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Aging but not dietary restriction alters the activation-induced apoptosis in rat T cells

M.A. Pahlavani*, D.A. Vargas

Geriatric Research, Education, and Clinical Center (182), South Texas Veterans Health Care System, Audie L. Murphy Veterans Hospital, and Department of Physiology, University of Texas Health Science Center, 7400 Merton Minter Blvd., San Antonio, TX 78284, USA

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Abstract The aim of this study was to determine if aging or dietary restriction (DR) alters activation-induced cell death, which is known to regulate cell proliferation and eliminate the high number of activated cells during an immune response. Splenic T cells were isolated from young (4–6 months) and old (25–26 months) Fischer 344 rats that had free access to food, ad libitum (AL), and from dietary-restricted (DR) old (25–26 months) rats that beginning at 6 weeks of age were fed 60% (40% food-restricted) of the diet consume by the AL rats. T cells were incubated with anti-CD3 antibody, or staphylococcal enterotoxin B (primary stimulus) for 72–96 h, followed by restimulation with anti-CD3 (secondary stimulus) for 72 h. Activation-induced apoptosis was assessed by DNA fragmentation and the expression of Fas/CD95 receptor and Fas ligand (Fas-L) was measured by flow cytometry. We found that the amount of DNA fragmentation was significantly ($P < 0.05$) higher in the stimulated and restimulated T cells from AL old rats and DR old rats compared to young rats. The increase in DNA fragmentation with age was paralleled by an increase in the proportion of the cells expressing Fas and Fas-L. However, DR had no significant effect on the age-related increase in DNA fragmentation or the expression of Fas or Fas-L. We also measured the levels of Bcl-2 and Bax protein and found that the level of Bcl-2 decreased and Bax increased with age and that DR had no effect on the age-related changes in the level of Bcl-2 or Bax protein. These results demonstrate that aging but not DR alters activation-induced apoptosis in rat T cells.

Key words: Activation-induced cell death; T cell; Aging; Dietary restriction; Rat

1. Introduction

Apoptotic cell death plays a key role in the maintenance of homeostasis in various physiological systems including the immune system (reviewed in [1]). Stimulation of T cells with activating agents such as concanavalin A, PHA (phytohemagglutinin), mitogenic antibody (anti-CD3), or superantigen, staphylococcal enterotoxin B (SEB), results in a strong proliferative response that usually leads to the expansion of cells and the expression of cytokines (e.g. IL-2). If the responding cells are allowed to revert to a resting phenotype and are subsequently restimulated, substantial amounts of cytokines are produced. Nevertheless, instead of entering another round of proliferation, the majority of the restimulated cells rapidly undergo activation-induced cell death (AICD) (reviewed in [2,3]). This process is of physiological importance because the presence of too many activated cells with different specificities might trigger excessive secondary immune responses leading to symptoms similar to autoimmune disorders and toxic shock [2,3].

Activation-induced apoptosis is mediated by Fas (CD95) receptor, a 48 kDa surface glycoprotein belonging to the tumor necrosis factor/nerve growth factor receptor family. Interaction of Fas with its ligand Fas/CD95 ligand (Fas-L) triggers a series of events leading to DNA fragmentation and cell disintegration [4,5]. In addition, gene products of some members of the Bcl-2 family of protooncogenes (e.g. Bcl-2 and Bcl-x_L) have apoptosis-inhibiting activity whereas other members (e.g. Bax and Bad) have been shown to promote apoptosis (reviewed in [6,7]). The Bcl-2 family members can bind to each other in various pairwise combinations (e.g. homodimers of Bcl-2:Bcl-2 or heterodimers like Bcl-2:Bax), and form ion-conducting channels in the mitochondria membrane [6,7]. How Bcl-2 family members function to promote or inhibit apoptosis is uncertain. The leading theory is that the anti-apoptotic Bcl-2-like proteins inhibit caspase activation either by binding directly to a protein called Apaf-1 (apoptotic promoting activating factor-1) or by preventing the release of cytochrome *c* from mitochondria, or both [6,7].

