Loss of Cell Cycle Controls in Apoptotic Lymphoblasts of the Bursa of Fabricius

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Lymphoblasts of the normal embryonic follicles of the chicken bursa of Fabricius undergo rapid apoptosis when exposed to γ -radiation or when cell-cell contacts are disrupted by mechanical dispersion in short term culture. We have observed previously that overexpression of *v-myc* sensitizes preneoplastic bursal lymphoblasts to induction of cell death, whereas resistance to induced cell death is acquired during progression to neoplasia. In this study we observed extensive DNA degradation in the large majority of the lymphoblast population within the first hour after dispersion-induced apoptosis. Paradoxically these cells continued to progress into S-phase with the bulk of DNA cleavage and death occurring in S-phase cells (i.e., in cells with more than 2C and less than 4C DNA content). We confirmed the S phase status of apoptotic cells by determining that detection of nuclear cyclin A in individual cells also corresponded with detection of DNA breakage. Levels of cyclin E, cyclin E-dependent H1 histone kinase, and p53 proteins were maintained during dispersion-induced DNA cleavage. γ -radiation failed either to inhibit cell cycle progression or to raise p53 levels in dispersed bursal lymphoblasts. In intact bursal follicles low doses of γ -radiation induced p53 whereas higher, apoptosis-inducing doses failed to induce p53 or prevent G1 to S-phase progression. These results suggest that normal DNA damage-induced cell cycle checkpoint controls are lost or overridden when apoptosis is induced in bursal lymphoblasts.

INTRODUCTION

The lymphocytes in embryonic follicles of the avian bursa of Fabricius include a small population of transplantable stem cells (Toivanen and Toivanen, 1973). These stem cells give rise to a predominant population of rapidly proliferating lymphoblasts with immunoglobulin (Ig)M on their surface engaged in preimmune diversification of their light and heavy chain Ig genes (Reynaud *et al.*, 1987; Thompson and Neiman, 1987). We have recently demonstrated that bursal stem cells and their progeny are exquisitely sensitive to induction of apoptotic cell death either by γ -radiation (and other DNA damaging agents) or by simple mechanical disruption of cell-cell contact in short term culture of bursal follicles (Neiman *et al.*, 1991). Either of these treatments induced extensive internucleosomal cleavage of cellular DNA and morphological changes typical of apoptosis within 4 to 8 h of exposure. These events were dependent upon protein synthesis in the bursal lymphoblasts.

Introduction of a constitutively expressed *v-myc* oncogene into bursal stem cells results in their developmental arrest and preneoplastic proliferation within bursal follicles (Thompson *et al.*, 1987). These *v-myc*-induced bursal stem cells were found to be hypersensitive to induction of apoptotic cell death by either radiation or mechanical dispersion (Neiman *et al.*, 1991). However, neoplastic progression of these cells to invasive bursal lymphomas was accompanied by the development of resistance to induced cell death (Neiman *et al.*, 1991). This observation suggests that modulation of an active cell death pathway may be a consequence of genetic change required for tumor progression in this neoplasia.

Several other reports have been published recently demonstrating enhanced frequency of cell death events in immortal and/or neoplastic cell lines induced by overexpression in these lines of exogenously introduced *myc* (Askew *et al.*, 1991; Evan *et al.*, 1992). In none of

these systems is the mechanism apparent by which *myc* modulates cell death. However, because one of the postulated roles for *myc* is in regulating cellular proliferation and cell cycle progression (Marcu *et al.*, 1992), it seems possible that defining a relationship between cell death events and cell cycle controls could help to provide a framework for understanding the role of *myc* in cell death. Furthermore, loss of cell cycle controls modulating genomic integrity may produce the genetic instability underlying tumor progression. Therefore it seems possible that any interactions between cell death and cell cycle regulation could provide the substrate for *myc*-induced tumorigenesis in the bursa.

