ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 2, 2004

Forum Original Research Communication

Vitamin E Inhibits Anti-Fas-Induced Phosphatidylserine Oxidation but Does Not Affect Its Externalization During Apoptosis in Jurkat T Cells and Their Phagocytosis by J774A.1 Macrophages

BEHICE F. SERINKAN,[1] YULIA Y. TYURINA,[1] HAREESH BABU,[1] MIRJANA DJUKIC,[1] PETER J. QUINN,[4] ALAN SCHROIT,[5] and VALERIAN E. KAGAN[1–3]

ABSTRACT

Apoptosis and phagocytosis of apoptotic cells provide for effective and harmless clearance of unwanted or damaged cells in the body. Preferential oxidation of one particular class of phospholipids, phosphatidylserine (PS), is a typical trait of both oxidant- and nonoxidant-induced apoptosis. PS oxidation is likely to play an important role in phagocytosis either by affecting PS externalization acting as an "eat me" signal or by more effective recognition of apoptotic cells by macrophage receptors. This implies that antioxidants effective in inhibiting PS oxidation may affect PS externalization and/or effective removal of apoptotic cells. Therefore, it is essential to determine whether vitamin E, the major lipid-soluble antioxidant of membranes, inhibits PS oxidation, and hence blocks apoptosis/phagocytosis. To test this, we studied the effects of vitamin E on PS oxidation and signaling using a model of anti-Fas-triggered apoptosis in Jurkat T cells. We found that incubation of cells with vitamin E (0.25–50 μ*M*) resulted in its integration into cells to reach physiologically relevant concentrations. Using labeling of cell phospholipids with oxidation-sensitive and fluorescent *cis*-parinaric acid (PnA), we found that anti-Fas exposure caused significant and selective oxidation of PnA-PS in Jurkat T cells (22 ± 2.1% of its content in nonexposed cells). Vitamin E protected PnA-PS against oxidation in a concentration-dependent way such that at 25 μ*M* and 50 μ*M*, a complete inhibition of anti-Fas-induced PS oxidation was achieved. At all concentrations used, vitamin E had no effect on either biomarkers of anti-Fas-induced apoptosis (PS externalization, nuclear fragmentation) or phagocytosis of anti-Fas-induced apoptotic cells by J774A.1 macrophages. We conclude that vitamin E does not significantly interfere with extrinsic (death receptor-triggered) pathways of apoptosis and does not affect phagocytosis of anti-Fas-triggered apoptotic cells. *Antioxid. Redox Signal.* 6, 227–236.

INTRODUCTION

APOPTOSIS is an effective way for the clearance of irreparably injured or dead cells without damaging surrounding tissue and without inflicting massive inflammatory response. Proper removal of apoptotic cells is achieved by expressing different "eat me" signals on the cell surface that are recognized by phagocytes (9). Universally, phosphatidylserine (PS) that undergoes transbilayer migration and appears on the outer leaflet of plasma membrane during apoptosis (14, 24) serves

Departments of [1]Environmental and Occupational Health and [2]Pharmacology, [3]Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A.
[4]Division of Life Sciences, King's College, London, U.K.
[5]Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

as an "eat me" signal (10) via its interactions with adapter proteins and the cognate receptors of macrophages (11). In addition to PS externalization, other surface changes are likely contributors to effective recognition and phagocytosis of apoptotic cells by macrophages (9). Among these, apoptotic manifestation of oxidized lipid epitopes on the surface of apoptotic cells has been identified as an important facilitator of phagocytosis (6, 33).

We have previously reported preferential oxidation of PS as compared with other more predominant classes of phospholipids, *e.g.*, phosphatidylcholine and phosphatidylethanolamine, as a typical trait of both oxidant- and nonoxidant-induced apoptosis (2, 17, 25). Although the specific mechanisms involved in selective PS oxidation remain to be elucidated, it is likely that positively charged cytochrome *c* (pI = 10.3) (22) released from mitochondria into the cytosol electrostatically interacts with negatively charged PS in the inner leaflet of plasma membrane and catalyzes preferential PS oxidation (18) utilizing hydrogen peroxide (H_2O_2) formed by disrupted mitochondria electron transport (4, 49).

