Massive Programmed Cell Death in Intestinal Epithelial Cells Induced by Three-dimensional Growth Conditions: Suppression by Mutant c-H-*ras* Oncogene Expression

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Abstract. Deregulation of molecular pathways controlling cell survival and death, including programmed cell death, are thought to be important factors in tumor formation, disease progression, and response to therapy. Studies devoted to analyzing the role of programmed cell death in cancer have been carried out primarily using conventional monolayer cell culture systems. However the majority of cancers grow as three-dimensional solid tumors. Because gene expression, and possibly function, can be significantly altered under such conditions, we decided to analyze the control and characteristics of cell death using a compatible three-dimensional tissue culture system (multicellular spheroids) and compare the results obtained to those using two-dimensional monolayer cell culture. To do so we selected for study an immortalized, but nontumorigenic line of rat intestinal epithelial cells, called IEC-18, and several tumorigenic variants of IEC-18 obtained by transfection with a mutant (activated) c-H-*ras* oncogene. The rationale for choosing these cell lines was based in part on the fact that intestinal epithelial cells grow in vivo in a monolayer-like manner and form solid tumors only after sustaining certain genetic mutations, including those involving the *ras* gene family. We found that the IEC-18 cells, which grow readily and survive in monolayer cell culture, undergo massive cell death within 48–72 h when cultured as multicellular spheroids on a nonadhesive surface. This process was accompanied by a number of features associated with programmed cell death including chromatin condensation (Hoechst 33258 staining) apoptotic morphology, DNA degradation, and a virtual complete loss of colony forming (clonogenic) ability in the absence of apparent membrane damage as well as accumulation of lipid containing vacuoles in the cytoplasm. Moreover, enforced over-expression of a transfected *bcl*-2 gene could prevent this cell death process from taking place. In marked contrast, three different stably transfected *ras* clones of IEC-18 survived when grown as multicellular spheroids. In addition, an IEC cell line (called clone 25) carrying its mutant transfected *ras* under a glucocorticoid inducible promoter survived in three-dimensional culture only when the cells were exposed to dexamethasone. If exposure to dexamethasone was delayed for as long as 48 h the cells nevertheless survived, whereas the cells became irreversibly committed to programmed cell death (PCD) if exposed to dexamethasone after 72 h. These results suggest that intestinal epithelial cells may be programmed to activate a PCD pathway upon detachment from a physiologic two-dimensional monolayer configuration, and that this process of adhesion regulated programmed cell death (ARPCD) can be substantially suppressed by expression of a mutant *ras* oncogene. As such, mutant *ras* oncogenes may contribute to tumorigenesis not only through their ability to promote cell proliferation, but also as direct, or indirect facilitators of cell survival and three-dimensional growth in certain types of solid tumor such as colon carcinoma.

THERE are several reasons for the current intense interest in the contribution of programmed cell death, or apoptosis, in cancer development and progression (4, 35, 52, 73, 87). The first is the recognition that the spontaneous expansion of tumor cell numbers in growing tumors represents the net outcome of the generation of new cells by proliferation, and the demise of existing cells, much of which can occur through programmed cell death (87). Hence, mechanisms responsible for relative suppres-

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sion of programmed cell death processes in tumor cells could be an effective means of conferring upon them a relative growth/survival advantage. Second, the toxic effects of many anti-cancer agents, including chemicals, radiation and various biologicals, can be mediated to a significant extent by induction of programmed cell death (2, 16, 24, 34, 73, 80). Consequently, genetic or epigenetic alterations in tumor cells that suppress their relative vulnerability to undergoing therapy-induced programmed cell death may be viewed as a generic therapeutic resistance mechanism, which might be therapeutically reversible, (24, 54, 73, 80).

The *bcl*-2 oncogene family in mammalian cells has attracted the greatest degree of attention in terms of regulators of programmed cell death in normal and cancerous cells (73, 80). Some members of this gene family, such as *bcl*-2, *bag*-1 and *bcl*-x_L, act as suppressors of programmed cell death whereas others, such as *bax*, *bcl*-x_s, *bak*, and *bad*, can promote the process (5, 12, 17, 36, 39, 60, 83, 89). Thus, experimental over expression of the *bcl*-2 or the *bcl*-X_L gene products in tumor cell populations can increase their relative resistance to a variety of anti-cancer therapeutic drugs, or other toxic agents (14, 15, 54). Similar to *bcl*-2 overexpression, the presence of mutant p53 tumor suppressor gene (50, 82), or the absence of wild-type p53 alleles is associated with increased resistance of tumor cells to apoptosis induced by DNA-damaging agents including drug or radiation treatment, either in vitro (49) or in vivo (50).

In comparison to the *bcl*-2 gene family, the cell survival promoting role of various other common dominantly acting oncogenes is thought to be relatively minor (11, 61). A case in point is the *ras* family of mutant oncogenes. They are thought to contribute to cancer development and progression mainly through their effects on promoting cell proliferation, which in turn is related to their pivotal role in key intracellular signal transduction pathways, particularly the raf-1/MAP kinase signaling pathway (65). However, there have been some reports that have suggested *ras* oncogenes may also function as regulators of programmed cell death, possibly acting indirectly in certain contexts as a suppressive influence. In particular, Wyllie and his colleagues have noted an association in transplantable mouse solid tumors between mutant *ras* oncogene expression and reduced levels of programmed cell death in vivo (1, 87, 88). In addition, the apoptotic death that is observed to take place in certain normal epithelial cells, as a result of disruption of integrin mediated interaction with a surrounding extracellular matrix, can be circumvented by enforced expression of v-H-*ras* (27), and mutant H-*ras* can prevent E1A-induced apoptosis in primary baby rat kidney (BRK)[1] cells (47). Dominant negative mutants of *ras* oncogenes have also been shown to enhance programmed cell death when transfected into erythroleukemia cells (76). In some cases the apoptosis inhibiting effects of mutant *ras* genes may be mediated indirectly through regulating members of the bcl-2 gene family, including *bcl-2* itself (41).

Much of the analysis dealing with the genetic regulation of programmed cell death in tumor cells has been undertaken using cell lines grown in conventional monolayer cell culture conditions. Given the fact that gene expression and function can be significantly altered by three-dimensional growth and multicellular contact (25, 38, 46, 48, 85, 86) and that most cancers grow as solid (three-dimensional) tumors, we decided to analyze the possible mechanisms regulating programmed cell death in tumors grown as three-dimensional, multicellular spheroids—as opposed to monolayer cell cultures only. Our decision to do so was influenced in part by recent findings in our laboratory that suggested that the survival of tumor cells after single or multiple exposures to toxic concentrations of various anti-cancer chemotherapeutic drugs could be enhanced by multicellular growth conditions (29, 38, 42). The basis of this multicellular drug resistance is unknown, but clearly could involve survival mechanisms that are regulated differently in three-dimensional (multicellular) versus two-dimensional (unicellular) tissue culture conditions (38).

The main purpose of the present study was to analyze the contribution of mutant *c*-H-*ras* to suppression of programmed cell death in transformed epithelial cells, using a rat intestinal epithelial cell line known as IEC-18. These cells grow in an immortalized fashion in monolayer culture but are not tumorigenic and cannot form colonies in soft agar; these traits are acquired, however, by transfection of an activated H-*ras* oncogene (9). We decided to address the following questions: (*a*) can the IEC-18 cells grow in a three-dimensional manner as multicellular spheroids? (*b*) If not, do the cells die of programmed cell death, and can this be prevented by expression of mutant c-H-*ras*?