Our point of departure for this analysis was the response of cell cycle controls to DNA damage. Normal eukaryotic cell populations respond to failure to complete DNA replication and/or DNA damage by arrest at specific positions in the cell cycle termed check points (Hartwell and Weinert, 1989). Clear examples in normal vertebrate cells are arrests in the G1 phase induced by the metabolic inhibitor of DNA synthesis PALA (Livingstone *et al.*, 1992; Yin *et al.*, 1992) and by radiation (Little, 1968). Loss of this normal response in human cells (together with a predisposition to cancer) accompanies specific mutations in Ataxia Telangiectasia and of P53 genes in the Li-Fraumini syndrome (Kastan *et al.*, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992). Furthermore, loss of this particular checkpoint function is very common during neoplastic progression in many tumor systems (Livingstone *et al.*, 1992). Therefore we examined the kinetics of DNA damage that accompanies apoptosis in relationship to parameters of cell cycle progression and G1 checkpoint function to obtain further insight into the relationships between these critical processes in the bursal system. We used nuclear DNA content determined by staining with propidium iodide to determine the distribution in the compartments of the cell cycle of populations of bursal cells entering apoptosis. Additionally, we confirmed the results of propidium iodide staining by correlating parameters of cell death and with detection of nuclear cyclin A, as a marker for S and G2-phase in single cells (Pines and Hunter, 1991). Finally, we measured the response of p53 and other cell cycle regulatory proteins to γ -radiation in normal and apoptotic cells. Our results suggested that normal bursal lymphoblasts appear to have normal G1 checkpoint function, but this function is lost or overridden during induction of apoptosis.

MATERIALS AND METHODS

Chickens and Dispersion of Bursal Cells in Culture

All bursal cells used for these experiments came from inbred specific pathogen-free White Leghorn SC chickens (Hyline Farms, Dallas Center, IA). Our techniques for short term bursal cell culture have been described (Neiman *et al.*, 1991). Briefly, bursas were excised between 18 d of embryogenesis and day of hatching, and follicles were released into Hann's culture medium (RPMI 1640 supplemented

with 3 μ M 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate 0.35% tryptose phosphate, 8% fetal calf serum, and 10% chicken serum) by gentle teasing with a scalpel. Gravity-sedimented follicles were dispersed at time 0 with a dounce homogenizer, and released cells were filtered through 40- μ m Nitex mesh (Tetko, Monterey, CA). Starting follicle cell populations, consisting of cycling bursal lymphoblasts, mixed with $\sim$ 10% noncycling medullary bursal epithelial cells, were cultured in concentrations of 10^6 /ml at 37°C in a CO₂ incubator. Cell viability was monitored by hand counting in a hemocytometer in 0.4% trypan blue. For cytologic analysis cells were deposited on glass slides in a Shandon cytofuge (Pittsburgh, PA) at 400 rpm for 5 min.

Analysis of DNA Fragmentation

Cytological detection of DNA 3' ends was carried out by tagging with terminal deoxynucleotidyl transferase (TdT) essentially as described by Gavrieli *et al.* (1992). Cytofuged preparations of bursal cells on aminoalkylsilane-treated glass slides were fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). The slides were digested for 15 min at room temperature with 20 μ g/ml of proteinase K in 0.1 tris(hydroxymethyl)aminomethane M (Tris); 0.05 EDTA M acid pH 8.0 and then washed four times for 2 min with distilled water. In experiments where TdT labeling was detected by fluorescence, slides were fixed in ice cold methanol for 10 min and digestion with proteinase K was omitted. The cytosols were incubated with TdT buffer as supplied by the manufacturer (Boehringer, Indianapolis, IN) for 5 min and then with 100 μ l of the same buffer with 10 μ M biotin-11-dUTP, 2.5 mM cobalt chloride, and 25 U of TdT in a humid chamber for 60 min at 37°C. The reaction was stopped in 0.3 M sodium chloride, 0.03 M sodium citrate pH 8.3 for 15 min at room temperature, and the slides blocked by incubation with 2% bovine serum albumin (BSA) in PBS for 10 min at room temperature. They were then rinsed in distilled water, soaked for 5 min in PBS, and incubated with 2 μ g/ml of Streptavidin-alkaline phosphatase conjugate (Pierce, Rockford, IL) at 37°C for 1.5 h. The slides were then washed five times for 10 min with PBS, soaked in 50 mM magnesium chloride, 100 mM sodium chloride, 100 mM Tris pH 9.5, and stained with 10 mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate and 50 mg/ml nitroblue tetrazolium chloride in the same buffer for 15 min to 1 h. For fluorescence microscopy a Streptavidin-texas red conjugate (Pierce) was used in place of the alkaline phosphatase procedure.

For visualization of fragmented DNA by agarose gel electrophoresis $\leq 10^7$ bursal cells were suspended in 0.5 ml RSB buffer (10 mM Tris, 10 mM sodium chloride, 5 mM magnesium chloride pH 7.4) and mixed with an equal volume of lysis buffer (1% sodium dodecyl sulfate, 20 μ M Tris, 100 mM potassium chloride, 10 mM EDTA, 16 mM dithiothreitol pH 7.4). Proteinase K was added to a concentration of 0.5 mg/ml, and the mixture was incubated at 55°C for ≥ 3 h. After addition of 0.5 vol of 7.5 M ammonium acetate, nucleic acids were precipitated with an equal volume of isopropyl alcohol, redissolved in 10 mM Tris, 1 mM EDTA, digested for 30 min with 150 mg/ml ribonuclease A at 37°C, and then again with proteinase A overnight. Aliquots of this digest were analyzed by electrophoresis on 0.7% SeaKem, 1.1% NuSieve (FMC Bioproducts, Rockland, Maine) agarose gels.

