Caspase-Dependent and -Independent Panc-1 Cell Death Due to Actinomycin D and MK 886 Are Additive but Increase Clonogenic Survival

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In human panc-1 pancreatic cancer cells, actinomycin D (act D) induces a type 1 (apoptotic, extrinsic, death domain, receptor-dependent, and caspase-positive) form of programmed cell death (PCD) and MK 886, a 5-lipoxygenase inhibitor serving among other functions as a surrogate for increasing oxidative stress, a type 2 form, defined as an intrinsic, mitochondria-dependent, autophagic form of cellular suicide. Using both agents simultaneously should allow for examination of their interaction in cells able to express either form of PCD. Activation of both forms might result in synergistic, additive, null, or inhibitory effects on the reduction in proliferation, PCD, and clonogenicity of surviving cells. Co-culture of panc-1 cells with act D and MK 886, which both inhibit their proliferation, had an additive effect on increasing the development of these forms of PCD, as determined by morphology, a nucleosome assay, and flow cytometry. Initially, laddering on agarose detected with propidium iodide, present in act D, and act D plus MK 886-treated cells was partially obscured by randomly degraded DNA. With the use of the more sensitive SYBR green dye and reduced exposure of detached cells to 37°C, a limited laddering of DNA from MK 886-treated cells was also detected. Caspase activity was present in act-D-cultured cells but was absent in cells cultured with MK 886. Combined culture reduced caspase activity in act D-treated cells, consistent with interference from type 2 of type 1 PCD. Removal after 48 hr of act D or MK 886 allowed regrowth of residual cells, the latter agent to a greater extent than the former. In combination, the number of clones was increased compared with act D alone. These features distinguish two forms of PCD. In therapeutic settings in which the modes of cell death have not been identified, unintentional activation of several cellular suicide pathways with "crosstalk" between them occurs. Their intentional simultaneous activation and responses, as modulated by the history of cells in or out of cycle, could reduce the intended therapeutic outcome with survival of additional clonogenic cells due to various forms of mutual interference. Exp Biol Med 228:915–925, 2003

Key words: pancreatic cancer; Panc-1; Actinomycin D; MK 886; programmed cell death type 1 and type 2

Depending upon the stimulus, cells can be induced to express either of what can be termed a Type 1 or a Type 2 form of programmed cell death (PCD) (1). Based chiefly on results with cultured cells, type 1 (apoptotic, extrinsic, receptor-dependent, death-domain, and caspase-dependent) PCD may occur more commonly in malignant hematopoietic cells (2, 3). Although the term has been applied to a receptor-dependent form of PCD (4), for our purposes, we would like to characterize a form of type 2 PCD that is intrinsic, autophagic, and mitochondria dependent, often expressed by epithelial-derived cells from solid tumors after exposure to chemotherapy or ionizing radiation (5, 6). The ability to respond to an agent with PCD requires the development during and after clonal evolution of components underlying the relevant signal transduction pathways. The absence of, for example, a functional FAS/FAS ligand (CD95/APO 1) receptor-signaling system in Panc-1 cells provides a basis for their lack of response to that ligand (7). In both of what may be the major forms of cellular suicide, an important role for participation of mitochondria in their expression is widely recognized (8–10).

The extent of "crosstalk" between the major modes of PCD, in response to agents inducing their activation, is an additional consideration (6, 10). Mitomycin C induces apoptosis in gastric cancer cells by a caspase-dependent (caspase 3 and 8) mechanism and by a FAS/CD95-independent, caspase 9-dependent mechanism (11). Whether apoptosis is due to independent activation of these pathways, possibly including the effect of one on the other is not

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Received June 25, 2002.
Accepted April 3, 2003.

1535-3702/03/2288-0915$15.00

certain. Although PCD associated with apoptosis can occur in the virtual absence of functional mitochondria (12, 13), the cellular response to many forms of "stress" includes perturbations of mitochondrial function contributing to cellular suicide and necrosis (14–16). Finally, the extent to which ATP continues to be synthesized has been implicated in defining a boundary condition between PCD and overt necrosis (17).

In addition to and perhaps for some examples included within what are believed to be two major categories of cellular suicide, there are a number of variants. These include multiple responses by the same type of cell to different inciting agents (18), other forms that have been termed "parapoptosis" (19), contributions from an autophagosomal-lysosomal mechanism (20–22), a ubiquitin-proteosome pathway (23), caspase 12-dependent responses by the endoplasmic reticulum to "stress" (24), and non-caspase-dependent forms of PCD (25–29), some of which tend to merge with overt necrosis. Delineating differences between the major identified PCD pathways or their variants, how they would interact were several activated simultaneously (11, 28), and their mutual effect on residual clonogenicity were reasons for examining the consequences of culturing panc-1 cells with MK 886 and/or act D.

Act D activates a type 1 PCD in panc-1 human pancreatic cancer cells (30), whereas MK 886, a 5-lipoxygenase inhibitor that at micromolar concentrations induces oxidative stress (31, 32) and probably other potential eicosanoid interactions (33), activates a form of "type 2" PCD, as defined previously (34). Combined culture with both agents could provide insight regarding interactions between these cell suicide pathways.

What effect on proliferation and the induction of PCD pathways would be expected if simultaneous activation of both major forms of cellular suicide occurred? This could result in synergism, additivity, a combination of augmentation and inhibition (potentially a "null" result), or direct interference. What is the effect on PCD induced by more than one agent, considered singly and in combination, on the clonogenicity of surviving cells (29, 35–38)? The experiments that follow address some of these questions, and provisional interpretations are discussed.

Materials and Methods

Cell Culture. Panc-1 cells originally obtained from the ATTC (Rockville, MD) were cultured either in 96-well microtiter plates or 25-cm^2 flasks at 37°C and 5% CO_2 for the times indicated. The RPMI 1640 medium contained 5% or 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 μg/ml streptomycin, and 25 m*M* HEPES. After attachment of cells, drugs were added after 6 hr (cells in early cycle) and culture continued for the times indicated. Media containing detached cells was collected and attached cells were released with 0.25% trypsin-0.03% EDTA and were combined with the media or not, as indicated. Viability was determined with trypan blue and a hemocytometer or with a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay according to the instructions provided with the commercial kit (Promega, Madison, WI.). Act D was prepared as a stock solution of 1 mg/ml (0.80 m*M*) in distilled water and MK 886 as a 40 m*M* stock in dimethyl sulfoxide (DMSO). Controls contained an identical concentration of the DMSO vehicle, 0.1% maximum, previously shown to have no effect on cell numbers (34).

