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Less death in the dying

Kornelia Polyak[1,2], Tsung-Teh Wu[3], Stanley R. Hamilton[2,3], Kenneth W. Kinzler[2] and Bert Vogelstein[1,2,4]

[1] The Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, 424. N. Bond St. Baltimore, Maryland 21231
[2] Oncology Center, The Johns Hopkins University School of Medicine, 424. N. Bond St. Baltimore, Maryland 21231
[3] Department of Pathology, The Johns Hopkins University School of Medicine, 424. N. Bond St. Baltimore, Maryland 21231
[4] corresponding author: The Johns Hopkins Oncology Center, 424 N. Bond Street, Baltimore, MD 21231, USA. tel: (410) 955-8878; fax: (410) 955-0548

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Abstract

Diseases associated with aging are now the primary causes of death in developed countries. This is in part due to the long recognized exponential association of cancer with age and perhaps to a deterioration of the immune system with advanced age. Both prevention of tumorigenesis and immune function are critically dependent on apoptosis. In this study we examined apoptosis in mice of various age following gamma irradiation. We found a striking age-dependent decrease in radiation-induced apoptosis in splenic lymphocytes but not in colorectal epithelial cells. These observations may therefore provide a clue to the decline of immune function with age.

Keywords: apoptosis, radiation, aging, immune system

Abbreviations: TdT, terminal deoxynucleotide transferase; TUNEL, terminal dUTP nick-end labeling

Introduction

Although the molecular basis of aging is largely unknown, it is likely the result of both genetic and environmental factors (Hart and Turturro, 1992; Cohen, 1994). Many cellular functions, including DNA repair, regulation of cell proliferation, endocrine and immune responses decline with age (Hart and Turturro, 1992; Cohen, 1994; Song *et al*, 1993; Miller, 1996). Partly as a consequence of these changes, certain diseases such as cancer and immune system disorders occur more frequently in older individuals (Cohen, 1994; Nakamura *et al*, 1992). Apoptosis has been shown to be involved in many physiologic and pathologic processes, including neoplasia and immune regulation. However, few studies have addressed whether the capacity for programmed cell death varies with age (Monti *et al*, 1992). To investigate the possibility that the elimination of damaged cells through programmed cell death declines with age, we examined the apoptotic response in mice following gamma irradiation.

Results and Discussion

One of the most well-studied experimental paradigms for investigating apoptosis involves its induction in lymphocytes by gamma irradiation (Howie *et al*, 1994). Eight hours after 5 Gy of whole body exposure, mouse spleens were examined with the TUNEL-staining assay (Gavrieli *et al*, 1992). This assay assesses the fraction of cells with DNA strand breaks, one of the hallmarks of apoptosis. Approximately half the lymphocytes in spleens from young mice (1–4 month old) stained positively following irradiation (Figure 1), with virtually no staining prior to irradiation (not shown). In contrast, many fewer cells in the spleens of older mice (32–34 month old) were stained with TUNEL, though the architecture of the spleen was histologically indistinguishable in the two groups of mice (Figure 1). Mice of the strain used (C57BL/6/CNNia) have a maximal life-span of 35–36 months.

To quantitate this effect, flow cytometric analysis was performed on the splenocytes. DNA from apoptosing cells degrades, resulting in cells with sub-G1 contents. Representative flow cytometric profiles of spleen cells from irradiated mice are shown in Figure 2a. The spleens from 34 month old mice contained many fewer sub-G1 cells than those from the 4 month old mice, consistent with the TUNEL-staining results. Fluorescence microscopy showed that a significant fraction of the cells from the younger mice exhibited condensed chromatin, and the cells were often micronucleated (Figure 2B). From 40–60% of the spleen cells from mice 1–22 months of age underwent apoptosis 8 h after irradiation, while in 28 month old mice, the fraction of apoptotic cells was reduced to $26 \pm 8\%$, and by 33 months, only $14 \pm 7\%$ of the cells underwent apoptosis (Figure 3A).