Analysis of Single Cells by Flow Cytometry

For determination of nuclear DNA content, bursal cell populations were suspended in 0.1% Triton X-100, 4 mM sodium citrate, 20 μ g/ml RNase A, and 50 μ g/ml propidium iodide pH7.8 for 10 min at 37°C. Sodium chloride was added to 0.014 M, and the suspension of nuclei held at 4°C for analysis of DNA content on a fluorescence-activated cell sorter (FACS Scan, Becton Dickinson, Fullerton, CA) employing a 15mW, 488 nm argon laser with emissions at 585 nm for propidium iodide, 530 nm for fluorescein, and 575 nm for phycoerythrin-labeled probes. The proportion of nuclei with G0/G1, S, and G2 DNA content was calculated using ReproMan flow cytometry

software (True Facts Software, Seattle WA). For experiments involving effects of γ -radiation newly hatched chicks or freshly prepared bursal cell cultures were exposed in a calibrated ^{137}Cs irradiator. For analysis of DNA synthesis and cleavage in single cells, incorporation of BrdU into bursal cell DNA was accomplished by inclusion of $10\ \mu\text{M}$ BrdU in the short term culture medium. Bursal cells were then fixed in 1% paraformaldehyde in PBS for 12 min at room temperature, washed twice in PBS, once in TdT buffer, and suspended in $10\ \mu\text{l}$ of TdT buffer containing dUTP-16-biotin and TdT, as described above, for 60 min at 37°C . After incubation in stop buffer for 15 min the cells were suspended in $100\ \mu\text{l}$ PBS, 0.1% BSA containing $2\ \mu\text{g}$ of anti-BrdU monoclonal antibody (Boehringer) at room temperature for 30 min and washed twice with PBS. Bound antibody was then detected with fluorescein-conjugated goat anti-IgG antibody (Pierce), and biotin-labeled DNA ends were detected with avidin-conjugated phycoerythrin (Pierce) by FACS. In all experiments controls included unlabeled, nonapoptotic bursal lymphoblasts and apoptotic preparations lacking one of the second reagents (anti-IgG or avidin) ruling out both cross-reactivity of reagents and overlap of signals as an artifactual source of doubly positive cells.

Analysis of Single Cells by Fluorescence Microscopy

Characterized antibodies to cyclin A (Koff *et al.*, 1991) were kindly provided by J. Roberts (Fred Hutchinson Cancer Research Center). Cold methanol-fixed cytosol preparations of bursal lymphoblasts were incubated with anticyclin A at a dilution of 1:30 for 3 h at 37°C , washed in PBS, 0.1% BSA, and incubated with fluorescein-conjugated goat anti rabbit immunoglobulin as recommended by the supplier (Pierce). Slides were then further processed by TdT labeling of DNA ends detected with Texas red-avidin, as described above. Double fluorescence analysis was carried out on a Biorad MRC-600 scanning laser confocal fluorescence microscope (Richmond, CA). Excitation was at 514 nm with an argon laser. Emission windows were 520–540 nm for fluorescein and $>600\ \text{nm}$ for Texas red, a separation that avoided detectable signal overlap in the experiments. Quantitation of fluorescence signals from individual cells was accomplished using Optimas software (Edmonds, WA). A cutoff threshold value was set using control samples, and positive nuclei were counted manually. The confocal apparatus included digital image collection and display that was used for recording results and for generating pictures such as shown in Figure 5.

Blot-Hybridization Analysis of Bursal Cell DNA

DNA was extracted as described and transferred from agarose gels to Hybond nylon membranes (Amersham, Arlington Heights, IL). BrdU was detected in the DNA on these Southern blots with anti-BrdU antibody as described by Colombel *et al.* (1992).

Immunoblot Detection of p53, Cyclin E, and Cyclin E-Dependent Kinase

Proteins were extracted from primary chicken embryo fibroblasts, and bursal cells were subjected to electrophoresis on 8% acrylamide gels and transferred to nitrocellulose membranes in a Biorad transblot cell as previously described (Sambrook *et al.*, 1989). Comparisons were made by loading extracts representing a constant number of bursal cells, 5×10^6 , in each lane. Previously characterized antibodies to cyclin E and cyclin A (Koff *et al.*, 1991) were kindly provided by J. Roberts. Detection of p53 was accomplished with a monoclonal antibody, PAb240, which detects, on immunoblots, a cryptic epitope conserved in wild-type p53 from many vertebrates including chicken (Stephen and Lane, 1992). Bound antibodies were reacted with peroxidase-conjugated anti-mouse IgG and detected with an electrochemiluminescence kit (Amersham). Immunoprecipitation of cellular proteins was carried out with the cyclin E antibodies, and histone H1 kinase activity in the immunoprecipitates was detected as previously described (Koff *et al.*, 1991).