Morphologic Procedures. Centrifuged pellets of detached cells washed with buffer were prepared for conventional transmission electron microscopy by fixation in 2.5% glutaraldehyde, pH 7.4, containing 4% sucrose, post-fixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in epon-araldite. Grids were stained with Reynold's lead and uranyl acetate and sections were examined with a 200 CX microscope (Siemens, New York, NY). The sections were also examined and photographed by light microscopy.

DNA Laddering. DNA was isolated with a DNA isolation kit (Easy-DNA kit; Invitrogen, Carlsbad, CA). It was found that continued incubation of detached cells at 37°C led to extensive nonspecific degradation of DNA that obscured laddering, when present. Detached cells were lysed and treated with the buffers provided. After addition of chloroform, DNA was precipitated from the aqueous layer with ethanol and was centrifuged at 4°C. Air-dried pellets were dissolved in Tris-EDTA buffer, incubated with 40 μg/ml DNase-free RNase at 37°C for 30 min, and stored at 4°C. Two- to 5-μg samples were electrophoresed in a mini-sub (Bio-Rad, Hercules, CA) or in DNA electrophoresis chambers (Gibco, Gaithersburg, MD) at 70 to 90 v for 1 to 2 hr on 1% agarose gels containing 0.5 μg/ml ethidium bromide or SYBR green dye at the same concentration.

Nucleosome Assay. PCD was induced by culture for 48 hr with 40 μ*M* MK 886 {3-[t-(4-chlorobenzyl-3-butyl) thiol-5-isopropylindol-2-yl]-2,3-dimethyl propanoic acid} or with 100 ng/ml act D; cells were detached, lysis buffer, sample diluent, and phenylmethylsulfonyl fluoride (PMSF) were added, and aliquots were frozen at –20°C for 18 hr. Following the instructions provided in the commercial kit (Nucleosome ELISA; Calbiochem, La Jolla, CA), 100 μl of diluted samples was added to each microtiter well, detector antibody was added, and the samples were incubated at room temperature for 1 hr and were washed three times with wash buffer. Diluted streptavidin conjugate was added, samples were incubated at room temperature for 30 min, and they were washed twice with wash buffer. Wells flooded with distilled water, and 100 μl of substrate solution was added. The samples were incubated in the dark for 30 min, 1200 μl of stop-solution was added, and plates were read on a spectrophotometric plate reader at 450 nm in a single wavelength machine within 30 min of adding the stop solution.

Assay of Multiple Caspases. Cells cultured with MK 886 or act D for 48 hr were adjusted to 1 million per

milliliter and 300 μl was added to the working dilution of FAM-VAD-FMK inhibitors, incubated for 1 hr at 37°C under 5% CO_2, washed several times in working dilution buffer, and analyzed by flow cytometry with an argon ion laser at 488 nm, according to the instructions supplied with the kit (CaspaTag fluorescein caspase [VAD] activity kit; Intergen, Purchaseville, NY). The kit is designed to detect activity from caspases 1 through 9 with the use of fluorescently labeled differential inhibitors.

Flow Cytometry. For cell cycle analysis, cells of interest were stained with 1% propidium iodide (1 μg/ml) and fluorescence was determined by flow cytometry on an XL machine (Coulter, Miami, FL) at the University of Illinois Flow Cytometry Laboratory.

Clonogenic Assay. Single-cell suspensions of 1000 trypan-negative panc-1 cells previously cultured with 40 μM MK 886 and/or 100 ng/ml act D for 48 hr and detached with trypsin were plated in T25 flasks with 5 ml of medium containing 10% fetal bovine serum; cells were allowed to attach for 6 hr, the medium was changed, and culture continued for 18 days until colony counts had stabilized. Fifty grouped cells were considered to represent a colony.

Chemicals, including MK 886 and act D, and tissue culture reagents and fluorescent probes were purchased from Sigma Biochemicals (St. Louis, MO); Calbiochem; Gibco-BRL, and Molecular Probes (Eugene, OR), and kits from the sources cited.

Results

Cell Proliferation and Survival. Table I presents the effect on viability of different concentrations of MK 886 and act D in various combinations on initially cycling panc-1 cells after 48 hr of subsequent culture, as judged by the extent of cellular metabolism with an MTT assay. At 40 μM, MK 886 reduced viability by some 30% compared with a 60% reduction by Act D at 100 ng/ml (0.083 μM). In combination, act D dominated the reduction in viability at concentrations of 50 and 100 ng/ml whether pairing the lowest concentration of one with the highest concentration of MK 886 or increasing the concentration of both agents. At intermediate concentrations of 30 μM MK 886 and 50 ng/ml of act D, reduced cell viability was essentially equal to the sum of their individual reductions. As long as neither agent completely dominated the inhibition of proliferation, depending upon the concentration used, an approximately additive contribution from both agents to the reduction in cell viability could be demonstrated.

Table I. Panc-1 Cells Cultured with Different Concentrations of MK 886 or Act D, either Alone or in Their Different Combinations, Compared with Controls = 1.0

MK 886 (μ*M*)	
10	0.95 ± 0.03
20	0.90 ± 0.07
30	0.75 ± 0.12
40	0.64 ± 0.11
Act D (ng/ml)	
1	0.90 ± 0.05
5	0.73 ± 0.08
50	0.48 ± 0.08
100	0.38 ± 0.05
MK 886 μ*M*/Act D ng/ml	
10/100	0.38 ± 0.08
20/50	0.42 ± 0.06
30/5	0.64 ± 0.05
40/1	0.62 ± 0.09
MK 886 μ*M*/Act D ng/ml	
10/1	0.85 ± 0.09
20/5	0.70 ± 0.04
30/50	0.31 ± 0.02[a]
40/100	0.24 ± 0.04[a]

Note. $n = 4$, with three data points per concentration. [a]$P < 0.01$.

When the direct effect of these agents on proliferation of cycling cells was determined, neither was markedly more effective, nor were they additive, at least not under the culture conditions used (Table II). The MTT metabolic assay was performed in 96-well microtiter plates and actual measurement of viable cell numbers was performed after culture in 25-cm^2 flasks. Although act D reduced cell viability to a greater extent (Table I), MK 886 in the presence or absence of act D was marginally more effective in reducing proliferation, as measured by viable cell numbers (Table II).

Morphological Studies. The next question addressed was to identify the type and extent of PCD these agents induced. Light microscopy of control Panc-1 cells (Fig. 1) are described elsewhere (34).

