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Age-Dependent Increases in Apoptosis/Necrosis Ratios in Human Lymphocytes Exposed to Oxidative Stress

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Unlike apoptosis, mechanisms leading to necrosis are less well understood. Moreover, changes in necrosis as a function of age have not been studied in human lymphocytes. H_2O_2-induced death of peripheral lymphocytes (56 healthy donors, 24–95 years) was evaluated by flow cytometry and propidium iodide staining, caspase activation, DNA laddering, and electron microscopy. H_2O_2-induced stress was associated with high levels of necrosis in young individuals (≤30 years), whereas progressively enhanced apoptotic death was observed in older donors, without changes in overall lymphocyte survival. Thus, apoptosis/necrosis ratios were inverted in young versus elderly (≥65 years) donors. Death was not accompanied by increased caspase activity and, accordingly, unaffected by caspase inhibition; however, it was almost completely prevented by poly ADP ribose polymerase inhibition. In summary, aging was associated with changes in the apoptosis/necrosis ratios, rather than susceptibility per se to H_2O_2-induced death, which was caspase independent but poly ADP ribose polymerase dependent. Understanding this switch in death modes may aid in understanding age-related disorders.

Key Words: Human lymphocytes—Aging—Apoptosis—Necrosis—PARP-1.

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AGING is a highly complex process associated with decreases in regenerative potential and adaptive responses to stress. In a broad sense, aging at the cellular level might be viewed as a process in which there is accumulation of damage that has not been repaired and/or a decrease in the capacity to replace damaged cells. Mechanistically, aging has been associated with increased oxidative stress, alterations in energy homeostasis, accumulation of damaged proteins, and DNA lesions. All these perturbations eventually lead to increased cell death and tissue atrophy (1–5). Two main categories of cell death are generally thought to exist, apoptosis and necrosis. Apoptosis, referred to as programmed cell death Type I (PCD), is necessary for the elimination of undesirable cells during development and disease and the best-characterized form of cell death. Necrosis, instead, is often considered a passive response to overwhelming physical or chemical stress and therefore unregulated and accidental. However, while perhaps appealing in simplicity, it is now becoming increasingly accepted that apoptosis is not the only form of PCD and, alternatively, that necrosis is not necessarily an unregulated event [reviewed in (6)—see also reviews by (7,8)]. On the other hand, clear distinctions between the different types of cell death can be difficult and features of both apoptosis and necrosis may coexist in the same cell population. Indeed, in response to a large number of cytotoxic agents, both apoptosis and necrosis are detected (9). In addition, the form of cell death may vary according to the concentration of the cytotoxic agent. For instance, hydrogen peroxide induces apoptosis at low doses and necrosis at elevated concentrations (10,11). Alternatively, inhibition of one type of death may promote activation of another type, to ensure that death occurs once the process has been initiated (12–14). Taken together, these data suggest that cell death is not the result of discrete, well separated pathways, but rather that these are interconnected at multiple levels and that a broad spectrum of death modes referred to as the "apoptosis–necrosis continuum" exists (6,15–17). To further increase complexity, caspase-independent cell death with features characteristic of both apoptosis and necrosis has been reported. These events may be accompanied by activation of poly ADP ribose polymerase (PARP-1), release of apoptosis-inducing factor from the mitochondria, and activation of endonucleases, which cleave DNA in larger DNA fragments than those observed in classic apoptosis.

As may be expected, links between apoptotic cell death and aging have been reported (4,18,19). For instance, aging in rats is associated with increased levels of caspases and

apoptosis in several tissues (5). On the other hand, the possibility that aging may affect caspase-independent death has received much less attention. Given the aforementioned evidence indicating that apoptosis and necrosis frequently occur side-by-side in the same cell population, we evaluated the possibility that the extent to which this occurs may vary as a function of age. A third type of cell death referred to as autophagy or PCD Type II also exists. This ancient lysosomal degradation pathway is involved in recycling of cellular proteins and organelles (20–22). However, given that it is considered by many a cell survival mechanism (21,22), it was not explored in this study. The death of lymphocytes from human donors of ages ranging from 25 to 94 induced by hydrogen peroxide (H_2O_2), a widely used agent to induce oxidative stress, was evaluated using flow cytometry, DNA laddering, and electron microscopy in addition to the use of caspase and PARP-1 inhibitors. Lymphocytes were chosen as a first approach because they are readily accessible and the changes occurring upon aging are thought to be generic (23).