Materials and Methods

Cell Lines and Culture Condition

Spontaneously immortalized IEC-18 cells were originally derived from the new born rat intestine as described by Quaroni and Isselbacher (68). This cell line and all its derivatives were maintained as monolayers in medium composed of αMEM base supplemented with 5% FBS (GIBCO BRL, Gaithersburg, MD), 4 mM L-glutamine, 20 mM glucose, 10 μg ml^{-1} insulin (Sigma Chem. Co., St. Louis, MO). All the transfectants were periodically treated with 200 μg/ml G418 (Geneticin; GIBCO BRL) or Hygromycin B (Boehringer Mannheim Corp., Mannheim, Germany). Derivation of clones ras-3, ras-4, and ras-7 constitutively expressing an activated human H-*ras* oncogene was described previously (9). Characteristics of clone 25 cells derived from IEC-18 cell line transfected with H-*ras* gene under control of the glucocorticoid-inducible MMTV long terminal repeat promoter were described elsewhere (23). In the latter case the expression of H-*ras* can be induced by addition of 10^{-6} M dexamethasone acetate (Sigma) into the culture medium. This produces a visible change in cell morphology within 3–6 d in monolayer and within 15–48 h in spheroid culture. The ras-7A3H clone (an antisense CD44 transfectant) was generated and characterized by Jamal and Filmus (unpublished observations). A pool of ras-7–derived clones has been screened in the homotypic aggregation assay and A3H cell line was selected on the basis of its nonaggregating properties. IEC-18 variants spontaneously resistant to programmed cell death were isolated through a serial passage of the parental IEC-18 cells on agarose-coated dishes (see below). The spheroids were initially incubated for four days and then pooled and replated on plastic until the cells were recovered and expanded. The cycle was repeated 4–10 times with increase in both duration of spheroid culture and yield of surviving cells. The resulting variants were designated as apoptosis-resistant (AR) cell lines. Three such cell lines were derived simultaneously (AR-1, AR-2, and AR-3) with some differences in duration of selection and recovery periods. For the purpose of this study the AR-1 cells were used after 7(AR-1.7) or 10 cycles (AR-1.10) of selection for survival in spheroid culture. Also cell lines AR-3.4 (4 cycles) and AR-2.10 (10 cycles) were used for some of our studies. Before such experiments the cells were passaged several times in monolayer to avoid transient alterations that could conceivably be induced in spheroid culture.

1. *Abbreviations used in this paper*: AR, apoptosis-resistant; ARPCD, adhesion-regulated programmed cell death; BRK, baby rat kidney; ECM, extracellular matrix; PCD, programmed cell death.

Three-dimensional Spheroid Culture

Multicellular spheroids were generated by the liquid overlay technique as described previously (42, 74). In brief, 96- or 24-well tissue culture plates or 100-mm plastic dishes (Costar Corp., Cambridge, MA) were coated with 50 μL, 250 μL or 5 mL, respectively, of prewarmed 1% SeaPlaque agarose solution in serum free αMEM (FMC Corp., Rockland, ME). After the agarose was allowed to solidify and to form a thin layer with a gentle meniscus on the bottom of the dish (~60 min), a single cell suspension of cells (usually an equivalent of 10^5 cells per well of the 24-well plate) was plated in complete growth medium into each well. The plates were swirled gently to allow cell-to-cell contact and incubated undisturbed in humidified atmosphere at 37°C, 5%CO_2 for at least 24 h. Clone 25 cells were induced to express mutant H-*ras* by addition of 10^{-6} M dexamethasone on the day of cell plating or at time points, as indicated. The drug was also added to parental IEC-18 cells (control) without any visible effect on the cells.

Monitoring of Cell Growth and Viability

The cells plated in spheroid culture and in parallel monolayers were inspected daily, photographed, and when indicated, analyzed for their colony forming ability (colony formation assay), DNA content and DNA integrity (FACS, gel electrophoresis), cell membrane integrity (trypan blue exclusion, morphology), metabolic activity, and other parameters of growth and viability.

Colony Formation (Clonogenic Survival) Assay

The ability of cells to form clonal colonies on plastic was analyzed as described previously (70). A putative single cell suspension was prepared from either pooled spheroids or corresponding monolayer cultures by treatment with trypsin-EDTA solution. In some cases the compact IEC-18 spheroids were dispersed by treatment with an enzyme cocktail containing 1 mg ml^{-1} Collagenase (CLS 3; Worthington Biochem. Corp., Freehold, NJ), 0.5 mg ml^{-1} Hyaluronidase, 0.5 mg ml^{-1} Collagenase type IV and 15 U ml^{-1} Elastase (all three purchased from Sigma). In the time course experiments the cell recovery was calculated as the total number of cells/well divided by the number of cells plated on day 0. Different numbers of trypan blue excluding cells from each sample were plated onto triplicate 100-mm tissue culture dishes (Costar) in complete growth medium and incubated for 7–10 d. The resulting clonal colonies were then fixed in Carnoy's fixative (methanol/acetic acid, 2:1 vol/vol), stained with crystal violet solution and counted (only those containing more than 30 cells) under dissecting microscope. The colony forming ability calculated as the number of colonies/plate divided by the number of cells plated (× 100%) was averaged across all the countable plates.

FACS Analysis

Single cell suspensions were prepared as described above. The cells were washed in $Ca^{2+}Mg^{2+}$-free PBS and fixed in cold ethanol/PBS (1:1 vol/vol). The fixative was removed by centrifugation and the cells washed with PBS, incubated with RNAseA (15 KU ml^{-1}) and stained with propidium iodide at the final concentration of 50 μg ml^{-1}. The samples were analyzed for the DNA content and cell cycle profile by using FACScan flow cytometer (Becton Dickinson, San Francisco, CA). The red fluorescence was excited at 488 nm wave length of the Argon laser beam. The data acquisition and analysis was performed by using the standard LYSIS II software.

DNA Electrophoresis

Spheroids were cultured for different periods of time and pooled (~10^7 cells per sample). The spheroid samples were pelleted and lysed with 1% Triton X-100, 3 uM $MgCl_2$ and 10 mM Tris, pH 8.0, digested overnight with proteinase K (200 μg ml^{-1}) in 0.5% SDS and with RNAse A. The DNA was extracted with phenol/chloroform and ethanol precipitated, and heated at 65°C for 10 min. The samples were resolved in 1.2% agarose gel containing ethidium bromide.

Morphological Analysis of Apoptotic Cells

The dying cells recovered from IEC-18 spheroids at different time points were examined in the presence of trypan blue under high magnification (400 ×) by using a Zeiss Axiovert 135 microscope (Carl Zeiss, Inc. Thornwood, NY). Separate cell aliquots were fixed in methanol/acetic acid solution (3:1 vol/vol) and stained for 10–30 min with the DNA-binding dye Hoechst 33258 (added directly to the fixative). The cells were subsequently washed with PBS placed on microscope slides and the fluorescence of the nuclei was examined using Zeiss Axiovert 135 microscope equipped with an appropriate UV filter. For the electron microscopy experiments, the single cell suspensions from monolayers or pooled intact spheroids were gently pelleted in Eppendorf tubes and fixed with buffered paraformaldehyde. The pellets were then removed, sectioned, and processed for electron microscopy. The specimens were viewed and photographed under different magnifications by using a Zeiss electron microscope.

MTT Assay

Cell survival was monitored indirectly by assessment of their metabolic activity in the standard MTT assay. The cells (1–2 × 10^5/well) were plated in agarose coated 24-well plates. At various time points the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS was added to triplicate wells to the final concentration of 1 mg ml^{-1}. After 4-h incubation individual spheroids surrounded with formazan crystals were transferred into 1.5-ml Eppendorf tubes, washed with PBS, and the crystals dissolved by addition of 100% DMSO for 20 min with occasional shaking. Aliquots of formazan solution (150 uL) were transferred into 96-well plates and their absorbance at 540 nm measured using MR5000 Multiscanner (Dynatech Labs., Inc., Chantilly, VA). The results obtained by using this assay were in agreement with measurement of histone-DNA complexes in culture supernatants of IEC-18 spheroids (Cell Death Detection ELISA; Boehringer Mannheim Corp.).

DNA Transfections

Transfections of IEC-18 cells with H-*ras* containing vectors were described elsewhere (23, 37). Human *bcl-2* was transfected into IEC-18 cells in the expression vector pBCL2-wt (64) containing the hygromycin resistance gene (a gift from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD) by using lipofectin reagent (GIBCO BRL). As a control, the corresponding empty vector pCEP4 (Invitrogen, San Diego, CA) was used. Clones resistant to 200 μg ml^{-1} Hygromycin B (Boehringer Mannheim Corp.) were isolated and screened for expression of BCL-2 protein by Western analysis (see below). Clones that did not express detectable levels of the protein were used as additional controls.