Research over the past three decades has shown that dietary restriction (DR) (i.e. 40% restriction in caloric intake) dramatically extends the life span and reduces the rate of occurrence of numerous physiological dysfunctions in laboratory rodents (reviewed in [8,9]). In addition, DR has been found to delay the incidence of most age-related diseases [8,9] and retard a wide variety of age-sensitive immunological parameters (reviewed in [10,11]). Our laboratory has previously shown that the induction of T cell proliferation and IL-2 expression is diminished with age in rat T cells and that DR attenuated the changes [10,11]. More recently, we found that the induction of Ras/MAPK (mitogen-activated protein kinase) activation decreased with age [12] and that DR reduced the age-related decline in the activation of MAPK but not Ras activity [13]. Although the effect of DR on aging immune system has been investigated by a number of laboratories including our laboratory [11], little is known about the impact of aging or DR on activation-induced apoptosis. Therefore, the objective of the present study was to determine if aging or DR affects AICD in T cells. We hypothesized that AICD increases with age and that DR might attenuate the changes. Our re-

*Corresponding author. Fax: (1)-210-617 5312.
E-mail: pahlavani@uthscsa.edu

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sults show that the activation-induced DNA fragmentation, Fas, Fas-L, Bax, Bcl-2 changes with age in T cells. However, DR had no significant effect on the age-related changes in the apoptotic parameters that were measured.

2. Materials and methods

2.1. Animals

Male Fischer 344 rats (specific pathogen-free) were obtained at 3 weeks of age from Harlan Sprague-Dawley (Indianapolis, IN, USA). The rats were caged individually in a barrier facility with a 12 h light/dark cycle and were fed a diet as we previously described [14]. At 6 weeks of age, the rats were randomly assigned to two groups. The control group was given free access to the diet and the restricted group received 60% of the diet consumed by the control rats [14]. Three groups of rats were used in these experiments: young (6 month) and old (24 month) rats that were fed ad libitum (AL) and old (24 month) rats fed a restricted diet (DR), 60% of calories consumed by AL rats.

2.2. Cell preparation and activation-induced apoptosis in vitro

Splenic T cells were obtained from rats as we previously described [15]. The cells were resuspended in culture medium, plated in a tissue culture flask, and incubated in the presence or absence of stimulating agent (described below).

Activation-induced apoptosis was performed as described by Radvanyi et al. [16]. The purified T cells (2 million cells/ml) were stimulated with mitogenic antibody (anti-CD3) or SEB (10 μg/ml) plus antigen presenting cells, irradiated adherent cells (B cells and macrophages, 1:1 ratio). After 72–96 h of anti-CD3 or SEB stimulation (primary stimulus), the cells were then harvested, re-plated in a tissue culture flask coated with anti-CD3 antibody (secondary stimulus). Cultured cells were supplemented with exogenous IL-2 (50 U/ml) and after 72 h of incubation, cells were harvested and the occurrence of the activation-induced apoptosis was measured.

2.3. DNA fragmentation analysis

Quantitation of DNA fragmentation was performed by determination of fractional solubilized DNA by diphenylamine (DPA) dye and spectrofluorimetric assay, as described by Duke and Cohen [17]. The DNA was precipitated with 0.5 ml of 25% trichloroacetic acid and was incubated for 15 min at 90°C to hydrolyze DNA. Each DNA sample was then supplemented with 160 μl of DPA reagents (Sigma), and color was allowed to develop for 4 h at 37°C. 200 μl of colored solution was transferred to a well of a 96-well flat-bottom enzyme-linked immunosorbent assay plate and the optical density was determined by a spectrophotometer at a wavelength of 620 nm. Percentage DNA fragmentation was calculated as the ratio of DPA fluorescence in the supernatant divided by the total fluorescence in the supernatant plus the pellet multiplied by 100 [17].

Qualitative DNA fragmentation analysis was performed by lysing the cells in lysis buffer, and the fragmented DNA was extracted following the procedure described by Herrman et al. [18]. Fragmented DNA with the loading buffer was electrophoresed on 1.2% agarose gel at 2 V/cm for 15 h. The DNA in the gel was visualized under UV after staining with 5 mg/ml ethidium bromide.