RESULTS

DNA Fragmentation Is an Early Event in Bursal Cell Apoptosis

In previous experiments our research (Neiman *et al.*, 1991) and the research of others (Motyka and Reynolds, 1991; Asakawa *et al.*, 1993) has noted extensive internucleosomal cleavage of DNA of embryonic bursal lymphocytes by 4 h after dispersion in short term culture. To determine more precisely when DNA degradation started, we repeated these experiments and examined the bursal cells at earlier time points. Normal embryonic bursal follicles were placed in culture, and cell contacts were disrupted by the dispersion protocol. Figure 1 shows that DNA degradation could be detected as early as 15–30 min after dispersion. A nucleosome ladder became discernible by 1 h and more clearly by 2 h after culture. There were no major cytological changes observable in simple Wright-stained preparations of the dispersed bursal cells at these early time points.

We sought to detect cytological evidence of DNA fragmentation using TdT to label the ends of DNA molecules as described by Gavrieli *et al.* (1992). Figure 2 shows that DNA ends are clearly labeled by this technique in a ring pattern in the vicinity of the nuclear membrane by 1 h after induction of apoptosis in most of the dispersed bursal lymphocytes. A similar pattern of labeling by this technique appears in published images of thymocytes induced to apoptosis with dexamethasone (Gavrieli *et al.*, 1992). It was clear that extensive DNA fragmentation occurred rapidly in the large majority of dispersed embryonic bursal lymphocytes.

Cell Cycle Kinetics of Dispersed Bursal Lymphocytes

Observation of normal cellular responses to DNA damage has contributed to the concept of regulatory checkpoints during cell cycle progression at which cells can arrest until the preceding phase of the cycle is completed (Hartwell and Weinert, 1989). For example irradiated normal vertebrate cells arrest in G1, presumably for purposes of repair, before entering S phase (Little, 1968).

Figure 1. Agarose gel analysis of DNA degradation after dispersion of embryonic bursal lymphoblasts in short term culture medium. Ethidium bromide-stained DNA is shown after electrophoresis in 1% agarose. Lane M shows size markers with positions indicated in kilobase. Lanes 1–5 show DNA extracted at 15, 30, 60, and 120 min after dispersion respectively.

Figure 2. Cytological analysis of DNA breakage by end labeling with TdT. Dispersed bursal lymphoblasts deposited on slides with a cytofuge were treated with TdT and biotin-11-dUTP followed by alkaline phosphatase-conjugated streptavidin. Preparations were made at 0 time (top) and 1 and 2 h (middle and bottom, respectively) after dispersion in culture.

We therefore examined cell cycle parameters of dispersed normal embryonic bursal lymphocytes undergoing extensive DNA damage. Figure 3 shows that over the first 4 h after dispersion the number of nuclei with 2C DNA content (i.e., G0/G1 cells) markedly decreased whereas the population with greater than 2C DNA content, presumably S and G2 phase nuclei, increased. Furthermore this tendency to shift from G1 into S and

G2 phases of the cell cycle over the first 4 h or more after dispersion was not inhibited by γ -radiation to 500R (Figure 3). By 6 h after dispersion >30% of the cells have already completed apoptosis and lysed. At this point in time Figure 3 shows a significant increase in the number of surviving cells with less than 2C nuclear DNA content presumably because of digestion and release from the isolated nuclei used for analysis (Ormerod *et al.*, 1992).

As noted extensive DNA damage was taking place, whereas the bursal cell population appeared to be continuing cell cycle progression. To determine in which cell cycle compartment DNA damage was occurring, nuclei with G0/G1(2C) and S-phase (between 2C and 4C) DNA content were separated by flow cytometry 2 h after dispersion. As shown in Figure 4, DNA from both fractions was analyzed by agarose gel electrophoresis for DNA fragmentation. DNA degradation was detected predominantly in S-phase cells.

