Age-adjusted antitumoral therapy based on the demonstration of increased apoptosis as a mechanism underlying the reduced malignancy of tumors in the aged

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Abstract

In view of the constant increase in the aged population, age-adjusted cancer therapy becomes an urgent target. Although cancer incidence rises with age, paradoxically, growth rate and metastasis often proceed at a slower rate in the aged. Determining the mechanism(s) underlying this reduced tumor progression in the old might have implications for a rational design of age-adjusted therapy. Thus far, decreased cell proliferation or immune response modifications were suggested as possible mechanisms. We show here that an increased tendency to apoptotic tumor cell death in the aged could constitute an additional mechanism. Based on this mechanism, we compared the therapeutic efficacy of two apoptosis inducers, hydrocortisone and adriamycin, on AKR lymphoma and B16 melanoma growth in young and old mice. Treatment with hydrocortisone acetate inhibited tumor growth practically only in old mice in the two tumor systems. Similar effects were obtained with adriamycin treatment of AKR lymphoma but opposite results were seen with B16 melanoma. We thus demonstrated, in three of the four tumor-therapeutic modality systems examined, an age-related antitumoral efficacy of two apoptosis-inducing agents, with tendency for a remarkably more pronounced effect in aged mice.
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Keywords: Cancer treatment; Aging; Apoptosis induction; Tumor progression; Age-dependent therapy design

1. Introduction

The research on tumor development and its treatment in the elderly should gain an importance in view of the constant rise of the fraction of aged individuals in the population of Western countries. Despite the steady increase of the aged population, which bears most of the cancer burden, only few studies have been devoted to the effect of age on tumor progression and on age-adjusted cancer therapy. Most experimental studies on cancer are (inappropriately) performed on young animals. Such studies may be irrelevant to the aged organism.

Cancer incidence is known to augment with age. Paradoxically, however, tumor growth and metastasis were often found to proceed at a slower rate in aged organisms, in both humans [1] and in experimental models. [2] For example, bronchogenic cancer [3] and cancers of breast [4] and colon [5] were reported to grow slower and metastasize less in old patients. With regard to experimental tumors, growth has often been shown to be slower and to display a reduced aggressiveness in aged as compared to young animals [2,6,7]. We have also reported this phenomenon in the B16 melanoma [8,9] and in the AKR lymphoma [10].

The mechanisms responsible for the reduced tumor progression in the aged compared to young organisms have not yet been established. Decreased proliferative capacity in the old has been suggested [11] as well as a decline in the availability of growth factors with age [12]. Modifications in anti-tumoral immune reactions with host age have been demonstrated by Kaesberg and Ershler [13] and by ourselves [9]. We have recently suggested that an increased apoptotic cell death could be responsible for the reduced malignant behavior of tumors in the aged [10].

Understanding the mechanism of the differential biological behavior of tumors in relation to host age is of

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importance in itself but it may also be important for the eventual exploitation of these mechanisms for a rational design of cancer therapy appropriate for the aged organism. The age-related differential biological behavior of tumors actually implies the necessity of a differential therapy for cancer patients of different ages. Very few experiments of this type have been performed [14–16].

In the present study, we demonstrate that the rate of apoptotic cell death in two tumors, B16 melanoma and AKR lymphoma, was higher in old mice than in those growing in young mice. We further examined the possibility, which seemed logical in view of the above, that apoptosis-inducing therapy could be more efficient against tumors in aged organisms than against those in young ones. As far as we are aware, this is the first study with such an approach (according to a search in Medline using as keywords: apoptosis, aging, cancer and therapy, 69 references were found but none was really relevant).

While for decades, the major goal of cancer treatment by cytotoxic agents was to inhibit proliferation of tumor cells, it now appears that the primary mechanism by which most (if not all) chemotherapeutic agents induce cell death is apoptosis [17]. It was, in fact, found that cytotoxic drugs with widely different modes of action and differing cellular targets (antimetabolites, alkylating agents, topoisomerase inhibitors or hormone antagonists) could induce apoptosis in susceptible cells [18]. Indeed, susceptibility or resistance to apoptosis of the tumor cells often determines the response to chemotherapy of particular cancers [18].

Although a large number of publications supporting this thesis are found in literature (4856 references according to Medline using the keywords: chemotherapy, apoptosis and cancer), curiously, in currently used screening assays, potential anti-cancer drugs are selected on the basis of growth inhibiting properties rather than on their ability to induce apoptosis [19].

To compare the efficacy of apoptosis-inducing antitumoral therapy in young and old mice, we used in the present study as apoptosis-inducing agents, hydrocortisone (HC) and adriamycin (ADR). We examined the possibility of an age-related differential efficacy of these treatments in two tumor systems, the B16 melanoma and the AKR lymphoma.

Adriamycin (doxorubicin) has been used for more than 30 years in treating a variety of malignancies [20]. Nevertheless, the mechanism of ADR has been a subject of controversy. A number of mechanisms have been suggested, such as intercalation into DNA, damage to DNA via free radical formation, interference with DNA unwinding or DNA strand separation by helicase, inhibition of topoisomerase II as well as effects on the cell membrane [21]. Elmore et al. [21] recently demonstrated that ADR treatment of breast tumor cells induced replicative senescence which involved telomere dysfunction. Anthracyclines were further shown to induce apoptotic cell death [22–24]. This could possibly represent a cell response resulting from previous

cellular events. Evidence proving that ADR induces apoptosis includes induction of DNA fragmentation and cell shrinkage [23,24]. Moreover, ADR-resistant multiple myeloma cell lines displayed various alterations in expression of genes involved in apoptosis-signaling pathways [25].

Glucocorticoids (GC) are used as immunosuppressive and anti-inflammatory agents in managing disorders of heightened immunity [26] as well as against lymphoproliferative diseases, mainly hematological cancers [27]. About 20 years ago, it was recognized that GC-induced cell death exhibits morphologic features characteristic of apoptosis [28]. Additional studies demonstrated that glucocorticoids are potent apoptosis-inducing agents, at pharmacological [28] and physiological levels [29].

Based on our experiments demonstrating a mechanism of the differential biological behavior of tumors in old as compared to young mice (modified apoptotic cell death), we show a differential age-related response to apoptosis-inducing therapy.

2. Materials and methods

2.1. Mice and tumors

Studies on animal models were performed according to the guidelines of Tel-Aviv University. C57BL/6J and AKR/J mice were purchased from the Tel-Aviv University Breeding Center. Two age groups were used: young mice, 6–8 weeks for both C57BL/6J and AKR/J mice; old mice, 16–24 months for C57BL/6J mice and 7–9 months for AKR/J mice. We chose this age as "old" for the AKR mice, because the life-span of this strain of mice is short, and in order to avoid the use of mice with spontaneous tumors which begin to appear at around 6 months. Mice were examined (by palpation of inguinal lymph nodes) for presence of spontaneous tumors, and those which developed such tumors were not included in the experiments. Tumor cell suspensions of both AKR lymphoma and B16 melanoma were prepared as previously described [30]. For experiments comparing the biological behavior of tumors and the various tests for apoptosis, the procedures were as follows: tumor cells (2×10^5 in 0.2-ml RPMI-1640 medium; Sigma-Aldrich, Rehovot, Israel) were inoculated subcutaneously (s.c.) in the back of mice. Tumor growth was evaluated by recording the incidence and by measuring two to three times a week the diameter of the tumors formed at the s.c. site of inoculation. Mice mortality was recorded daily.

For the tests related to apoptosis, tumors were taken from mice when they reached diameters of 15–20 mm in the young, which occurred around day 20 after tumor inoculation in the case of B16 melanoma and around day 12 in the case of AKR lymphoma. The size of the tumors was chosen so that necrosis could be avoided as far as possible but so that they could be large enough (in old mice as well) to

enable us to perform the different tests. The time points could slightly differ from experiment to experiment but tumor sizes were comparable among experiments.

Treatment of tumor-inoculated mice with apoptosis-inducing agents was performed as follows: AKR/J mice were inoculated s.c. with 1×10^4 cells of AKR lymphoma and C57BL/6J mice were inoculated s.c. with 2.2×10^5 cells of the B16 melanoma. This inoculum was chosen since we recently demonstrated that at this particular inoculum, the melanoma displayed maximal differential biological behavior between young and old mice (unpublished results). Treatment was performed with single doses of 500 or 25 μg per mouse of HC or ADR, respectively, 24 h following tumor inoculation. Each experimental group consisted of five mice and each experiment was repeated four to seven times.

