Disruption of Epithelial Cell–Matrix Interactions Induces Apoptosis

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Abstract. Cell–matrix interactions have major effects upon phenotypic features such as gene regulation, cytoskeletal structure, differentiation, and aspects of cell growth control. Programmed cell death (apoptosis) is crucial for maintaining appropriate cell number and tissue organization. It was therefore of interest to determine whether cell–matrix interactions affect apoptosis. The present report demonstrates that apoptosis was induced by disruption of the interactions between normal epithelial cells and extracellular matrix. We have termed this phenomenon “anoikis.” Overexpression of bcl-2 protected cells against anoikis. Cellular sensitivity to anoikis was apparently regulated: (a) anoikis did not occur in normal fibroblasts; (b) it was abrogated in epithelial cells by transformation with v-Ha-ras, v-src, or treatment with phorbol ester; (c) sensitivity to anoikis was conferred upon HT1080 cells or v-Ha-ras–transformed MDCK cells by reverse-transformation with adenovirus E1a; (d) anoikis in MDCK cells was alleviated by the motility factor, scatter factor. The results suggest that the circumvention of anoikis accompanies the acquisition of anchorage independence or cell motility.

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Materials and Methods

Anoikis Assay

Cell lines were grown to confluence in 100-mm tissue culture dishes, unless otherwise indicated. Trypsinized cells (3 x 10^6) were then counted and plated onto 100-mm petri dishes which had been coated with polyHEMA (Roisman and Moscona, 1976) (polyHEMA plates were made by applying 4 ml of a 10 mg/ml solution of polyhydroxyethylmethacrylate [Aldrich Chemical Co., Milwaukee, WI] in ethanol onto the dish, drying in the tissue culture hood and repeating once, followed by extensive PBS washes). After 12 h (MDCK), 20 h (HaCat), or indicated times, cells were collected from polyHEMA dishes by pipetting or from tissue culture dishes by scraping cells into the medium in which they had been incubated. (In all tissue culture dish controls, floating cells were combined with the attached cells before DNA extraction, to determine spontaneous apoptosis.) Low molecular weight genomic DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4 (0.6 ml), phenol-chloroform extracted three times, ethanol precipitated and analyzed on a 1.5% agarose gel in TBE buffer. For the experiment in Fig. 1, MDCK cells were plated on tissue culture dishes containing 1 mg/ml of the peptide GRGDSP (Ruoslahti and Pierschbacher, 1986) (RGD) or the peptide GRGESP (CON) (JLCRF protein chemistry facility). Cells were harvested and processed after 8 h as above.

Antibodies and Western Blotting

Antibodies were obtained from the following sources: anti-lamin antibody 818A (Glass and Gerace, 1990), provided by L. Gerace, (scripps Research Institute, La Jolla, CA); anti-bcl2 9716 (Reed et al., 1991), provided by J. Reed (La Jolla Cancer Research Foundation); anti-Ela M73 (Harlow et al., 1985) (Oncogene Science Inc., Manhasset, NY), anti-Elb 517 (White et al., 1988) provided by E. White (Rutgers University, New Brunswick, NJ). Except for nuclear lamin analysis, protein extracts were made by lysing directly in SDS sample buffer. Soluble lamin was obtained by clearing 0.5% Triton X-100 cell extracts for 10 min in a microfuge. Proteins were electroblotted on Immobilon (Millipore Corp., Bedford, MA). After reaction with secondary antibody blots were developed using ECL reagent (Amersham Corp., Arlington Heights, IL).

Flow Cytometry

Flow cytometric analysis of DNA degradation in response to disruption of cell-matrix interactions. MDCK or HaCat cells (2 x 10^6) were incubated on tissue culture or on polyHEMA-coated 100-mm dishes for 20 h, collected and fixed with 70% ethanol. After staining with 10 μg/ml propidium iodide, cells were analyzed on a Becton Dickinson FACS (Scripps Institute).