Aging is generally associated with increased exposure to noxious stimuli, cell death, and augmented predisposition to pathologies as a consequence. Surprisingly, we found that overall viability of lymphocytes exposed to oxidative stress was similar for those from young and elderly donors. However, in the latter case, lymphocytes were less prone to necrosis than apoptosis when compared with those from young individuals. Understanding the processes that lead to this shift in the apoptosis/necrosis ratios may have important implications for understand aging-related disorders.

Experimental Procedures

Study Participants

The study was approved by the Ethics Committee of the Hospital Clínico Universidad de Chile. Healthy donors were recruited from the hospital staff, medical students, and healthy relatives of patients attending the hospital. After providing informed consent, each participant filled out a brief questionnaire containing questions related to their health, tobacco consumption, and demographics. Also, each donor provided 10–12 mL of whole blood, extracted between 9 and 11 AM in non-fasting conditions. Donors with signs or a history of recent infection were excluded. Fifty-six participants were recruited and divided into three groups: young (Y), ≤30 years (n = 16), old (O), ≥65 years (n = 22), and middle aged (M) between 30 and 64 years (n = 18). Eight participants of ages belonging to the three groups consented to repeated extractions of blood samples to assess intrasubject variability. Variability was less than 10% in repeated experiments on the same participant (data not shown).

Minimum sample size calculations were based on the results of (24) that showed a significant difference in apoptotic death ($p < .001$) in 10 young and 10 old participants.

Cell Culture

Peripheral blood mononuclear cells were separated from whole blood by Ficoll-Hypaque density centrifugation. Cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum 1×10^6 cells/mL and subsequently cultured for 4, 8, 12, or 20 hours at 37°C in the presence of H_2O_2, at concentrations ranging from 10 μM to 1 mM. Higher concentrations were avoided because of cell disintegration which interfered with the analysis. No antibiotics were added. The presence of 2 mM Tiron, a free radical scavenger, attenuated H_2O_2-induced death (not shown), indicating that the H_2O_2 concentrations used in our experiments were indeed causing oxidative stress. For inhibition experiments, cells were preincubated for 30 minutes in either the absence or the presence of the poly (ADP-ribosyl) polymerase 1 (PARP-1) inhibitor 3-Aminobenzamide (3-ABA) at 5 mM or the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD.fmk) at 10 μM. Cells were harvested and stained with 10 μg/mL propidium iodide (PI) to determine cell viability. PARP inhibition via 3-ABA in the same cells was demonstrated previously by showing that this inhibitor reduces loss of ATP and NADPH upon H_2O_2 treatment (25). Caspase inhibition by zVAD was confirmed by measuring the activity and showing that it was completely abolished in the presence of this inhibitor (see Figure 4).

Viability Assays

Cell viability was analyzed by fluorescence-activated cell sorting (FACS) as described previously (19,20). Briefly, based on PI fluorescence intensity, cells impermeable to PI were considered as viable (PI negative) and PI-permeable (PI positive) cells, composed of two populations corresponding to either apoptotic (hypodiploid cells) or necrotic (diploid cells), were considered dead. The fluorescence associated with total DNA content was defined by the permeabilization of cells with methanol. Samples containing roughly 1×10^5 cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) using the software program FACSDiva. Necrotic death has been validated in previous publications using a variety of approaches (19). Additionally, electron microscopy analysis was performed (see below) and necrotic features such as cell swelling and the loss of organelle and plasma membrane integrity were observed.

Caspases 3 and 7 Determination

Caspases 3 and 7 activity was measured in multiwell plates following the procedure of the Caspase-Glo 3/7 Assay using a luminometer. The activity measured in H_2O_2 or staurosporine-treated lymphocytes was expressed relative to the untreated wells to avoid changes in reagent activity due to storage.

DNA Laddering

Lymphocytes (1×10^6 cells per condition) obtained from old or young participants were treated for 20 hours with either H_2O_2 or staurosporine with or without zVAD.fmk. Cells were harvested by brief centrifugation at 4°C and DNA extracted with a phenol:chloroform:isoamylalcohol mixture (25:24:1). After RNAse treatment, samples were analyzed in a 2% agarose gel.

Hoechst Staining

Cell nuclei were counter stained with Hoechst 33342 1:1000 (Sigma Aldrich, St. Louis, MO). Images were captured using inverse fluorescence microscopes (DMIL and DMI4000; Leica Wetzlar, Germany).

Electron Microscopy

Lymphocytes were washed in phosphate-buffered saline and fixed in 3% glutaraldehyde, 100 mM Na-cacodylate at 4°C for 90 minutes. After postfixation in 1% OsO4 and dehydration, cells were embedded in EPSON 812 resin and were observed in a Zeiss TEM Electron Microscope (Electron Microscopy Center, Instituto de Ciencias Biomédicas, Department of Morphology, University of Chile).