DNA Degradation Occurs in Cyclin A Positive Bursal Lymphoblasts

The preceding analysis is based on the supposition that increases in nuclear staining with propidium iodide correspond to the progressive increases in nuclear DNA content of S-phase cells. However, nuclease digestion of thymocyte nuclei in vitro has been reported to increase the DNA staining of isolated nuclei (Crompton *et al.*, 1992). Therefore the increased propidium iodide staining we observed in apoptotic bursal lymphoblasts could have been an artifact of DNA degradation rather than an index of S-phase. Cyclin A has been detected by immunofluorescence only in the nuclei of S and G2-phase cells (Pines and Hunter, 1991). We therefore exploited double immunofluorescence technology to examine cells with cyclin A positive nuclei for evidence of apoptotic DNA damage after dispersion in culture. Figure 5 shows a double immunofluorescence experiment where individual bursal lymphoblasts were probed for both DNA damage by TdT end labeling and cyclin A content. Over 200 cells were examined at time 0 and at 4 h after dispersion in culture. Cells positive for TdT DNA end labeling increased from <5% at 0 time to >70% at 4 h. The percentage of cyclin A positive cells also increased in that time interval to 54% of the population examined. About 91% of the cells with cyclin A positive nuclei were also positive for DNA damage by TdT labeling. Figure 5 shows a representative field of cells 4 h after dispersion illustrating nuclei specifically fluorescent for DNA damage and cyclin A. These data were completely consistent with the flow cytometry findings using propidium iodide staining as an index of S phase DNA content. We concluded that apoptotic DNA damage occurred predominantly in S-phase cells, although the presence of some TdT positive nuclei that were negative for cyclin A suggested that DNA damage also occurred in non-S-phase cells.

Figure 3. DNA content analysis by flow cytometry of dispersed bursal lymphoblasts. Propidium iodide-stained nuclei from bursal lymphoblasts were prepared 0, 2, 4, and 6 h after dispersion in culture as marked. The right panels show analyses after γ -radiation immediately after dispersion of follicles in culture. The vertical axis of each panel indicates numbers of cells analyzed. The horizontal axis shows an index of the intensity of propidium iodide staining. The positions of cells with 2C (G0 and G1) and 4C (G2) DNA content are indicated.

S-Phase DNA Synthesis and DNA Degradation Occur in the Same Cell

By including BrdU in the medium DNA, synthesis could be detected in these fractions by transferring the DNA to membranes and probing with anti-BrdU antibody. Some BrdU incorporation was detected in DNA from

the G0/G1 fraction, but at 2 h after culture in BrdU containing medium, the vast preponderance ($\sim 90\%$) of incorporation was in DNA from cells with an S-phase DNA content (Figure 4A). At short intervals of labeling, ≤ 2 h as shown in Figure 4A, the BrdU labeling was detected in fairly high molecular weight DNA. However, after longer periods (4–6 h) the label appeared in oligonucleosomal fragments (Figure 4B) suggesting that DNA synthesis occurred in relatively intact replicons that were subsequently digested.

In bursal cells, as in other cell types, apoptotic nuclei show decreased staining with propidium iodide because of digestion and loss of DNA (Omerod *et al.*, 1992). Therefore, we could not determine whether the BrdU labeling in the G1 fraction represented a repair response to apoptotic DNA damage or was contributed by a relatively small number of S-phase cells that had, by 2 h after dispersion, sustained sufficient DNA digestion and loss to have contaminated the G1 fraction (i.e., to have pseudo-G1 DNA content). Nevertheless, it was clear that the preponderance of BrdU labeling occurred in cells with S-phase DNA content. Therefore BrdU incorporation into nuclei could reasonably be used as an index of S-phase DNA synthesis. This fact provided an opportunity to analyze individual apoptotic bursal lymphoblasts for both S-phase DNA synthesis and DNA breakage using antibody to BrdU and TdT labeling of DNA ends respectively. By flow cytometry we were able to enumerate cells that entered S-phase without detectable DNA breakage, cells that sustained DNA damage without DNA synthesis (e.g., DNA damage in G1), and cells in which both events occurred within the first 4 h after induction of apoptosis. Figure 6 shows that the majority of bursal lymphocytes scored positively for both DNA synthesis and breakage during the 4-h interval. In addition, there appeared to be positive relationship between the amount of the signals for breakage and synthesis producing a distinct diagonal on the graph. A possible artifact might result, however, from an increase in access by the anti BrdU antibody because of the digestion of DNA (Dolbeare and Gray, 1988) occurring in apoptotic nuclei. Therefore it is premature to conclude that a simple linear relationships exists between the extent of S phase DNA synthesis and DNA damage. The base of the diagonal appeared to be shifted along the axis for DNA breakage consistent with the interpretation that at least some of the cells that remained in the G0/G1 compartment during the first 4 h after induction of apoptosis had sustained DNA damage, albeit less than those with extensive BrdU incorporation, i.e., in S phase.