2.4. Flow cytometric analysis of Fas and Fas-L

The proportion of the activated T cells expressing Fas and Fas-L was assessed by flow cytometry as previously described [19]. Briefly, T cells (1–2 million) were washed and resuspended in FACS buffer (phosphate-buffered saline with 5% fetal calf serum, and 0.1% sodium azide). Cells were stained with optimal concentration (10 μg/ml) of FITC-anti-Fas antibody (PharMingen, San Diego, CA, USA). The analysis of Fas-L expression was performed by incubating the cells in permeabilized solution (0.33% saponin at 4°C) for 60 min followed by staining with FITC-anti-Fas-L antibody (PharMingen, San Diego, CA, USA). An isotype-matched negative control was used to determine the background of fluorescence. Cells were incubated for 30 min at 4°C, washed twice with FACS buffer, and were analyzed with a flow cytometer (FACScan, Becton Dickinson). The samples were gated using forward versus 90° light. For each test sample, 10 000 cells were analyzed and the results were expressed as percentage of the gated cells.

2.5. Western blotting

The levels of Bcl-2 and Bax proteins in the activated T cells were determined by immunoblotting as we previously described [12]. Protein samples (30 μg) were electrophoresed on 10 or 12% SDS–polyacrylamide gel for 3 h at 30 mA. Separated proteins were transferred to a nitrocellulose membrane and probed with an appropriate dilution of the primary antibody for Bcl-2, Bax or actin (control). The immune complex was detected using an enhanced chemoluminescence detection system (ECL) (Amersham, Arlington Heights, IL, USA).

2.6. Statistical analysis

The Mann–Whitney *U* test was used to make comparisons between the two independent samples, young and old AL rats or young and old DR rats. The Wilcoxon signed rank test was used to determine the significant difference between matched groups, i.e. AL old rats vs. DR old rats.

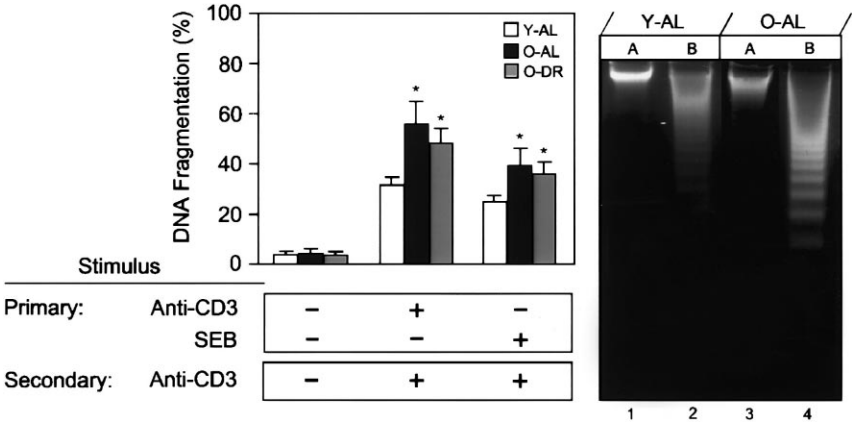

Fig. 1. Effect of age and DR on activation-induced DNA fragmentation in T cells from rats. Splenic T cells were isolated from young (6 month) and old (24 month) rats fed AL or old rats fed a restricted diet (DR). Cells were stimulated with anti-CD3 or SEB for 72–96 h (primary stimulus) followed by restimulation with anti-CD3 (secondary stimulus). DNA fragmentation was assessed 72 h after restimulation and the ratio of low to high molecular weight DNA was determined (left panel) and expressed as percentage as described in Section 2. Each point represents the mean ± S.E.M. for data obtained from AL young (eight spleens) and old (six spleens) rats and from DR old (six spleens) rats. *The values for AL and DR old rats were significantly different from the value for the AL young rats at $P < 0.05$. DNA gel electrophoresis was used to detect DNA ladder formation (right panel). Splenic T cells pooled from three young and two old rats were incubated without stimulatory agent (A) or stimulated and restimulated with anti-CD3 for 72 h (B). DNA extract was prepared and DNA ladder formation was determined by gel electrophoresis as described in Section 2. Lanes 1 and 2, DNA isolated from unstimulated and restimulated T cells from AL young rats, respectively. Lanes 3 and 4, DNA isolated from unstimulated and restimulated T cells from AL old rats, respectively.