Data Analysis

Results were expressed as mean ± *SEM*. Data sets were analyzed with analysis of variance and Student's *t* test. Comparisons yielding $p = .05$ or less were considered indicative of statistically significant differences.

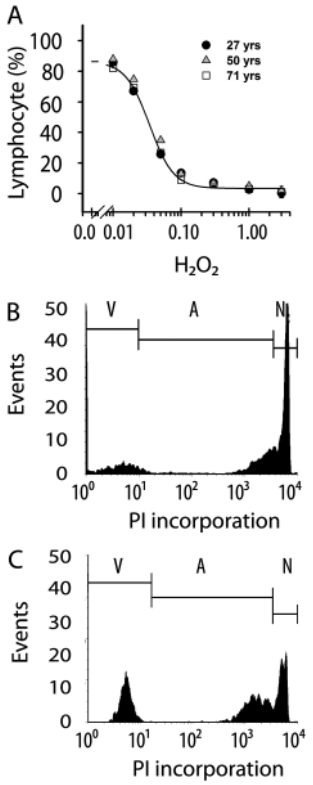

Figure 1. Viability of lymphocytes from donors of three different ages after exposure to H_2O_2. (**A**) Lymphocytes obtained from individual donors aged 27, 50, and 71 years were incubated for 20 hours with the indicated concentrations of H_2O_2. No difference in cell viability was observed. (**B**) and (**C**) Flow cytometry histograms of lymphocyte number versus propidium iodide (PI) fluorescence showing live (PI-negative) apoptotic (hypodiplod PI positive) and necrotic (diploid PI positive) cells, measured after 20-hour exposure to 50 μM H_2O_2 of the (**B**) 27- and (**C**) 71-year-old donors are shown.

Results

Apoptosis and Necrosis Induced by Hydrogen Peroxide in Human Lymphocytes as a Function of Age

Lymphocytes obtained from healthy individuals of ages ranging from 24 to 95 years were exposed to increasing concentrations of H_2O_2 for 20 hours, after which cell viability was assessed by flow cytometry following PI staining. Figure 1A shows a representative experiment of lymphocyte viability from donors of 27, 50, and 71 years of age. Exposure to increasing H_2O_2 concentrations (10 μM–3 mM) induced dose-dependent increases in death starting at around 10 μM. Overall susceptibility to H_2O_2-induced cell death was very similar in the three individuals (Figure 1A). Viable lymphocytes (PI negative) were additionally distinguished from lymphocytes that died either by necrosis (diploid PI positive) or apoptosis (hypodiploid PI positive) at each H_2O_2 concentration, as shown for the 27- and 71-year-old donors at 50 μM H_2O_2 (Figure 1B and C). Interestingly, a pronounced increase in apoptotic death was observed for lymphocytes from the elderly patient.

Dose–response curves of H_2O_2-induced death of individual patients of different ages were pooled into three age groups: young (Y) ≤30 years; old (O) ≥65 years, and middle age (M) between 31 and 64 years (Figure 2A–D). As previously shown for individual patients (Figure 1A), there were no significant differences in overall viability of lymphocytes upon exposure to increasing concentrations of H_2O_2 (Figure 2 A). Beyond 10 μM H_2O_2, a decrease in survival due to both types of cell death, apoptosis and necrosis, was observed at all concentrations tested. The H_2O_2 concentration that induced 50% death (LD50) obtained from the adjusted data was 38.2 ± 2.5 μM. Maximal levels of death were observed following exposure to concentrations greater than approximately 0.3 mM H_2O_2 (Figure 2A). At H_2O_2 concentrations higher than 3 mM, lymphocytes disintegrated making the analysis more difficult. Interestingly, the proportion of apoptotic and necrotic death differed according to donor age. In the Y group (Figure 2B), necrosis predominated over apoptosis at all H_2O_2 concentrations tested. Conversely, in the O group (Figure 2D), the opposite was observed with a predominance of apoptosis over necrosis. For the M group of donors, an intermediate situation was observed, with very similar proportions of apoptosis and necrosis at all H_2O_2 concentrations (Figure 2C).