2.2. DNA flow cytometry

Cells derived from primary tumor growths of AKR lymphoma and B16 melanoma, grown in young or old mice, were incubated with propidium iodide (50 μg/ml) (Sigma-Aldrich) following the procedure of Vindelov [31]. The data were analyzed on a Cell Quest Software BP, MultiCycle Phoenix Flow Systems (Phoenix, Arizona).

2.3. ApopTag staining

Apoptosis was identified by labeling the DNA 3′-OH nick-ends using a variant of TUNEL staining. ApopTag staining was carried out according to the manufacturer's instructions using the materials provided in the kit (Apop-Tag, Intergen Company, New York). After deparaffinization in xylene and rehydration in a series of decreasing concentrations of alcohol, the 5-μm-thick tissue sections were incubated at 37 °C for 1 h in the presence of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled nucleotides. After washing, anti-digoxigenin-peroxidase was added to the slides which were incubated in a humidified chamber for 30 min at room temperature. After washing in PBS, the slides were stained with diaminobenzidine (Sigma-Aldrich) and then counterstained with 1% methylgreen (Vector, Burlingame, California). The percentage of apoptotic bodies was determined at high power fields. A total of 300 cells was counted.

2.4. DNA fragmentation analysis by agarose gel electrophoresis

The purification of genomic DNA was done with Puregene DNA Isolation Kit (Gentra, Minneapolis, Minnesota).
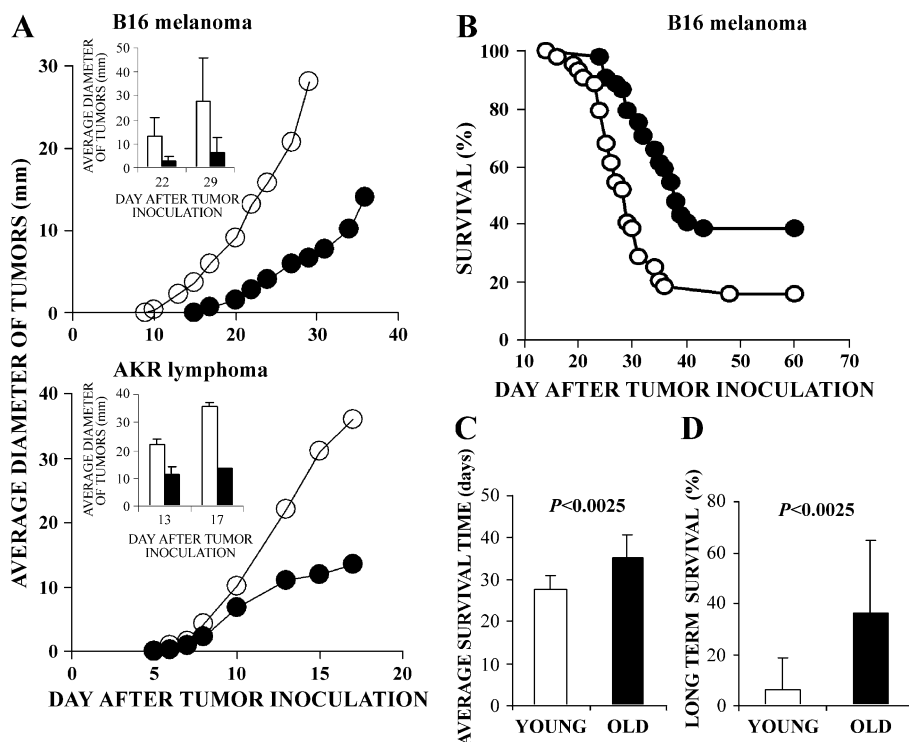

Fig. 1. Differential biological behavior of B16 melanoma and AKR lymphoma in young and old mice. (A) Average tumor size of B16 melanoma and AKR lymphoma in young (○) and aged (●) mice in representative experiments. Inserts: Average diameters of tumors for B16 melanoma on days 22 and 29 ($P < 0.0125$ and $P < 0.025$, respectively) and for AKR lymphoma on days 13 and 17 ($P < 0.001$ and $P < 0.0005$, respectively). (B) Kinetics of mortality of young (○) and old (●) mice inoculated with B16 melanoma. (C) Average survival time of young and aged B16 melanoma-bearing mice. (D) Long term survival of young and old mice bearing B16 melanoma.

DNA from AKR lymphoma primary tumors, grown in young or old mice, was analyzed by horizontal electrophoresis during 2 h on 2% agarose gel (Talron, Rehovot, Israel) and visualized by UV fluorescence after staining with ethidium bromide (0.5 μg/ml) (GibcoBRL, Rockville, MA). As DNA marker, pUC18 DNA marker *Hae*III digest, 102–587 b.p. (Sigma-Aldrich), was used in this study.

2.5. Analysis of Bcl-2 protein expression by flow cytometry

Single cell suspensions were washed with PBS and red blood cells were removed by RBC buffer (Epicentre, Addisson, WI). Cells were suspended at a concentration of 2×10^7 cells/ml in saponine buffer (SB) (0.03% saponine in 1% bovine serum albumin (BSA) in PBS, pH 7.4) (Sigma-Aldrich) in order to permeabilize the cells [32]. The cells were incubated with hamster anti-mouse-Bcl-2 mAb

(clone 3F11) (PharMingen, San Diego, CA), 1:250 for 30 min at 4 °C. After washing with SB, cells were incubated with FITC-conjugated goat anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:50 for 30 min at 4 °C. After two additional washings, samples were analyzed on a FACSort Becton Dickinson, San Jose, CA, with WinMDI 2.8 Joseph Trotter Scripps data processing.

2.6. Analysis of Fas protein expression by flow cytometry

Cells prepared as for Bcl-2 analysis were washed in cold buffer (1% FCS in PBS) and were suspended to a concentration of 2×10^7 cells/ml. In order to reduce Fcγii/iii R-mediated antibody binding, the cells were incubated with 0.25-mg Fc Block (clone 2.4G2) (PharMingen) per 10^6 cells for 3 min at 4 °C. After washing, the cells were incubated
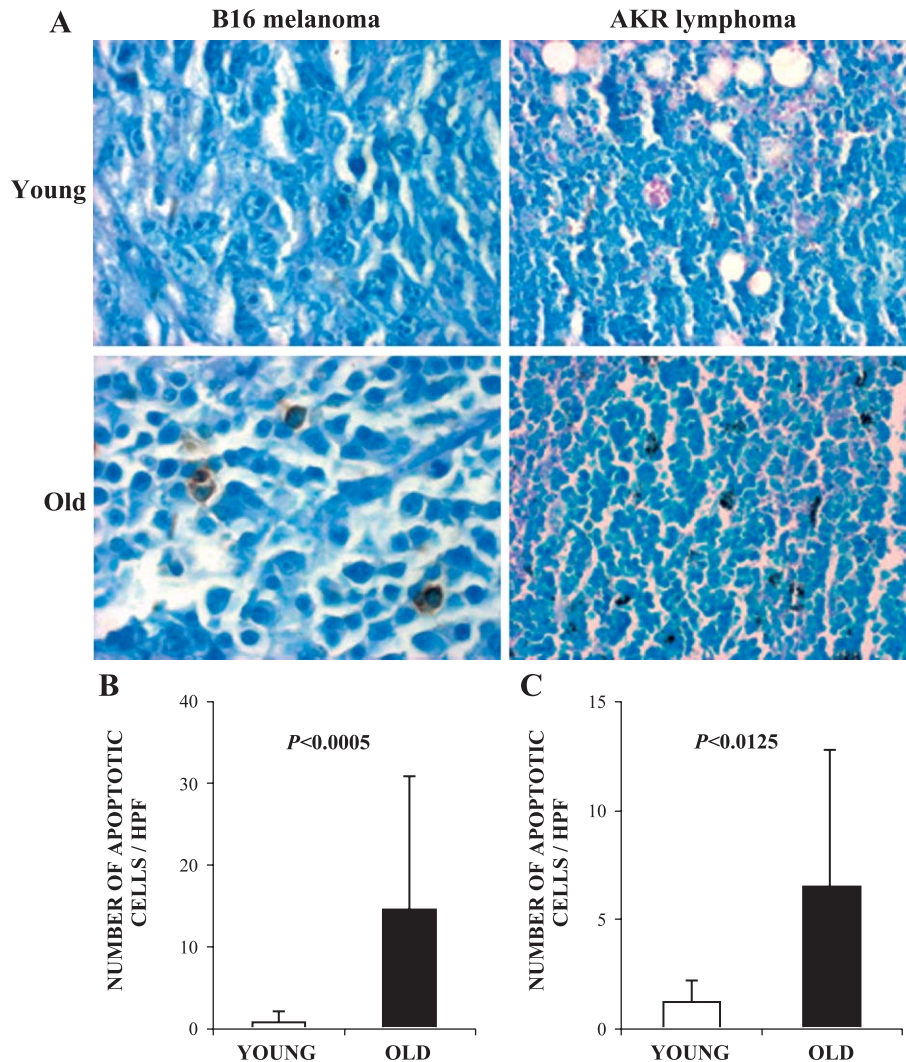

