Regulation of Apoptosis by Low Serum in Cells of Different Stages of Neoplastic Progression: Enhanced Susceptibility after Loss of a Senescence Gene and Decreased Susceptibility after Loss of a Tumor Suppressor Gene

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ABSTRACT

A cell culture model system has been used to study the susceptibility of cells to apoptotic cell death during different stages of neoplastic progression. This system consists of normal diploid Syrian hamster embryo (SHE) cells, two preneoplastic cell lines [tumor suppressor stage I (sup') and non-tumor suppressor stage II (sup'')], and hamster tumor cell lines. Stage I preneoplastic cells are nontumorigenic immortal clones that suppress tumorigenicity when hybridized to tumor cells, whereas stage II cells have lost the ability to suppress tumorigenicity in cell hybrids. We refer to these two types of preneoplastic cells as sup' and sup'', respectively.

Neoplastic progression is generally associated with cellular alterations in growth factor responsiveness. Therefore, to study the regulation of apoptosis in the system described above, cells were cultured in low serum (0.2%) as a means of withdrawing growth factors. In low serum, normal SHE cells were quiescent (labeling index of 0.2%), with little cell death. The sup' cells showed a relatively low labeling index (1.6%) but, in contrast to the normal cells, died at a high rate (55% cell loss after 48 h) by apoptosis, as evidenced by morphology, DNA fragmentation, and in situ end-labeling of fragmented DNA. The apoptotic cells did not go through a replicative cycle while in low serum, implying that apoptosis was initiated in the G0/G1 phase of the cell cycle. The sup'' cell line showed a high labeling index (40%) after 48 h, but cell growth was balanced by cell death that occurred at approximately the same rate. The cells died, however, predominantly by necrosis. The tumor cell lines continued to proliferate in low serum, with high labeling indices (ranging from 27% to 43%) and a low level of apoptotic or necrotic cell death. To determine the relative ability of these cells to survive in vivo, normal SHE cells, sup' cells, and sup'' cells were injected s.c. into nude mice. At 5 or 21 days after injection, the normal cells were retrieved readily from the mice and grew well in culture. In contrast, few sup' cells were retrieved 5 days after injection and no viable cells were retrieved after 21 days. Sup'' cells were not retrieved at either the 5-day or 21-day harvest, and histological examinations of the sites of injection showed the presence of macrophages, eosinophils, and neutrophils, indicating an inflammatory response associated with necrotic cell death. These results indicate that normal SHE cells, although nontumorigenic, were quiescent in vivo and in vitro, whereas sup' and sup'' cells died in vivo and in vitro. These findings suggest that in vivo conditions are similar to limited serum conditions in culture. Normal cells in low serum undergo growth arrest and do not die. Cells that have lost a senescence gene and are immortal but at an early stage of preneoplastic progression activate apoptosis when placed in low serum. Cells that have lost a tumor suppressor gene and are at a later stage of preneoplastic progression have decreased susceptibility to apoptotic death.

INTRODUCTION

Two distinctly different forms of cell death have been described, apoptosis and necrosis (1, 2). Apoptosis is an active form of cellular suicide that typically involves single cells and results in a variety of ultrastructural changes including cell shrinkage, cytoplasmic membrane blebbing, nuclear fragmentation, and DNA degradation (2). Necrosis is the classical pathological form of cell death that results from noxious injury or trauma. It often affects groups of cells and is usually associated with an inflammatory response. Necrosis is characterized by breakdown of the plasma membrane, loss of ion transport, cell swelling, and lysis. Apoptosis can occur under normal physiological circumstances such as embryonic development, metamorphosis, and hormone-dependent atrophy of tissues, thus providing a method for selective removal of cells from a tissue without evoking an inflammatory response (1, 2). Apoptosis is a mechanism for maintenance of a constant cell number in tissue homeostasis as a counterbalance to cell division. Dysregulation of apoptosis may lead to altered cell number within the tissue and, eventually, to tumor formation and malignancy.

Tumor growth can occur either by disruption of cell cycle arrest, decreased rates of cell death, or both (3, 4). New studies support the idea that the processes are interrelated. For example, the multifunctional tumor suppressor p53 can transduce antiproliferative signals and can also serve as an inducer of apoptosis (5, 6). The proto-oncogene product of the c-myc gene is implicated as serving a dual role in proliferation and regulation of apoptosis (7). Furthermore, the product of the chimeric homeobox gene E2A-PBX1, observed in childhood leukemia, induces proliferation, apoptosis, and malignant lymphomas in transgenic mice (8). The only known example of an oncogene that, when overexpressed, can result in neoplasia without apparent effects on cell proliferation is bcl-2, an inhibitor of apoptotic death (9–11).