Levels of p53 and Cyclin E During Apoptosis of Dispersed Bursal Lymphoblasts

The tumor suppressor gene p53 was, potentially, of major importance in this system. It has been reported

Figure 4. DNA fragmentation and BrdU incorporation in different compartments of the cell cycle. (A) Apoptosis was induced by dispersion of bursal lymphoblasts in culture, and 2 h later nuclei were prepared in propidium iodide and subjected to analysis of DNA content by flow cytometry (thin line). About 2×10^6 nuclei were sorted into G1 (2C) and S-phase (between 2C and 4C) populations by DNA content (bold line). DNA was extracted from each fraction, subjected to agarose gel electrophoresis, and stained with ethidium bromide (EtBr). The DNA was transferred to nitrocellulose and probed with anti-BrdU antibody. (B) Anti BrdU probe of DNA from bursal lymphoblasts 6 h after dispersion and culture in the presence of BrdU (lanes 1 and 2). Lane 3 shows control DNA from normal bursal lymphoblasts at time 0.

to enhance cell death when introduced and expressed in murine erythroid and myeloid cell lines lacking wild type p53 (Yonish-Roach *et al.*, 1991; Ryan *et al.*, 1993) and is implicated in mediating the G1 check point because this function is defective in the presence of loss-of-function mutations in p53, such as in the Li-Fraumeni syndrome, and restored by introduction of wild-type p53 (Livingstone *et al.*, 1992; Yin *et al.*, 1992). In many types of normal mammalian cells, DNA damage induced by radiation and other agents causes a rapid increase (within 1 h) of levels of p53 because of stabilization of normally rapid protein turnover preceding arrest in the G1 phase of the cell cycle (Kastan *et al.*, 1991). In bursal lymphoblasts p53 RNA levels remained stable, and p53 protein levels remained stable or decreased somewhat in the first few hours after induction of apoptosis by dispersion (Figure 7A, lanes 1 and 2). Furthermore, in repeated experiments, we could not detect a significant induction of p53 protein by a range of doses of γ -radiation of dispersed bursal lymphoblasts (Figure 7A, lanes 3–7). In these experiments the radiation exposure was completed within 10 min after dispersion of bursal lymphoblasts in culture. The levels of p53 detected were either the same (compare Figure 7, lane 3 with lanes 5 and 6) or decreased (compare lane 3 with lanes 4 and 7) as found in zero time controls. This variability was independent of the dose of radiation but is otherwise unexplained. Importantly, however, we in no case have observed an induction of the level of p53 in dispersed bursal lymphoblasts. Therefore apoptotic dispersed bursal lymphoblasts failed to demonstrate expected responses (p53 induction and G1 arrest) to radiation or to apoptosis-induced DNA damage associated with normal G1 check point function.

Recently a pathway has been defined whereby p53 may negatively regulate cyclin E dependent kinase ac-

tivity thought to be critical for G1 to S transition (El-Diery *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). Immunoblot analysis of cyclin E (Figure 8A) showed levels of this protein to be maintained or only slightly reduced after induction of apoptosis. Significantly, the level of cyclin E dependent H1 histone kinase also was not affected (Figure 8B). These observations were also consistent with the absence of a p53 response to apoptosis induced DNA damage.

P53 Induction and Cell Cycle Response After γ -Radiation of Intact Bursal Follicles

The apparent lack of checkpoint responses to γ -radiation and DNA damage could have been because

Figure 5. Double immunofluorescence analysis of cyclin A and DNA breakage in dispersed bursal lymphoblasts. The same field of cells showing coincidence of nuclear fluorescence for cyclin A (left) and TdT-labeled DNA breaks (right) 4 h after dispersion in culture.

Figure 6. Flow cytometric analysis of DNA breakage and DNA synthesis in single cells. Bursal lymphoblasts were analyzed at 0 time (A and C) and 4 h (B and D) after induction of apoptosis by dispersion in culture. DNA breakage was probed by TdT end labeling (horizontal axis) and DNA synthesis by BrdU incorporation (vertical axis) as described in MATERIALS AND METHODS. Data in top panels is the same as corresponding bottom panel except for the three-dimensional display.

