

# Inverse Susceptibility to Oxidative Death of Lymphocytes Obtained From Alzheimer's Patients and Skin Cancer Survivors: Increased Apoptosis in Alzheimer's and Reduced Necrosis in Cancer

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A paucity of cancer in individuals with Alzheimer's disease (AD) and low rates of AD in cancer survivors has been reported in epidemiological studies. Deregulation in opposite directions of biological mechanisms, such as susceptibility to cell death, might be shared in the two disorders. We analyzed lymphocytes from AD and skin cancer patients as well as healthy controls and found significantly increased vulnerability of AD lymphocytes to H<sub>2</sub>O<sub>2</sub>-induced apoptotic death and higher resistance to death of skin cancer lymphocytes, due to reduced necrosis, as compared with healthy controls by pairwise comparisons adjusted for age and sex. H<sub>2</sub>O<sub>2</sub>-induced death in lymphocytes was caspase independent and significantly reduced by PARP-1 inhibition in all three groups. These differences in the susceptibility to cell death observed for lymphocytes from AD and skin cancer patients may be one of the mechanisms that help explain the inverse correlation detected between these diseases in epidemiological studies.

**Key Words:** Alzheimer—Cancer—Cell death—Apoptosis—Necrosis.

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**I**NCREASES in life expectancy are associated with an augmented prevalence of Alzheimer's disease (AD) and cancer, two devastating diseases in the elderly patients. AD is a neurodegenerative disorder associated with widespread deposits of beta amyloid peptides and intraneuronal hyperphosphorylated tau that ultimately lead to progressive synaptic dysfunction and neuronal death (1). Alternatively, cancer generally is associated with enhanced cell proliferation and a reduction in susceptibility to cell death, which reflects changes in the opposite sense to those occurring in neurodegenerative diseases (2). In previous epidemiological studies, we and others have shown an inverse association between AD and cancer (3–8), such that patients with AD have less cancer, and cancer survivors have less AD. Interestingly, this inverse relationship was not found in patients with vascular dementia, which is not thought to be neurodegenerative (7). Therefore, cancer and AD may be associated with changes in partially overlapping functions

at the cellular level. We have proposed that perturbations of mechanisms involved in cell survival/death regulation might be involved in the genesis of both disorders, whereby cell death is favored in degenerative diseases and cell survival is privileged in cancer (9,10).

In our previous epidemiological study, the inverse association between AD and cancer was also found when the group of skin cancer patients was analyzed separately (9). Interestingly, this is in accordance with the unexpected finding of an increased risk of skin cancer found in the participants of a recent clinical trial for AD treatment with a gamma secretase inhibitor (11). To explore the hypothesis of a differential susceptibility to cell death as an underlying mechanism that may explain the inverse association between cancer and AD, we sought to compare the susceptibility to H<sub>2</sub>O<sub>2</sub>-induced death of lymphocytes extracted from AD patients with those obtained from patients with a previous history of skin cancer as well as healthy controls (HC).

Table 1. Demographic Data of the Study Participants

	AD <i>n</i> = 17	Skin Cancer* <i>n</i> = 17	Healthy Controls <i>n</i> = 26
Age, <i>M</i> ± <i>SE</i>	79.3 ± 1.0	77.9 ± 2.5	75.3 ± 1.7
Age, range	68–83	61–93	61–95
Sex F (%)	12 (70.6)	13 (76.5)	21 (80.8)
Education, y	11.3 ± 1.6	12.7 ± 1.4	13.6 ± 1.7
CDR 0	0	17	26
CDR 1	13	0	0
CDR 2	4	0	0

Notes: CDR = Clinical Dementia Rating; AD = Alzheimer's disease.

\*Basal cell carcinoma (*n* = 12), squamous carcinoma (*n* = 3), and melanoma (*n* = 2).