To determine whether this age-related decrease in apoptosis was cell autonomous, we isolated splenocytes from mice of different ages and irradiated them *in vitro*. The fraction of apoptotic cells in all mice was higher following *in vitro* irradiation compared to irradiation *in vivo*, consistent with previous studies (Durand, 1994) (Figure 3B). Nevertheless, the fraction of apoptotic cells was considerably less in 33 month-old mice ($38 \pm 3\%$) than in 4 month old mice ($75 \pm 2\%$; $P < 0.001$) following 5 Gy irradiation (Figure 3B). The *in vitro* assays also afforded the opportunity for dose response analyses, which were difficult to perform *in vivo* because of the expense and limited availability of the older mice. As shown in Figure 3B, the apoptotic response of the old mice was decreased to that of young mice at all doses, with the most significant difference observed at 5 Gy, the same dose used *in vivo*. To exclude the possibility that the decreased apoptosis in older mice was a result of increased phagocytosis, macrophages were removed from the isolated splenocytes in some experiments prior to radiation; this depletion had no effect on the fraction of apoptotic cells.

We also attempted to determine whether decreased apoptosis could be observed in the intestinal epithelium of

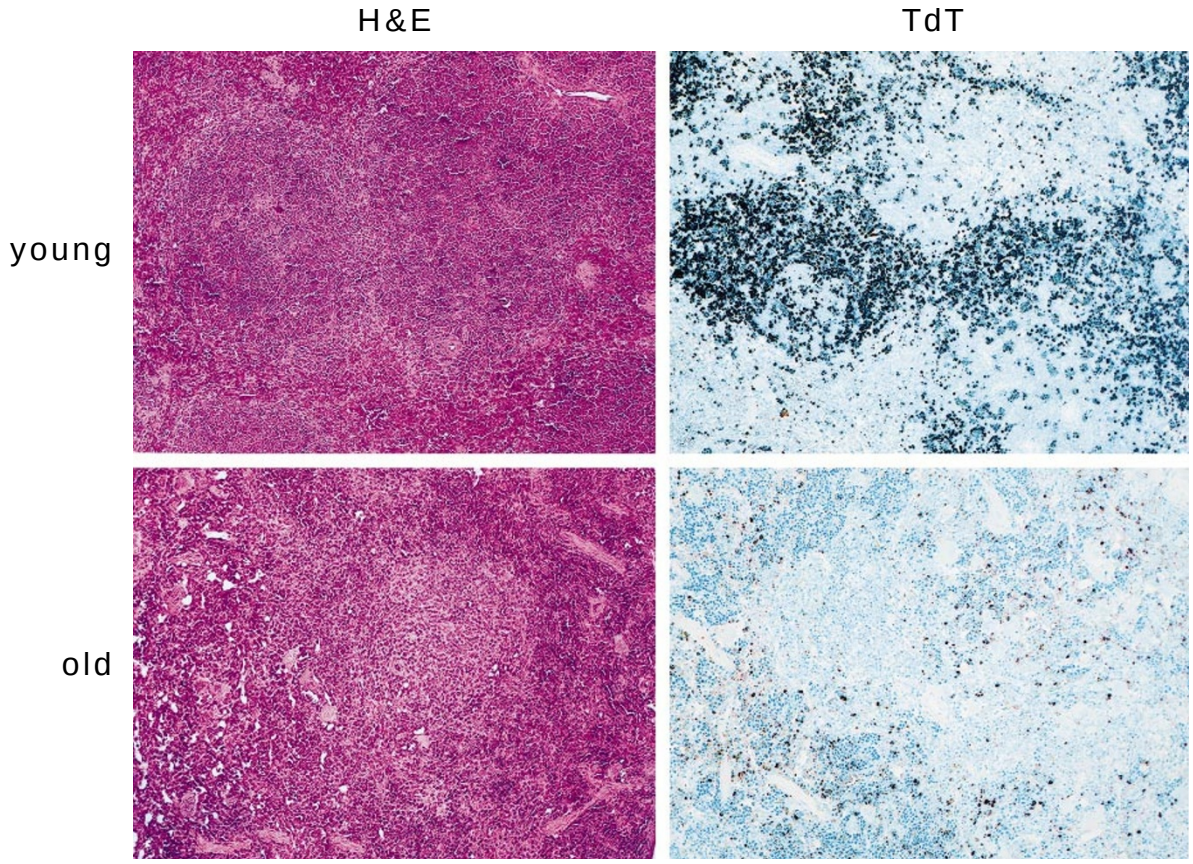

Figure 1 TUNEL staining of irradiated spleen. Hematoxylin-eosin (H&E) and TUNEL (TdT) stained sections of spleen from young (4 months old) and old (33 months old) mice 8 h after receiving 5 Gy of γ-irradiation. The number of apoptotic cells (stained black on the TdT slides) was significantly lower in the old mice, while the morphology of the spleen was comparable. Similar results were obtained in three independent experiments.