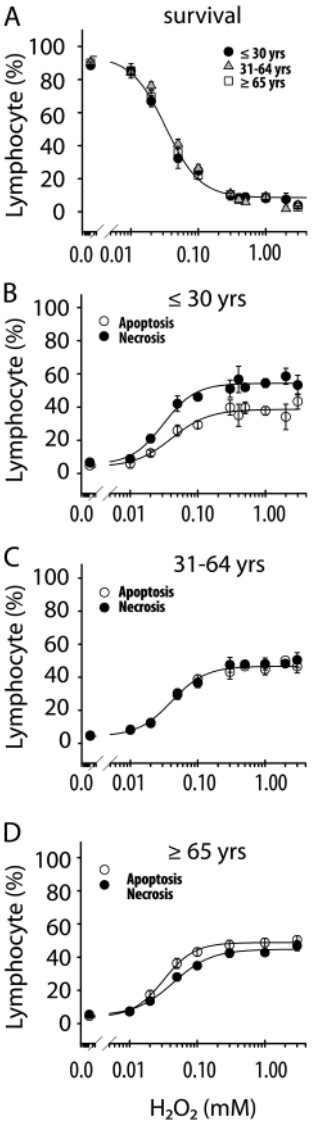

Figure 2. Dose–response curves of H_2O_2-induced death of lymphocytes from participants of three age groups. Lymphocytes from 56 healthy donors were grouped in young (Y, ≤30 years, n = 16 donors), middle age (M, between 31 and 64 years, n = 18 donors), and old donors (O, ≥65 years, n = 22 donors). (**A**) Survival curves for Y (black circles), M (gray triangles), and O (open squares). (**B**–**D**) Death by apoptosis (open circles) and necrosis (black circles) shown as a function of increasing concentrations of hydrogen peroxide in the Y, M, and O groups, respectively. Note the logarithmic abscissa. The curves were fitted using a sigmoidal Hill equation with four parameters. The LD50 value calculated from curve (**A**) was 38.2 ± 2.5 μM. The LD50 values were not significantly different between the three age groups. However, maximal values for apoptotic (p = .006) and necrotic (p = .03) death were significantly different among the three groups analyzed by analysis of variance.

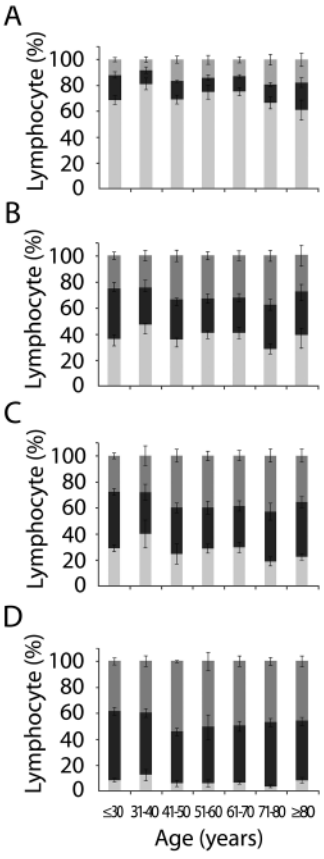

Figure 3. Variations in the type of death of human lymphocytes as a function of age. The percentage of viable (light gray), necrotic (black), and apoptotic (dark gray) lymphocytes determined after exposure for 20 hours to 0.02 (**A**), 0.05 (**B**), 0.1 (**C**), and 1 (**D**) mM H_2O_2 divided into 10-year age groups are shown: ≤30 years n = 16, 31–40 years n = 6, 41–50 years n = 4, 51–60 years n = 6, 61–70 years n = 11, 71–80 years n = 8, ≥80 years n = 5.

To further investigate age-dependent changes in H_2O_2-induced death, the numbers of viable, necrotic, and apoptotic cells of individual donors were clustered into 10-year age groups (Figure 3). Interestingly, as shown in Figure 2, in lymphocytes from donors younger than 30 years, increasing H_2O_2 concentrations led to reduced survival that was attributable to greater levels of necrosis rather than apoptosis at all concentrations tested (Figure 3A–D, first column to the left). In the groups between ages 31–80 years, aging was accompanied by a progressive increase in the extent of apoptosis (Figure 3A, dark gray); with increasing H_2O_2 concentrations, apoptosis was higher than necrosis at all concentrations (Figure 3B–D). For the oldest group (≥80 years), the levels of apoptosis and necrosis were similar at the different H_2O_2 concentrations. The ratio of necrotic to apoptotic death measured at H_2O_2 38.2 μM (=LD50) was calculated for the three age groups. The apoptosis/necrosis ratios in the Y group was 0.60 ± 0.09 (mean ± *SE*), whereas in the O group, the ratio was essentially reversed (1.37 ± 0.18). For the M group, a ratio of 1.11 ± 0.14 was observed. These results indicate that upon exposure to oxidative damage by H_2O_2, lymphocytes of individuals from the Y group die predominantly by necrosis and that susceptibility to death via apoptosis increases with age. Here, it is important to mention that necrosis detected in these experiments was not of the type that occurs secondary to apoptosis because onset of both apoptosis and necrosis was detected at the same time points for both young and elderly individuals (Supplementary Figure 1, data not shown).

