival on ECM proteins may involve cell type–specific mechanisms, the phenomenon is likely to be widespread. In so far as most cells are dependent on adhesion for growth, these results extend the analogy with growth factors, which also function as survival factors for most cell types.

The concept that cell survival requires appropriate contacts with ECM may be relevant for several events in vivo. For one, it provides a mechanism for highly localized elimination of unneeded or improperly targeted cells during development, irrespective of the hormonal environment. For another, degradation of ECM is known to be an early and critical event in organ regression in several systems. For example, apoptosis during mammary gland involution is dependent on basement membrane degradation (Talhouk *et al.*, 1991, 1992). Our results suggest that matrix degradation could be an important signal for the PCD that occurs in the mammary gland and in other instances of organ regression.

Table 1. Suspension-induced apoptosis in other cell types

Cell type[a]	Apoptosis[b]
Gut epithelial	Yes
Mesothelial	No
Ureteral epithelial	??
Fibroblast	No

[a] Specific cell types are listed in MATERIALS AND METHODS.

[b] Cells were detached and incubated in suspension for up to 50 h. Apoptotic cell death was determined by cell morphology and nuclear staining with acridine orange.

Finally, our results suggest that invasion and metastasis by tumor cells, processes that involve loss of normal ECM contacts, should require independence from such control mechanisms. The result that vanadate treatment prevents cell death indicates that inappropriate tyrosine phosphorylation can substitute for integrin-dependent signals. This result supports the idea that oncogenes could potentially substitute for integrin-dependent signals, in much the same way that oncogenes can substitute for growth factor–dependent signals (Schwartz, 1993). In summary, adhesion of cells to ECM is required for integrin-mediated production of a signal that prevents entry into a cell suicide program. Further investigation into the mechanism of this process is likely to yield insight into a number of developmental events and possibly tumorigenesis.

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