Retroviruses

A retrovirus bearing the wild-type 243 aa EIA gene was constructed as follows. The BstXI partial-PstI fragment from the plasmid 252wt (Mornan et al., 1986) (adenovirus map positions 510-1835) was religated with a synthetic oligonucleotide containing adenosine map positions 555-610 (with a synthetic 5' HindIH end) and Bluescript which had been incubated with Klenow enzyme and subcloned into the Stu site of the retrovirus vector pNSX (Miller and Rosman, 1989). The packaging cell line genomic DNA was then transfected with this plasmid, and the resulting virus stock used to infect the amphotropic packaging cell line pGEM12. G418 (500 μg/ml)-resistant producer cell lines were screened by Western blotting for ability to confer EIA expression on infected HT1080 cells. The amphotropic v-src retroviruses MOneoMT211 v-src (Warren and Nelson, 1987) and the bcl-2 retrovirus, ST44-1 (Hanada et al., 1993) were provided by S. Warren (Yale University, New Haven, CT) and J. Reed, respectively.

Hoechst Staining

MDCK or HaCat cells (2 x 10^6) were incubated on tissue culture or on polyHEMA-coated 100-mm dishes for 20 h, collected, fixed on glass coverslips with methanol acetic acid (3:1), stained with 2.5 μg/ml of Hoechst 23388 and photographed on a Zeiss Axiovert microscope (Carl Zeiss Inc., Thornwood, NY).

Cell Lines

MDCK cells (Taub and Saier, 1979) were from ATCC. The M8 subclone of MDCK cells (Nelson and Vensnick, 1987; provided by W. J. Nelson, Stanford University, Stanford, CA) was used for the experiments with scatter factor, as it was found to be more morphologically responsive than MDCK from ATCC. HaCat cells (Ryle et al., 1989) were from R. Fusenig (German Cancer Research Center, Heidelberg, Germany) via A. Pentland (Washington University, St. Louis, MO); the bcl-2- Ela-, and v-src-expressing cell lines were constructed by infection with the retroviruses described above. Infections were done for 8 h in medium containing 4 μg/ml of polybrene. After G418 selection (500 μg/ml, 2-3 wk), cell clones stably expressing the gene were identified by Western blotting of SDS sample buffer lysates. The Elb-expressing derivative of Ela/H4 was made by cotransfection of the plasmid pCMVElb (White and Cipriani, 1990; provided by E. White, Rutgers University), with pSV40hyg (Frisch, S. M., unpublished data), selection in 250 μg/ml hygromycin and screening of clones by Western blotting using the antibody 317. Ras-transformed MDCK cells (Scollin et al., 1976) were obtained from P. Insel (University of California, San Diego, CA).

Scatter Factor

Purified, bacterially expressed human scatter factor was generously provided by G. Van de Woude (National Cancer Institute, Frederick, MD). (Bottaro et al., 1991), and used at 25 ng/ml final concentration in complete growth medium. Cells were pretreated for 36 h with scatter factor, at which time extensive scattering and appearance of fibroblastoid cell morphologies were seen.

Results

Disruption of Normal Epithelial Cell-Matrix Interactions Results in Apoptotic Cell Death

Madin-Darby canine kidney epithelial cells (MDCK) or virus. A HindIII–HpaI fragment (555-1575) from this plasmid, (which excluded 3' polyadenylation sequences from Ela) was then made blunt-ended with Klenow enzyme and subcloned into the Stu site of the retrovirus vector pNSX (Miller and Rosman, 1989). The packaging cell line genomic DNA was then transfected with this plasmid, and the resulting virus stock used to infect the amphotropic packaging cell line pGEM12. G418 (500 μg/ml)-resistant producer cell lines were screened by Western blotting for ability to confer EIA expression on infected HT1080 cells. The amphotropic v-src retroviruses MOneoMT211 v-src (Warren and Nelson, 1987) and the bcl-2 retrovirus, ST44-1 (Hanada et al., 1993) were provided by S. Warren (Yale University, New Haven, CT) and J. Reed, respectively.