Act D-treated samples included small dark cells (Fig. 2) and cells with the classic "teardrop" nuclear DNA, in addition to marginated chromatin, cytoplasmic vacuolization without evidence of secretory product, and some darkly staining ovid bodies that may represent apoptotic bodies. A limited number of act-D-treated cells manifest classic patterns of type-1 PCD. Figure 2 illustrates the type-1 processes with cytoplasmic organelles coalesced and segregated into distinct membrane-bound regions (i.e., microbodies). Extensive swelling of the endoplasmic reticulum-cisternae was seen, with increased numbers of lamellar bodies and distorted organelles (e.g., mitochondria; Fig. 3A). Viewing the cell's cytoskeletal network at higher mag-

Table II. Viable Cell Numbers after Culture with 40 μ*M* MK 886 and/or 100 ng/ml Act D for 48 hr

Control ($\times 10^{-3}$)	MK 886	Act D	MK + Act D
44	13	12	7
46	16	23	18
39	15	16	12
30	10	13	14
39 ± 7.1	13.5 ± 2.6	16 ± 4.9	13 ± 4.6

Note. All numbers were determined by conventional hemocytometer counting.

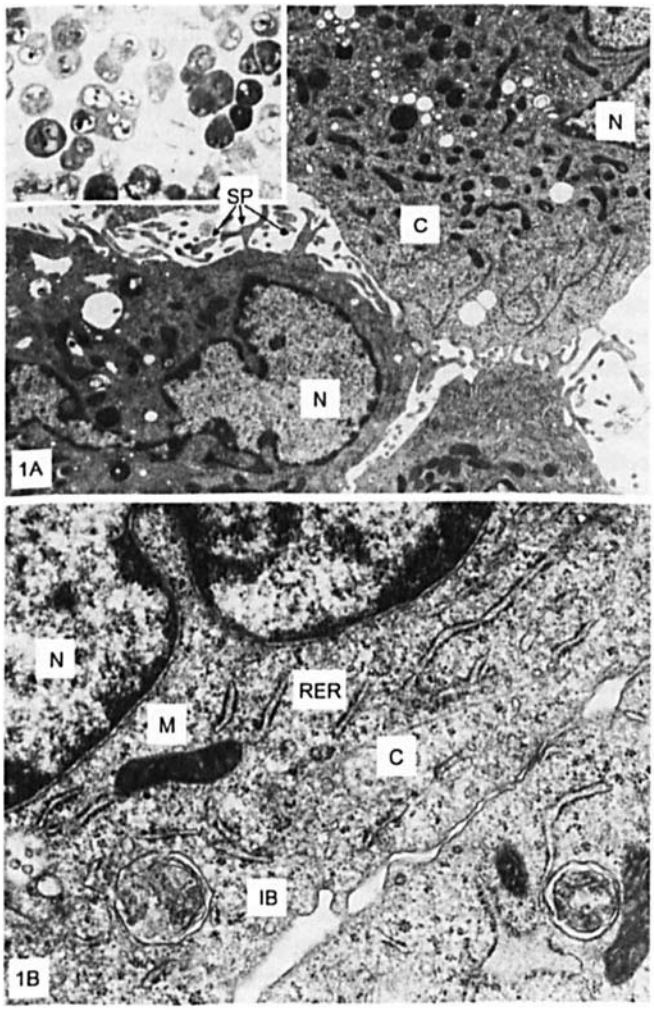

Figure 1. Electron and light microscopy of sections from control Panc-1 cells. Low- and high-power electron micrographs (A and B), along with a low power light micrograph (A, inset) illustrate the overall structural features of control cells. Note the polymorphic nuclei (N) with a large central area of euchromatin and peripheral band of heterochromatin. The cytoplasmic/nuclear ratio is generally high, as the voluminous cytoplasm is filled with dense granules, inclusion bodies (B), numerous mitochondria (M), strands of rough endoplasmic reticulum (RER), ribosomes, matrix fibers, and ground substance. The cell periphery is generally active as evidenced by the presence of numerous microvilli and various sized protrusions (SP). Magnifications of A, A, inset, and B are 5,000×, 400×, and 20,000×, respectively.

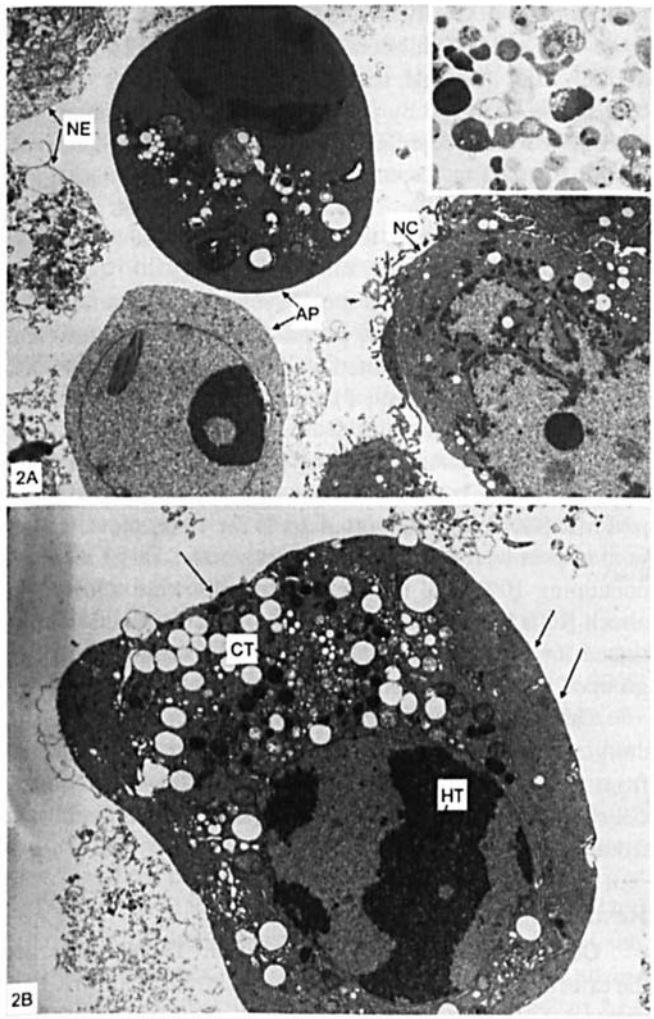

Figure 2. Electron and microscopic sections from Act D-treated cells (200 ng/ml) for 48 hr. Low- and high-power electron micrographs (A and B) and a light micrograph (A, inset) illustrate the overall structural changes noted in Panc-1 cells treated for 48 hr with act D (200 ng/ml). Note the presence of small, round cells with apoptotic features (AP), alongside cells in advanced stages of necrosis (NE) and cells of nearly normal structural features (NC). Micrograph B at a higher magnification shows Act D-treated apoptotic cells. Note the massive patches of heterochromatin (HT) within the nucleus, the consolidation and segregation of cytoplasmic organelles (CT), and the smoothness of the cell's surface, i.e., lack of surface microprojections (arrows). Magnifications of A, A, inset, and B are 3,300×, 400×, and 20,000×, respectively.

nification revealed the presence of increased number and order of microfibrillar bundles (Fig. 3B). In concert with these cytoplasmic changes, the nuclei of the act D-treated panc-1 cells show typical increases in patches of heterochromatin, the larger of which often serves as a hallmark of the apoptotic process within cells.