Fig. 2. Effect of host age on apoptosis in B16 melanoma and AKR lymphoma: evidence at the cellular level. (A) Apoptotic cell content in representative pictures of ApopTag staining sections of B16 melanoma (× 400) and AKR lymphoma (× 200) in mice of different ages. (B and C) Quantitative comparison of Apoptag+ cell content in tumors from young and old mice.

with hamster anti-mouse Fas mAb (clone Jo2) (PharMingen), 1:50 for 30 min at 4 °C in the dark. After washing with cold buffer, cells were incubated with the same secondary antibody as for Bcl-2 and analyzed by FACS.

2.7. Western blot analysis for caspases 8, 3 and 9

Total cell protein lysates were prepared as follows: cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl (pH 8.0) and 1 mM

Sodium vanadate) (Sigma-Aldrich) supplemented with protease inhibitor mixture (0.1 mM PMSF and 2 μg/ml of leupeptin and apoprotein) (Sigma-Aldrich) for 15 min on ice. Lysates were centrifuged at 10,000 rpm for 5 min at 4 °C. Forty micrograms of total proteins was dissolved by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked for 2 h at room temperature with 5% BSA. Primary antibodies reactive to caspase-8 (1:1000), caspase-3 (1:2500) and caspase-9
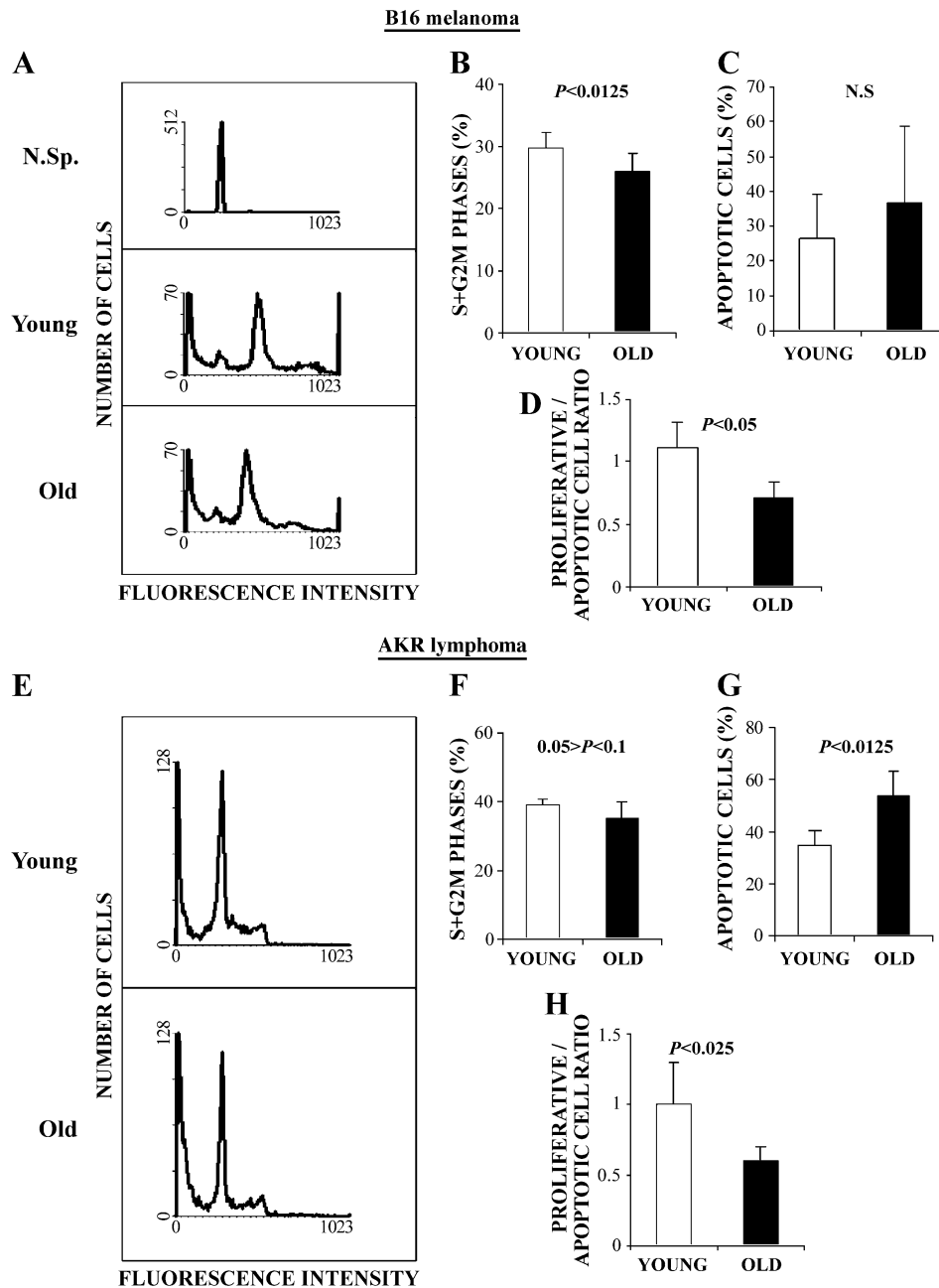

Fig. 3. Profile of DNA flow cytometry in B16 melanoma and AKR lymphoma growing in young as compared to old mice. A and E show representative experiments with B16 melanoma and AKR lymphoma, respectively. Quantitative evaluations of the data of four experiments with regard to cell proliferative

(1:500) and secondary antibody-HRP-conjugated (1:10,000) (StressGen Biotechnologies Corp, Victoria, British Columbia, Canada) were used for identification, incubating for 1 h with each of the antibodies. Five washes in TTBS (10 mM Tris–HCl, 0.15 M NaCl, 0.1% Tween, pH 7.4) followed each incubation. The caspases were detected by enhanced chemiluminescence (ECL) using the substrate of Pierce (SuperSignal West Pico Trial Kit; Pierce, Rockford, IL). HeLa cell lysates (2 μg/lane) (StressGen Biotechnologies) served as positive control for caspases 8, 3 and 9. A quantitative analysis was performed using the KODAK 1D 3.5 software.

2.8. Statistical evaluation

Statistical evaluation was performed for most data by Student's t test. The Log-rank test [33] was used for the cumulative incidence and mortality summarized from all experiments. The χ^2 test was used to test the significance of the differences between the treated and nontreated groups of mice in long-term survivors.