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Madin-Darby canine kidney epithelial cells (MDCK) or
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HaCat cells (spontaneously immortalized but nontransformed human keratinocytes) were plated on tissue culture plastic or on petri dishes that had been coated with polyhydroxyethylmethacrylate (polyHEMA). polyHEMA has previously been used to prevent cell attachment because its uniformly nonionic nature prevents matrix deposition (Folkman and Moscona, 1978). Agarose gel analysis of low molecular weight DNA (Fig. 1 a) demonstrated a ladder of ~200-bp multiple DNA fragments occurring in suspended but not attached cells. This ladder has been shown to reflect internucleosomal DNA degradation, and is the currently accepted hallmark of apoptosis (Wyllie, 1980). The DNA ladder from ~3 × 10^6 cells was detectable within 7.5 h for MDCK or within 8.5 h for HaCat, and increased with incubation time on polyHEMA. Flow-cytometric analysis of propidium iodide-stained cells (Fig. 1 b) indicated that ~20% (MDCK) or ~60% (HaCat) of the cells contained <2n DNA content after 18 h on polyHEMA. (These numbers could in principle underestimate the fraction of apoptotic cells because mildly degraded DNA could score positively in the flow cytometer, resulting in apparent 2n cells.)

To confirm that the extracellular signal for apoptosis was the lack of cell–matrix interactions, fibronectin and vitronectin receptors of MDCK cells on tissue culture plastic were functionally blocked by the addition of the peptide GRGDSP (Ruoslahti and Pierschbacher, 1986). This peptide-induced apoptosis that was detectable in 8 h (Fig. 1 a) whereas a control peptide that does not bind integrins (GRGESP) had no effect. We propose the term “anoikis” (an-o-EE-kis; Greek, meaning the state of being without a home) to describe the cells’ apoptotic response to the absence of cell–matrix interactions.

Nuclear fragmentation, another diagnostic feature of apoptosis (Wyllie, 1980), was also observed in cells incubated on polyHEMA (Fig. 1 c). Nuclear lamins have been reported to convert to a soluble form during apoptosis (Ucker et al., 1992) or to partially degrade into a 45-kD product (Kaufmann, 1989), representing the α-helical domain common to lamins A, B, and C. Although increased soluble lamin level was not observed in suspended MDCK and HaCat cells (data not shown), the appearance of the 45-kD lamin degradation product was seen (Fig. 1 d), indicating some breakdown of the nuclear envelope. Generalized protein degradation was not observed on stained Western blot filters (data not shown).

Nonadherence has been shown to block DNA replication (Folkman and Moscona, 1978). To determine whether this inhibition sufficed to induce apoptosis, adherent MDCK cells were exposed to aphidicolin and analyzed for DNA degradation. No degradation was observed (Fig. 1 e) even when DNA replication was inhibited by >95% (data not shown). Inhibition of DNA replication appeared to be the inducer of anoikis; it also did not protect cells against anoikis.

Certain forms of apoptosis are alleviated by inhibition of protein synthesis with drugs such as cycloheximide (Martin et al., 1988), implicating de novo expression of gene product(s) involved in cell suicide. Cycloheximide did not protect MDCK cells against DNA degradation in response to matrix detachment (data not shown). Several other forms of apoptosis are resistant to cycloheximide (for example see Cotter et al., 1992) predicting the existence of both gene regulation-dependent and posttranslational pathways.

Figure 2. Effect of overexpression of bcl-2 on anoikis. Cell clones overexpressing bcl-2 were derived from MDCK or HaCat by infection with the bcl-2 retrovirus and screening with anti-bcl-2 antibodies (results shown in the lower panel). Two clones of each were assayed for anoikis as described in Materials and Methods.