Although tumor cells often retain the intrinsic ability to undergo apoptosis under certain conditions, the process of induction by specific signals can be defective (12). A more complete understanding of cellular resistance to apoptotic homeostatic processes will require the elucidation of when and how, during neoplastic progression, tumor cells lose their sensitivity to apoptotic signals. Few studies have been described in which preneoplastic cells rather than tumor cell lines have been used to determine the mechanisms by which cells respond or fail to respond to apoptotic signals. Alterations in these pathways during neoplastic progression can be an early event in carcinogenesis, as evidenced by the studies of Schulte-Hermann and coworkers (13). Rats treated with phenobarbital showed growth stimulation of hepatocytes in the form of hyperplastic foci. Upon withdrawal of phenobarbital the foci were largely eliminated by the process of apoptosis. The reversibility of the effect demonstrated that hyperplastic growth was achieved through inhibition of apoptosis by the tumor promoter at an early stage of neoplastic progression.

An integral component of the studies presented here is the utilization of a cellular system composed of clonal cell lines that are representative of different stages of neoplastic progression. The system provides a method to follow changes in apoptotic regulation in association with other phenotypic alterations identified with tumorigenesis. The cellular system was derived from carcinogen-treated...
SHE² cells (14–16). As depicted in Fig. 1, the system includes a normal diploid cell line (SHE), stage I preneoplastic cell lines, stage II preneoplastic cell lines, and tumor cell lines. Each group has a distinctively different phenotype. The stage I preneoplastic cell line is represented by cells that have lost one or more senescence genes and have become immortal with an indefinite life span, in contrast to the normal SHE cells that senesce after <40 population doublings. Stage I preneoplastic cells retain the ability to suppress tumorigenicity, as determined by cell fusion assays, and are therefore termed sup" I. The stage II cells, which represent a later stage of neoplastic progression, have lost the tumor suppressor phenotype and are termed sup" II. Anchorage-dependent growth regulation has been altered in the sup" II cells, as determined by growth in soft agar in the presence of mitogens (17). Both sup"I and sup"II preneoplastic cells have wild-type p53 and RB genes, indicating that the "sup" gene is another, as yet unidentified, suppressor gene. Candidates for this gene include the putative tumor suppressor genes H19 (18) and TM-1 (19), which are differentially expressed in sup"I but not sup" II cells (20).

During carcinogenesis, cellular changes occur that result in a growth advantage for the neoplastic cells. We determined whether the cellular transitions in the progression towards neoplasia were associated with quantitative variations in the relative proportions of cell proliferation and cell death. The inducer of cell death used in these studies was growth factor reduction produced by culturing the cells in low serum (0.2%). This choice was based on evidence that the loss of senescence can be induced in cytokine-dependent immune cells (21) and apoptosis can be induced in hormone-dependent tumors (22–24). Other studies have shown that as cells become tumorigenic they evolve toward reduced growth factor dependence and increased autonomy from host influences (25).

The second issue addressed in this study concerns whether quiescent cells enter an inappropriate or aberrant DNA replicative phase (S-phase) before apoptosis is initiated. This was investigated by incubating cells with [³H]-TdR for 48 h prior to labeling (26) of apoptotic cells to identify radioactively labeled apoptotic bodies. If the apoptotic bodies were radioactively labeled, i.e., replicated prior to apoptosis when conditions would normally induce a quiescent state, it could be proposed that aberrant DNA replication was the activator of the apoptotic signal, whereas if the apoptotic bodies were not radioactively labeled the data would suggest that the initiation of apoptosis was triggered when neither a G2/M arrest state nor a replicative phase could be successfully achieved. In this case, replication would rescue cells from apoptosis.