Western Analysis

To detect the expression of BCL-2 or BCL-X_L proteins, the cells were harvested from monolayer cultures, pelleted, and lysed at 4°C with 1% Triton X-100 in PBS in the presence of PMSF. The lysates were centrifuged at 12,000 *g* for 20 min, supernatants removed and assayed for protein concentration using the Bradford method. After the protein concentrations were adjusted, the samples were mixed with 2 × nonreducing SDS containing sample buffer and boiled for 3 min. Equal amounts of protein were resolved on 12.5% SDS-PAGE and electro-transferred to nitrocellulose membrane. The membranes were blocked with 5% skim milk and incubated with anti-human BCL-2 rabbit polyclonal antibody CN-19 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). This antibody is also able to recognize rat BCL-2 protein that in parental IEC-18 cells was not detectable. As a positive control for BCL-2 expression the human neuroblastoma cell line SHZ-3-2-5 was used (15) (gift from Dr. V. P. Castle, University of Michigan, Ann Arbor, MI). Alternatively, blots were probed with mouse monoclonal anti-human BCL-X antibody that detected appreciable levels of the BCL-X_L protein in both IEC-18 cells and AR cell lines or with anti-RAS mouse monoclonal antibody (Transduction Labs., Lexington, KY). The signal was detected by using Horseradish Peroxidase conjugated, goat anti-rabbit IgG (1:1000, Bio-Rad) antibody (or for bcl-x an appropriate anti-mouse IgG antibody) and the chemiluminescence ECL kit (Amersham Corp., Arlington Heights, IL).

Results

Intestinal Epithelial Cells Expressing Oncogenic ras Acquire the Ability to Grow in a Three-dimensional Manner

The intestinal rat epithelial cell line, IEC-18, is nontumorigenic in nude mice upon subcutaneous (9, 23, 37) or even

orthotopic (intracecal) injection (Rak J., J. Filmus, R. S. Kerbel, unpublished observations). Overexpression of the mutant H-*ras* oncogene (Fig. 1, *bottom*), alone, resulted in the acquisition by these cells of the tumorigenic phenotype. This change in in vivo behavior was paralleled by an alteration in cell morphology (Fig. 1, *A* and *D*) and a moderate (25%) increase in growth rate in vitro (91). However, both parental IEC-18 cells and their tumorigenic counterparts grow readily under conventional monolayer tissue culture conditions (Fig. 1, *A* and *D*). In confluent cultures we observed the *ras* transfectants, but not the parental IEC-18 cells, frequently detached from the bottom of tissue culture wells, spontaneously aggregated and continued to grow three-dimensionally (Fig. 1, *C* and *D*). To elucidate the nature of this difference in behavior, various tumorigenic and nontumorigenic sublines of IEC-18 cells were generated and examined for their ability to grow in a three-dimensional culture system i.e., as so-called multicellular spheroids (42, 81) (Fig. 2). In such culture conditions, cell adhesion, spreading, and monolayer formation on the bottom of the tissue culture dish is prevented by the presence of a thin layer of solid agarose; this forces the cells to remain in suspension or to form tumor-like homotypic multicellular aggregates (74). Under these conditions the initial aggregation of parental IEC-18 cells was rather poor with numerous single cells and small clumps surrounding a larger central aggregate. Within six days, the free-floating single cells and small clumps were replaced by cellular debris and, the remaining compact, central aggregate gradually disintegrated. In contrast, the H-*ras* transformed, tumorigenic variants, ras-3, ras-4 and ras-7 readily aggregated and within the first 24 h in culture formed three-dimensional spheroids in which all of the cells in the well were incorporated. These cells remained viable and metabolically active for several weeks, and in the case of high *ras* expressers (for example ras-7 clone; compare Fig. 1, *bottom* and Fig. 2) an apparent rapid increase in size of spheroids over time was consistently observed.

To examine whether the change in aggregation, spheroid morphology and growth was directly related to expression of the oncogenic *ras* we used the IEC-18-derived clone carrying an activated H-*ras* under control of the glucocorticoid inducible MMTV promoter (IEC-25 or clone 25 cells). The clone 25 cells formed spheroids similar to those of parental IEC-18 cells in the absence of dexamethasone (Fig. 2). However, when plated in the presence of dexamethasone the cells aggregated and grew on agarose in a manner indistinguishable from the stable *ras* transfectants. Dexamethasone itself had no effect on parental IEC-18 cells (Fig. 2).

It was puzzling that the ability of *ras* transfectants to grow in the spheroid culture was associated with a remarkable alteration in their aggregation properties. In order to elucidate whether there is a causal relationship between these two characteristics, a nonaggregating variant (ras7A3H cell line) was generated (Filmus, J., unpublished observations). These cells, when plated on agarose, formed only a layer of single cells or loose clusters rather than spheroids, but they still retained the ability to proliferate (Fig. 2). This observation suggests that aggregation is not essential for enhanced three-dimensional growth capacity of the H-*ras* transformed IEC-18 cells.

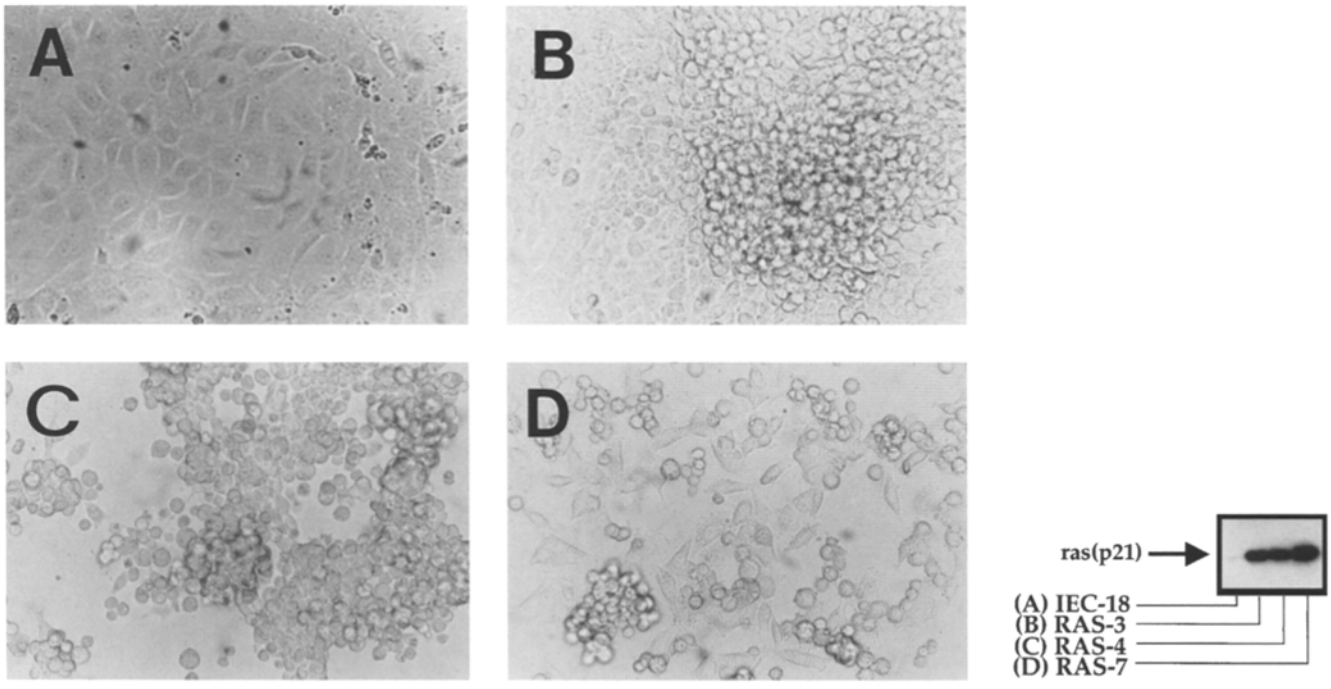

Figure 1. Morphological features of intestinal epithelial cells in monolayer culture (phase contrast microscopy). (*A*) Typical epithelial appearance of parental IEC-18 cells. These immortalized rat intestinal epithelial cells are nontumorigenic but grow readily in monolayer culture. Transfection of IEC-18 with an activated H-*ras* oncogene resulted in the derivation of transformed clones ras-3 (*B*), ras-4 (*C*), and ras-7 (*D*) expressing increasing levels of malignancy in vivo. These *ras* transformed cells display spindle shaped or round morphology, easily detach from the bottom of the dish and aggregate; bottom panel: Western analysis showing the relative levels of $p21^{ras}$ protein expression by transfected and parental IEC-18 cells.