older mice. Classic morphologic criteria following routine hematoxylin and eosin staining was used (Clarke *et al*, 1994), as the TUNEL assay was found to be a somewhat unreliable indicator of apoptosis in the intestinal tract (Hall *et al*, 1994 and our unpublished data). In the small intestine, irradiation increased apoptosis from 1 ± 1 cell per 100 crypts (in all mice) to 97 ± 25 and 111 ± 32 cells per 100 crypts in the young and old mice, respectively. Gamma irradiation increased the number of apoptotic cells in the colon to a similar degree, also with no significant differences between old and young mice.

These studies demonstrate a profound difference between irradiation-induced apoptosis of the splenic lymphocytes in young and old mice. The apoptosis 'defect' in the older mice was reproducibly observed in lymphocytes of the spleen, but did not affect the gastrointestinal epithelium. It is perhaps relevant in this regard that radiation-induced apoptosis in the gastrointestinal epithelium is p53-dependent while that of cycling splenocytes is not (Clarke *et al*, 1993; Lowe *et al*, 1993; Clarke *et al*, 1994; Merritt *et al*, 1994; Strasser *et al*, 1994; Tamura *et al*, 1995). The decreased apoptotic response in the older mice was not due to a microenvironmental factor present in the spleen, as similar differences were also observed following irradiation of isolated splenic lymphocytes (Figure 3B).

The mechanism underlying the age-related differences in apoptosis is not known, but two hypotheses can be formulated. First, it is possible that the intrinsic ability of cells to undergo apoptosis declines with age, due to changing patterns in chromatin or in other determinants of gene expression (Issa *et al*, 1994). Second, it is possible that the decreased apoptosis reflects an age-specific difference in lymphocyte subtypes that populate the spleen (Song *et al*, 1993). It is known that radiation sensitivities vary with cell type, and it is possible that differences in lymphocytic subtypes could account for our results. The very large differences in apoptosis in the older mice would mandate an equally substantial change in lymphocytic population. It has been shown that non-functional T cells increase with age in both mouse and man, though the molecular and physiologic bases for this dysfunction is not yet clear (Song *et al*, 1993; Nagel *et al*, 1988). It will be interesting to determine in the future whether specific lymphocyte subsets, defined by surface markers, occupy a major portion of the aging peripheral immune system, and whether the same cells are deficient in their response to antigenic stimulation and in apoptosis.

What is the significance of the decreased apoptosis in aging mice? Though this can only be a topic of speculation at present, it is important to point out that the magnitude of the decreased apoptosis was considerable, representing a three- to fourfold reduction, and that a significant difference in the degree of apoptosis was observed at least 6 months prior to death, when mice were 28 months old. These two observations (magnitude and time of occurrence) distinguish the apoptotic-induction differences from many of the other age-related phenomenon that have been reported in the literature (Hart and Turturro, 1992; Cohen, 1994; Song *et al*, 1993). We hypothesize that an age-related difference in apoptosis induction could make older mice more susceptible to cancer or to autoimmune diseases. Studies to determine whether apoptotic induction varies with age in the human should prove of interest in this regard.

Materials and Methods

In vivo irradiation and flow cytometry

Male C57BL/6/CNNia mice of various ages (1–34 months) were irradiated (5 Gy) with a ^{137}Cs γ source (dose rate, 0.9 Gy/min) and sacrificed 8 h later. For morphologic measurements of apoptosis and for TUNEL assays, small intestine, colon and spleen were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline) at 4°C overnight and embedded in paraffin the next day. Three μm sections of the intestine and spleen were either stained with hematoxylin and eosin for morphologic assessment of apoptosis or processed for TUNEL assays. Apoptotic cells were counted in coded slides assessing 100 crypts per section, and triplicate sections were analyzed from each animal (3–4 mice/age group). Analysis was restricted to crypts where the plane of the section included the crypt axis. For flow cytometric analysis, spleen cells were resuspended in