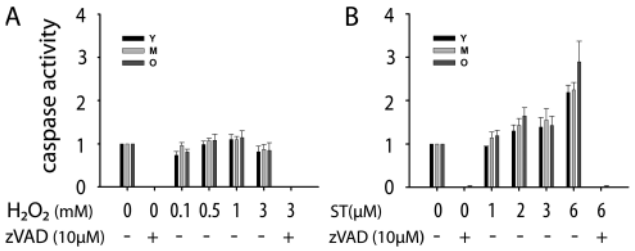

Figure 4. Caspase activity in lymphocytes from healthy donors of the three age groups. Caspases 3 and 7 activity were measured in lymphocyte lysates from Y (n = 11), M (n = 13), and O (n = 18) participants, after 4-hour exposure to H_2O_2 (**A**) or staurosporine (**B**) at the indicated concentrations, in the absence or presence of zVAD.fmk, as indicated. Values are expressed relative to untreated samples.

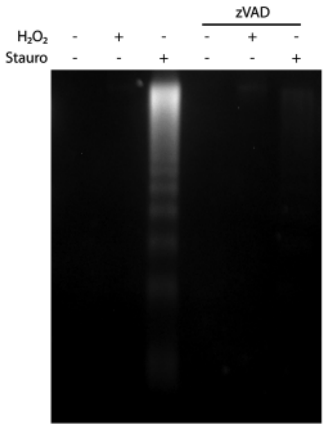

Figure 5. Absence of DNA laddering after H_2O_2-induced death of lymphocytes. Lymphocytes from a healthy donor aged 70 years were exposed for 20 hours to 100 μM H_2O_2 or staurosporine 2 μM (ST), as a positive control in the absence or in the presence of zVAD.fmk. No laddering was obtained after H_2O_2 exposure, whereas exposure to staurosporine induced a characteristic DNA laddering pattern, which was diminished by 30-minute preincubation with 50 μM zVAD.fmk.

Characterization of the Type of Cell Death

Executioner caspases 3 and 7 activity.—One of the hallmarks of apoptotic death is its dependence on caspase activity. Therefore, combined caspases 3 and 7 activity was measured in individual experiments with lymphocytes from donors of the three different age groups to investigate whether differences in the type of death observed for cells from young and old individuals were due to different levels of caspase activity. Exposure to increasing H_2O_2 concentrations (up to 3 mM) for 4 hours was not accompanied by significant increases in caspase activity in donors from the O, M, or Y groups (Figure 4A). Extended exposure to H_2O_2 up to 10 hours did not result in significantly greater levels of caspase activity (data not shown). As a positive control, caspase activity was measured after the treatment of lymphocytes with staurosporine, a known inducer of caspase activity and apoptosis (Figure 4B). As anticipated, the exposure to 1, 2, and 6 μM staurosporine for 4 hours induced up to a 2.9-fold increase in activity of the executioner caspases 3 and 7 that did not differ significantly between the three age groups (Figure 4B). As with H_2O_2, extended exposure to staurosporine was not accompanied by greater levels of caspase activity (data not shown). Preincubation of lymphocytes with 10 μM zVAD.fmk, a known broad-spectrum caspase inhibitor, reduced caspase activity to undetectable levels both in untreated lymphocytes and after the incubation with H_2O_2 or staurosporine (Figure 4A and B). These results indicate that death of lymphocytes induced by H_2O_2 exposure was not accompanied by a significant increase in caspases 3 and 7 activity.

DNA Laddering

Another well-known characteristic of apoptotic death is DNA fragmentation that becomes apparent as DNA "laddering" following gel electrophoresis. To further investigate whether apoptosis contributes to death of lymphocytes induced by the exposure to H_2O_2, we evaluated DNA laddering in healthy young and old donors. Consistent with the absence of an increase in caspase activity, no DNA laddering was detected after H_2O_2 exposure in either lymphocytes from young or old donors (Figure 5). Alternatively, staurosporine induced characteristic DNA laddering that was diminished by the generic caspase inhibitor zVAD.fmk (Figure 5, right lanes).