Light microscopic studies of cells cultured with MK 886 revealed highly segmented nuclei with regions of marginated heterochromatin, some cells with a washed-out nucleoplasm, extensive cytoplasmic vacuolation, a distended endoplasmic reticulum lined with cisternae filled with secretory product, and mitochondria that included small dense to large swollen forms (Fig. 4). Phagolysosomal-like inclusions could be seen in some sections, as were occasional necrotic cells. The ultrastructure of MK-treated cells appears to lack many of the changes elicited by act D. For example, the panc-1 nuclei after MK treatments do not show the large patches of heterochromatin or large-scale nuclear fragmentation. However, the number of nuclear inclusion bodies appears to be increased (Fig. 4A). The cytoplasm of these cells also includes a number of distinctive features: first, lesser coalescence of cytoplasmic organelles; second, the endoplasmic reticulum-cisternae, near the cell's periphery, appeared greatly expanded without clear signs of cytoplasmic segregation and fragmentation; and third, the fine structure of the cytoskeleton appears distinctly different in

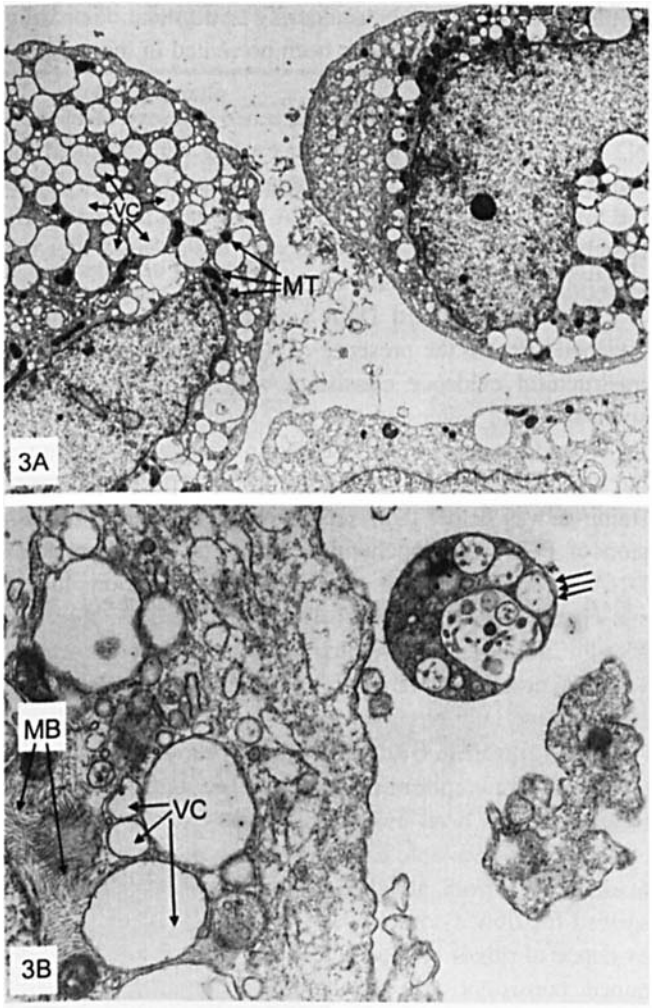

Figure 3. Electron microscopy of sections from Act D-treated Panc-1 cells. The low- and high-power micrographs (A and B) illustrate the overall structural changes noted in cells cultured with 200 ng/ml of Act D for 48 hr. Note the vacuolation of the cell's cytoplasm (VC) due to swelling of the ER cisternae, and the dispersed laminar bodies and distorted mitochondria (MT). In B, the Act D treatment-related increase in microfibrillar bodies (MB) is illustrated, along with the presence of extruded apoptotic microbodies (arrows). Magnifications in A and B are 3,300× and 20,000×, respectively.

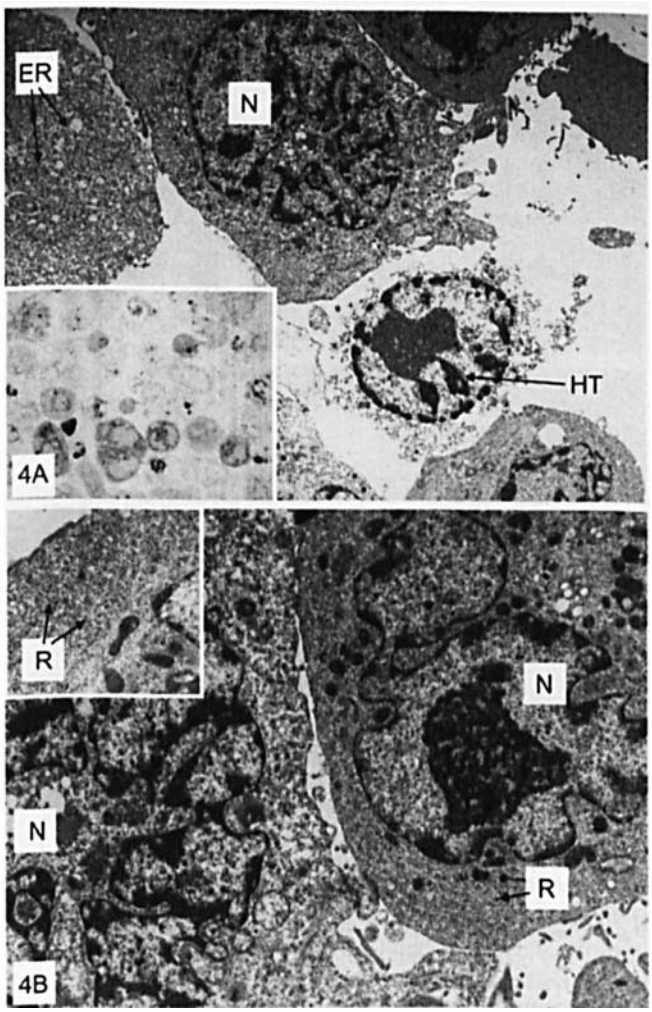

Figure 4. Electron micrographs of sections from MK-886-treated Panc-1 cells. Low- and medium-power electron micrographs (A and B), along with a higher power micrograph (B, inset) illustrate the overall structural changes in panc-1 cells treated for 48 hr with 40 µg/ml of MK 886. Note in nuclei (N) of cells pleomorphic shapes and slightly increased banding of heterochromatin (HT) at the nuclear periphery, and in the cytoplasm, extended ER cisternae and increase in subplasmalemmal space (B, inset, arrows). Magnification of A is 2000×, and of B, insert and B, 5000×.