3. Results

3.1. Differential biological behavior of B16 melanoma and AKR lymphoma in young and old mice

The biological behavior of the B16 melanoma and the AKR lymphoma in young and old mice is presented in Fig. 1. A reduced tumor growth rate was observed in aged as compared to young animals in both tumors (Fig. 1A and B). As seen in the inserts, the differences between tumor size in young and old mice were statistically significant in both the B16 melanoma ($P < 0.0125$ on day 22) and the AKR lymphoma ($P < 0.001$ on day 13). Mortality was markedly inhibited in old as compared to young B16 melanoma-bearing animals (Fig. 1B). The difference between young and old mice, according to the average survival time (Fig. 1C), was statistically highly significant ($P < 0.0025$). Moreover, a significant number of long-term survivors (LTS) were observed in both young and old mice, but the number of LTS was higher in old than in young mice (Fig. 1D), again statistically highly significant. The number of long-
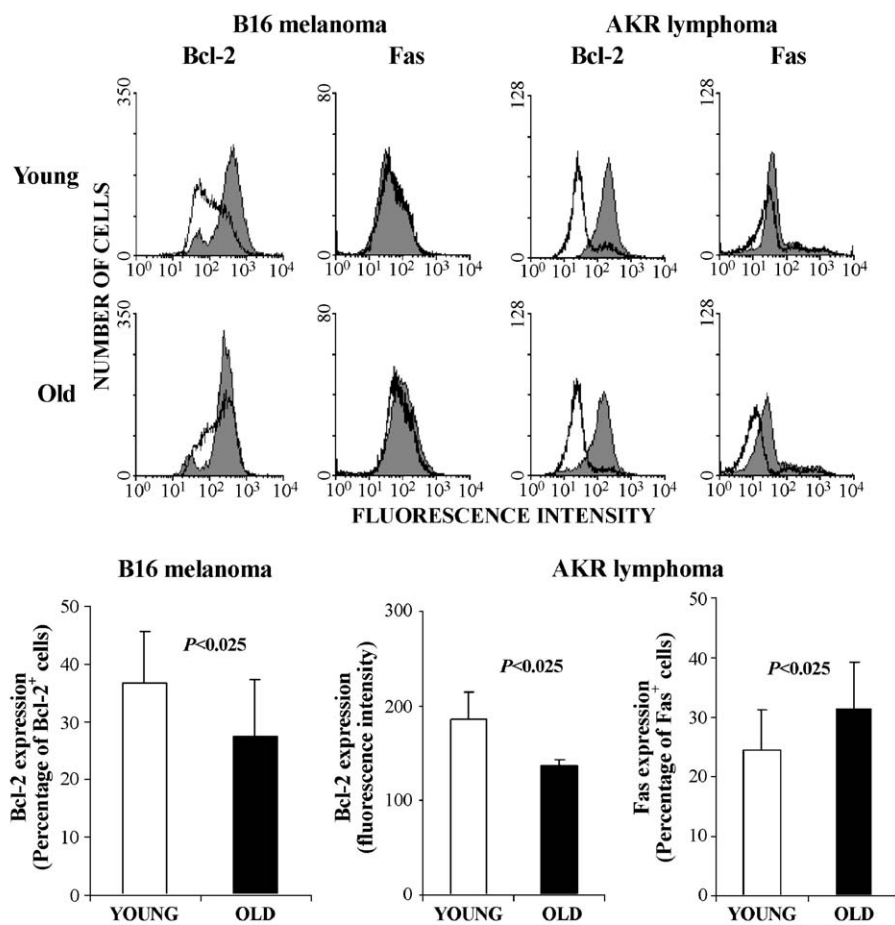

Fig. 4. Effect of host age on apoptosis of tumors: evidence at the molecular level. Comparison of Bcl-2 and Fas protein expression in B16 melanoma and AKR lymphoma from young and old mice. Upper part: representative experiments. Empty areas: secondary antibody only; gray areas: anti-Bcl-2 or anti-Fas antibody-positive cells. Lower part: quantitative evaluation of six experiments with regard to Bcl-2$^+$ and Fas$^+$ cell populations.

term survivors was 3/44 (6.8%) and 15/44 (34%) in young and old mice, respectively. Thus, in old mice a fivefold increase was found in long-term survivors as compared to young animals. In AKR lymphoma, the differential age-related reduced tumor growth was demonstrated by both cumulative tumor incidence (not shown) and tumor size (Fig. 1A). This was not seen, however, with regard to mice mortality (see Figs. 6 and 7).

3.2. Evidence indicating that increased apoptosis constitutes a mechanism of the age-related reduced tumor growth rate in aged mice

Figs. 2–5 adduce evidence that tumors from old mice have a higher propensity for undergoing apoptosis than tumors in young animals. Staining of tumor cells by Apop-Tag showed a higher content in apoptotic bodies in tumor
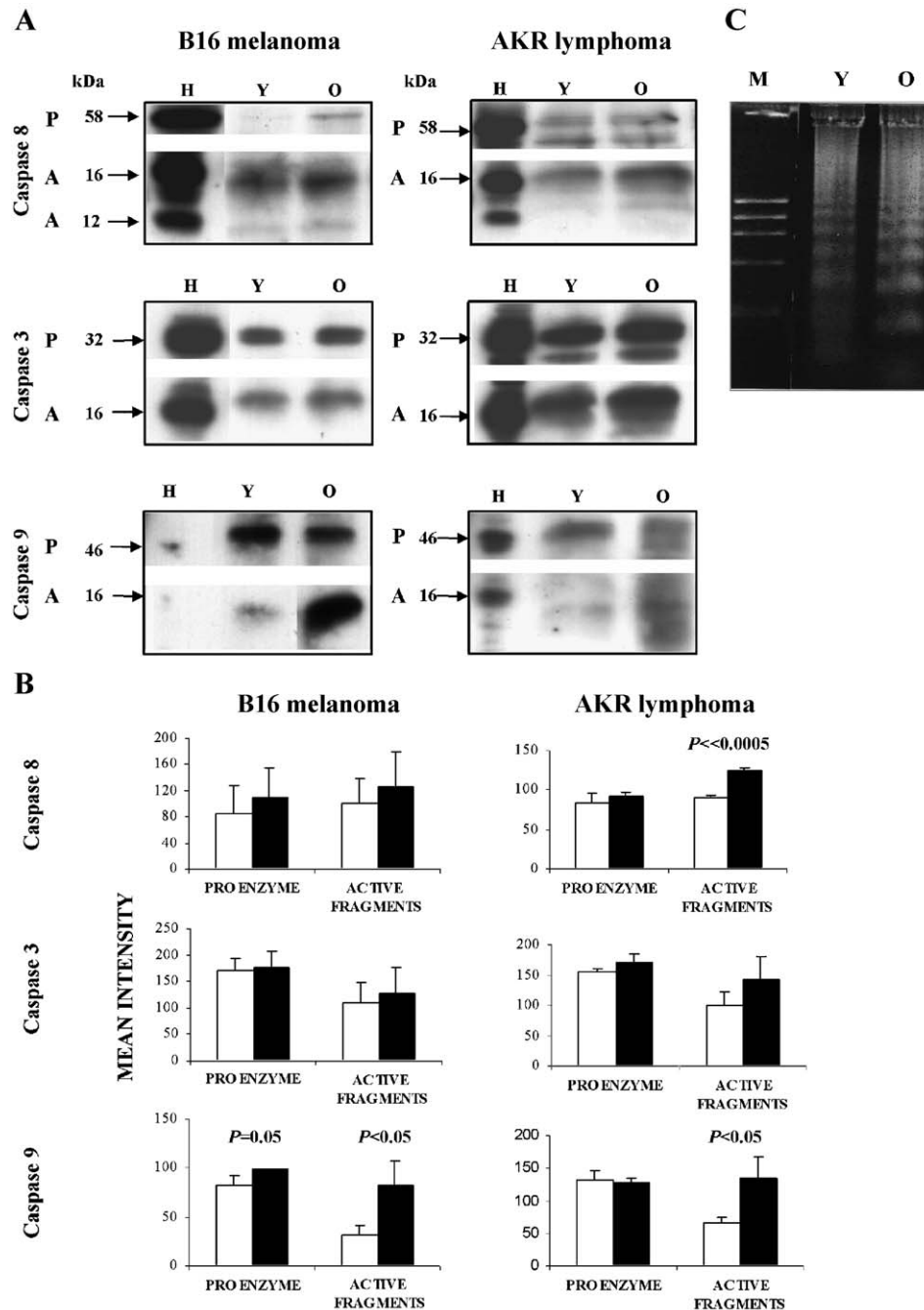