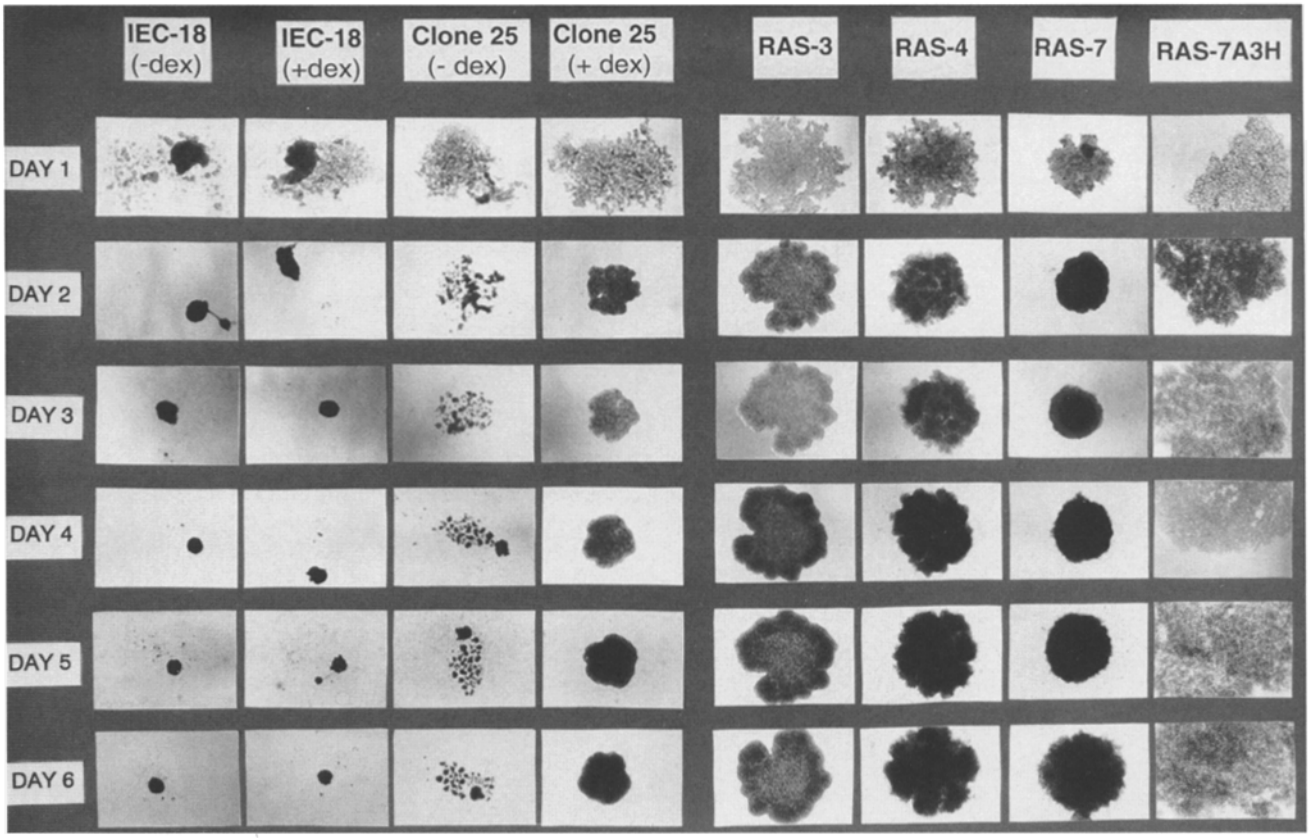

Figure 2. Time dependent changes in morphology of spheroids generated by IEC-18 epithelial cells and their various *ras* transfected sublines (10^5 cells/well of the 24-well plate were plated on day 0). (*IEC-18*) The cells are either incorporated into a larger central spheroid or remain dispersed as single cells or small aggregates. *-dex*, no dexamethasone; *+dex*, cells plated in the presence of 10^{-6} M dexamethasone (no change); (*Clone 25*) IEC-18 cells transfected with H-*ras* under control of glucocorticoid inducible promoter. *+dex*, Addition of dexamethasone (10^{-6} M) on day 0 resulted in dramatic increase in aggregation, growth and viability of cells; *-dex*, in the absence of dexamethasone the spheroids morphology resembles that of IEC-18 cells. (*RAS-3, RAS-4, RAS-7*) These tumorigenic variants of IEC-18 cells, stably transfected with the H-*ras* oncogene, rapidly aggregated and formed large spheroids in which all the cells are incorporated. The increase in spheroid volume is clearly visible in the case of ras-7 cells that expressed the highest level of transfected *ras* and that are tumorigenic and metastatic in vivo (71). (*RAS-7A3H*) A nonaggregating variant of ras-7 cells grows in suspension or as loose clusters.

Nontumorigenic Intestinal Epithelial Cells Undergo a Form of Programmed Cell Death in Spheroid Culture

Despite residual cell–cell adhesion and abundance of growth factors in the medium, IEC-18 cells maintained in three-dimensional culture displayed signs of spheroid disintegration and spontaneous cell death, as described above. FACS analysis revealed time dependent abnormalities in the DNA profile of cells recovered from disintegrating IEC-18 spheroids. Such alterations were not found in cells from parallel monolayers or cultures of *ras*-transformed cells (Fig. 3 *A*). Within 3–4 d of the initiation of the IEC-18 spheroid culture the majority of the cells moved to the sub-G1 region. In contrast, monolayer cultures as well as the ras-7 cell line grown as spheroids continued to express a normal DNA profile, with some reduction in S phase and marginal fraction of dead cells in the latter case. The time-dependent DNA degradation in IEC-18 spheroids was also observed when the intact spheroids were collected and their DNA resolved on a 1.2% agarose gel (data not shown). No oligonucleosomal sized DNA "ladders" were detected, which is not necessarily surprising in this case considering the rather asynchronous nature of the DNA degradation in IEC-18 cells and similar characteristics of apoptosis described for other systems (13, 59, 62, 77, 80).

Changes in DNA integrity were accompanied by the loss of the clonogenic capacity of IEC-18 cells but not, at least initially, by any appreciable cell membrane damage (Fig. 3 *B*). While the spheroids still contained trypan blue excluding cells after 78–100 h in culture, the capability of these cells to give rise to clonal colonies was almost entirely eliminated. No colonies were detected at these later time points even after the plating of as many as 10^5 trypan blue excluding cells on plastic. Again, this observation suggests that the functional death of epithelial cells in spheroid culture precedes and initially exceeds signs of celi membrane damage (Fig. 3 *B*). Morphological features of IEC-18 cells in spheroid culture seem to support this conclusion (Fig. 4). Thus, initially, the cells recovered from IEC-18 spheroids were indistinguishable in appearance from their monolayer derived counterparts (Fig. 4 *A*). However, after 24–72 h on agarose a gradual change in morphology began to take place (Fig. 4, *B* and *D*). At first a moderate enlargement of some of the cells was observed. The cytoplasm of these cells frequently contained com-