that the microfibrillar network is less pronounced (Fig. 4B). The density of the fibril mesh, the length of the composite fibrils, and the size and frequency of fibril bundles all appear to be reduced in the MK-treated cells compared with act D- or DMSO vehicle-treated cells. When the process is more advanced, there is considerable cytoplasmic vacuolization, distension of the endoplasmic reticulum with secretory product, and aberrant mitochondria, including small dense to large, swollen forms (not shown; see Fig. 4 in Ref. 34).

In addition, MK 886 elicited a subplasmalemma band of linear-arrayed microfibrils, seemingly in concert with the generalized loss of rough endoplasmic reticulum and concentration of polyribosomes (Fig. 4B). Neither the subplasmalemmal band or the loss of rough endoplasmic reticulum (RER) or of polysomes was seen in the act D-treated cells, which did express a swollen and vesiculated RER (Fig. 3, A and B). In combined treatments, necrotic cells predominated, accompanied by act D or MK 886-type responses in non-necrotic cells (Fig. 5). A unique nuclear response of dissolution of the nuclear structure occurred in dual-treated cells.

Thus, the conclusion after these studies was that ultrastructural changes induced by the separate treatments of panc-1 cells with act D or MK 886 resulted in cells with distinct forms of PCD that could be termed "type 1" (act D) and "type 2" (MK 886; Table III).

DNA Laddering. Results from DNA agarose electrophoresis were found to depend upon the length of time detached cells were allowed to continue at 37°C. In detached cells that were not removed for 48 hr, DNA laddering was marginally present in act-D and act D plus MK

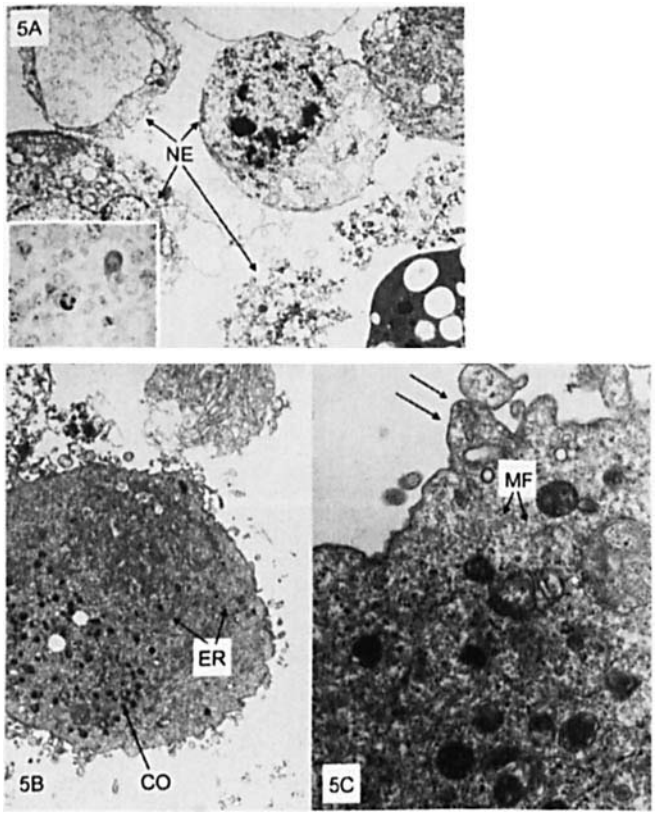

Figure 5. Electron and light microscopy of sections from Act D- plus MK 886-treated Panc-1 cells. Low-power light and electron micrographs (A, A, inset, and B) along with a higher-power electron micrograph (C) illustrate the overall structural changes noted above in Panc-1 cells treated for 48 hr with either drug alone, i.e., in this case, Act D (100 ng/ml) and MK 886 (40 μ*M*). Note the large number of necrotic cells following the combined drug treatment (A and A, inset). The few remaining intact cells show features common to both type 1 and 2 PCD. A shows a portion of small, dark apoptotic cell, whereas B and C demonstrate cytoplasm of a cell with combined apoptosis-like (PCD type 1) coalescence type cytoplasmic organelles (CO), but with expanded ER cisternae (ER), evidence of microfibrillar arrays (perinuclear, granular, and subplasmmalemmal forms; MF), and normal to blunted surface microprojections (arrows; PCD type 2). Magnification of A, B, and C are 2000×, 2600×, and 5000× respectively.

886-treated cells, but was not observed in MK 886-treated cells (Fig. 6A). These samples were accompanied by considerable nonspecific, presumably single-stranded DNA degradation, rendering their detection and documentation difficult. The results were altered by limiting how long detached cells remained at 37°C, 24 hr or less and with the use of the more sensitive SYBR dye for detection of low-molecular-weight DNA (Fig. 6B). Laddering was clearly present in cells exposed to act D and was marginally present at a much lower concentration in cells cultured with MK 886 alone. No laddering or degradation of DNA from attached cells was detected with either ethidium bromide or SYBR green dye (neither shown). In these studies, the response of detached panc-1 cells to the agents included a mixture initially of PCD with necrosis developing during continued culture, the net result depending upon the time of sampling and the sensitivity of the dye used. Other studies of the dependence of DNA laddering or degradation on drug concentration and time have been presented in the publications cited (30, 34).

Flow Cytometry. We examined this extent of sub-N2 DNA with flow cytometry of cycling cells attached for 6 hr before addition of drugs. Figure 7 and Table IV depict the limited extent of sub-N2 DNA present in attached cells remaining after 48 hr of culture with the agents under the conditions described in the legends.

The lack of robust DNA laddering or degradation of attached cells, in the presence of light microscopic and ultrastructural evidence consistent with cellular suicide in some of them, suggested that the extent of early, minimal DNA laddering or DNA degradation, analyzed by agarose gel electrophoresis and ethidium bromide or SYBR green staining, was below their sensitivity of detection. Expression of PCD with much more complete development of DNA laddering and the less specific degradation due to subsequent necrosis occurs after cells detach. Pancreatic Mia-PaCa-2 cancer cells cultured with eicosapentaenoic or with polyunsaturated fatty acids acid exhibit DNA laddering (39, 40) (the latter with some difficulty).