PS oxidation may play a key role in PS externalization via either inhibition of aminophospholipid translocase (APT) activity (known to possess redox sensitivity) or augmentation of transbilayer diffusion of oxidized as well as nonoxidized aminophospholipids. This implies that antioxidants effective in inhibiting PS oxidation may affect PS externalization, and hence modulate phagocytosis of apoptotic cells and resolution of inflammation. Indeed, our previous work has established that a phenolic antitumor drug, etoposide (a topoisomerase II inhibitor), can act as a potent lipid antioxidant (16) and dissociate PS-dependent signaling from the final common pathway for apoptosis by inhibiting PS oxidation (42). Etoposide was able to block oxidation of all major phospholipids, including PS, during both oxidant- (H_2O_2) and nonoxidant-(etoposide) induced apoptosis. The blockage of phospholipid oxidation correlated with the etoposide-dependent inhibition of PS externalization and phagocytosis of apoptotic cells (42). Moreover, PS oxidation can also function to generate additional signals on the plasma membrane surface for more effective recognition of apoptotic cells by macrophage receptors, particularly those known to bind oxidized epitopes, such as CD36 (13) or lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) (29). In fact, we have recently reported that partial inhibition of PS oxidation during anti-Fas-induced apoptosis in Jurkat T cells by a combination of antioxidant enzymes, superoxide dismutase (SOD) plus catalase, was sufficient to decrease their phagocytosis by different macrophages (murine macrophage cell line J774A.1 cells, human macrophage-like cell line THP-1, and human monocyte-derived macrophages) despite the fact that no changes in apoptotic biomarkers were detected after exposure to the antioxidant enzymes (17).

Overall these findings suggest that natural and pharmacologically used antioxidants can affect key mechanisms of oxidative apoptotic signaling, and hence impede the effective clearance of apoptotic cells by macrophages via PS-dependent pathways. As a consequence, vital physiologic functions that are governed by programmed cell death and phagocytosis of apoptotic cells, such as immunologic surveillance, inflammatory response, or embryogenesis, would be impaired. In this regard, the potential role of vitamin E is of the greatest concern due to its role as the major lipid-soluble membrane antioxidant (27, 37). To test this calamitous possibility, we chose a model of anti-Fas-triggered apoptosis in Jurkat T cells and studied the effects of vitamin E on PS oxidation and signaling. We found that vitamin E, integrated into cells to reach physiologically relevant concentrations, completely blocked anti-Fas-induced PS oxidation. Most importantly, however, vitamin E had no effect on either biomarkers of cell death receptor-induced apoptosis, such as PS externalization, nuclear morphology of apoptosis, or phagoytosis of anti-Fas-induced apoptotic cells by macrophages.

MATERIALS AND METHODS

Reagents

Cell Tracker Orange™ (CMTMR) [5-(and-6)-[[(4-chloromethyl)benzoyl]amino]tetramethylrhodamine],*cis*-parinaric acid (PnA), and JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) were from Molecular Probes (Eugene, OR, U.S.A.). α-Tocopherol (α-TOH), HPLC grade solvents, fetal bovine serum (FBS), glutathione (GSH), Hoechst 33342, human serum albumin (hSA), and phenylmethylsulfonyl fluoride were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), penicillin, streptomycin, and gentamycin were purchased from Invitrogen Inc. (Carlsbad, CA, U.S.A.). ThioGlo™-1 was from Calbiochem (San Diego, CA, U.S.A.). 1-Palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]hexanoyl]-*sn*-glycero-3-phosphoserine (NBD-PS) was from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). Anti-Fas monoclonal antibody (CH-11) was obtained from MBL (Nagoya, Japan). Annexin V-FITC Apoptosis Detection Kit was from Biovision (Mountain View, CA, U.S.A.).