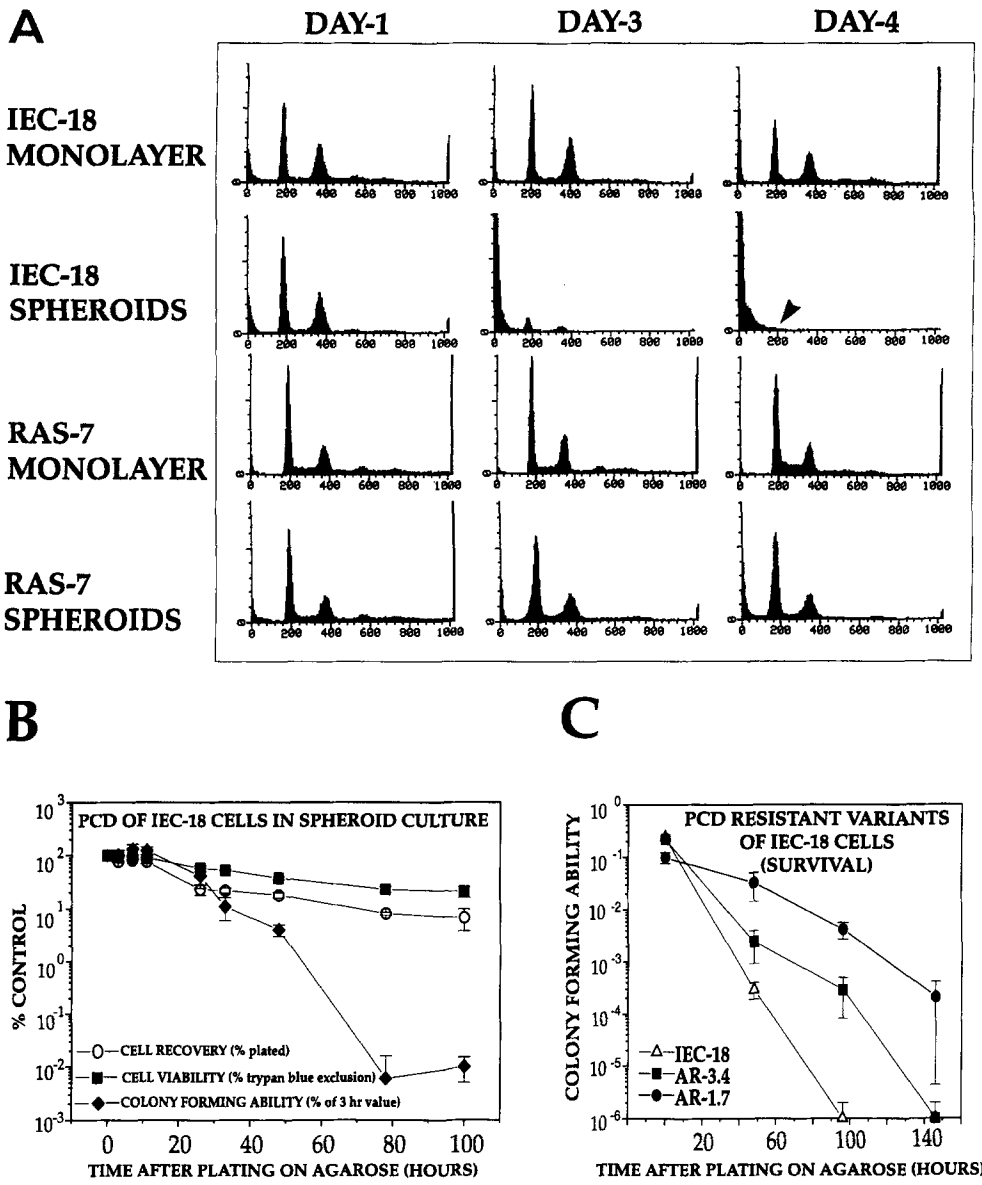

Figure 3. (*A*) Programmed cell death of IEC-18 cells in spheroid culture. Time dependent DNA degradation of IEC-18 cells maintained in spheroid culture. FACS profiles of the DNA content in either monolayer or spheroid cultures of IEC-18 and ras-7 cells (staining with propidium iodide). By day four, IEC-18 cells maintained in spheroid culture display sub-G1 DNA content; arrowhead indicates the position of the G1 peak; (*B*) Time-dependent decrease in colony forming ability precedes the cell membrane damage as indirectly measured by trypan blue exclusion and drop in total cell recovery. The viable cells were isolated from spheroids and plated at densities ranging from 160–10^5 cells per 100-mm dish in triplicates; (*C*) Derivation by serial selection of programmed cell death resistant variants of IEC-18 cells. The parental population was subjected to several rounds of selection in spheroid culture alternated with cell recovery in monolayer. Independently derived cell lines AR-3.4 and AR-1.7 have undergone four and seven selections, respectively, and acquired corresponding levels of resistance to PCD as measured by colony forming efficiency.

partments that no longer excluded trypan blue while the remaining cytoplasm continued to do so. It is possible that this was a result of an engulfment of dead cells by their still viable neighbors (87). At the same time a large number of cytoplasmic vacuoles was formed. These changes were followed by cell shrinkage and the resulting smaller cells upon, staining with the DNA binding dye, Hoechst 33258, displayed features of chromatin condensation and marginalization around the nuclear membrane (Fig. 4 *C*). Finally, after 100 h in culture the ability to exclude trypan blue by the IEC-18 cells was entirely compromised and the spheroids disintegrated into dead cells, cellular fragments and debris. This sequence of events triggered by the three-dimensional culture conditions was clearly indicative of programmed cell death and morphologically reminiscent of so-called type II apoptosis (10).

As may have been expected, at the ultrastructural level the cells present within dying IEC-18 spheroids were vastly different from cells recovered from monolayer cultures (Fig. 5). Within the monolayer cell population multiple mitoses were observed and the cells contained numerous mitochondria and surface microvilli (Fig. 5, *A* and *C*) whereas none of these features was present in spheroids. In the latter case the cells were loaded with cytoplasmatic vacuoles containing a lipid-like material or in some cases membrane-like structures. Finally, the cells maintained in spheroid culture for 48–72 h manifested signs of an apparent apoptotic death with chromatin condensation, nuclear fragmentation and numerous apoptotic bodies scattered throughout the low power field (Fig. 5, *B* and *D*).

Temporal Nature of the Cell Death Process in IEC-18 Cells Grown as Spheroids

After ~70 h in spheroid culture the IEC-18 cell "suicide" program reached an irreversible stage. Addition of dexamethasone to clone 25 cells before, but not after, that stage resulted in a recovery of the clonogenic capacity and