Nucleosome Studies. The appearance of nucleosomes in the cytoplasm of cells undergoing type 1 "apoptotic" PCD has been used to assess its extent. We used a commercially available ELISA kit to assess the presence of nucleosomes from attached cycling panc-1 cells as described for flow cytometry. As presented in Table V, little evidence of oligo- or mononucleosomal formation was obtained, consistent with the inability to detect laddering or degradation by PI or SYBR staining of DNA from attached act D or MK 886-cultured cells. For comparison, a panel of detached MK 886-treated panc-1 cells is presented in Figure 1 of Reference 34.

Caspase Assays. To further differentiate the form of PCD induced by act D from that due to MK 886, we examined caspase activity in detached cells originally attached for 6 hr before addition of the drugs and cultured subsequently for 48 hr, as described in the legend to Figure 7. The commercial kit used detects the presence of caspases 1 through 9. Act D-treated cells were caspase positive (Fig. 8A, control compared with F), and MK 886-treated cells were negative (Fig. 8, A compared with D). After combined culture with both agents, the extent of caspase activation due to act D was reduced by at least 90% (Fig. 8, F, act D compared with H, act D + MK 886).

Clonogenic Assays. It was suggested that therapeutic induction of apoptosis and PCD in cells from cancers may or may not include those that were potentially clonogenic (29, 35–38). If significant numbers of clonogenic cells are not included among those undergoing suicide, controlling or even curing a malignancy should be reduced. We measured the extent to which clonogenic cells survived induction of either form of PCD in cycling cells exposed to

Table III. Features Characterizing Cells Cultured with MK 886, Act D, or Their Combination, as Described in Figures 2 through 5

No. attribute	+MK	+Act D	MK + Act D
Cytoplasmic			
1. Microfibrillar network[a]	Minimized	Variable bundles	Variable bundles
2. Cytoplasmic organelles	Normal distribution	Regionalized	Regionalized
3. Lamellar bodies	Increased nos.	Increased AC[b]	Increased nos.
4. Mitochondria	Var. nos, distorted	High nos., distorted	Few, high nos., distorted
5. Cisternae network	Extensive chanellin	Extensive swelling	Extensive swelling
6. RER cisternal network	Swollen, extensive	Swollen	Stacked arrays
7. Polysomes	Reduced	Normal	Variable
8. Ribosomes (free)	Increased nos.	Few/normal nos.	Few/normal nos.
Nuclear			
1. Chromatin condensates	Normal	Increased	Increased
2. Fragmented nuclei	Normal	Increased	Increased
3. Nuclear bodies	Increased nos.	Few/normal nos.	Few/normal nos.

[a] Cytoskeletal elements + fibrils.
[b] AC, type 1 apoptotic cells.

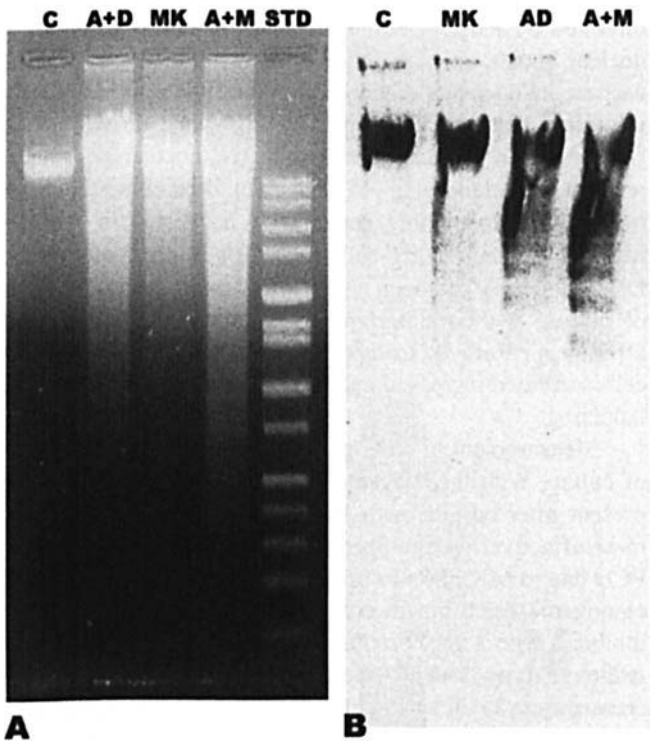

Figure 6. DNA extracted from "spontaneously" released cells that had been cultured alone or in combination with Act D (100 ng/ml) and/or 40 μ*M* MK 886. (A) DNA was electrophoresed on agarose in an apparatus (Bio-Rad) and stained with propidium iodide, or (B) in an apparatus (Gibco-BRL) and stained with the more sensitive SYBR green dye. C, control; STD, molecular weight standard.

conditions previously described (Fig. 9 and Table VI). Viable MK 886-treated cells were associated with a greater number of residual clonogenic cells, defined as those groups of 50 or more cells, compared with the same number of viable, act D-treated cells. Viable cells cultured with both agents yielded an increased number of colonies compared with the least numerous number associated with either agent alone, in these studies, act D.

Discussion

Panc-1 cells, susceptible to induction by two mechanistically distinct agents of what can be defined as a type 1 and a type 2 PCD, provide an experimental system with which to examine their mutual effect on cell proliferation, PCD, and clonogenicity. Act D or MK 886 inhibit panc-1 cell proliferation and initiate distinct forms of cell death, characterized by differences in ultrastructure and biochemistry. The combined effect of act D and MK 886 on cell "growth" was approximately additive, as estimated with an MTT assay. This assay reflects the viability of remaining cells and is not necessarily a direct measure of their number. Table I represents a composite of these features. Cell counts after culture in flasks with the single agents yielded a comparable reduction in cell numbers; the two assays measure different end-points that in some studies may be closely correlated.

As described in the morphologic studies summarized in Table III, cell death induced by these agents was dissimilar, with distinct features characterizing either form consistent with identifying an act D-induced type 1 and an MK 886-induced type 2. The presence of necrosis, especially prevalent in combined cultures, is believed to result from direct cytotoxicity, continued culture at 37°C for 48 hr, and the lack of phagocytosis of affected cells occurring normally *in vivo*. The extent of laddering of DNA from act D alone or in combined culture was considerably greater than that marginally detected from MK 886-treated cells. We believe that it was not a primary feature of MK 886-induced death in these cycling cells and may reflect some form of "crosstalk" between type 2 and 1 PCD, as defined earlier. The morphology of nuclear chromatin and the lack of caspase activity in MK 886-treated cells are consistent with this view.