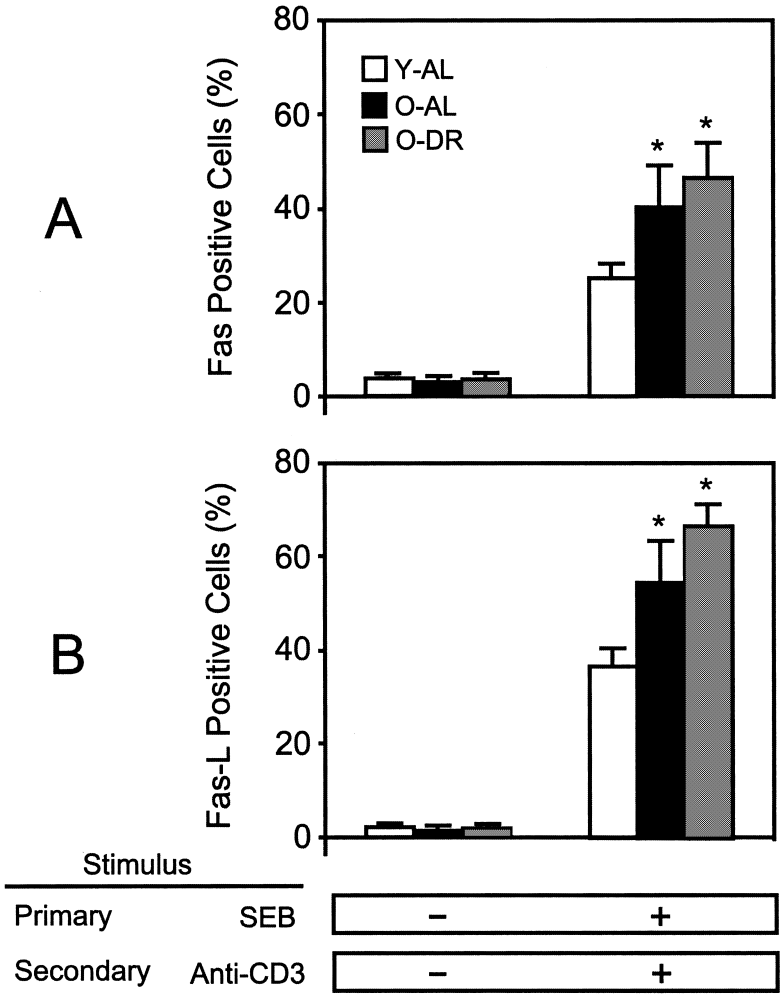

Fig. 2. Effect of age and DR on activation-induced Fas and Fas-L expression. Splenic T cells from AL young and old rats and from DR old rats were incubated with SEB for 96 h, followed by restimulation with anti-CD3 for 72 h. Cells were stained with anti-Fas or anti-Fas-L antibody and the percentages Fas positive cells (A) and Fas-L positive cells (B) were assessed by flow cytometry as described in Section 2. Each point represents the mean ± S.E.M. for data obtained from AL young (six spleens) and old (four spleens) rats and from DR old (four spleens) rats. *The values for AL and DR old rats were significantly different from the value for the AL young rats at $P < 0.05$.

3. Results and discussion

It has been demonstrated that reactivation of T cells after a short period of resting triggers the cells to undergo apoptosis. This phenomenon, which is known as AICD, is believed to limit cell proliferation and to eliminate the high number of activated cells during an immune response (reviewed in [2,3]). In the apoptotic cells, DNA is cleaved into oligonucleosomal fragments [20–22], probably through the activation of an endogenous endonuclease [23,24]. It has been demonstrated that micrococcal nuclease-induced cleavage of DNA in isolated nuclei causes ultrastructural changes similar to those seen in apoptotic nuclei, and therefore DNA fragmentation may be the trigger for such nuclear changes in apoptosis [21]. Immunologic functions decrease with age [25,26] and DR has been shown to reduce/retard the age-related decline in immune function, e.g. mitogen-induced proliferation and IL-2 expression (reviewed in [10,11]). In view of the known age-related decline in immune function and the beneficial effect of DR on immunosenescence and given the potential important role of apoptosis in lymphocyte homeostasis, it was of interest to determine whether aging or DR alters the events that regulate activation-induced apoptosis in T cells. Fig. 1 shows that DNA extracted from resting T cells from AL young or old rats or DR old rats had a very low level of DNA fragmentation. However, the amount of fragmented DNA was increased after stimulation with anti-CD3 or SEB (primary stimulus), followed by restimulation with anti-CD3 (secondary stimulus). For example, after primary and secondary stimulation with anti-CD3, the percentage of fragmented DNA was 66% and 45% higher for activated T cells isolated from AL old rats and DR old rats, respectively, than for T cells isolated from young rats. Although DNA fragmentation was approximately 12% lower in the activated T cells from DR old rats compared to the activated T cells from AL old rats, this difference was not statistically significant. Our results demonstrate that regardless of whether cells were stimulated with anti-CD3 or SEB, the activation-induced DNA fragmentation was increased with age and that DR had no significant effect on the age-related increase in activation-induced DNA fragmentation. The activation-induced DNA fragmentation was also confirmed by conventional DNA ladder analysis, which is frequently used to assess apoptosis [18]. As shown in Fig. 1, DNA ladder formation was undetectable in the unstimulated