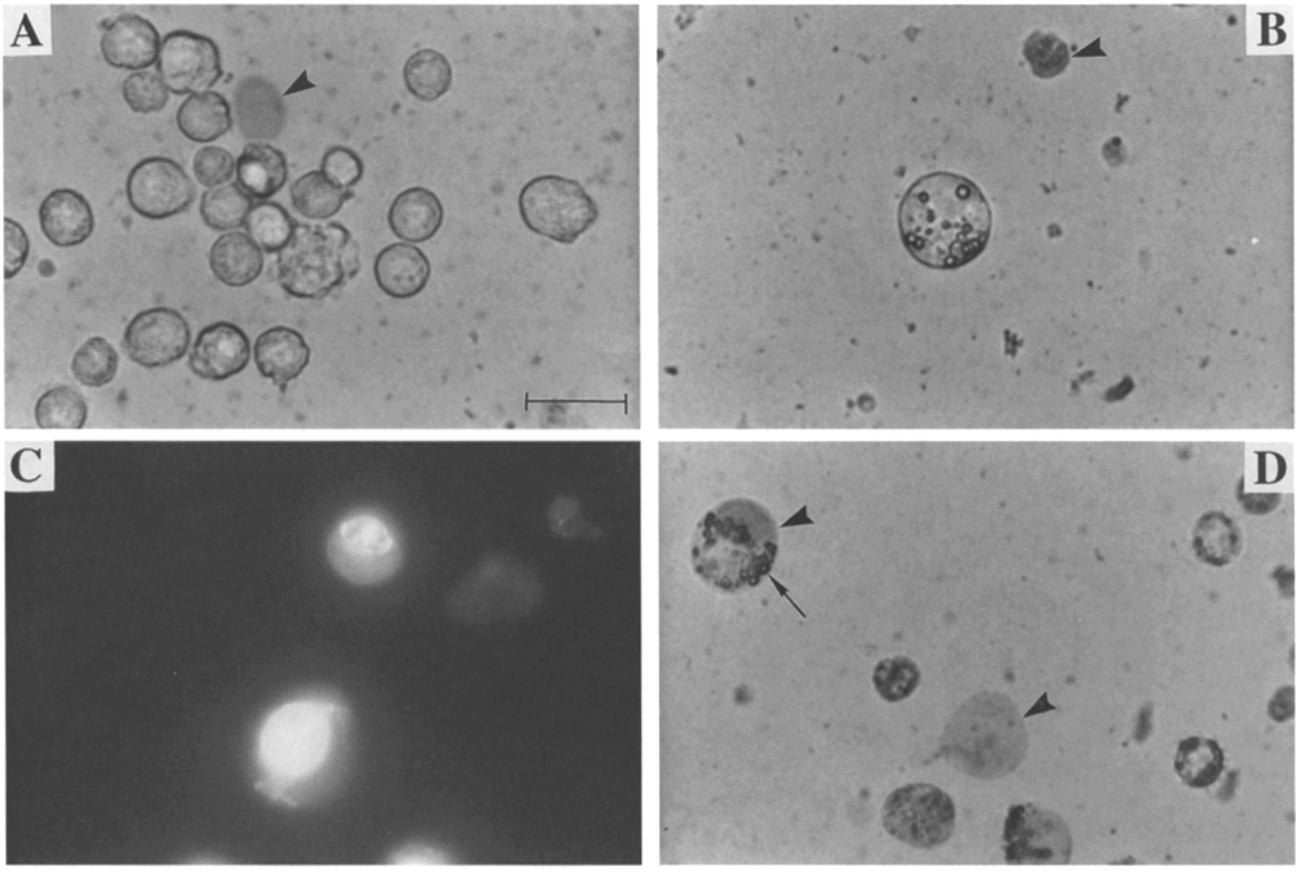

Figure 4. Morphological features of IEC-18 cells cultured for 72 h as monolayer (*A*) or spheroids (*B–D*). (*A*) Single cell suspension of monolayer cells; the majority of the cells are viable and do not stain with trypan blue or display morphological abnormalities. (*B* and *D*) Cells recovered from spheroid culture display features reminiscent of type II apoptosis (e.g., presence of multiple intracellular vacuoles) (10). Some trypan blue excluding cells contain compartments containing the dye possibly due to engulfment of dead cells by viable cells. (*C*) Staining of the spheroid derived IEC-18 cells with DNA binding dye Hoechst 33258; smaller cells display signs of chromatin condensation and marginalization around the nuclear membrane. Arrowheads, trypan blue positive cells; arrow, cytoplasmic vacuoles in the "viable" compartment of the cell. Bar, 10 μM.

prevented morphological disintegration of the spheroids (Table I, Fig. 2). Thus up to 48 h in spheroid culture the induction of oncogenic *ras* was able to abort the cell death program triggered by three-dimensional culture conditions. Since this type of programmed cell death (PCD) seems to be unique in that it is initiated in nontransformed epithelial cells by the absence of normal adhesion and spreading, we will refer to this phenomenon as adhesion regulated programmed cell death (ARPCD) to distinguish it from PCD induced by other stimuli.

Selection of Cellular Variants Expressing Spontaneous Resistance to ARPCD

After four days in spheroid culture the colony forming ability of IEC-18 cells is usually undetectable (Fig. 3, *B* and *C*). We noticed however that when a large number of such spheroid derived cells were plated, single colonies could occasionally be found. We hypothesized that these surviving cells may represent a rare, "silent," PCD-resistant variant present within the IEC-18 cell population. The latter population was therefore subjected to a series of selections to enrich it for such putative ARPCD resistant subpopulations. The cells were repeatedly incubated in spheroid culture, which was alternated with periods of recovery in monolayer. The results shown in Fig. 3 *C* suggest that indeed ARPCD-resistant variants of IEC-18 cells could be isolated in this manner. In this experiment the time dependent survival capacity of two out of three independent variants was evaluated by the colony formation assay. These variants designated AR-3.4 and AR-1.7 have undergone four and seven cycles of selection, respectively. It is apparent that in both cases the survival is visibly extended, although unlike the *ras* transfectants, the spontaneous ARPCD-resistance has a limited duration (compare Table II). This may be due to contamination of the variant cell lines with parental ARPCD-sensitive cells, especially since a longer selection resulted in greater resistance and presumably higher purity of the late passage AR cell lines. Alternatively, the level of expression of the gene(s) antagonizing ARPCD in these cells may have been increased during the course of the selection process. By using this protocol we were able to obtain variants that survived up to three months in three-dimensional culture (data not shown). Again, in contrast to the *ras* transfectants, these cells did not display elongated morphology nor did they express any obvious growth advantage in monolayer culture. In contrast, on agarose coated surfaces they formed

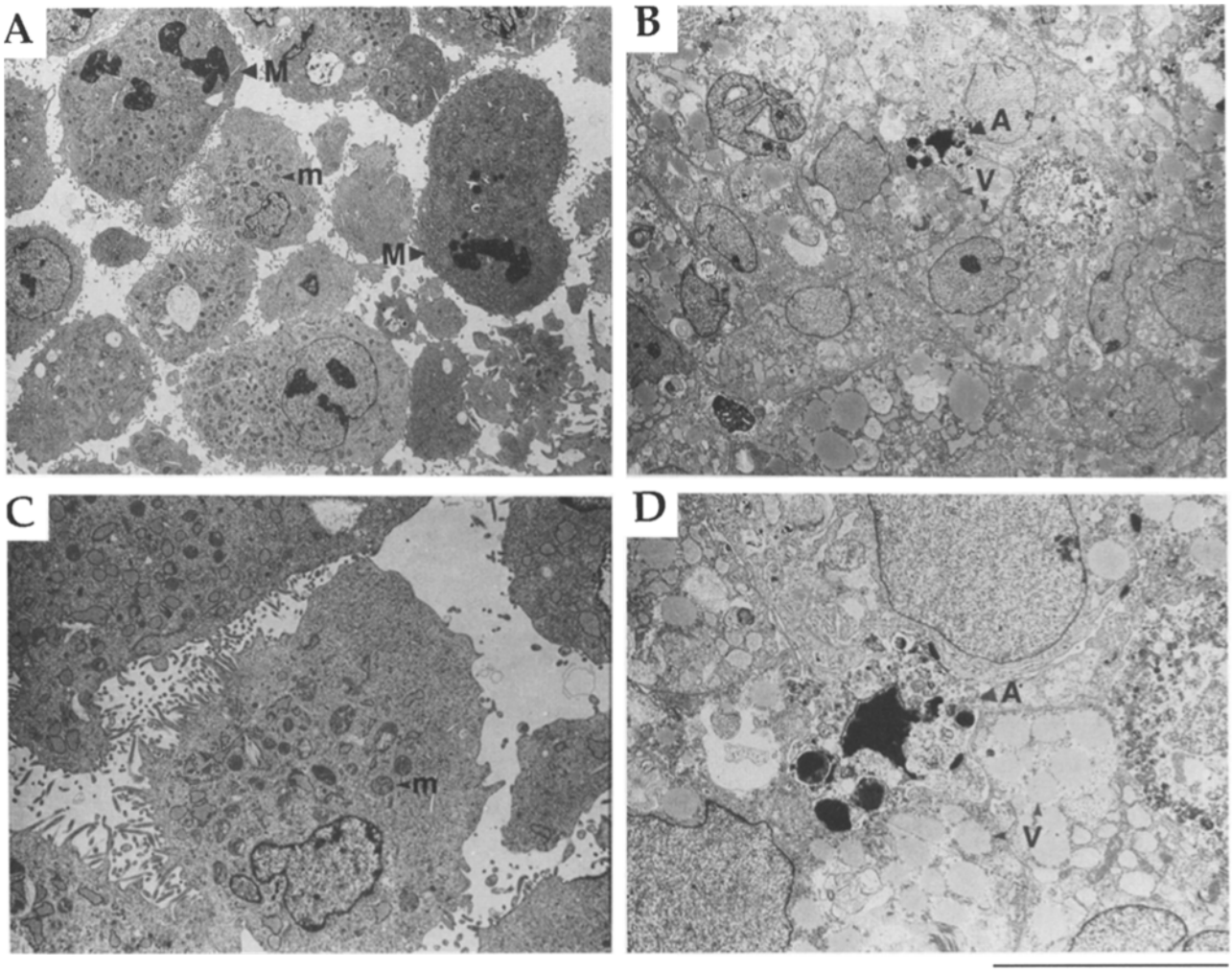

Figure 5. Electron micrograph of IEC-18 cells cultured for 72 h as monolayer (*A* and *C*) or spheroids (*B* and *D*). (*A*) Cells recovered from monolayer are rich in microvilli, mitochondria (*m*) and display multiple mitotic figures (*M*) (magnification 3,850×). (*B*) Section through IEC-18 spheroid, magnification 3,850×. The cells are filled with vacuoles (*V*) containing lipid- or membrane-like material, some manifesting different degrees of apoptotic death 'A'. (*C*), monolayer cells at the magnification of 10,500×. (*D*) Details of the apoptotic cell from the field shown in *B* (magnification 10,500×). Bar, (*A* and *B*) ~8 μm; (*C* and *D*) 3 μm.