Lastly, because we observed differences in growth arrest and survival in low serum between normal and preneoplastic cells in vitro, we were interested in the relative ability of these cells to survive in vivo. Normal and preneoplastic cells, both of which are nontumorigenic, were injected s.c. into nude mice. At various times after injection, the cells were recovered and growth abilities were determined, allowing the in vivo results to be compared to those obtained in vitro.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. Normal diploid SHE cell lines were established as described by Koi and Barrett (16). The preneoplastic stage I (10W and DES4) immortalized cell lines were derived after treatment of normal SHE cells with asbestos (10W) (16) or diethylstilbestrol (DES4) (27). Early passage 10W and DES4 cells retained the ability to suppress tumorigenicity (sup") when cell hybrids with tumor cells were examined. Later passage cell populations were subcloned, and cell variants were isolated that no longer displayed the tumor suppressor phenotype (sup") when hybridized with tumor cells (26). Concomitant with the loss of suppressor gene function was the loss of the dependency for attachment for cell growth, demonstrated by growth in soft agar (17). Tumorogenic cell lines utilized in these studies were 10W2T, DES4T, and BP6T (derived from benzopyrene-treated hamster cells). Cells were maintained in Dulbecco's modified IBR medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin and were incubated at 37°C in 10% CO₂ in air.

Analysis of Growth Arrest and Cell Death in Low Serum Conditions. Analyses of cell number, flow cytometry, DNA fragmentation, in situ end-labeling, electron microscopic morphology, and DNA labeling index were conducted in parallel. Several different sup"I cell lines (three derived from asbestos-treated populations and one from a DES-treated population) and sup" II cell lines (two from asbestos-treated populations and one DES-derived line) were examined and the data recorded to eliminate the possibility that the observations presented are specific to a particular clonal cell line, although the data presented in this paper are results obtained using 10Wsup" (stage I cells) and 10Wsup" (stage II cells). The data presented are an average of at least two independent experiments for each cell line. Cells (5 × 10⁵/100-mm dish) were plated in medium supplemented with 10% serum for 24 h. The monolayer of cells was rinsed twice with CMF-PBS and placed in medium containing 0.2% fetal bovine serum. Analyses were performed at time points of 0, 24, 48, 72, and 96 h. Cells that remained attached to the dish at the respective time points were trypsinized and counted with a Coulter Counter. Flow cytometric analysis was performed on cells isolated from plates by trypsinization combined with detached cells in the medium. The cells were pelleted, washed in CMF-PBS, fixed with methanol:CMF-PBS (65:35, v/v), and stored at 4°C. For flow cytometry analysis, the fixed cells were pelleted and resuspended in CMF-PBS at a final concentration of 1 × 10⁶ cells/ml. The DNA flow

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2 The abbreviations used are: SHE, Syrian hamster embryo; sup", tumor suppressor; sup", non-tumor suppressor; sup"I, tumor suppressor stage I; sup" II, non-tumor suppressor stage II; TdR, thymidine; CMF-PBS, calcium/magnesium-free phosphate-buffered saline; DES, diethylstilbestrol; PBS, phosphate-buffered saline; PALA, N-(phosphonomethyl)-L-aspartate.
cytometric analysis reagent kit (Boehringer Mannheim) was used for final preparation and the cells were analyzed using a Becton-Dickinson FACScan fluorescence-activated cell scanner. Data were analyzed using CellFIT software. Cell viability was assayed by trypan blue exclusion. The cells were grown on microscope slides, stained with 0.4% trypan blue for 5 min, and counted in situ.

Electrophoretic analysis of DNA fragmentation was performed on combined attached and detached cells. Cells were collected, rinsed with PBS, lysed in 50-μl volumes of lysis buffer (10 mM EDTA, 50 mM Tris, pH 8.0, 0.5% sodium lauryl sarcosine, 0.5 mg/ml proteinase K), and then incubated at 50°C for 1 h. RNase A (0.5 mg/ml) was added and lysates were incubated for an additional 1 h. Two phenol extractions (equal volume) were performed, followed by one chloroform extraction. DNA was precipitated with 2 volumes of ice-cold ethanol and incubated at −70°C for at least 2 h. DNA was pelleted by centrifugation at 14,000 rpm for 10 min at 4°C. Pellets were air-dried for 30 min, resuspended in 50 μl of Tris-EDTA, pH 8, and incubated overnight at 4°C. DNA was electrophoresed in a 1% agarose gel in 1X TBE running buffer (0.05 M Tris base, 0.05 M boric acid, 1 mM disodium EDTA) for 1 h at 90 V. Table 1 Growth arrest and apoptosis of Syrian hamster embryo cells in low serum (0.2% fetal bovine serum)

<table>
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<th>Cell line</th>
<th>Time (h)</th>
<th>Labeling index</th>
<th>Apoptotic fragments</th>
<th>[3H]-TdR-labeled fragments</th>
<th>DNA ladders</th>
<th>Cells in G0/G1 (%)</th>
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In situ end-labeling of fragmented DNA was accomplished by adaptation of the TUNEL method described by Gavrieli et al. (26). Cells were seeded on microscope slides, and 24 h prior to fixation [3H]-TdR (1 pCi/ml) was added to the medium. After treatment, slides were fixed with 4% formaldehyde in PBS. In situ end-labeling was performed, the slides were dipped in NTB-2 emulsion, and the slides were developed after 3–5 days. Labeling indices were calculated as the number of radiolabeled cells divided by total cells counted (~500 cells/point). Quantiﬁcation of apoptotic cells was accomplished by counting the number of apoptotic bodies sighted in the microscopic ﬁelds while counting 500 cells for determination of the labeling index.