To distinguish between events occurring in cells released into the media from attached cycling cells, we detached the latter with trypsin. These cells exhibited little low-molecular DNA detected with flow cytometry. Culture with either agent alone increased this DNA, and when com-

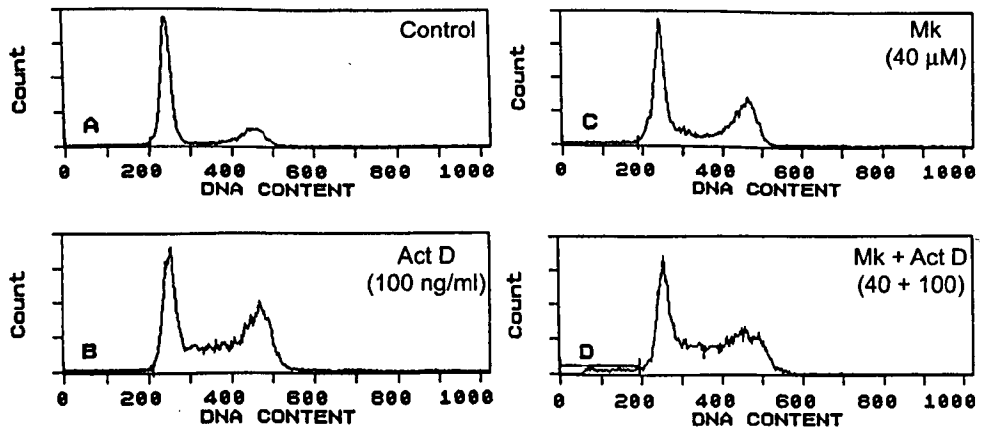

Figure 7. (A-D) Flow cytometry of attached Panc-1 cells detached with trypsin and cultured for 48 hr with 40 μ*M* MK 886 and/or 100 ng/ml Act D. A, control; B, 100 ng/ml Act D; C, 40 μ*M* MK 886; D, 100 ng/ml Act D + 40 μ*M* Mk 886. Little sub-N2 DNA was present and there was no evidence for more than its approximate additivity in samples cultured with both agents (D). The standard deviation of measurements in these three experiments varied from 20% to 30%.

Table IV. Percentage of Sub-N2 DNA Detected by Flow Cytometry in Samples from Attached Cells Cultured with Act D and/or MK 886

	Experiment no.		
	1	2	3
Control	0.3	0.0	1.4
MK 886	1.0	0.7	2.0
Act D	2.5	2.2	3.4
MK + Act D	3.9 (3.5)	3.6 (2.9)	4.7 (5.4)

Note. The standard deviations varied by 20% to 30%. Number in parenthesis represents the individual sums of MK (40 μ*M*) + Act D (100 ng/ml) to be compared with the combined measured values.

bined, the measured amounts were essentially additive. Nucleosome assays performed on attached cells under comparable conditions yielded similar conclusions. It was after cells detached that they developed the observed changes in DNA distribution; DNA laddering in act D or in act D plus MK 886-treated cycling cells and marginal laddering in cells cultured with MK 886 alone. If detached culls continued to be cultured from 24 to 48 hr, these changes were rendered difficult to observe due to extensive random DNA degradation. Examination of any "crosstalk" between these several forms of PCD and whether MK-866-induced cells express DNA laddering as an integral component of their cellular suicide is a subject for further study. "Anoikis," a form of apoptosis after detachment of primary human intestinal epithelial cells has been described in which caspase 8 is not a major component and the release of cytochrome C is delayed (41).

Several reports contribute to understanding differences in the nuclear morphology of types 1 and 2 PCD (42, 43). Activation of apoptosis protease activating factor-1 (APAF-1), caspase 3, caspase-activated Dnase (CAD), the latter inhibited by ICAD yields the classic nuclear morphology, nuclear bodies, and DNA laddering of type 1 form (44). A caspase-independent response that depends upon apoptosis inhibitory factor (AIF) results in large-scale nuclear DNA fragmentation and condensation at the nuclear periphery, without DNA laddering (45) This would be consistent with our inability to reliably detect DNA laddering in cycling, MK 886-treated panc-1 cells (34). In a number of studies, DNA laddering has been successfully examined after 48 hr of culture without interference from randomly degraded DNA (e.g., Ref. 3). Compared with those studies, panc-1 cells exhibited increased necrosis, obscuring clearly defined laddering.

Measurement of clonogenic cells remaining after 24 hr of culture with these agents demonstrated that more were present after culture with both drugs, compared with the most effective anticlonogenic agent, act D alone. Type 2 PCD due to MK 886 was less effective in reducing residual clonogenic cells, but in combination with act D, partially inhibited type 1 act D-induced cell suicide (Figs. 8 and 9). Although type 1 act D-induced cell death may have increased type 2 cell suicide, the obverse also appears to have occurred, with a net increase in surviving clonogenic cells after the combined culture. Suppression by MK 886 of caspase expression or activity in cells cultured with act-D could have contributed to the emergence of additional clo-

Table V. Measurement of Nucleosome Concentration in the Attached Cells Cultured with MK 886 or Act D as in Figure 7, Using an ELISA Assay

	Experiment		
	1	2	3
Control	0.092 ± 0.005	0.071 ± 0.002	0.068 ± 0.002
MK	0.091 ± 0.007	0.079 ± 0.005	0.068 ± 0.001
Act D	0.199 ± 0.018	0.210 ± 0.015	0.197 ± 0.010
MK + Act D	0.299 ± 0.005 (0.29)	0.360 ± 0.006 (0.29)	0.269 ± 0.017 (0.265)

Note. Values represent duplicate optical density measurement. Standard curves are not shown. MK 886, 40 μ*M;* Act D, 100 ng/ml. Number in parenthesis is the sum of MK and Act D determined separately to be compared with the measured combined values.