parental-like, small, compact spheroids composed of mostly viable but nonproliferating cells (data not shown).

Transient Rescue of Epithelial Cells from ARPCD by Overexpression of bcl-2 Proto-oncogene

A series of *bcl-2* transfectants was generated to examine whether the overexpression of this gene could (*a*) abort or mitigate the IEC-18 cell death process in spheroid culture thus confirming its apoptotic nature or (*b*) mimic the behavior of the *ras*-transfected or spontaneous ARPCD-resistant variants. Indeed, the resulting *bcl-2* transfectants shared some degree of phenotypic similarity with the latter cell lines. The morphology of both types of variants in monolayer was indistinguishable from parental IEC-18 cells (not shown). Also, in spheroid culture, both *bcl-2*–transfected and spontaneous ARPCD resistant cells were unable to expand significantly, manifested compact morphology and had extended but limited survival capability (Fig. 6, Table II). Somewhat surprisingly, neither BCL-2 (Fig. 6, *bottom*) nor BCL-X_L protein (not shown) was overexpressed in spontaneous ARPCD-resistant variants implying that other genes may be responsible for their phenotype. The latter possibility is currently under study.

Discussion

Two main conclusions can be drawn from our results. First, when nontumorigenic intestinal epithelial cells are grown in a nonphysiologic circumstance, i.e., as a three-dimensional, tumor-like multicellular aggregate, the cells die spontaneously by a form of programmed cell death. Second, this adhesion regulated programmed cell death process can be prevented by expression of a mutated *ras* oncogene. Because oncogenic *ras* mutations are a common genetic aberration in cancer, especially colorectal carcinomas, the results have a number of implications. These include how *ras* mutations may contribute to the development and progression of solid tumors, the manner in which programmed cell death may be regulated, especially by adhesion, in normal and tumor cell physiology, and for how

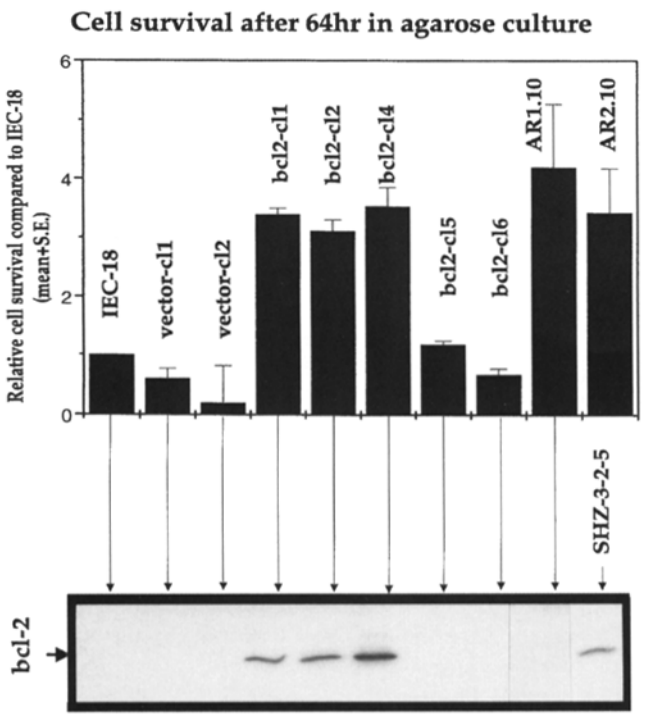

Figure 6. Adhesion-regulated programmed cell death of IEC-18 cells is mitigated by overexpression of the bcl-2 protooncogene. Parental IEC-18 cells, control transfectants (vector-cl1 and vector-cl2) and nonexpresser clones (bcl2-cl5, bcl2-cl6) display an expected, low survival rate after 64 h in agarose culture. In contrast, clones expressing BCL-2 protein acquired an increased survival capability similar to the spontaneous ARPCD resistant cell lines (AR-1.10 and AR-2.10). The latter cell lines do not express endogenous BCL-2 protein. In this experiment cell viability and metabolic activity was evaluated by MTT assay. SHZ-3-2-5 is a human neuroblastoma cell line expressing *bcl-2* (which was used as a positive control).

drugs that interfere with the function of mutant *ras* genes or RAS proteins may suppress the growth of solid tumors in vivo.

ARPCD

In a broad sense our observations point to the fact that not only growth but also cellular life and death are under the continuous influence of both intracellular and extracellular contexts. The specific features of such contexts would likely be determined by cell type, the presence of other cells, cell shape, tissue topology and the types of adhesive interaction with surrounding extracellular matrix (ECM) molecules (6, 75, 79). With respect to cell-ECM interactions in regulating apoptosis, it is known, for example, that adhesion and spreading on ECM is essential for survival of endothelial cells (7, 55, 72), certain types of epithelial cells in culture (6, 27, 55), or tumor cells (40, 58). ECM mediated inhibition of apoptosis may be regulated through integrin-dependent modulation of survival or death genes, e.g., upregulation of *bcl-2* (90) or suppression of putative cell death promoting elements, e.g., interleukin-1β converting enzyme (6). The presence of an adhesive substrate has also been shown to be required for completion of the cell cycle (30). In this context our recent observation that

Table I. Time-dependent Rescue of IEC-18/Clone 25 Cells from PCD in Spheroid Culture by Induction of ras Oncogene Expression

Time of dexamethasone addition after plating cells in 3-D culture	Number of colonies per 10^4 cells cultured for 124 h on agarose
h	
0	⩾250, ⩾250, ⩾250
30	⩾250, ⩾250, ⩾250
48	>250, >250, >250
70	4, 1, 1
96	4, 2, 0
No dexamethasone	0, 0, 0
2-D control	⩾250, ⩾250, ⩾250

Single cell suspension was prepared 124 h after plating cells in 3-D and viable cells were seeded in triplicate wells. Colony forming efficiency was determined after 6 d.

cyclin D1 is upregulated in *ras* transformed anchorage-independent IEC-18 cells may suggest that this cyclin also perhaps plays a role in regulating three-dimensional cell survival (22).