Electron microscopic analysis of cell morphology was performed on combined attached and detached cells. Cells were pelleted and rinsed in cold 0.1 M piperazine-N,N’-bis(2-ethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, MO). Pellets were covered with 0.7 ml of fixative [2% paraformaldehyde and 2% glutaraldehyde in 0.1 M piperazine-N,N’-bis(2-ethanesulfonic acid)] and refrigerated overnight. Samples were rinsed (0.1 M phosphate buffer, pH 7.4) and refrigerated for 2 days. Thin sections (60–90 nm) were stained with 5% uranyl acetate and 2.7% lead citrate. Sections were examined using a Phillips 400 transmission electron microscope.

**In Vivo Analysis of Cell Survival.** Normal SHE cells, sup^I^ cells, or sup^II^ cells were injected s.c. (6 sites/cell type, i.e., utilizing 3 mice given injections at 2 sites/mouse) into nude mice at 5 × 10^6^ cells/site. After 5 or 21 days, cells were retrieved by sacrificing the animal, opening the injected site, and scraping the area. Retrieved cells were placed in culture for 3 days, at which time a portion of the plates were stained with Giemsa stain. Remaining plates were subcultured and metaphase chromosomes were prepared by the methods described previously (28).

**RESULTS**

**Growth Arrest/Cell Death of Normal, Preneoplastic, and Transformed Cells under Low Serum Conditions.** Cell lines representative of normal, preneoplastic, and neoplastic stages of progres-
sion (SHE, sup*I, sup*II, 10W2T tumor, BP6T tumor, and DES4T tumor cells) were placed in low serum conditions to test the response to withdrawal of growth factors. Calculation of cell survival for normal SHE cells showed an increase in cell number of ~1% during the first 24 h, dropping slightly after 72 h. The estimated total cell loss during 72 h in low serum was 2% (Fig. 2). The majority of cells were arrested in G0/G1, as determined by flow cytometry (Table 1). After a 72-h growth arrest period, the normal cells were restimulated with 10% serum and >90% of the cells entered S-phase (as determined by [3H]-TdR labeling) and divided, which indicated that the low serum arrest was reversible. In contrast to the normal cells, approximately 40% of the sup*I preneoplastic cells were lost within the first 24 h after the cells were placed in low serum conditions and the cell number decreased progressively from 24 h to 72 h (Fig. 2). After 96 h in low serum <15% of the starting sup*I population remained (data not shown). Recoverability of these cells, when replated into 10% serum, was >90%, as assessed by uptake of [3H]-TdR. Importantly, when the recovered population of cells was placed again in low serum, they showed the same rate of death as the original sup*I cells (data not shown). In contrast to the sup*I cells, the number of sup*II preneoplastic cells remained relatively constant, showing an increase of ~2.5% during the first 24 h, increasing to 4% after 48 h, and dropping during the 48–72-h incubation (Fig. 2). Overall cell loss was estimated to be 1.5%. The 10W2T tumor cells showed a 140% increase in cell number in low serum for 48 h. Cell number decreased during the 48–72-h incubation.

Quantitation of the labeling index in low serum was achieved by autoradiographic evaluation of [3H]-TdR incorporation during a 24-h incubation (Table 1). The labeling index for normal SHE cells at
while the flow cytometry data showed a higher percentage of cells arrest in S-phase when placed in PALA (pyrimidine deprivation) (79—83%). Why this increase in labeled cells was observed will be the subject of later studies; however, other studies have shown that SHE cells arrest in S-phase when placed in PALA (pyrimidine deprivation) and the percentage of S-phase-arrested cells diminishes with time. An S-phase-arrested population of cells could be ultimately responsible for the increase in radioactively labeled cells in low serum. S-phase-arrested cells may be re-entering the cell cycle with time, incorporating [3H]-TdR, and then stopping in G1/G0 (note the increased percentage of G1/G0 cells at 72 h). Sup"1 cells showed low labeling indices of 19% at 24 h, 1.6% at 48 h, and 10% at 72 h (similar to normal SHE cells). Sup"II cells showed a relatively high labeling index of 42% at 48 h and 41% at 72 h, as did the tumorigenic cell lines (10W2T, 32% at 48 h; BP6T, 43% at 48 h; DES4T, 27% at 48 h). Flow cytometric analysis of the sup"I cells showed 72% of the cells with a DNA content of <2N (histogram shown in Fig. 3A), which was not observed in treated groups of SHE cells, sup"II cells (Fig. 3B), or tumor cells.