## PATIENTS AND METHODS

A total of 60 patients (17 AD, 17 skin cancer, and 26 HC) participated in the study after providing an informed consent approved by the Ethics Committee of the Hospital Clínico Universidad de Chile. AD diagnosis was established according to the National Institute of Neurological and Communicative Diseases and Stroke—AD and Related Disorders Association (12) and the Clinical Dementia Rating (CDR) scale (13). AD patients had a CDR of 1 or 2 (mild or moderate) and no clinical evidence of any cancer (Table 1). Patients with a history of any type of skin cancer (basal cell carcinoma [*n* = 12], squamous carcinoma [*n* = 3], and melanoma [*n* = 2]) and without evidence of any cognitive impairment (CDR = 0) were selected as the cancer group. Healthy participants without both a history of any type of cancer and cognitive deterioration (CDR = 0) were included as HC. Patients were followed for 2–6 years to assure their group assignment. Demographic characteristics of participants are described in Table 1.

### Cell Culture

Peripheral blood mononuclear cells were extracted as previously described (14). Briefly, lymphocytes, separated by Ficoll-Hypaque density centrifugation, were resuspended in RPMI 1640 medium containing 10% fetal bovine serum  $1 \times 10^6$  cells/mL and exposed to H<sub>2</sub>O<sub>2</sub> (Merck & Co, diluted in sterile phosphate buffered saline) at concentrations ranging from 10  $\mu$ M to 3 mM for 20 hours at 37°C. For inhibition experiments, cells were preincubated for 30 minutes in either the absence or the presence of 5 mM 3-aminobenzamide (3-ABA), a poly (ADP-ribosyl) polymerase 1 (PARP-1) inhibitor, or the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.fmk) at 10  $\mu$ M. Cells were harvested and stained with 10  $\mu$ g/mL propidium iodide (PI) to determine cell viability by fluorescence-activated cell sorting as described previously (14). Briefly, based on PI fluorescence intensity, cells impermeable to PI were considered as viable (PI negative) and those permeable to PI (PI positive) as dead cells. In the latter case, two populations corresponding to either apoptotic (hypodiploid cells) or necrotic (diploid

cells) were considered. Total DNA content was defined by the fluorescence associated with permeabilization of cells with methanol. Samples containing roughly  $1 \times 10^5$  cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) using the software program FACSDiva. Caspase-3/7 activity was measured with the Caspase-Glo 3/7 Assay. Results were expressed as mean  $\pm$  standard error of the mean.

### Statistical Analysis

Analyses were conducted using PROC MIXED (SAS 9.1, Cary, NC) to test whether differences existed between the three experimental groups at each dose with regard to lymphocyte survival, apoptosis, and necrosis. In these analyses, dose was treated as a repeated measure, and the models included a term representing the interaction of dose and experimental group. The analyses were adjusted for age and sex. Comparisons yielding *p* = .05 or less were considered indicative of statistically significant differences.

## RESULTS

A significant interaction between dose and group was found for survival (*p* = .0019), apoptosis (*p* = .0024), and necrosis (*p* < .0001). Pairwise comparisons indicated that upon exposure to H<sub>2</sub>O<sub>2</sub>, lymphocytes from AD patients showed increased susceptibility to death compared with lymphocytes from patients with a history of skin cancer and HC. This was apparent in a shift of the dose–response curve to the left for AD lymphocytes and to the right for skin cancer lymphocytes with respect to HC lymphocytes (Figure 1A). The concentration inducing half-maximal death (LD50) was  $23.7 \pm 1.2 \mu$ M in AD lymphocytes compared with  $40.2 \pm 3.2 \mu$ M in skin cancer and  $35.6 \pm 1.8 \mu$ M in HC (Table 2). Comparison between AD and skin cancer lymphocytes showed significant differences with increased susceptibility to death for AD lymphocytes at 10, 20, and 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> concentrations. HC lymphocytes showed intermediate survival values between those of AD and skin cancer patients, being significantly higher than those of AD patients at 10 and 20  $\mu$ M and significantly lower than those of skin cancer patients at 50 and 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Figure 1A).

The enhanced susceptibility to death of AD lymphocytes compared with HC was due to increased apoptosis; AD lymphocytes showed significantly higher levels of apoptosis than HC at 20 and 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Figure 1B and Table 2). Compared with lymphocytes from cancer patients, the increased death of AD lymphocytes at 10, 20, and 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> concentrations was attributable to higher levels of both apoptosis and necrosis. On the other hand, the higher resistance to H<sub>2</sub>O<sub>2</sub>-induced death observed for skin cancer lymphocytes was associated with significantly lower necrosis values at all H<sub>2</sub>O<sub>2</sub> concentrations higher than 50  $\mu$ M when compared with HC and at 20 and 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> when compared with AD (Figure 1C and Table 2).