Fig. 5. (A) Western blot analysis of caspases 8, 3 and 9 in B16 melanoma and AKR lymphoma derived from young (Y) and old (O) mice. P—precaspase; A—active caspase. H—HeLa cell lysate. (B) Quantitative evaluation of four different experiments. Empty columns—young mice; black columns—old mice. (C) Comparison of DNA fragmentation in AKR lymphoma cells derived from young and old mice. Lane "M" represents the DNA marker of different sizes. Y—young; O—old.
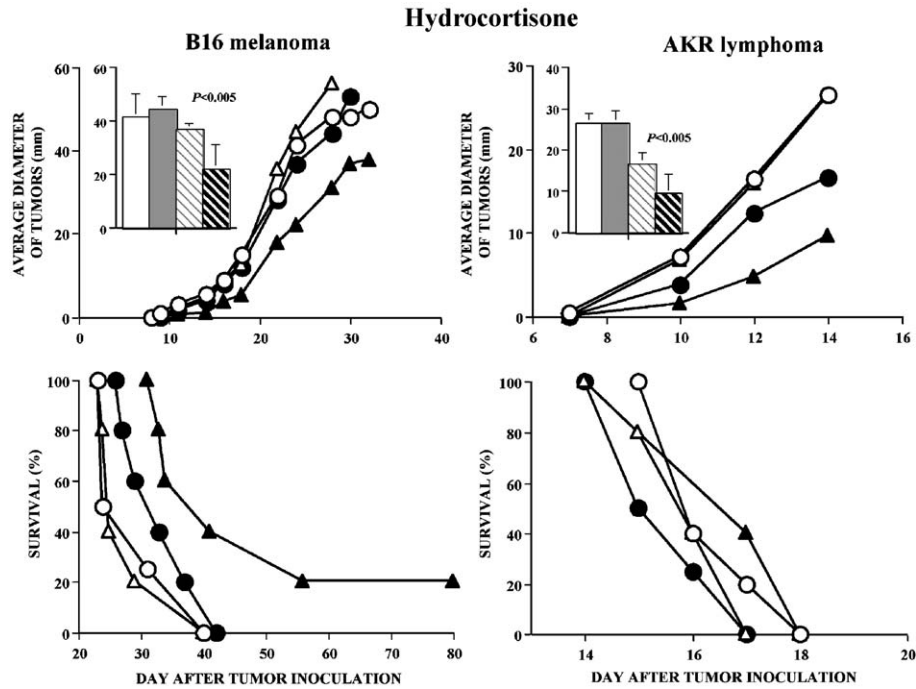
Fig. 6. Differential effect of hydrocortisone treatment on B16 melanoma and AKR lymphoma in young and old mice. Nontreated young (○) and old (●) tumor-bearing mice; hydrocortisone-treated young (△) and old (▲) tumor-bearing mice. A comparison of the average diameter of tumors on day 24 (for B16 melanoma) and on day 14 (for AKR lymphoma) is shown in the inserts. Inserts: Clear area—young nontreated; gray area—young treated; striated—old nontreated; striated bold—old treated. The number of mice in each group was 5.

cells from old mice as compared to those derived from young ones, both in B16 melanoma and in AKR lymphoma (Fig. 2). The increase in the average apoptotic cell content in old as compared to young mice was 18-fold in B16 melanoma and about 11-fold in the case of AKR lymphoma, as was calculated from Fig. 2B and C, respectively.
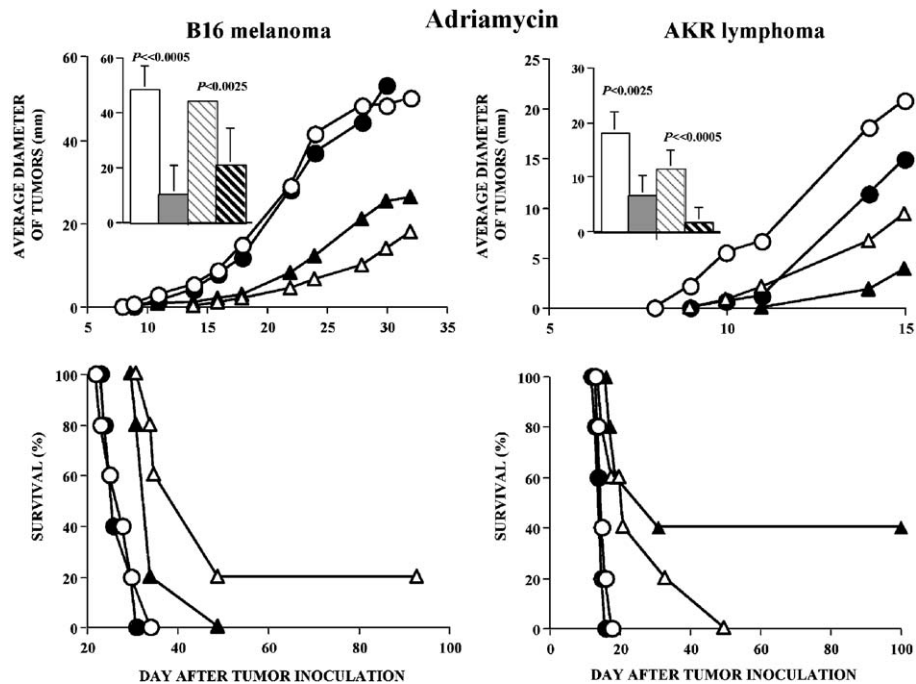

Fig. 7. Differential effect of adriamycin treatment on B16 melanoma and AKR lymphoma in young and old mice. Nontreated young (○) and old (●) mice; ADR-treated young (△) and old (▲) mice. Inserts show the average diameter of tumors on day 28 for B16 melanoma and on day 14 for AKR lymphoma. Clear area—young nontreated; gray area—young treated; striated—old nontreated; striated bold—old treated. The number of mice in each group was 5.
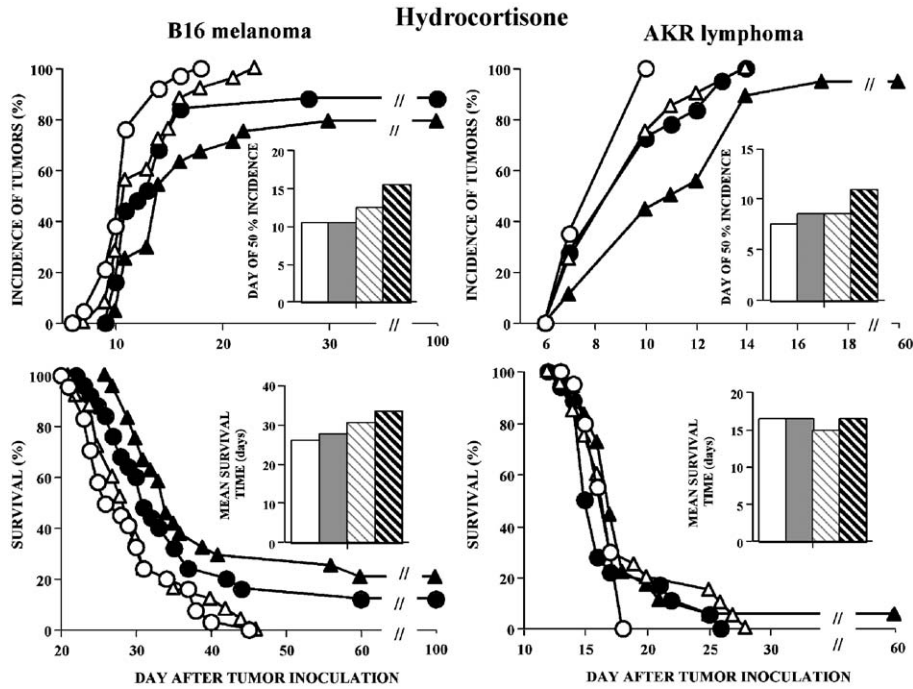
Fig. 8. Effect of hydrocortisone treatment on the B16 melanoma and AKR lymphoma in young and old mice—cumulative incidence and mortality of all experiments. Nontreated young (○) and old (●) tumor-bearing mice; treated young (△) and old (▲) tumor-bearing mice. The inserts show the day of 50% incidence and the mean survival time in young nontreated mice (clear area), in young treated (gray area), in old nontreated (striated area) and in old treated mice (striated bold). The number of mice in each group is that appearing as total number of mice in Table 1.