of either an intrinsic property of normal bursal lymphoblasts or a consequence of dispersion such as induced apoptosis. To obtain evidence in support of one or the other of these alternatives, we examined the response to γ -radiation of the cells of intact embryonic bursal follicles. In these intact follicles relatively few bursal lymphoblasts undergo apoptosis at low doses of γ -radiation but demonstrate increasing evidence of apoptosis by morphology and DNA degradation at doses >200 rads (Neiman *et al.*, 1991). As shown in Figure 7B, p53 protein levels were induced at dose levels of 40 to 100 rads, but we failed to detect induction at doses of 160 rads and above. Figure 9 shows results of cell cycle analysis of bursal lymphoblasts from these same experiments. At 4 h after radiation (Figure 9A) doses <100 rads induced small increases in the proportion of cells in G1 and recip-

rocal decreases in the proportion of cells in S and G2 phases. Taken together with the induction of p53 at these doses of γ -radiation, these data were consistent with a normal G1 checkpoint response. However, with increasing doses of radiation (and increasing apoptosis in the intact follicles), the proportion of cells in G1 decreased concordant with reciprocal increases of cells in S and G2 phases in a fashion similar to that observed in dispersion-induced apoptosis (Figure 3). By 8 h after the higher doses of γ -radiation, when apoptotic cell death was extensive, the proportion of surviving S-phase cells markedly diminished (Figure 9B). This observation was again consistent with the predominance of DNA damage and cell death of S-

Figure 7. Levels of p53 in dispersed and intact bursal follicles after γ -radiation. (A) Immunoblot analysis of p53 in extracts of 5×10^6 dispersed bursal lymphoblasts at time 0 (lanes 1) and after 2 h in culture (lane 2). From a separate experiment the panel shows p53 levels in dispersed bursal lymphoblasts at time 0 (lane 3) and 1 h after 40, 100, 160, and 560 rads of γ -radiation at time 0 (lanes 4, 5, 6, and 7, respectively). This blot was exposed for ~ 5 min longer than that in lanes 1 and 2. (B) Results of an immunoblot assay of p53 levels from intact bursal follicles 1 h after 0, 40, 100, 160, and 480 rads of total body γ -radiation of newly hatched chicks.

Figure 8. Levels of cyclin E and cyclin E-dependent H1 histone kinase after induction of apoptosis. (A) Immunoblot analysis of cyclin E at 0 time (lane 1), 30 min of dispersion (lane 2), 2 h of dispersion (lane 3), and 2 h of dispersion plus 500 rads of γ -radiation. (B) H1 histone kinase assay in immunoprecipitates with antibody to cyclin E. Lane 1 is a positive control with a previously characterized cyclin E rich extract of primary human T-cells (Palacino *et al.*, 1993). Lane 2 is a preimmune serum control. Lane 3 is normal bursal lymphoblasts at time 0. Lanes 4 and 5 represent extracts from apoptotic bursal lymphoblasts at 1 and 2 h after dispersion, respectively.

Figure 9. Proportion of bursal cell nuclei with G0/G1, S, and G2 DNA content (vertical axis) in intact bursal follicles at 4 h (A) and 8 h (B) after increasing doses of whole body γ -radiation of newly hatched chicks (horizontal axis).

phase cells after dispersion-induced apoptosis. The reciprocal increase of the proportion of surviving cells in the G2 compartment at 8 h (Figure 9B) suggested both preservation of G2 checkpoint response to radiation and relative resistance of bursal lymphoblasts in G2 phase to apoptotic cell death.

DISCUSSION

Apoptosis in Embryonic Bursal Lymphoblasts

Dispersion-induced apoptosis of embryonic bursal lymphoblasts is a remarkably efficient process associated, as documented in these experiments, with early and extensive DNA cleavage. Despite this DNA damage dispersed bursal lymphoblasts continued to enter S-phase, as measured by both nuclear DNA content and cyclin A content, and to synthesize DNA. The drop in the proportion of G0/G1 cells relative to S-phase cells in the first 4 h (Figure 3) was presumably because of the failure of cycling cells to complete S-phase (where extensive DNA damage was detected) or subsequent G2 phase and mitosis in numbers sufficient to maintain the population of G1 cells during that period of time. Maintenance of levels of cyclin A, cyclin E, and cyclin E-dependent kinase (Figure 6), thought to be important for S-phase and G1 to S transition, are consistent with that interpretation. In addition, in other unpublished experiments, we found that levels of *c-myc* protein were maintained at a constant level during the first 4 h after induction of apoptosis. Significantly, apoptotic bursal lymphoblasts showed no response to γ -radiation in terms of either G1 cell cycle arrest (Figure 3) or acute elevation of p53 protein levels (Figure 7). Therefore, paradoxically, dispersed normal bursal lymphoblasts continued to transit from G1 into S-phase despite DNA damage, a phenotype in vertebrate cells previously as-

sociated only with neoplasia (Kastan *et al.*, 1991; Livingstone *et al.*, 1992) and heritable defects affecting p53 and/or its response to radiation (Kastan *et al.*, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992).