These data taken in toto show that normal SHE cells arrested the cell cycle under serum deprivation conditions, as indicated by the relatively constant cell number over a 72-h period, the accumulation of the cells in G1/G0, and the failure of cells to incorporate [3H]-TdR. In contrast, a large percentage of the sup"I preneoplastic cells died in low serum, as determined by the large decrease in cell number and the appearance of cells with <2N DNA content. Sup"II cells, representative of a later preneoplastic stage of progression, showed a relatively high level of [3H]-TdR incorporation; however, the cell number remained relatively constant. Therefore, the rate of proliferation approximately equaled the rate of cell loss. Tumorigenic cells showed a high labeling index during the 72-h time period, which was similar to the sup"II cells, but, unlike the sup"II cells, an overall increase in cell number was observed, suggesting that cell death was less frequent.

Evidence that, in Low Serum, Sup"I Cells Undergo Apoptosis, Whereas Sup"II Cells Die by the Process of Necrosis. Because cell death was indicated in the two preneoplastic cell lines, sup"I and sup"II, further analyses were done to determine whether the cells were dying by the process of apoptosis or necrosis. The mechanism of cell death was determined by the presence of oligonucleosomal DNA fragmentation, combined with electron microscopic analysis of distinctive morphological changes. Fig. 4 shows the agarose gel prepared from samples taken at the 48-h time point. Normal SHE cells showed no DNA fragmentation, in contrast to the sup"I cells, which showed nucleosomal fragmentation characteristic of apoptosis (also observed in the 24- and 72-h time point; data not shown). Fragmentation was not detected in DNA from sup"II cells at any time examined (0, 24, 48, 72, or 96 h). Electron micrographs from the 48-h treatment groups of sup"I cells showed morphological changes characteristic of apoptotic cells, with condensation of chromatin and dilated cisternae of the rough endoplasmic reticulum (Fig. 5, A and B). Fig. 5C shows a surviving sup"I cell that has engulfed an apoptotic body. Sup"II cells showed a distinctly different morphology, characteristic of necrotic death (Fig. 5D). To quantitate necrotic cell death in the sup"II cell line, the cells were stained with a viability stain (trypan blue). The percentages of cells that took up the stain were 0.4% in the 0-h control and approximately 17% (87 of 520) at the 48-h low-serum time point.

In contrast, the normal SHE cells showed 0% at the 0-h time point and 1.1% (5 of 440) after 48 h in low serum. Quantitation of Apoptotic Cell Death. To quantitate the relative number of cells undergoing apoptosis in low serum, the terminal transferase end-labeling method of Gavrieli et al. (26) was used. Fragmented DNA of apoptotic bodies was end-labeled by transferring one or more biotinylated dUTP molecules to free 3'-OH ends. The addition of avidin-conjugated horseradish peroxidase enabled colorimetric staining through use of the horseradish peroxidase substrate 3-aminio-9-ethylcarbazole (red color). Cells undergoing DNA synthesis were radioactively labeled through incorporation of [3H]-TdR. The remaining cells were stained with Wright's stain. By combining these labeling techniques, apoptotic cells (labeled red), cycling cells (with black nuclei in [3H]-TdR autoradiography), and arrested cells (stained blue with Wright's stain) could be discriminated. Cells or apoptotic bodies were counted and categorized per microscopic field. The requirement of counting at least 500 intact cells determined the number of microscopic fields evaluated. An example is given in Fig. 3A, showing sup"I cells after 48 h in low serum, with apoptotic cells (Fig. 3A, arrows 1, 2, and 3 as examples), S-phase cells (Fig. 3A, arrow 4), and arrested cells (Fig. 3A, arrow 5). In striking contrast, Fig. 3B shows sup"II cells (also after 48 h in low serum) with no apoptotic fragments but instead remnants of necrotic cells (Fig. 3B, arrow 1) and a large number of [3H]-TdR-labeled cells (Fig. 3B, arrow 2). After 48 h in low serum, 28 apoptotic fragments were observed in the normal SHE cell line, in contrast to >1400 fragments observed in the sup"I cell line. While the number of apoptotic bodies in the SHE cell group increased to 379 by 72 h, there was a slight decrease in the sup"I cell group to 1162, but the contrast remained remarkable. No apoptotic fragments were seen in the sup"II cells at the 48-h time point, but 13 apoptotic bodies were observed after 72 h in low serum. Tumor cells (10W2T) showed only 5 apoptotic fragments after 48 h in low serum, and this number increased to 69 after 72 h. The detection of apoptotic cells in each of these stages of neoplastic progression demonstrates that the cells have the capacity to undergo apoptosis but that the different responses observed in these cellular groups are probably due to differences in the regulation of the apoptotic process.