Electron Microscopy and Hoechst Staining

To further analyze the type of death induced by H_2O_2 exposure, the morphological features of dying or dead lymphocytes were investigated by electron microscopy. Figure 6A shows an electron micrograph taken after a 20-hour exposure of human lymphocytes of an old healthy donor. Apoptotic features such as chromatin condensation, preservation of organelles, and plasma membrane blebbing (apoptosis) were present in some cells, whereas for other cells, disintegration of the plasma membrane and organelles, characteristic of necrosis, were detected. Hoechst staining of H_2O_2-treated lymphocytes showed some cells with fragmented nuclei (Figure 6B) similar to what was observed in the staurosporine-treated lymphocytes where apoptosis prevailed (Figure 6C). A similar result on electron microscopy and Hoechst staining, but with more frequent lymphocytes with necrotic features, was present in a young donor (data not shown).

PARP-1–Dependent H_2O_2-Induced Death of Human Lymphocytes

Because death by H_2O_2 was independent of caspase activity, we investigated the role of the caspase-independent, PARP-1–dependent pathways in H_2O_2-induced death of lymphocytes from young and old donors. Preincubation with 3-ABA, a potent inhibitor of PARP-1 activity, offered substantial protection of lymphocytes from young and old individuals against H_2O_2-induced death (Figure 7A and B). In both age groups, greater than 60% viability was observed

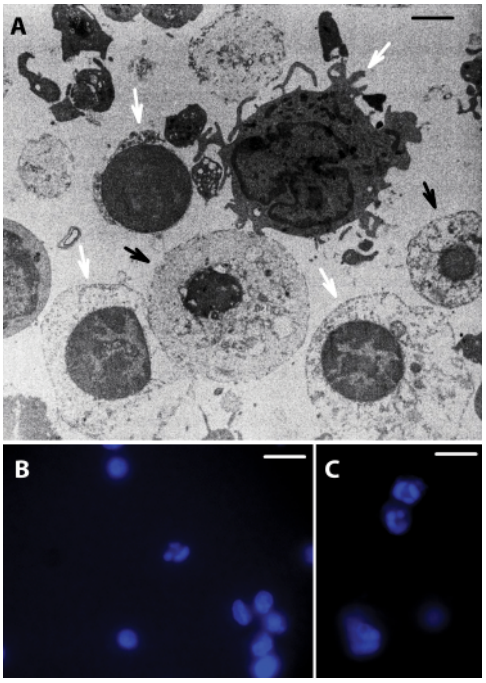

Figure 6. Electron microscopy and Hoechst staining of lymphocytes exposed to H_2O_2 reveal apoptotic and necrotic features. (**A**) Lymphocytes from a 65 years old donor after exposure to 50 μM H_2O_2 for 20 hours. Apoptotic features such as chromatin condensation (white arrows) are seen. However, necrotic features such as disintegration of plasma membrane and organelles (black arrows) are also present; bar: 3 μm. (**B**) and (**C**) Lymphocytes from a 65 years old donor exposed for 20 hours to 100 μM H_2O_2 (left panel) or staurosporine 2 μM (right panel) and submitted to Hoechst staining. (**B**) A cell with fragmented nuclei is seen among other lymphocytes with diffuse staining of the nucleus. (**C**) Fragmented nuclei are present in staurosporine-treated lymphocytes; bar: 7 μm.

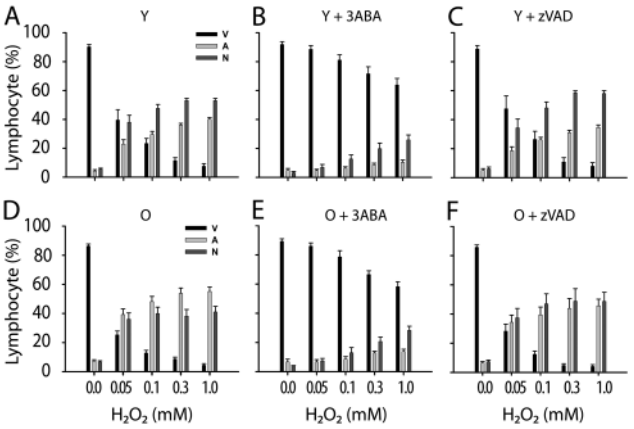

Figure 7. H_2O_2-induced death of lymphocytes from the three age groups in the presence of PARP-1 or caspase inhibitors. Viable (black bars), apoptotic (light gray bars), and necrotic (dark gray bars) lymphocytes from Y (**A**–**C**; n = 7) and O participants (**D**–**F**; n = 9) measured after 20-hour exposure to the indicated concentrations of H_2O_2 in the absence (**A** and **D**) or in the presence of 5 mM 3-ABA (**B** and **E**) or 10 μM zVAD.fmk (**C** and **F**). 3-ABA almost completely protected from H_2O_2-induced death, whereas zVAD.fmk had no effect.