Despite the apparent lack of requirement for new protein synthesis, detachment did not commit the cells to apoptosis instantaneously. MDCK cells were incubated on polyHEMA dishes for various lengths of time followed by reattachment onto tissue culture dishes. The earliest detectable clear DNA ladders were visible at 5 h on polyHEMA (Fig. 1 f). This indicated that commitment to apoptosis required a rate-limiting process, such as posttranslational modification of a regulatory protein, that was complete within 3–5 h after detachment.

The bcl-2 gene has been reported to confer resistance to apoptosis in certain systems (reviewed in Reed, 1993). MDCK or HaCat cells were infected with a bcl-2 retrovirus and bcl-2-overexpressing clones were selected. bcl-2 overexpression conferred partial or complete resistance to anoikis (Fig. 2). This supported the concept that DNA degradation occurring in cells detached from matrix resulted from apoptosis.

Transformation Confers Resistance to Anoikis

Transformation frequently confers anchorage-independent growth potential (Stoker et al., 1968). The circumvention of anoikis would presumably be a prerequisite for anchorage independence. To test this hypothesis, we transformed MDCK cells to an anchorage-independent state with the v-src oncogene and assayed for anoikis induction; v-Ha-ras–transformed MDCK cells (Scolnick et al., 1968) were
also tested. Transformed clones were less susceptible to anoikis than parental mdck cells (Fig. 3A). This demonstrates that loss of susceptibility to anoikis can accompany the acquisition of anchorage independence.

Reverse-Transformation Confers Sensitivity to Anoikis

In principle, one of the mechanisms of reverse-transformation by a tumor suppressor gene might be to predispose cells to anoikis. The Ela gene of adenovirus-5 has previously been shown to reverse the transformed phenotype of HT1080 and other tumor cells to an anchorage-dependent state (Frisch, 1991). A homogeneous subclone of HT1080 cells (H4) was infected with a recombinant retrovirus encoding the 243-amino acid form of Ela. Ela-expressing clones were, as previously reported for transfection of the complete Ela gene (Frisch, 1991), anchorage dependent for growth (by soft agar colony formation; data not shown). Ela-mediated reverse-transformation predisposed HT1080 cells to anoikis (Fig. 3B). In contrast to other cell systems reported (Debbas and White, 1993; Lowe and Ruley, 1993), Ela did not significantly induce apoptosis under normal, matrix-attached conditions (see attached-cell control lanes, Fig. 3B).

To examine the effects of Ela in the MDCK system, the v-Ha-ras-transformed cell line was infected with Ela retroviruses and Ela-expressing clones were selected. The resulting fully reverse-transformed phenotype of these clones will be documented elsewhere (Frisch, S. M., manuscript in preparation). Ela expression restored the sensitivity of transformed MDCK cells to anoikis (Fig. 3A), although in this cell system some spontaneous apoptosis was stimulated as well.

As with the MDCK or HaCat cells, anoikis could be alleviated by overexpression of the bcl-2 protein in cells reverse-transformed with Ela (Fig. 3C). Expression of the adenovirus Elb gene has previously been reported to protect certain cells against certain apoptotic stimuli (Debbas and White, 1993; White et al., 1992). In the case of HT1080 cells reverse-transformed by Ela, Elb expression also conferred resistance to anoikis (Fig. 3C).

Cell–Cell Interactions Program Cellular Sensitivity to Anoikis

As described above, transformation to an anchorage-independent phenotype conferred resistance to anoikis. We rea-
Cells were then assayed for anoikis, in the continued presence or absence of scatter factor. (d) Effect of disruption of cell-matrix interactions on apoptosis in fibroblasts. 3.7 x 10^6 lung fibroblasts (IMR90) or MDCK cells were plated on polyHEMA dishes; genomic DNA was analyzed as above after 12 or 24 h.

Sonned that cell migration might also require the circumvention of anoikis. Because cell migration and transformation have in common the breakdown of intercellular adhesion (for review see Behrens et al., 1991, 1992), we reasoned that this breakdown might confer resistance to anoikis.