The comparison of DNA flow cytometry of B16 melanoma and AKR lymphoma derived from young and old animals is presented in Fig. 3. Fig. 3A and E shows representative experiments of B16 melanoma and AKR lymphoma, respectively. The figure further summarizes the quantitative evaluation of several experiments done with B16 melanoma (Fig. 3B–D) and AKR lymphoma (Figs. 3F–H).
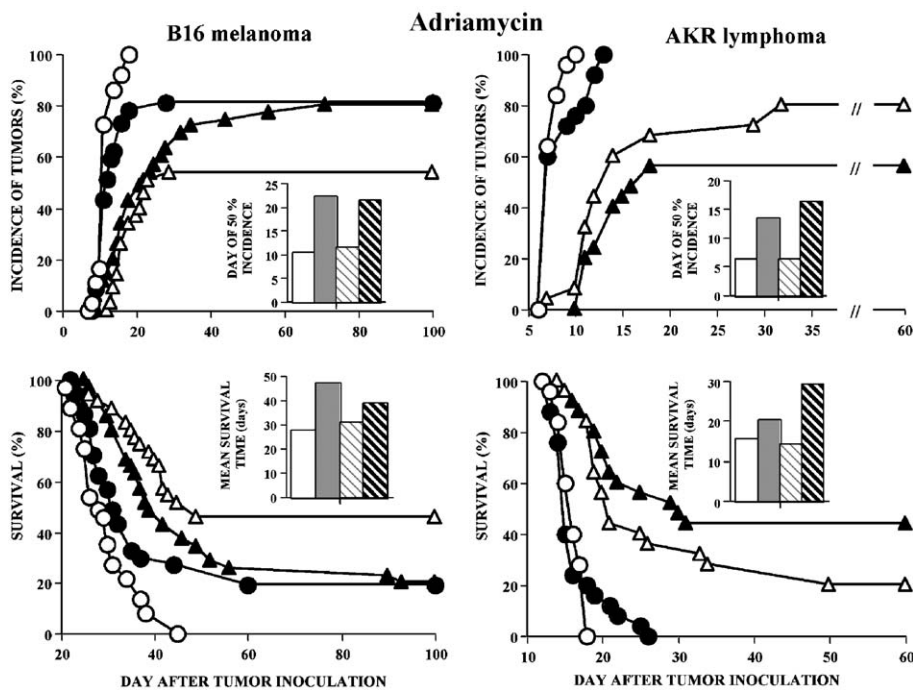

Fig. 9. Effect of adriamycin treatment on the B16 melanoma and AKR lymphoma in young and old mice—cumulative incidence and mortality of all experiments. Details as in Fig. 8.

Table 1
Age-dependent effect of hydrocortisone and adriamycin treatment on B16 melanoma and AKR lymphoma: effect on long-term survivors

B16 melanoma				AKR lymphoma			
Age	Treatment	S/T	%S	Age	Treatment	S/T	%S
Hydrocortisone treatment							
Young	Control	0/24	00.0	Young	Control	0/20	0.0
Young	HC	0/25	00.0	Young	HC	0/20	0.0
Old	Control	3/25	12.0	Old	Control	0/18	0.0
Old	HC	5/24	20.8	Old	HC	1/18	5.6
Adriamycin treatment							
Young	Control	0/37	00.0	Young	Control	0/25	0
Young	ADR	16/35	45.7	Young	ADR	5/25	20
Old	Control	7/37	18.9	Old	Control	0/25	0
Old	ADR	7/35	20.0	Old	ADR	11/25	44

Two age groups of mice were used: young mice, 6–8 weeks for both C57BL/6J and AKR/J mice; old mice, 16–24 months for C57BL/6J mice and 7–9 months for AKR/J mice. C57BL/6J mice were inoculated s.c. with 2.2×10^5 cells of B16 melanoma and AKR/J mice were injected by the same route with 1×10^4 cells of AKR lymphoma. Control—nontreated tumor-bearing mice; HC—the mice were treated with 500 µg/mouse of hydrocortisone at a single dose, injected intratumorally on day 1 after tumor cell inoculation; ADR—the mice were treated with 25 µg/mouse of adriamycin at a single dose, injected intratumorally on day 1 after tumor cell inoculation. S/T=surviving/total number of mice; %S=percentage of surviving mice.

The comparison of DNA flow cytometry of B16 melanoma as compared to normal spleen (Fig. 3A) shows that the majority of the cells is tetraploid. This has been reported for the murine B16 melanoma [34,35] as well as for highly malignant human melanoma [35,36]. The AKR lymphoma displays a near diploid cell population. In both tumors, a decrease in proliferative cell fraction (S + G2M) (significant in B16 melanoma and at the limit of significance in AKR lymphoma) concomitant with an increase in apoptotic cell fraction (sub G1 population) (significant in AKR lymphoma, nonsignificant in B16 melanoma) is seen in old as compared to young mice. When the ratio of proliferative cell fraction per apoptotic cell fraction of the different experiments is calculated, a sharp decrease in this ratio is seen in

aged as compared to young mice with both tumors, statistically significant in both cases.

Examination of the expression of apoptosis-related genes in B16 melanoma and AKR lymphoma (Fig. 4) supports the results at the cellular level. Tumors from aged mice express a lower level of Bcl-2 protein as compared to those derived from young animals. According to the average of six experiments, the difference was statistically significant ($P < 0.025$) in both tumors. Contrary to bcl-2, the expression of the fas gene was higher in lymphoma cells originating from the old than in those grown in young mice in the AKR lymphoma. The difference was statistically significant ($P < 0.025$). No Fas receptor expression was detected in B16 melanoma cells, whether derived from young or from old mice.

Comparison of caspases 8, 3 and 9 activities, analyzed by Western blot in cells from B16 melanoma and AKR lymphoma cells derived from mice of different ages (Fig. 5), generally demonstrates a higher activity of the three enzymes in B16 melanoma and AKR lymphoma cells from aged as compared to tumors from young mice. This difference was lower in the case of caspase 3 in the B16 melanoma. The content in procaspase was higher in caspase 8 in B16 melanoma and in caspase 3 in AKR lymphoma. The quantitative evaluation shows that all three caspases are more active in tumors derived from old mice than in those originating from young ones in both tumors. The differences reached statistical significance in the case of caspases 8 and 9 in the case of AKR lymphoma and in the case of caspase 9 in B16 melanoma. The lack of significance may be due to two reasons: first, although the differences between young and old were consistently observed in each experiment, there were large differences in band intensities between the different experiments, resulting in high standard deviations; second, in fact, the differences, in most cases, are underestimated by the quantitative evaluation because high intensities appear to be "seen" much below linear relation.

A comparison of ladder type DNA degradation, as seen by agarose gel electrophoresis, between tumors derived from young and aged animals shows that degradation of

Table 2
Assessment of statistical significance of treated versus nontreated tumor-bearing mice according to different statistical tests

Tumor	Age of mice	Treatment	Incidence of tumors		Mice mortality		
			Average day of 50% incidence of tumors (*t* test)	Significance according to Log-rank test	Average survival time (*t* test)	Significance according to Log-rank test	Significance of LTS according to the χ^2 test
B16 melanoma	Y	HC	$P < 0.05$	$0.05 < P < 0.1$	N.S.	N.S.	N.S.
B16 melanoma	O	HC	N.S.	$0.05 < P \leq 0.1$	N.S.	N.S.	N.S.
AKR lymphoma	Y	HC	$0.05 < P < 0.1$	N.S.	$P < 0.05$	$0.05 < P < 0.1$	N.S.
AKR lymphoma	O	HC	$P < 0.025$	$P < 0.025$	N.S.	N.S.	N.S.
B16 melanoma	Y	ADR	$P \ll 0.0005$	$P \ll 0.00025$	$P \ll 0.0005$	$P \ll 0.00025$	$P \ll 0.00025$
B16 melanoma	O	ADR	$P \ll 0.0005$	$P < 0.05$	$P \ll 0.0005$	$0.05 < P < 0.1$	N.S.
AKR lymphoma	Y	ADR	$P \ll 0.0005$	$P \ll 0.00025$	$P \ll 0.0005$	$P \ll 0.00025$	$P < 0.0125$
AKR lymphoma	O	ADR	$P \ll 0.0005$	$P \ll 0.00025$	$P < 0.0025$	$P \ll 0.00025$	$P < 0.0005$

Y = young; O = old. Other details as in Table 1.