even with the highest doses of H_2O_2 evaluated in these experiments (Figure 7C and D). Protection was mediated by decreases in both apoptosis and necrosis (Figure 7A, B, D, and E). Therefore, PARP-1-dependent pathways seem to mediate both apoptotic and necrotic human lymphocyte death. Interestingly, in the presence of 3-ABA, H_2O_2-induced death of the O group acquired a pattern very similar to that in the Y group. Survival values were almost the same, and necrosis predominated over apoptosis. Consistent with the previous results showing no change in caspase activity after oxidative damage with H_2O_2 (Figure 4A), the addition of zVAD.fmk had no significant effect on the survival of lymphocytes from donors of the different age groups (Figure 7C and F).

Glutathione Levels

As H_2O_2 is a strong oxidizing agent, lower level of necrotic death detected in old individuals could be due to the presence of higher contents of glutathione, a cellular antioxidant, in lymphocytes from this age group. Only total glutathione levels were determined since the oxidized levels were undetectable in the lysate. No significant differences with a slight decrease in total glutathione levels with age was observed for the different age groups (Supplementary Figure 2S), indicating that reduced necrotic death observed in lymphocytes of old individuals was not due to elevated glutathione levels.

Discussion

Aging at the cellular level is thought to reflect the accumulation of damage in essential macromolecules due to exogenous (ionizing or ultraviolet radiation, cytotoxic drugs) and endogenous (oxidative and nitroxidative damage, energy failure, anomalous protein folding) insults. A frequent cellular response in such situations is the increase in reactive oxygen species, whereby H_2O_2 is one of the most common oxidants produced in cells either physiologically, as a by-product of mitochondrial activity and several other metabolic stress conditions, or pathologically, mediating the action of polymorphonuclear cells against pathogens and the killing of tumor cells by lymphocytes (26,27). Here, we evaluated whether the exposure to oxidative stress (H_2O_2) leads to variations in cell death of lymphocytes obtained from human donors of ages ranging from 24 to 95 years. Lymphocytes of healthy donors showed a progressive decline in lymphocyte viability upon exposure to H_2O_2, whereby features of both apoptotic and necrotic cell deaths were detected in all age categories. When comparing individuals younger than 30 to those more than 65, significant variations in the necrosis/apoptosis index were detectable. Young donors had higher necrosis-to-apoptosis ratios, whereas the inverse was observed for donors of the older age group. This change in the ratio of apoptosis-to-necrosis with age was detected despite very similar overall cell viability. In addition, even though H_2O_2-induced death of human lymphocytes showed features characteristic of

apoptosis, this cell death program was executed in a manner independent of caspase activity but instead was dependent on PARP-1, as evidenced by using the inhibitor 3-ABA.

Interestingly, H_2O_2-induced death of lymphocytes displayed features characteristic of both apoptosis and necrosis, as shown by flow cytometry, DNA laddering, and electron microscopy. A hallmark of apoptotic cell death is the activation of a group of proteases referred to as caspases, which are responsible for the organized disassembly of cells (28). Surprisingly, apoptotic death in our experiments was not accompanied by increases in caspase activity, and the characteristic DNA laddering pattern was also not observed, although typical morphological features, such as peripherally condensed chromatin and preservation of the nuclear and plasma membrane, were evident. Furthermore, H_2O_2-induced death of lymphocytes was neither modified by the addition of the broad-spectrum caspase inhibitor zVAD.fmk nor was it accompanied by significant increases in the activity of the executioner caspases 3 and 7. Thus, although some traits typical of apoptosis were maintained, others were not.

Examples of apoptotic forms of cell death that are essentially caspase independent and mediated by the mitochondrial oxidoreductase, apoptosis-inducing factor, have been described previously (29,30). The death process is initiated by the cleavage of apoptosis-inducing factor yielding a soluble form that migrates from the mitochondria to the nucleus where, in conjunction with endonucleases, large-scale DNA degradation is induced (31). In addition, other forms of PCD, with morphological characteristics typical of necrosis, have been described, such as necroptosis (28). For tumor necrosis factor-α–mediated necrosis (12), parthanathos (32), and "programmed necrosis," the sequential involvement of proteins that also participate in classical apoptosis has been described (33). Recent studies propose that extensive DNA damage initially activates PARP-1, resulting in calpain and then Bax activation. The latter, a member of the bcl-2 family, migrates from the cytosol to the mitochondria to release apoptosis-inducing factor, which transfers to the nucleus to form a complex with phosphorylated histone 2AX and triggers large-scale DNA degradation (33,34). In our experiments, both apoptotic and necrotic characteristics were associated with H_2O_2-induced death. The observation that all forms of death observed were abrogated by PARP-1 inhibitors suggests that caspase-independent forms of cell death were being detected.