cells from young and old rats. However, DNA ladder formation increased in T cells from both young and old rats that were stimulated and restimulated with anti-CD3. The data in Fig. 1 show that the DNA ladder formation was more intense in the activated T cells from old rats than in the activated T cells from young rats.

The mechanism by which activation-induced DNA fragmentation increases with age is yet to be determined. The conventional understanding is that T cells can be induced in vitro to up-regulate Fas and Fas-L when cells are subjected to prolonged stimulation with mitogen or antigen [27–31]. Fas surface protein is highly expressed on activated T cells, and it has been suggested that it may play a crucial role in activation-induced apoptosis in T cells [4,5]. Fas-L is not expressed on resting cells but increases in expression during T cell activation, and it can cross-link to Fas, leading to apoptosis [3–5]. In addition, an increase in Bax protein has been associated with promotion of apoptosis and converse effects occur when the level of Bcl-2 protein increases [6,7]. To establish that the increase in activation-induced DNA fragmentation that we have observed with age was associated with an increase in the proportion of the activated T cells expressing Fas or Fas-L, we measured the percentage of Fas^+ and $Fas\text{-}L^+$ cells in the activated T cells using flow cytometry. Fig. 2 shows that in unstimulated T cells the proportion of cells expressing Fas and Fas-L was very low in T cells isolated from either AL young and old rats or DR old rats. However, the proportion of T cells expressing Fas and Fas-L increased after primary (SEB) and secondary (anti-CD3) stimulation. The percentage of Fas^+ T cells was significantly ($P < 0.05$) higher in the activated T cells from AL old rats and DR old rats compared to the activated T cells from young rats. The expression of Fas surface protein was 62% and 88% higher in the activated T cells from AL old and DR old rats, respectively, than activated T cells from young rats. Similarly, the expression of Fas-L was 46% and 78% higher in the activated T cells from AL old and DR old rats, respectively, than activated T cells from young rats (Fig. 2). Although the proportion of Fas^+ and $Fas\text{-}L^+$ cells was approximately 12–18% higher in the activated T cells from DR old rats compared to AL old rats, the difference was not statistically significant.

Because Bcl-2 and Bax proteins have been implicated in the regulation of apoptosis [1,4–7], we also measured the levels of Bcl-2 and Bax proteins in the resting and activated T cells by Western blot analysis using specific antibodies that recognize each protein or an antibody against actin as a control. Fig. 3 shows a representative Western blot of protein extracts isolated from the cells after primary (SEB) and secondary (anti-CD3) stimulation. A very low level of the Bcl-2 and Bax proteins was detected in the unstimulated cells from AL young and old rats and DR old rats. However, 72 h after secondary stimulation the levels of Bcl-2 protein decreased and Bax protein increased with age. For example, the Bcl-2 protein level was approximately 60% and 65% lower for the activated T cells from AL old and DR old rats, respectively, compared to the activated T cells from young rats. In contrast, Bax protein level was 62% and 75% higher for the activated T cells from AL old rats and DR old rats, respectively, than the activated T cells from young rats (Fig. 3). Although the level of Bcl-2 and Bax protein was slightly higher in the DR old rats compared to the AL old rats, this difference was not statistically significant. Thus, our results for the first time