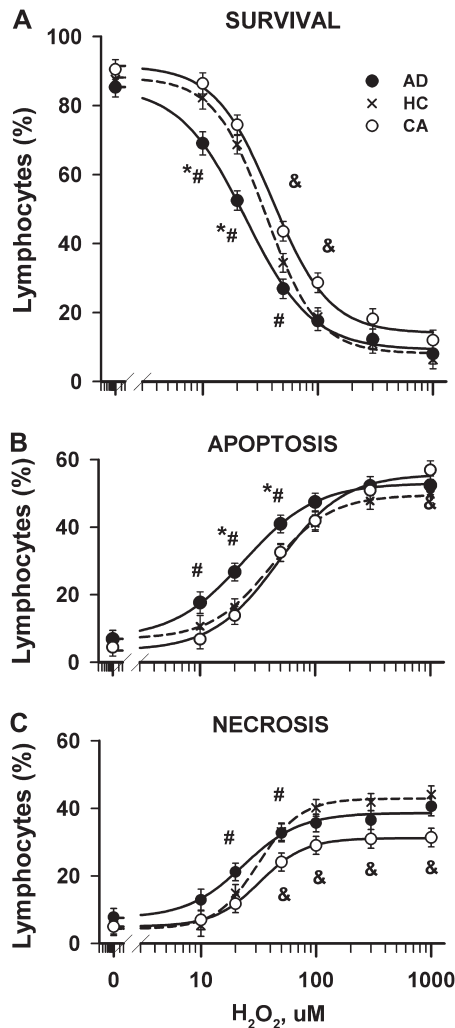


Figure 1.  $H_2O_2$ -induced death of lymphocytes. Lymphocytes from 17 Alzheimer's disease (AD) patients (filled circles), 17 skin cancer patients (CA, open circles) patients, and 26 healthy controls (HC; crosses) were exposed to  $H_2O_2$  for 20 hours. (A) Survival, (B) apoptosis, and (C) necrosis. Means  $\pm$  SE. Curves adjusted with Sigmoidal Hill 4-parameter equation. # $p < .0001$  AD versus cancer; \* $p < .005$  AD versus HC; & $p < .05$  CA versus HC.

We previously reported (14) that  $H_2O_2$ -induced death of lymphocytes from HC donors was independent of caspase activity, and accordingly, death was neither prevented by caspase inhibition nor accompanied by DNA laddering. Instead, lymphocyte death was very effectively prevented by PARP-1 inhibition. We therefore tested the effect of PARP-1 inhibition in lymphocytes from AD and cancer patients. Inhibition of PARP-1 activity with 3-ABA markedly protected from  $H_2O_2$ -induced death in the three groups of patients (Figure 2A). The protection was smaller in AD lymphocytes, although the difference was not statistically significant, possibly due to the small number of patients. The protective effect of 3-ABA was due to inhibition of both apoptosis and necrosis (Figure 2B and C). In turn, the addition of the caspase inhibitor Z-VAD had no effect on lymphocyte death

Table 2. LD50 and Maximal Death Values Obtained from the Adjusted Curves of Survival and Death by Apoptosis and Necrosis After  $H_2O_2$  Exposure

	Survival		Apoptosis		Necrosis	
	Alive Cells at Maximum		Maximum		Maximum	
	LD50, $\mu$ M	[ $H_2O_2$ ], %	LD50, $\mu$ M	Death, %	LD50, $\mu$ M	Death, %
AD	23.7 $\pm$ 1.2	9.2 $\pm$ 1.1	24.1 $\pm$ 0.7	46.1 $\pm$ 0.6	23.4 $\pm$ 3.1	31.1 $\pm$ 2.1
HC	35.6 $\pm$ 1.8	8.2 $\pm$ 1.3	41.1 $\pm$ 1.5	42.6 $\pm$ 0.8	31.1 $\pm$ 2.4	38.6 $\pm$ 1.6
Cancer	40.2 $\pm$ 3.2	14.0 $\pm$ 2.0	47.4 $\pm$ 5.5	52.2 $\pm$ 3.1	32.2 $\pm$ 0.4	26.2 $\pm$ 0.2

Note: Values were obtained from experimental data from Figure 1, adjusted with a Sigmoidal Hill 4-parameter equation. AD = Alzheimer's disease; HC = healthy controls.