Equally important in the aging process, besides stress and reactive oxygen species–induced damage, are cellular pathways that maintain and repair damage to proteins and also DNA. In the latter case, the enzyme PARP-1 is one of the most important mechanisms involved in repairing single- and double-strand breaks. PARP-1 catalyzes poly(ADP-ribosyl)ation of nuclear proteins using NAD+ as a substrate and is the fastest known cellular response to genotoxic stress (35). PARP-1 is also involved in the regulation of telomere length and the expression of inflammatory proteins (35,36). At low levels of activation, PARP-1 has a protective role. However, at high activation levels, such as those induced by excessive reactive oxygen species, PARP-1 consumes large amounts of nicotinamide dinucleotide (NAD+) and ATP, thereby depriving the cell of energy and promoting cell death by caspase-independent pathways. Consistent with the view that PARP-induced pathways play an important role in cell death processes triggered here in our experiments, the PARP-1 inhibitor 3-ABA–protected cells from H_2O_2-induced death by decreasing both apoptosis and necrosis.

A clear relationship between PARP-1 and aging has been demonstrated because PARP−/− mice age prematurely (37). Furthermore, the maximum life span of 13 mammalian species strongly correlates with the poly(ADP-ribosyl)ation capacity of their mononuclear leucocytes (38). Additionally, poly(ADP-ribosyl)ation capacity declines with age in several cell types in humans, as well as rodents, and centenarians—reflecting very successful aging—display significantly higher poly(ADP-ribosyl)ation capacities than individuals of the average elder population (39,40). Our results showing more necrosis in younger individuals is in accordance with reports where higher poly(ADP-ribosyl)ation capacity was detected in this age category. Furthermore, the observation that elevated proportions of PARP-dependent death in the young individuals is associated with more necrotic death coincides with the role PARP-1 is thought to play in inflammation (41,42).

Limitations of this study include the small number of donors in certain age groups, that is 31–40, 41–50, 51–60 years old. However, the number of donors in the Y and O groups was large enough to show the inverse ratio of apoptosis-to-necrosis, in the absence of a significant change in overall survival. Also, even for the small number of cases in the intermediate age categories, it was sufficient to observe the tendency toward an increase in the proportion of apoptosis with age. Because we measured all lymphocytes and did not distinguish between specific subtypes, it is possible that the results may not be applicable to all lymphocyte populations.

Deregulation of PCD is associated with major diseases, such as cancer and neurodegeneration. Thus, in addition to its important role in aging, PARP-1 is attracting considerable attention as a potential chemotherapeutic target for certain cancers due to its proposed action as a tumor suppressor (43). Understanding how PARP-1–dependent mechanisms contribute to death of human lymphocytes is an interesting future avenue of research that is likely to help in the development of strategies to fight age-related disorders, including cancer and neurodegeneration (44).

Our results showing higher levels of apoptosis with increasing age is in agreement with previous studies demonstrating an increased susceptibility of human T lymphocytes to apoptosis induced by Fas or CD95 ligands (24,45–47). Increased expression of Bax (pro-apoptosis) and decreased

expression of Bcl-2 (anti-apoptotic) in CD4+ and CD8+ T cells from elder individuals compared with young controls and increased levels of caspases 8 and 3 in naive and memory CD8+ T cells have been described (45). In our study, we also found an increase in apoptosis with aging, and in addition, we demonstrate that the necrosis-to-apoptosis index is modified with aging. Moreover, we show that the necrosis-to-apoptosis index decreases with age. In young donors, necrosis prevailed over apoptosis, whereas the opposite was observed with aging. Thus, changes in the mode of cell death rather than susceptibility per se are associated with aging. Necrosis, as opposed to apoptosis, is characterized by an early rupture of the plasma membrane with the concomitant release of cytoplasmic contents to the extracellular media that then triggers an inflammatory response. Hence, an increase in the degree of necrosis in younger individuals might be associated with higher inflammation and more effective immunological responses to a variety of stress situations, including infection by pathogens. Future studies will seek to unravel how depressed immunity in the elderly individuals can be reverted. Again, PARP-1 may represent an attractive target in this respect.

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Supplementary Material

Supplementary material can be found at: http://biomed.gerontologyjournals.org/

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