DNA is more intense in the tumors taken from old than in those derived from young mice in AKR lymphoma (Fig. 5C). Similar results were obtained with the B16 melanoma (not shown).

3.3. Differential effect of apoptosis-inducing agent therapy (hydrocortisone and adriamycin) on tumors in young and old mice

Fig. 6 presents the effect of hydrocortisone treatment on B16 melanoma and AKR lymphoma growth in young and old mice in a representative experiment. The results demonstrate a very marked inhibition of the growth of both tumors in aged animals. The difference in the average tumor size between the HC-treated as compared to nontreated aged tumor-bearing mice was statistically highly significant ($P < 0.005$) in both malignancies. In young mice, by contrast, AKR lymphoma was not inhibited by hydrocortisone. Moreover, in the B16 melanoma, a slight tumor enhancement by HC was seen. Hydrocortisone markedly delayed the mortality of old mice in both B16 melanoma and AKR lymphoma. Mice mortality in young mice was, by contrast,
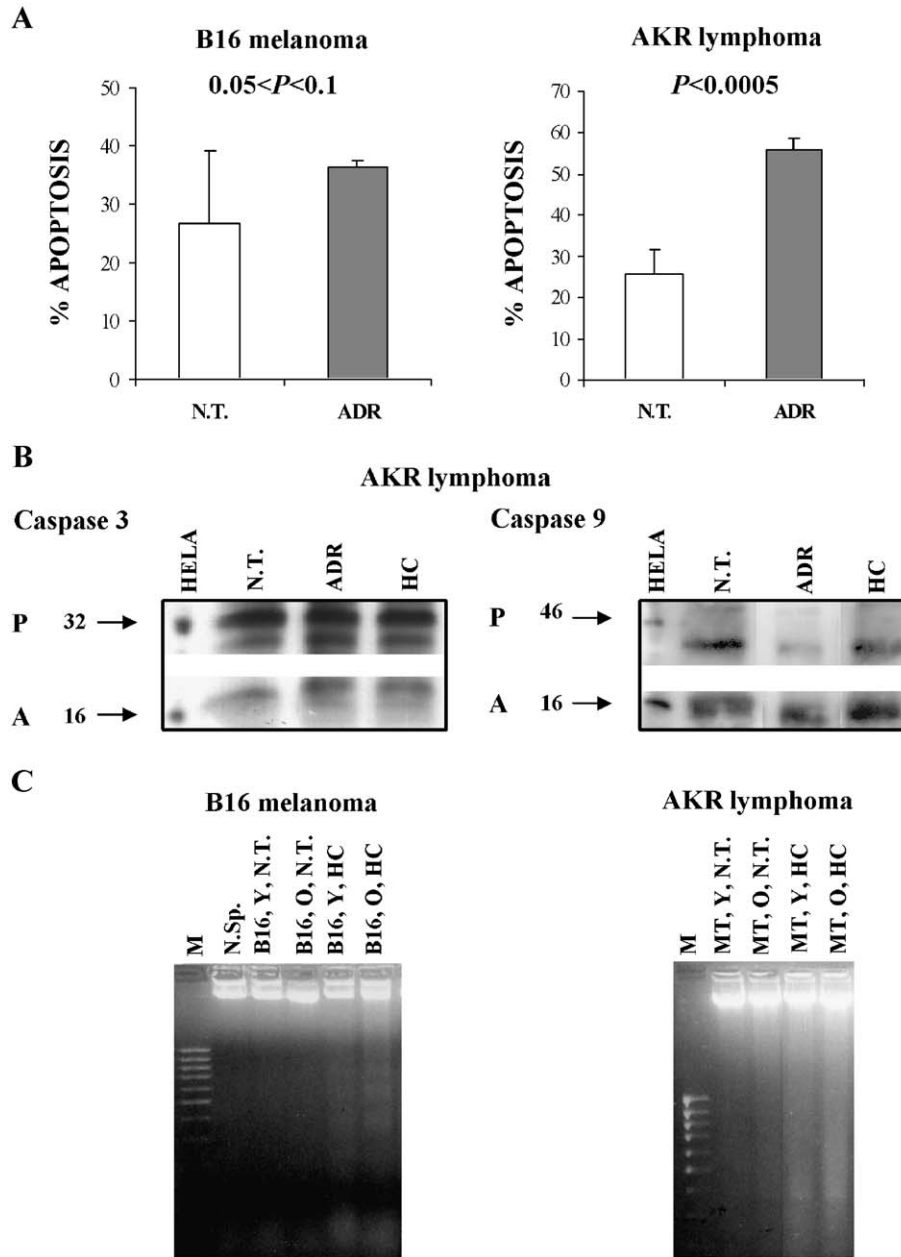

Fig. 10. Induction of cell apoptosis by hydrocortisone (HC) and adriamycin (ADR) in B16 melanoma and AKR lymphoma. (A) Effect of in vitro ADR treatment on apoptotic cell fraction. (B) Effect of ADR and HC on caspases 3 and 9 activities on AKR lymphoma of young mice. (C) Effect of HC on apoptotic cell death in young and old mice according to DNA degradation. HELA—HeLa cells; P—procaspase; A—active caspase; M–marker; N.Sp.—normal spleen; MT—metastatic tumor; Y—young mice; O—old mice; N.T.—nontreated; HC—hydrocortisone-treated mice; ADR—adriamycin-treated mice.

slightly accelerated by the corticosteroid treatment, again in both tumors.

Treatment by adriamycin, shown in a representative experiment (Fig. 7), contrary to our anticipation, inhibited the B16 melanoma more markedly in young than in old mice, in terms of tumor incidence (not shown), tumor size and mice mortality. In AKR lymphoma, nevertheless, as with hydrocortisone, ADR had a higher tumor inhibitory effect on tumors from aged animals than in those from young ones. This was demonstrated by tumor incidence (not shown) and size as well as by mice mortality rate, including the number of long-term survivors.

Figs. 8 and 9 summarize the data of all experiments done with regard to hydrocortisone and adriamycin treatment, respectively, in relation to tumor incidence and mice mortality, for B16 melanoma and AKR lymphoma. Table 1 summarizes the data regarding long-term survival (LTS) for the two treatments for both tumors. Table 2 presents the statistical evaluation of the efficiency of treatments according to different statistical tests.

The effect of hydrocortisone on B16 melanoma appears to be slightly more efficient in old than in young mice (Fig. 8), according to the mean day of 50% incidence and according to the mean survival time (see inserts). The differences between treated and nontreated mice were, however, nonsignificant according to most statistical tests in both young and even less so in old mice (see Table 2).

As for the AKR lymphoma, old-mice-derived tumors were more affected by hydrocortisone than young ones according to the cumulative incidence of tumors (see insert in Fig. 8). This difference between the effect of HC on the lymphoma in young and old mice is statistically more significant in aged mice, both according to the average day of incidence and according to the Log-rank test (Table 2). Mortality, however, was more rapid in old than in young mice (same table).

The effect of adriamycin on B16 melanoma in young and old mice, as summarized in Fig. 9, shows (contrary to our expectation) that ADR affected more efficiently the tumors of young mice than those from old animals. This is seen according to both the Log-rank test and the χ^2 test for the comparison of the long-term survivors (Table 2).

The AKR lymphoma was, however, clearly more markedly affected in old than in young animals: see inserts in Fig. 9 and statistical evaluation of the significance of the long-term survivors data in Table 2.

Fig. 10 shows that both hydrocortisone and adriamycin induce apoptosis in vitro, in both B16 melanoma and AKR lymphoma. The effect of ADR was more pronounced on the AKR lymphoma than on the B16 melanoma cells (Fig 10A). Both adriamycin and hydrocortisone treatments increased activation of caspases 3 and 9 (Fig. 10B).