Cell–cell adhesion and aggregation have also been demonstrated to play a role in multicellular growth and survival in various normal or tumor cell systems (3, 33, 38, 67). This includes our own earlier studies suggesting that a drug-induced cell death pathway can be modified by three-dimensional cell growth (38, 42). In these and a number of other studies it was postulated that specific signaling events are triggered by cell–cell interaction, cell adhesion, and cell shape changes that in turn regulate cell growth, or else inhibit programmed cell death (3, 27, 69, 70, 75). Our present observations, suggest that the type and molecular consequences of cell adhesion may be more important than the adhesion itself. Even a rather tight cell–cell adhesion in IEC-18 spheroids could not prevent activation of apoptotic cell death in three-dimensional culture, although it should be noted that the IEC-18 cells that were incorporated into spheroids retained their viability longer than cells that were not (compare Fig. 2 and Results). In this regard it is noteworthy that in the spheroid model used in this study, the analysis of cell growth and survival is performed in a multicellular context where the potential for intercellular adhesion and cell–cell interaction are preserved. In most other three-dimensional cul-

Table II. Transient Rescue of IEC-18 Cells from PCD by Expression of bcl-2 Proto-oncogene

	Relative viability of cells in spheroid culture after		
Cell line examined*	24 h	48 h	72 h
		mean ± SEM	
IEC-18	29.5 ± 3.8‡	12.7 ± 0.9	7.3 ± 0.3
IEC-bcl2-cl1	60.3 ± 6.1	35.4 ± 1.6	20.0 ± 4.4
IEC-bcl2-cl2	67.2 ± 1.5	39.8 ± 2.2	28.9 ± 2.8
IEC-bcl2-cl4	64.1 ± 2.5	32.0 ± 1.6	21.7 ± 2.4
AR1.10	47.3 ± 0.9	28.5 ± 2.4	21.3 ± 2.0
AR2.10	46.6 ± 6.0	33.6 ± 0.2	26.9 ± 0.8
Ras4	97.2 ± 4.1	ND	201.1 ± 5.9

*IEC-18 refers to parental cell line: IEC-bcl2-cl1; IEC-bcl2-cl2 and IEC-bcl2-cl4 are bcl-2 transfectants of IEC-18 cells; AR1.10, AR2.10 are spontaneous selected (adhesion regulated) cell death-resistant variants of IEC-18 cells; ras-4 is a *ras* transfected clone of IEC-18.

‡Relative viability of cells as measured by MTT assay expressed as percentage of the absorbance detected at the time of plating (0 h).

ture systems, single cells are either embedded in soft agar or plated onto a nonadhesive polymer (e.g., polyHEMA) without direct contact with one another (27, 67, 81). Thus our results may be pertinent to gaining a better understanding of classical anchorage-independent growth. Ability of single tumor cells, unlike nontransformed epithelial (such as IEC-18) or mesenchymal cells, to grow into small colonies when plated in soft agar has long been known to be an in vitro hallmark of cancer (26). However, the basis for the inability of such single nontransformed cells to grow anchorage independently is unknown and intrinsically difficult to evaluate: it could represent a failure to survive due to induction of apoptosis, or a simple inability of the cell to divide. Our results with preformed spheroids suggest that, at least for epithelial cells, the former is the more likely possibility.

Ras Oncogenes as Suppressors of Programmed Cell Death in Tumors

How ARPCD is activated and how oncogenic *ras* mutations abort this process remain to be determined. The suppressive influence of *ras* in programmed cell death has not been widely appreciated, partly because the experimental data on the role of mutant *ras* genes in apoptosis have been somewhat conflicting (1, 20, 21, 27, 57, 76, 84) and because of the great emphasis placed on its direct contribution to cell division. In addition, molecules acting upstream of normal RAS such as v-src and Grb-2 were shown to affect apoptosis in different systems (18, 27, 57). Activated components of the *ras* signaling pathway such as mutant *ras* itself, activated *raf* and MAPK kinase invariably transform cells, frequently leading to contact independent growth and formation of three-dimensional solid tumors in vivo (8, 51, 53). Moreover, in the case of two human colon cancer cell lines called DLD 1 and HCT 116, in which the mutated copy of K-*ras* was inactivated by homologous recombination, the ability of the cells to form tumors in nude mice or colonies in soft agar was reduced to much greater extent than their mitogenic activity in monolayer culture (78). Virtually all these results have been interpreted exclusively in the light of the role of the ras/MAPK signaling pathway in mitogenesis. However, in the case of IEC-18 cells, simple activation of the mitogenic pathway seems to be insufficient for survival under three-dimensional conditions. For example, the cells cannot be rescued from ARPCD by growth factors present in the medium or by conditioned medium from H-*ras* transfected (ras-7) cells (data not shown).

Formation of solid tumors by transformed epithelial cells is associated with a drastic change in tissue topology. For such a change to occur the ARPCD induction would have to be compromised thereby allowing the transition from physiological, monolayer-like alignment of epithelial cells to the three-dimensional configuration characteristic of solid tumor masses. Presumably the apoptosis-antagonizing effect of BCL-2 or BCL-X_L proteins would be expected to facilitate this process. Paradoxically, in breast cancer, melanoma and some other types of solid tumor, high levels of bcl-2 expression are associated with a more favorable prognosis rather than tumor progression (31). Such observations cast some doubt as to the dominant or exclusive role of the aforementioned, classical apoptosis repressor genes, at least insofar as the growth of some types of solid tumor in vivo is concerned. It is of course conceivable that activated *ras* may act to suppress apoptosis through its stimulatory effect on transcription or function *bcl*-2 like survival genes (41). However, we have been unable to detect any elevation of bcl-2 expression in H-*ras* transfected IEC-18 cells (not shown). Thus, we favor the hypothesis that oncogenic *ras* itself may play a more direct role in cell survival under three-dimensional growth conditions, both in vitro and in vivo. Consequently the ARPCD suppressing and not just the mitogenic effect of *ras,* may be partly responsible for its role in tumorigenesis in many instances. In this regard it is of interest to note that during the development of colorectal neoplasia there seems to be a direct correlation between the susceptibility of epithelial cells to undergo apoptosis and the stage of the disease progression (4). Moreover, mutations of the K-*ras* oncogene are found at high frequency in colorectal cancers, and are temporally associated with the transition from early adenoma to intermediate adenoma stage of the disease (19) and coincide with the three-dimensional expansion of the tumor mass. Consistent with this notion, recent studies have demonstrated that although K-*ras* mutations are found in ~50% of all cases of colorectal adenocarcinomas, such mutations were detectable in only 16% of cases of superficial-type or "flat" colon tumors (32, 56). This suggests that K-*ras* mutations are not strongly selected for unless the lesion assumes a three-dimensional growth pattern, which would be nonphysiologic for colonic mucosal cells and thus require the expression of genes inhibiting spontaneous programmed cell death. However, it is also known that in a sizable proportion of solid tumors *ras* mutations are not detectable (19, 63). This suggests that other similarly dominantly acting genes such as v-src, for example, may also confer resistance to ARPCD; indeed, there is some preliminary evidence for this hypothesis (27, Mitsuhashi Y., J. Rak, and R. S. Kerbel, unpublished observation).

Mutant ras Oncogenes as Inhibitors of Programmed Cell Death: Implications for Targeting RAS as a Strategy for Anti-Tumor Therapy

For a number of reasons the apparent anti-apoptotic effect of mutated *ras* may be of practical importance. For example, it could help explain why a relatively potent in vivo antitumor effect on established mutant *ras* expressing tumors, is exerted by recently described inhibitors of *ras*, namely, the protein farnesyltransferase inhibitors, such as the compound known as L-739,749 (44). This drug, thought to inhibit posttranslational processing and hence membrane localization of RAS proteins, was shown to exert a moderate cytostatic effect on *ras*-transformed cells in monolayer culture (28, 43, 66). It is possible that in vivo, this and other similarly acting compounds (45), may have a much more dramatic anti-tumor effect by virtue of their ability to induce a significant level of apoptosis in tumor cells which, under three-dimensional growth conditions, may depend on mutated *ras* oncogene for their survival. This may help explain why such drugs, despite being nontoxic to tumor cells in vitro (in monolayer culture) may

nevertheless cause striking regressions of extremely large, established solid tumors in vivo (45). This could also occur through such inhibitors having anti-angiogenic properties, by preventing ras-mediated upregulation of angiogenic growth factors (71).

In summary, three-dimensional, multicellular configuration can spontaneously activate an adhesion regulated process of programmed cell death in epithelial cells that normally grow (in vivo) in a monolayer-like manner. Activated *ras* can abort this process. These findings implicate *ras* oncogenes as agents in the development of solid tumors through their suppressive influence in programmed cell death, and not just by their ability to promote cell proliferation per se. Our results also serve to emphasize the need to use three-dimensional culture systems when studying in vitro the molecular and cellular biology of cell survival in solid tumors, and provide a basis for the putative suppressive influence of activated *ras* oncogenes on spontaneous apoptosis in vivo observed in transplanted solid tumors (1, 87, 88).

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