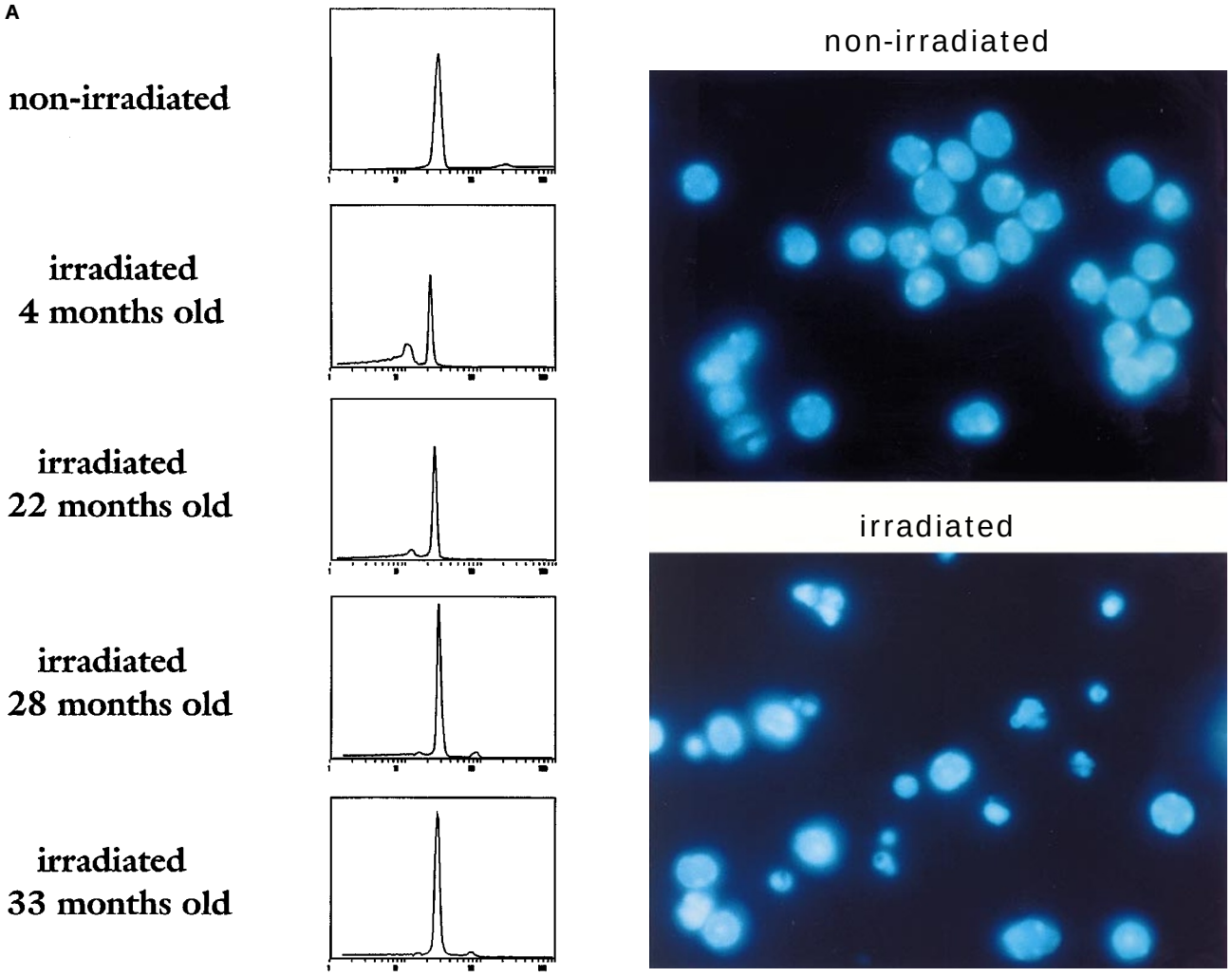

Figure 2 Flow cytometric and microscopic analysis of splenocytes. (**A**) Flow cytometric analysis of splenocytes isolated from mice of various ages (4–33 months old, as indicated) without treatment (non-irradiated) or 8 h after 5 Gy of γ-irradiation. The splenocytes of non-irradiated mice are almost exclusively in G0/G1 with 2n DNA content. The apoptotic fraction (< G1) of cells after irradiation decreases with increasing age. Representative samples from three independent experiments are shown. (**B**) Splenocytes isolated from mice prior to or after irradiation were fixed, stained with the DNA-binding dye Hoechst 33258, and observed under fluorescence microscopy. Representative cells are shown. Condensed chromatin characteristic of apoptotic cells were observed in the irradiated cells.