To determine whether interactions between cells served to modulate their sensitivity to anoikis, MDCK cells plated at either low or high density were trypsinized and replated onto polyHEMA plates (Fig. 4a). The sparse cells were resistant to anoikis. This suggested that sensitivity to anoikis was programmed by interactions among MDCK cells.

To further explore this effect, intercellular adhesions were disrupted in two other ways. First, confluent MDCK cultures were pretreated with the phorbol ester TPA, which has been previously shown to cause the disappearance of adherens junctions and tight junctions in MDCK cells (Ojakian, 1981). Second, cells were pretreated with scatter factor, which is a motility factor (Stoker et al., 1987) that also causes the breakdown of epithelial intercellular adhesions (Schmidt et al., 1993; Behrens et al., 1991). Both of these treatments conferred resistance to anoikis (Fig. 4b and c).

Finally, these results would predict that cells which naturally lack intercellular adhesions, such as fibroblasts, might be resistant to anoikis. Ladder formation was not detected in DNA from 3.5 x 10^6 fibroblasts (IMR-90) plated on polyHEMA (Fig. 4d). This is consistent with previous reports (for example see Ben-Ze'ev et al., 1980) that growth arrest caused by non-adherence in fibroblasts is reversible upon readherence. It also indicates that anoikis is a cell type-specific property.

**Discussion**

Raff (1992) has proposed that "... just as a cell seems to need signals from other cells in order to proliferate, so it needs signals for other cells in order to survive; in their absence, the cell kills itself by activating an intrinsic suicide program. ..." Survival signals have been identified for certain cell types, for example, adrenocorticotropic hormone for adrenal cells, testosterone for ventral prostate cells, IL-2 for T lymphoblasts, nerve growth factor for sympathetic neurons (for review see Raff, 1992; Tomei and Cope, 1991).

Extracellular matrix regulates many aspects of cell phenotype such as gene expression, differentiation state, and proliferation (Adams and Watt, 1993; Blau and Baltimore, 1991; Ingber, 1993). It is crucial for the development and stabilization of tissue structure. This paper reports that extracellular matrix is also a survival factor for certain epithelial cells. This suggest a new mechanism by which matrix stabilizes tissue structure: for the appropriate cell types, detachment from matrix causes apoptosis. It has long been appreciated that matrix attachment is important for cell proliferation. Proliferation is normally restricted to matrix-attached cells according to classical anchorage dependence (Folkman and Moscona, 1978; Ben-Ze'ev et al., 1980) which is an important way that matrix prevents dysplasia (Stoker et al., 1968). However, under this model, cells that have detached from matrix (e.g., in cut skin) might reattach to inappropriately located matrices, perhaps including the matrix that they would eventually synthesize themselves, and resume growth. Apoptosis in detached cells (anoikis) provides for more stringent control by abrogating this escape mechanism.

Apoptosis plays a particularly important role in the control of cell numbers where cells compete for limiting amounts of the survival factor (Raff, 1992). This occurs, for example, in development of the sympathetic nervous system. Anoikis may operate under such conditions to determine tissue organization. For example, confluent cultures of MDCK cells continuously shed floating, apoptotic cells (data not shown). These cells presumably result from anoikis due to the shortage of accessible matrix for their attachment. The resulting growth/death equilibrium results in a constant number of cells in the monolayer. This state of affairs resembles actual skin: only those keratinocytes interacting with basement membrane in skin are division competent, while matrix-distal keratinocytes are apoptotic (McCall and Cohen, 1991). While this manuscript was under review, an apoptotic response resembling anoikis was reported in another system, capillary endothelial cells (Meredith et al., 1993).
The acquisition of anchorage-independent growth during transformation (Stoker et al., 1968) would presumably require that anoikis is abrogated. Transformation accomplished this in the MDCK system. Elucidation of the mechanisms by which transformation abrogates anoikis may be critical for understanding the basis of anchorage (in)dependence. Clustering or ligand binding of $\beta_1$ integrin has been shown to result in the phosphorylation of a protein kinase known as p125$\text{\textgamma}$; the latter is thus a candidate integrin signal transducer (for review see Juliano and Haskill, 1993). V-src expression has been shown to cause constitutive hyperphosphorylation of this molecule, perhaps playing a role in anchorage-independent growth. The involvement of p125$\text{\textgamma}$ in anoikis is currently being tested; hyperphosphorylation was observed in v-src-transformed MDCK cells (data not shown).