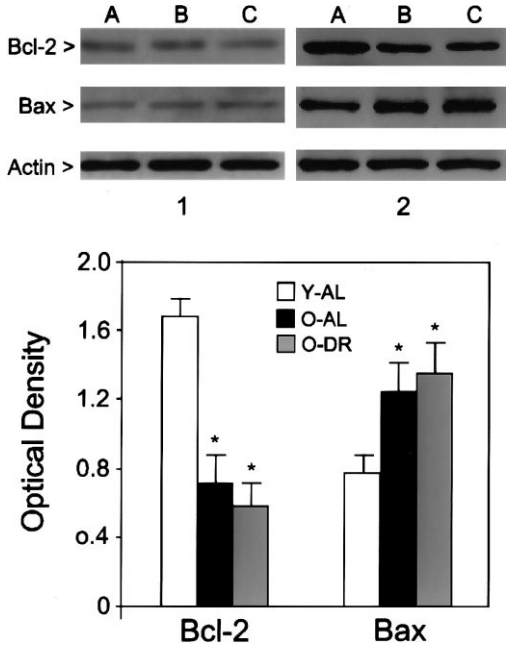

Fig. 3. Effect of age and DR on the levels of Bcl-2 and Bax proteins in T cells from rats. Splenic T cells from AL young (A) and old (B) rats and from DR old (C) rats were incubated without stimulatory agent (panel 1) or with SEB for 96 h, followed by restimulation with anti-CD3 for 72 h (panel 2). Bcl-2 and Bax protein levels were determined by Western blot analysis as described in Section 2. The blots were probed with anti-actin antibody as a control. The bands corresponding to activation-induced Bcl-2 and Bax expression were quantified by densitometry, and the data are presented in the graph. Each point represents the mean ± S.D. for data obtained from three experiments and each experiment was pooled from two rats. *The values for AL and DR old rats were significantly different from the value for the AL young rats at $P < 0.05$.

show that activation-induced apoptosis as assessed by DNA fragmentation, Fas, Fas-L, Bcl-2, and Bax expression alters with age and that DR had no significant effect on the age-related changes in the apoptotic parameters that were measured.

Our results are in agreement with recent studies showing that activation-induced Fas/CD95 expression is increased with age in T cells [32–37]. In addition, this study suggests that activation-induced apoptosis in rat T cells is mediated through the Fas/Fas-L signaling pathway. Our data also show that activation-induced apoptosis changed with age but not with DR. The lack of influence of DR on activation-induced apoptosis in rat T cells was somewhat unexpected in light of a study in mice that show the inducibility of apoptosis by dexamethasone decreases with age and that DR significantly reversed the age-related decline in apoptosis [38]. The difference between this study and our findings may reflect differences in the experimental design, the assay system, cell population analyzed and/or species of rodents studied. For example, in the study reported by Reddy et al. [38], T cells were stimulated only once with anti-CD3 for 24 h. Apoptosis was assessed by measuring the expression of annexin V as an indicator of apoptosis. These differences in experimental design could contribute to the discrepancy between this study and our results. A recent study has demonstrated that aging accelerated and DR suppressed apoptosis-related Fas expression in mice hepatocytes [39]. In addition, DR has

been shown to enhance p53 expression but not increase apoptosis in rat hepatocytes [40].

Apoptosis has been proposed to play an important role in inhibiting tumor development by eliminating damaged and genetically transformed cells from tumor-susceptible tissues [41–46]. Cancer cells die at a higher rate than normal cells, suggesting that environmental modulators of apoptosis can influence the rate of tumor growth. For example, DR reduces cancer progression by stimulating apoptosis of precancerous cells [47,48]. DR has also been shown to increase the spontaneous apoptotic rate and decrease cell proliferation rate in mice hepatocytes [48]. The fact that in our study the apoptotic parameters that were measured changed with age but not with DR suggests that the influence of DR on activation-induced proliferation and cell death can vary considerably from one type of cell to another (e.g. normal cells vs. cancer cells). The increased proliferation rate observed in cancerous cells is often accompanied by a parallel increase in apoptosis [49,50]. It has been suggested that this adaptive apoptotic response may serve an important protective role in countering aberrant hyperplasia [49–51]. Cellular homeostasis depends on an integrated balance between cell proliferation and cell death such that during normal cell turnover the rates of these two processes are counterbalanced and equivalent [52,53]. The homeostatic maintenance of cell proliferation and cell death may also contribute to a critical defense mechanism in response to endogenous and exogenous stressors. Future studies on the molecular events that regulate AICD may help to elucidate the role of diet and aging on cell proliferation and cell death.

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