Evidence that Cells Do Not Undergo DNA Synthesis Prior to Induction of Apoptosis. The combination of in situ end-labeling and [³H]-TdR labeling facilitated the determination of whether a particular apoptotic cell had progressed through S-phase 24 h prior to undergoing apoptosis. As an example, Fig. 6 shows DES4T tumor cells after 24 h in low serum. The apoptotic bodies A and B were first visualized using horizontal illumination (Fig. 6, top) and then switched to a reflected light source (epi-illumination) (Fig. 6, bottom). Apoptotic body A underwent DNA replication prior to initiation of apoptosis, while apoptotic body B did not. The total number of [³H]-TdR-labeled apoptotic bodies/fragments observed (Table 1) was 2 of 409 in the normal SHE cells (both at the 24-h time point) and 5 of 91 in the DES4T tumor cells (also at the 24-h time point). No other radioactively labeled apoptotic bodies were detected among 3325 fragments observed.

In Vivo Analysis of Cell Survival. Because we observed that normal cells undergo growth arrest and survive in low serum, whereas sup⁺I preneoplastic cells die under these conditions, we were interested in the relative ability of these cells to survive in vivo. Cells were injected s.c. into nude mice and, after 5 or 21 days, cells from the injected area were retrieved (by scraping the s.c. area of injection) and grown in culture (Fig. 7). The results show that viable normal SHE cells were consistently retrieved from the mice after 5 days or 21 days and confluent cultures were obtained after 3 days of in vitro growth (Fig. 8). Metaphase chromosome analysis of the cells showed that >90% of the metaphases were hamster cells (Fig. 8, bottom, A and B).
In contrast, sup II cells retrieved 5 days after injection showed relatively few colonies; no colonies were observed in vitro after 21 days in vivo (Fig. 8). The few cells in the cultures from the 21-day in vivo period were mouse cells only, as determined by karyotyping (Fig. 8, bottom, C and D). Injected sup II cells were not retrievable 5 or 21 days after injection. Histological examination showed the presence of eosinophils, neutrophils, and macrophages at the site 3 days after injection (data not shown), which was not observed in animals given injections of SHE cells or sup I cells. Since sup II cells predominantly undergo necrotic death in low serum in vitro, it is hypothesized that this process evoked an inflammatory response in vivo, eliminating the sup II cells from the site. The data indicate that normal SHE cells can survive in vivo for at least 3 weeks. The sup II cells were partially recovered after the short incubation time in the animals but were not recovered at the later time point, and we interpret these results to indicate that sup I preneoplastic cells in the animals die, presumably by activation of the apoptotic process. The sup II cells evoke an inflammatory response in the animals and are eliminated by 3 days after injection, implying necrotic cell death in vivo as was observed in vitro.

Fig. 6. DES4T tumor cells after 24 h in low serum conditions. [3H]-TdR labeling of S-phase cells is shown with autoradiographic grains, and apoptotic bodies are labeled by the TUNEL technique (red). Top, apoptotic bodies A and B visualized using horizontal illumination; bottom, the same apoptotic bodies visualized using a reflected light source (epi-illumination). Apoptotic body A underwent DNA replication prior to initiation of apoptosis, while apoptotic body B did not.