Altered cell–cell interactions could also be important for the effect of transformation on anoikis. Resistance to anoikis was conferred not only by transformation, but also by low cell density, TPA and scatter factor. All of these conditions cause the breakdown of normal epithelial cell–cell interactions (Zuk et al., 1989; Behrens et al., 1991, 1992; Schmidt et al., 1993; Ojakian, 1981). Although TPA and scatter factor may affect cells in other unrelated ways, conferral of resistance by low cell density supports the role for cell–cell interactions. This breakdown and the ensuing resistance to anoikis is presumably important for tissue remodeling events involving conversions of epithelial cells to temporarily or permanently motile states, such as epithelial–mesenchymal transitions. Alterations of cell–cell interactions by oncogenes could contribute to carcinogenesis through the abrogation of anoikis.

Reverse transformation by a tumor suppressor gene could in principle involve the conferral of cellular sensitivity to anoikis. We reported that the Ela gene of adenovirus is a tumor suppressor gene in a wide variety of human tumor cells (Frisch, 1991), as well as in v-ras-transformed MDCK cells (Frisch, S. M., manuscript in preparation). Results reported in this paper show that conferral of cellular sensitivity to anoikis is a possible mechanism for tumor suppression by Ela. The Ela gene has previously been reported to induce apoptosis independently of matrix effects (Debbas and White, 1993; Rao et al., 1992) and to sensitize cells to the apoptosis induced by tumor necrosis factor-$\alpha$ (White et al., 1992). As with the sensitization to anoikis, these effects are also blocked by bcl-2 or Elb. However, the Ela-induced apoptosis effect in rodent fibroblasts is mediated by wild-type 53 and blocked by mutant p53 (Debbas and White, 1993). By contrast, the anoikis effect occurred in Ela-expressing HT1080 cells, which are devoid of wild-type p53 (Anderson, M. J., and E. Stanbridge, manuscript submitted for publication), and expression of mutant p53 did not block anoikis in MDCK cells (data not shown). Anoikis therefore differs from previously reported Ela-induced apoptosis in that it is contingent upon matrix detachment and appears to use a p53-independent apoptosis pathway.

The results identify a new function, regulation of anoikis, for several proteins whose contributions to carcinogenesis have previously been demonstrated. Cellular proteins that complex with Ela, to mediate both transformation (for review see Moran, 1993) and tumor suppression (Frisch, 1991) effects are likely to be key regulators of anoikis. The integrins (Ruoslhati, 1991), which can act as tumor suppressors, (Giancotti and Ruoslhati, 1990) initiated or prevented the anoikis response, depending upon their ligand occupancy. Activated protein kinase C (Colburn, 1980), the scatter factor receptor c-met (Rong et al., 1992) and bcl-2 (Reed et al., 1990) can contribute to transformation; each of these blocked the anoikis response. Proteins involved in intercellular adhesion, such as E-cadherin, are likely to be involved in programming sensitivity to anoikis, which is currently being tested. Cellular adhesion molecules such as E-cadherin are important suppressors of carcinogenesis (Behrens et al., 1991, 1992). Re-examination of these classes of proteins in terms of anoikis regulation, as well as identification of new proteins and elucidation of the signaling pathways involved, could prove important in understanding and controlling cancer.

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