Fig. 10C shows, according to DNA degradation, that hydrocortisone induces a higher rate of apoptosis in tumors of old than in those of young mice, in both AKR lymphoma and B16 melanoma. In the case of AKR lymphoma, metastatic tumors are presented in Fig. 10, while in Fig. 5 DNA degradation of primary tumors is shown. We have previously shown that metastatic cells have a much lower tendency to apoptosis than primary AKR lymphoma cells [37]. In the case of metastatic cells, DNA degradation appears partly typical for necrotic cells. However, apoptotic cells can become necrotic [38].

Both hydrocortisone and adriamycin induce apoptosis in AKR lymphoma and B16 melanoma. Moreover, hydrocortisone provokes a more marked effect on tumors growing in old than in those derived from young mice.

4. Discussion

The increasing number of elderly cancer patients imposes a more complex therapeutic approach in this population, in view of the decreased homeostatic reserve and the increased prevalence of concomitant diseases so often encountered in the old. Functional status and comorbidity have to be taken into account in determining the eligibility of elderly cancer patients for chemotherapy [39].

Aged cancer patients are often offered suboptimal treatment. In fact, the elderly have been routinely underrepresented or excluded from most cancer studies [40]. Recently, however, agreement has been reached that age alone should not be the only consideration in making treatment decisions [39].

In addition to the abovementioned differences between young and old cancer patients (which must be taken into account), it seems that the tumor itself may behave differently in hosts of different ages. Various authors [1–7] as well as ourselves [8,9] demonstrated such an age-dependent differential biological behavior of tumors.

Our previous studies [8] were related to B16 melanoma. Here we showed that the AKR lymphoma as well grows at a markedly slower rate in old as compared to young mice. This was seen according to cumulative incidence and size of tumors. Mice mortality was, however, in most experiments, accelerated in old as compared to young mice. This finding is difficult to explain and merits further research. It is possible that the high apoptotic cell level and possibly necrotic cells derived from the apoptotic bodies [38] of the tumor induce some systemic damage provoking an earlier mortality. Indeed, tumor suppression was recently found to be accompanied by accelerated aging in p53-overexpressing mice [41] and antitumoral intervention has been suggested to eventually affect aging [42], possibly via apoptosis.

We suggest that the age-related differential tumor biology should also be taken into account in the design of treatment modalities for the aged. Understanding the mechanism(s) responsible for the age-related biological behavior of tumors may permit a logical design of age-adjusted anti-tumoral therapy.

In the present study, we demonstrated in B16 melanoma and in AKR lymphoma that increased apoptotic cell death is at least one of the mechanisms responsible for the age-related reduced malignancy of tumors. We demonstrated an increase in apoptotic cell death in tumors derived from old as compared to young mice, according to cell morphology, DNA fragmentation, modification in apoptosis-related gene expression (bcl-2 and fas) and increased caspase 8, 3 and 9 activities.

Various groups [43,44] and ourselves [45] have shown increased apoptosis with increasing age in normal tissues. We suggest that apoptosis-inducers may be present in normal aged organs and these might affect tumor cells situated in this environment, thereby reducing tumor growth. Indeed, healthy cells can be induced to die by apoptosis if exposed to the medium conditioned by senescent cells of the same or even different species [46].

We postulated that it may be possible to design age-adjusted treatment modalities based on the mechanism(s) responsible for the reduced tumor progression rate in the aged. One of these mechanisms is a decline in the proliferative capacity of tumor cells with age [11]. However, decreased proliferative ability implies decreased sensitivity to drugs. Classical antiproliferative chemotherapy is therefore expected to be less efficient against cancer in the old. This in fact constitutes an additional hindrance in cancer treatment of the aged. For instance, breast tumors in elderly patients display an indolent behavior, implying a lower proliferative rate and therefore a lower susceptibility to cytotoxic agents [40]. Inducing apoptosis, instead of acting on cell proliferative ability, might therefore have an additional advantage of avoiding this age-related drug-resistance.

Based on our results, pointing to an increased apoptotic cell death as being one of the mechanisms responsible for the reduced tumor aggressiveness in the old, we attempted to discover whether treatment with apoptosis-inducing drugs might result in differential treatment efficacy in old and young tumor-bearing mice. We compared the treatment efficacy of two apoptosis-inducing agents, hydrocortisone and adriamycin, on tumors growing in mice of different ages. Hydrocortisone, which acts as a potent specific inducer of apoptosis, was more efficient against tumors growing in old than in those growing in young mice, in both B16 melanoma and more so in the AKR lymphoma, according to incidence and size of tumors. While this same tendency was observed with ADR treatment of AKR lymphoma, opposite results were obtained in B16 melanoma treatment: ADR therapy was more efficient in young than in old mice. This was evident according to incidence and size of tumors as well as according to the data regarding long-term survivors. We have also demonstrated that the differential effects of both hydrocortisone and adriamycin can, at least partly, be due to induction of apoptotic cell death.

It is now well established that many chemotherapeutic drugs, including adriamycin, exert their effect, at least partially, by apoptosis [47]. The cytotoxic effect of chemotherapeutic agents depends on Fas and Fas ligand [48]. Loss of Fas expression during chemotherapy constitutes one of the mechanisms of cancer drug resistance.

Due to their "lympholytic effect", adrenal corticosteroids have been used to treat lymphomas since the late 1940s [49]. Lymphoid and leukemic cells are generally prone to apoptosis and, moreover, uniquely sensitive to the lytic action of glucocorticoid hormones [50].

It is believed that the caspase cascade activated by GC occurs through mitochondria and not through membrane death receptors such as Fas [51]. In AKR lymphoma and B16 melanoma, both the extrinsic (involving caspase-8) and the intrinsic (mitochondria-mediated, involving caspase-9) pathways of caspase-activating cascades [52] were more active in old than in young mice (Fig. 3A).

An age-related increase of glucocorticoid levels has been reported in the literature [53]. Elevated corticosteroids, as well as elevated tumor necrosis factor [54] with aging, might be the reason for the increased apoptosis that we have shown to occur in normal tissues derived from aged mice [45] as well as in tumors growing in aged mice (shown here). It is therefore possible that tumors in old organisms are more prone to apoptosis due to the presence of apoptosis-inducers in their microenvironment. Apoptosis-inducing therapy may prove to be far less toxic than antimitotics [55], which in itself is of importance for the aged cancer patient.

To explain the different action exerted by ADR on B16 melanoma, the following can be said: GC-signaling to apoptosis is essentially different from that of cytostatic drugs or ionizing radiation. Glucocorticoids can kill their sensitive target cells only by inducing programmed cell death whereas the contribution of apoptosis to the total level of tumor cell kill by cytostatic drugs such as adriamycin and radiation is a matter of debate [56]. Thus, mechanisms other than apoptosis-induction (such as antiproliferative effect) may be responsible for the higher effect of ADR on B16 melanoma of young mice, which may not be efficient against tumors of old mice. In this sense, adriamycin (and cytotoxic agents in general) is not a purely apoptosis-inducing agent. In addition, the B16 melanoma appears to be less prone to apoptosis than the AKR lymphoma. Indeed, it is not expected that all apoptosis-inducing treatments will be more effective in old mice against each tumor. However, in view of the rapidly increasing aged population and the paucity of efficient antitumoral treatments in the aged, it may be of importance if such a mechanism of treatment will be effective in some tumors.

Since elderly cancer patients are usually considered unsuited for the often aggressive therapeutic modalities now applied to cancer patients, it may be of high importance to find that milder treatments could be appropriate for patients of advanced age. Our finding that certain apoptosis-inducing agents, in certain tumors, might be even more efficient against tumors in old organisms is particularly

encouraging. This in view of the accepted dogma (and of the massive data published in this field) that cancer treatment is less efficient and more dangerous for the elderly patient.

The differential malignant behavior of tumors in old as compared to young patients might impose a new concept in the treatment of elderly cancer patients. While aging is considered to preclude current (mainly antiproliferative) antitumoral treatments, therapeutic modalities based on other mechanisms (those shown to be responsible for the reduced tumor progression rate in the old) might be as efficient or even more efficient in aged than in young organisms.

The understanding of the mechanisms underlying the reduced aggressiveness of tumors in the aged might be of high importance since it may shed some light on yet unknown physiological means able to inhibit or confine tumor growth. Moreover, information about these presumed natural antitumoral means might suggest new therapeutic targets not only for old but also for young people.

Acknowledgements

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