DISCUSSION

It is believed that one possible key event in tumorigenic conversion may be an alteration that permits cells to escape apoptotic cell death and thus grow to form a tumor. Unexpectedly, our data suggest that cells at an early preneoplastic stage show a sharp increase in the activation of apoptosis in low serum. Normal cells in low serum are reversibly growth arrested primarily in the G0/G1 phase of the cell cycle. Prenesoplastic sup I cells die progressively under these conditions. Only 15% of the sup I preneoplastic cells survived after 96 h in low serum. These cells can be rescued by addition of 10% serum. However, if the serum is reduced again, the cells die, again primarily by the process of apoptosis. The preneoplastic cells also died in vivo when placed into the s.c. area of nude mice, whereas the normal cells survived, indicating that our findings are not limited to in vitro conditions. Other blocks to the proliferative cycle, for example, isoleucine deprivation, PALA-induced deprivation of purines, and treatment with aphidicolin, a DNA polymerase inhibitor, also induced apoptosis in sup I cells, whereas growth arrest was observed in normal cells. Other immortal preneoplastic cell lines die by apoptosis when deprived of serum (29).

These findings are also consistent with observations of carcinogen-induced preneoplastic cells in vivo. For example, preneoplastic foci of rat hepatocytes continue to proliferate in the presence of a tumor promotor (phenobarbital), but when administration of the drug is stopped the preneoplastic cells in the focus die by apoptosis, whereas the normal cells are not affected (13). Regression of preneoplastic cells is often observed in chemically induced tumors, for example, 12-O-tetradecanoylphorbol 13-acetate-induced papillomas in mouse skin (30) and 7,12-dimethylbenz(a)anthracene-induced dysplastic lesions in trachea (31). These published findings can be explained by an increased rate of apoptosis in these lesions, although this was not directly measured.

4 G. A. Preston and J. C. Barrett, unpublished observations.
It has been proposed that tumor suppressor p53 is a molecular sensor of growth deregulation (34). Attempts to define the activities of wild-type p53 as a positive regulator of apoptosis have led to the hypothesis that p53 does not have a role in apoptosis in normal cells. Such a function may be recruited in cells that have undergone oncogenic genetic alterations such as those that occur in the early steps of neoplastic conversion (34). Support for this idea has been provided by studies which show that oncogene-transformed cells are often much more responsive to the inhibitory effects of wild-type p53 than are their normal counterparts (35, 36). In fact, the threshold level of p53 required to exert antiproliferative effects appears to be much lower in cases where oncogenic deregulation has occurred. We find that sup"I and sup"II cell lines carry a wild-type p53 tumor suppressor gene (determined by DNA sequencing of the complementary DNA) and that the gene coding for the p53-associated protein mdm2 is not amplified. Therefore, it is possible that p53 plays a role in apoptosis in the sup"I cells. The block in apoptosis in the sup"II cells in low serum is analogous to the failure of cells with mutant p53 to die by apoptosis. Because the sup"II cells have a wild-type p53 (determined by sequencing of the complementary DNA and immunoprecipitation of the protein with a wild-type protein-specific antibody), a mutation in another gene in the p53 pathway to apoptosis may be involved in the block. It is important to note that these cells are capable of apoptosis if induced by the appropriate stimulus, i.e., okadaic acid.

Furthermore, when the activity of a candidate apoptotic endonuclease, NUC18 (37), was examined, the protein levels and activity were comparable to those of normal SHE cells or sup"I cells (data not shown). These cells may represent an interesting model to elucidate the role of other genes in apoptosis.

Interestingly, the sup"II cells show a relatively higher degree of genetic instability, as measured by an increased frequency of CAD gene amplification (38), suggesting that inappropriate rescue from apoptotic death can result in or be associated with an increased probability for tumorigenicity.

An important aspect of apoptosis and the pathways involved relates to the phase of the cell cycle in which the activation of apoptosis is initiated. The cellular system described here provides a good model to study this question because both a high labeling index and a high frequency of cell death are observed. Our data support the hypothesis that cells committed to apoptotic death fail to enter into the DNA synthesis phase of the cell cycle. Similar to observations made in prostatic G0 glandular cells (39), the majority of the apoptotic cells observed had not undergone DNA synthesis prior to initiation of cell death. However, a very small number of radioactively labeled apoptotic cells were observed at the first 24-h time point. These cells may have been in S-phase when serum was reduced, progressed through G2/M, and undergone apoptosis in G1 as labeled cells. We propose that cells initiate apoptosis in the G2/M-phase of the cell cycle. This proposal is supported by the following observations. The state of quiescence (G0) or replication rescues cells from apoptotic cell death. Sup"II cells and tumor cells continued to progress through S-phase and were also rescued from apoptosis. Sup"I cells could not successfully maintain quiescence in low serum, probably due to the genetic changes that conferred immortalization, nor could they replicate; therefore, the alternative of choice was the apoptotic pathway while in early G1.