(not shown), and accordingly, it was not accompanied by changes in caspase activity, either in AD or skin cancer lymphocytes (Figure 3A), as found for HC donors in our previous report (14). As a positive control, exposure to staurosporine, a known inducer of apoptosis, resulted in a significant increase in caspase activity that was completely prevented by Z-VAD (Figure 3B).

## DISCUSSION

Our results indicate that under oxidative stress, lymphocytes from AD patients showed increased susceptibility to death, but those from patients with a history of skin cancer showed increased resistance to  $H_2O_2$ -induced death, when compared with HC. Compared with HC lymphocytes, the increased death observed for AD lymphocytes was mainly due to higher levels of apoptosis, whereas the reduced death of skin cancer lymphocytes was due to reduction of necrosis. Compared with cancer lymphocytes, the augmented death of AD lymphocytes at lower  $H_2O_2$  concentrations was due to higher levels of both apoptosis and necrosis. These results may provide an explanation for the reportedly inverse association found in epidemiological studies between cancer and AD. Additionally, the results are consistent with our hypothesis proposing that the machinery regulating cell death/survival mechanisms might be deregulated favoring death in neurodegenerative diseases such as AD but favoring survival/proliferation in patients with cancer (9,10,15). Furthermore, these observations are also in agreement with the increase in skin cancers reported in participants in a recent clinical trial for AD treatment with a gamma secretase inhibitor to decrease beta amyloid production (<http://www.alzforum.org/drg/drc/detail.asp?id=108>). We chose to study skin cancer patients because it is the most prevalent cancer, it is relatively benign, and the inverse association between cancer and AD was also seen when the analysis was performed in this group of patients. We have observed a similar resistance to  $H_2O_2$  death in a small group of patients with other types of cancer (prostate, breast, lung, and colon); however, the number of cases is insufficient to perform a statistical analysis (M. I. Behrens, M. Silva, A. F. Q. Quest, unpublished results).

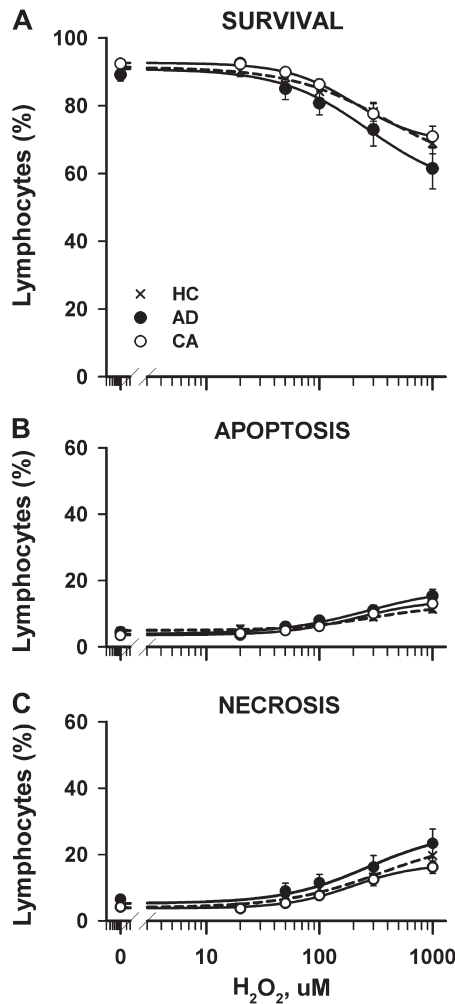


Figure 2. PARP-1 inhibition protected lymphocytes against H<sub>2</sub>O<sub>2</sub>-induced death. Lymphocytes from 12 Alzheimer's disease patients, 16 CA, and 14 healthy controls were exposed to H<sub>2</sub>O<sub>2</sub> in the presence of 5 mM 3-ABA, a PARP-1 inhibitor. There was marked inhibition of lymphocyte death. (A) Survival, (B) apoptosis, and (C) necrosis.