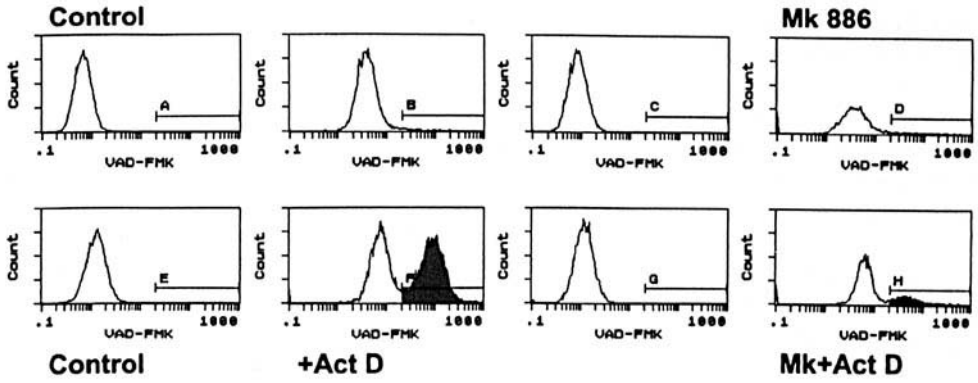

Figure 8. Representative examples of caspase activity in the Panc-1 cells cultured alone or together with either 100 ng/ml Act D or 40 μ*M* MK 886 or combined and measured by flow cytometry. A, B, C, E, and G are different controls. Shaded areas in F (Act D) and H (Act D + MK 886) represent caspase activity; D, (MK) caspase activity is absent; H, reduced compared with F.

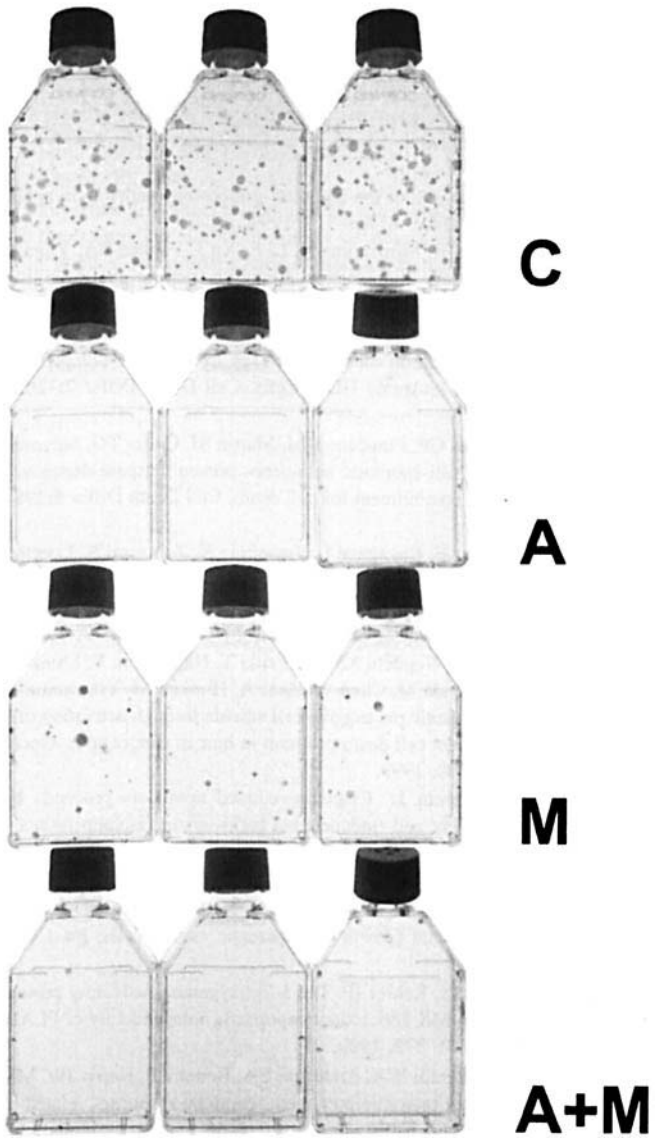

Figure 9. Clonogenicity assays of viable cells that had been cultured with MK 886, Act D, or their combination as Described in Figure 8 and Table IV. Assays were performed as described in "Materials and Methods." Experiment 3 is depicted.

nogenic cells compared with act D alone. Interactions between both activated pathways due to "crosstalk" (46) could have important implications for the outcome of "therapy."

Table VI. Clonogenicity Assays of Cells Cultured with 40 μ*M* MK 886 and/or 100 ng/ml Act D as Described in "Methods" and "Results" Section

	Experiment		
	1	2	3
Control	86 ± 17	105 ± 8.7	92 ± 7.2
MK 886	48 ± 7.9	10 ± 1.5	25 ± 5.5
Act D	2 ± 0	7 ± 2.6	1 ± 1.5
MK + Act D	15 ± 2.5	27 ± 3.8	4 ± 2.3

The inability to activate caspase 3 in phorbol ester-resistant U937 cells resulted in an apoptotic-resistant phenotype devoid of DNA laddering (47). The mechanism of MK 886-induced suppression of act D-induced caspase activity, whether due to events of transcription or translation, is not established.

After castration, involution of rat prostate cells involves both type 1 and 2 forms of PCD (1). MK 886, a 5-lipoxygenase inhibitor at nanomolar concentrations, at micromolar concentrations induces oxidative stress and a classic type 1 apoptotic PCD with activation of caspases in U937 monoblastoid cells (Ref. 48 and work in progress) and an type 2, autophagic, caspase negative-form of PCD in panc-1 cells (Ref. 34 and this work). The absence of a functional FAS (or related) receptor system or of Bcl-2 in panc-1 cells (7) and their presence in U937 cells may contribute to these differences. When HL-60 Bcl 2 was downregulated, a non-caspase-dependent form of PCD resulted (25). Oncogenic RAS activated a caspase-independent cell death program in human glioma and gastric carcinoma cell lines (28).

If it were generally true that type 2 PCD is strongly dependent upon the mitochondrial mass (16), that generally both diminish with cancer cell clonal evolution, and that PCD is therefore less effective in reducing residual clonogenic cells, this association could provide an additional reason why treatment of solid epithelial-derived cancers with forms of therapy inducing it is often less successful than the initial type 1 responses of hematopoietic cells. Possibly, pretreatment assessment of epithelial-derived cancer cell cytochrome C content would allow an estimate of likely "global" tumor response to therapies primarily initiating nontype 1 responses.

In most studies, the modes of death of cells after different polytherapies, the interactions of these therapies, and the resultant defects in cell suicide pathways as they may impact on clonogenicity are unclear (49). In clinical studies using combinations of chemotherapy, radiation, or biological response modifiers, coincident or nearly simultaneous activation of major cellular suicide pathways or their variants may have inadvertently occurred. It will be a challenge to distinguish useful from deleterious interactions between these various expressions of PCD, necrosis, and reproductive death as more specific, targeted therapies become available.

We thank the Weinberg Foundation, the Seidel Family Trust, and the Wadsworth Memorial Trust for their continued support.

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