Therefore, because it appears that cells commit to apoptosis in G1, it is likely that proteins generally present during this stage of the cell cycle are involved in the process. Immediate early genes c-fos, c-myc, and hsp70 are candidate regulators of apoptosis (40). Constitutively expressed c-myc has been shown to induce apoptosis in Rat-1 fibro-

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Our data confirm and extend the model that apoptosis is triggered by conflicting cellular signals. Umanosky (32) proposed in 1982 that cells contain a built-in program that initiates apoptosis as a default option for the elimination of cells that have incurred oncogenic mutations. Our results demonstrate that the genetic changes involved in immortalization (loss of a senescence gene) of the sup"I pre-neoplastic cells impart a requirement for mitogenic stimulation by growth factors and the cells are destined to undergo apoptosis unless stimulated with proliferative stimuli from serum. Unlike normal cells, the sup"I cells cannot successfully maintain a growth arrest state in the absence of growth factors. In low serum, the immortal cells may generate intrinsic mitogenetic signals due to constitutive activation of one signaling pathway but may not receive extrinsic signals from serum-derived mitogens. Apoptosis is initiated due to an imbalance in growth signals or to conflicting signals. Mutually incompatible signals were shown to result in apoptotic elimination of cells in studies described by Askew et al. (33). Those investigators used an interleukin-3-dependent 32D mouse myeloid cell line and showed that, when deprived of interleukin-3, the cells initially arrested in G0/G1 but, in the continued absence of the factor, the cells ultimately underwent apoptosis. Retrovirus-mediated gene transfer of c-myc into these cells resulted in the loss of the capability for cellular arrest and forced them to initiate the apoptotic program as an immediate consequence of interleukin-3 deprivation.

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4 A. C. Barrett, unpublished observations.
blast cells (7, 41), and antisense oligonucleotides corresponding to c-myc blocked activation-induced cell death of immature T-cells (42). Work done in this laboratory using a reporter construct to study c-fos activation in the presence of okad酸ic acid showed induction of the c-fos gene to be coincident with the initiation of apoptosis (43). Interestingly, activation of apoptosis was not accompanied by induction of DNA synthesis, providing further support for the hypothesis that initiation of apoptosis is a G1 event. As to the functional aspects of c-Fos involvement in the initiation of apoptosis, preliminary studies using the cellular system described here have shown that apoptosis can be induced in the sup- II cells in low serum by increased levels of exogenous Fos protein. Similar observations were reported by Smeyne et al. (44) using c-Fos-transformed rat fibroblast cells. Conditional expression of c-fos in serum-deprived cultures resulted in DNA fragmentation. Although the data favor the hypothesis that apoptosis is initiated in G1, there still remains the possibility that apoptosis could be initiated in G2, and the sup-I cells (low labeling index and high incidence of apoptosis) could activate the apoptotic cascade while in the quiescent state, with no progression into the G2 proliferative cycle. There is precedence for such a pathway in thymocyte apoptosis (45).

The studies presented here show a direct correlation between the deregulation of apoptosis and the early stages of neoplastic development (Fig. 9). When placed in low serum, the normal SHE cells undergo growth arrest, as shown by a low labeling index and accumulation of cells in G1/G0, and the incidence of apoptosis is low. Coincident with the escape from senescence and acquisition of immortality, the sup-I cells exhibit a surprising increase in the incidence of apoptosis in low serum. Neoplastic progression can result from the progressive loss of the cell death signals. The data generated using this model support the concept that cancer arises due to alterations in the regulation of DNA synthesis, providing further support for the hypothesis that the same genes may be involved in regulating the two processes. Unfortunately, many of these important genes have not been cloned.

Research to develop methods for the elimination of neoplastic cells before they become malignant is critical. Although avoidance of identified cancer-causing agents is one approach to cancer prevention, the component of the genetic makeup of individuals complicates these efforts. There are likely to be a variety of strategies that can be used for cancer prevention. One such strategy is implicated by our model system. Presumably, with modulation of growth factors for a short period of time, early neoplastic cells initiate apoptosis while normal cells remain unaffected.

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APOPTOSIS DURING NEOPLASTIC PROGRESSION


Regulation of Apoptosis by Low Serum in Cells of Different Stages of Neoplastic Progression: Enhanced Susceptibility after Loss of a Senescence Gene and Decreased Susceptibility after Loss of a Tumor Suppressor Gene

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