The inhibition of PARP-1 significantly protected against H<sub>2</sub>O<sub>2</sub>-induced death of lymphocytes in all three groups (AD, skin cancer, and HC) studied. Protection tended to be lower in cells from AD patients, although the differences were not statistically significant. PARP-1 belongs to a family of nuclear enzymes that catalyze poly(ADP-ribosylation) of DNA-binding proteins. Upon DNA damage, PARP-1 acts as a nick-sensor protein that uses beta-nicotine amide dinucleotide(+) to generate polymers of ADP-ribose that participate in DNA repair. PARP-1 has been implicated in maintenance of genomic integrity and mammalian longevity. However, excessive activation of PARP-1 induced by oxidative stress results in the depletion of NAD(+) and adenosine triphosphate and consequently may trigger necrotic cell death and organ dysfunction (16). Therefore, PARP-1 has two opposing functions, one related to cell survival by stabilizing the mitochondrial respiratory complex and another as a promoter of cell death when released into the cytosol (parthanatos (17–19)). Caspase-independent but PARP-1-dependent forms of apoptotic cell death (20–22) are increasingly recognized as important in the control of cell death in cancer (23), stroke (24), and AD (25). Currently, PARP-1 inhibitors are being tested as potential chemotherapeutic drugs for the treatment of certain cancers, whereby the most promising results were obtained in breast cancer patients (26). In addition, a role for PARP-1 in AD was also reported in a recent study showing that beta amyloid-induced neuronal death of hippocampal/glia cocultures was paralleled by accumulation of polymers of ADP-ribose and decreases in NAD levels and mitochondrial membrane potential in astrocytes, all of which were prevented by PARP inhibitors (25). To underscore this PARP connection, recently significant differences in PARP haplotype distributions were observed between AD patients and controls in a case-control study (27).

In this context, it should be mentioned that autophagy, a type of cell death not studied in this report, represents an

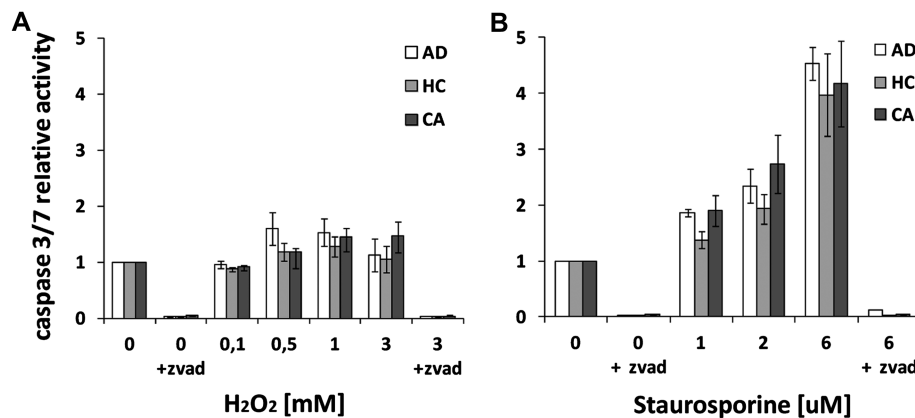


Figure 3. Caspase-3/7 activity in lymphocytes from Alzheimer's disease (AD), CA, and healthy controls (HC). (A) Caspase-3/7 activity was measured after 4 hours of incubation with H<sub>2</sub>O<sub>2</sub>. Relative values compared with untreated samples are shown for AD (n = 12), skin cancer patients (CA, n = 11), and HC (n = 15), mean ± SE. (B) As a positive control, staurosporine induced an increase in caspase activity that was completely inhibited by Z-VAD (a caspase inhibitor).

additional mechanism that may potentially participate in the AD and cancer connection reported on here (28–31). Although analyzing this possibility exceeds the scope of the current study, it certainly represents an interesting topic that could be addressed in the future.

It remains to be determined whether the observed enhanced susceptibility to H<sub>2</sub>O<sub>2</sub>-induced death is detectable in patients at very early stages of AD (CDR 0.5 or mild cognitive impairment). Measurement of lymphocyte death by H<sub>2</sub>O<sub>2</sub> exposure is a simple procedure, which employs readily accessible cells and could eventually prove useful as a diagnostic test for AD. Understanding the mechanisms underlying the differential susceptibility to cell death should help in the development of strategies to treat these devastating disorders. With this in mind, PARP inhibitors might represent an interesting approach worth exploring in the treatment of AD patients.

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