100 μl PBS and were added to 500 μl of fix/staining solution containing 0.6% NP40, 3.7% formaldehyde, 11 μg/ml Hoechst 33258 in PBS. The fixed and stained cells were directly used for flow cytometry analysis. In each experiment 50,000 cells were counted and the sub-G1 population was quantitated using the Multicycle software package.

TUNEL assay

Paraffin sections of the spleen or intestine were dried overnight at 80°C and deparaffinized in xylene for 30–60 min (three changes) at room temperature. Sections were rehydrated through descending ethanol washes and stored in PBS. Sections were then treated with 60 μg/ml proteinase K (Gibco BRL, DNase and RNase free) in PBS for 15 min at room temperature. Protease digestion was stopped by consecutive washes in PBS and TdT buffer solutions (5 min each). TUNEL assays were performed at 37°C for 1 h in 1 × TdT buffer (USB), 150 mM NaCl, 2–5 μM biotin 16-dUTP (Boehringer) and 50–100 U/ml TdT (USB). After washing in PBS, labeled cells were visualized with the ABC horseradish peroxidase method (Vector laboratories, Burlingame, CA). Sections were counterstained with 0.5% methyl green, dehydrated, then mounted in Cytoseal 60 (Stephens Scientific, Riverdale, N.J.).

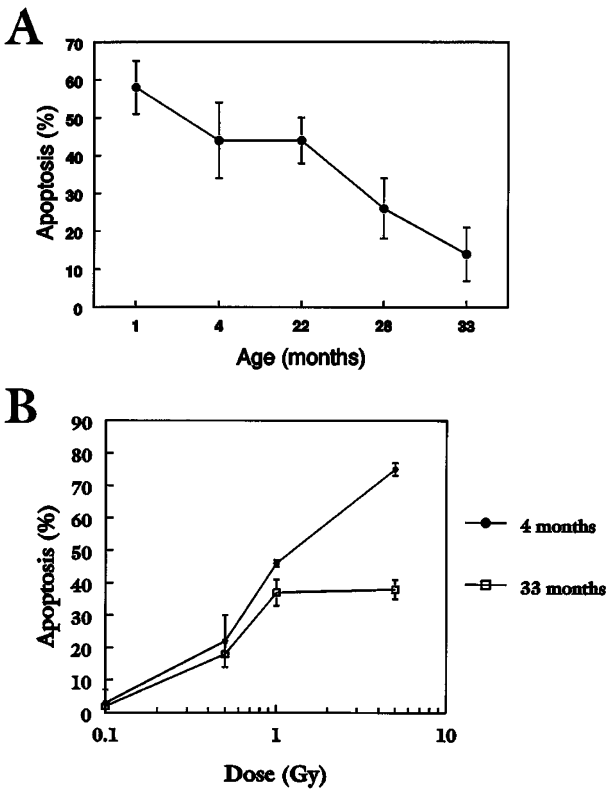

Figure 3 Relationship between apoptosis and age. (**A**) The fraction of apoptotic cells (<G1) was quantitated based on flow cytometry analysis of splenocytes isolated from mice of various ages 8 h after 5 Gy of γ-irradiation. Numbers are based on results of two independent experiments, each experiment employing 5 mice/age group. Error bars represent standard deviations. The difference in apoptosis between young (1–4 months) and old (28–33 months) mice was highly statistically significant ($P<0.0001$). (**B**) Splenocytes were isolated from mice of various ages and irradiated *in vitro* as described in Materials and Methods. Flow cytometric analysis was performed 8 h after radiation and apoptotic cells (<G1) were quantitated. Numbers are based on results of two independent experiments each employing 2 mice/age group. Error bars represent standard deviations of the mean. The difference in apoptosis between the two age groups approached statistical significance at 1 Gy (P0.08) and reached statistical significance at 5 Gy ($P<0.01$).

In vitro irradiation and flow cytometric analysis

Splenic lymphocytes were isolated from untreated animals essentially as described (Kruisbeek, 1993) using Histopaque-1083 (Sigma). Freshly isolated splenocytes were resuspended at 1×10^6 cells/ml in RMPI-1640 medium supplemented with 10% fetal bovine serum and irradiated using a ^{137}Cs source. In some experiments phagocytic cells were removed from the splenocyte suspension by incubating the cells in T75 tissue culture flasks for 1 h. Non-adherent cells were used for subsequent treatment and analysis (Waldman *et al*, 1995). Cells were collected by centrifugation 8 h after irradiation and prepared for flow cytometry as described above.

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