In contrast to the findings in mechanically dispersed follicles, bursal lymphoblasts within intact follicles responded to low dose γ -radiation by induction of p53 (Figure 7). However, at increased radiation doses that trigger apoptosis p53 induction were diminished or absent (Figure 7), and bursal cell populations accumulated in S and G2 compartments of the cell cycle like apoptotic bursal lymphoblasts from dispersed follicles (Figure 8). These observations suggest that both G1 and G2 checkpoint functions are active in bursal lymphoblasts but that the p53-mediated G1 checkpoint may be abrogated by some component of the cell death pathway in this system. More proof of this hypothesis might be obtained if experimental suppression of the cell death pathway leads to restoration of normal cell cycle controls in dispersed and intact follicles.

The role of apoptotic cell death in the bursa is not yet apparent. Because it is induced by simple mechanical disruption of cell-cell contact, it may reflect loss of constitutive signals essential for suppressing the rapidly activated cell death pathway in bursal cells. The need for such an active cell death mechanism could be associated with negative selection of immunoglobulin receptors, the elimination of B-cell clones with autonomous growth potential and/or some presently unknown function. In any case it is reasonable to speculate that the lethality generally associated with cell cycle progression in the presence of unrepaired DNA damage may enhance the cell death mechanism and serve to ensure efficient removal of cells in response to relatively subtle cell surface changes in intact bursal follicles.

Cell Cycle Controls and Bursal Cell Death

Despite these observations in the bursa, the general relationship between apoptotic cell death and cell cycle regulation is complex and by no means limited to S phase. The data presented here, in fact, do not rule out some cell bursal cell deaths occurring in non-S-phase compartments of the cell cycle. Furthermore, there are examples, such as corticosteroid induction of apoptosis in thymocytes, T-cell hybridomas, and leukemias, where cell death is preceded by decreases in *c-myc* expression (Yuh and Thompson, 1989) and arrest in G1 (Harmon *et al.*, 1979). In the case of mouse myeloid- and erythroid leukemia-derived cell lines that lack p53 introduction of wild-type p53 produces G1 arrest followed by apoptotic cell death (Yonish-Roach *et al.*, 1991; Ryan *et al.*, 1993). In fact apoptotic cell deaths induced by radiation or corticosteroids in the thymus (Clarke *et al.*, 1993) or by E1A in cultured rodent cells (Debbas and White, 1993; Lowe and Ruley, 1993) appear to be dependent upon wild-type p53. As in G1 arrest (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991, 1992), p53 dependent apoptosis has been associated with an acute elevation of wild-type p53 protein levels.

In the case of the bursa, low levels of p53 protein were present early after induction of cell death, but we did not detect an increase as a result of apoptosis-induced DNA cleavage or additional γ -radiation (Figure 7). Therefore, we cannot deduce from our observations so far whether p53 plays a role in bursal cell death. However, the failure of radiation to induce p53 responses and cell cycle arrest in apoptotic bursal lymphoblasts suggests the possibility of the presence of dominant regulators that can act opposite to the protective check point response to DNA damage in this system. In such a setting genes that promote cell cycle progression such as *c-myc* might be expected to enhance cell death. We have observed such a sensitization of the bursal cell death pathway by over-expression of *v-myc* (Neiman *et al.*, 1991), and it is possible that the enhancing effects of *c-myc* expression on cell death in other settings (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992) could be similarly explained.

Tumor Progression and the Cell Death Pathway

Both cell death and cycle controls are modified during lymphomagenesis in the bursa. Deregulation of *myc* expression in the stem cell compartment of embryonic bursal lymphocytes arrests development and sensitizes these cells to induction of cell death by either radiation or dispersion (Thompson *et al.*, 1987; Neiman *et al.*, 1991). Tumor progression in this system clearly results in loss of sensitivity to induced cell death (Neiman *et al.*, 1991) as might be expected for a neoplastic phenotype requiring viability and growth outside the follicular environment. Neoplasms of many kinds may involve maturation arrest in cell lineages at positions in

which gene-directed cell death mechanisms are very active. In such cases, as in the bursa, active suppression of this mechanism may be a prerequisite for successful tumor progression. This may well explain why tumor sensitivity to relatively nonspecific cytotoxic chemotherapeutic agents frequently appears to involve apoptosis (Eastman and Barry, 1992). Furthermore, such cell death pathways, if they interact with p53 and/or other components of the checkpoint mechanism (as seems plausible from the data in the bursal system we describe here), could contribute significantly to the genetic instability that appears to underlie tumor progression even in the absence of structural mutations in p53.

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