Cell cultures

Jurkat T cells purchased from ATCC (American Type Culture Collection) were maintained in RPMI 1640 medium supplemented with 12.5% heat-inactivated FBS at 37°C in a humidified atmosphere (5% CO_2 plus 95% air). For vitamin E supplementation, cells were preincubated with different concentrations of vitamin E for 24 h under the cell culture conditions. After incubation, cells were harvested and washed with RPMI medium and anti-Fas stimulation started by adding 250 ng/ml anti-Fas monoclonal antibody.

Macrophages J774A.1 (from ATCC) were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin sulfate in a humidified atmosphere (5% CO_2 plus 95% air) at 37°C.

HPLC assay of vitamin E (α-tocopherol) content in Jurkat T cells

After 24 h of incubation with different concentrations of vitamin E, cells were washed with PBS; α-tocopherol (α-TOH) was extracted from cells by chloroform/methanol (2:1, vol/vol) and measured by HPLC using a reverse-phase C18 column (5 µm, 4.6 mm × 15.0 cm; Hewlett–Packard). A Shimadzu LC-10A HPLC system was used with an LC-10 pump and RF-551 fluorescent detector. Methanol was used as a mobile phase.

The wavelengths used in the assay were 292 nm (excitation) and 324 nm (emission). Flow rate was 1 ml/min. Under these conditions, the retention time for α-TOH was 7.2 min. The data acquired were exported from the RF-551 detector using Shimadzu EZChrom software.

Assay of phospholipid peroxidation

PnA was incorporated into Jurkat T cells as described previously (15). In brief, Jurkat T cells enriched with varying concentrations of vitamin E were incubated in serum-free RPMI 1640 medium without phenol red in the presence of PnA (1 μg of PnA/10^6 cells) for 2 h at 37°C. At the end of incubation, cells were washed with PBS containing fatty acid-free hSA (0.5 mg/ml) to remove any excess of unbound PnA. PnA-labeled cells were treated with anti-Fas (250 ng/ml) for 2 h at 37°C in 25 m*M* HEPES buffer, pH 7.4, containing 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, and 10 m*M* glucose. At the end of the incubation, lipids were extracted by the Folch procedure (12). The lipid extracts were separated by normal-phase HPLC using a 5-μm microsorb MV column (4.6 × 250 mm; Rainin Instrument Co. Inc.) as described previously (15). The separations were performed using a Shimadzu LC-600 HPLC system with an in-line configuration of RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). Fluorescence of PnA was measured at 420 nm emission after excitation at 324 nm. Data were processed and stored in digital form with Shimadzu EZChrom software. Lipid phosphorus was determined by a micro method (5).

Assays of GSH and protein sulfhydryl contents

GSH content in the cells was determined fluorometrically using ThioGlo™-1 as previously described (36). In brief, cells treated with anti-Fas for 2 h at 37°C were collected by centrifugation, washed, and resuspended in PBS. GSH was measured in cell lysates prepared by freezing and thawing cells. Immediately after addition of ThioGlo™-1 to the cell lysates, fluorescence was measured in a Packard Fusion™ Multifunctional Plate Reader (PerkinElmer, Boston, MA, U.S.A.) using excitation 390 ± 15 nm and emission 515 ± 30 nm.

Total protein sulfhydryls relative to controls were determined as an additional fluorescence response at the same wavelength 1 h after addition of 3.3 m*M* sodium dodecyl sulfate to the ThioGlo™-1-treated lysates kept at room temperature in the dark.

Apoptotic nuclear morphology

Jurkat T cells were incubated with anti-Fas for 2 h at 37°C. At the end of the incubation, cells were washed and resuspended in PBS. Hoechst 33342 (2 μg/ml) was added, and cells were examined by fluorescent microscopy. Results are expressed as the percentage of the cells showing characteristic nuclear morphological features of apoptosis (nuclear condensation and fragmentation) relative to the total number of counted cells (>200 cells).

Determination of mitochondrial membrane potential (MMP) using fluorescence probe JC-1

Changes of MMP are one of the earliest events during apoptotic process. Disruption of mitochondrial integrity results in release of some mitochondrial content such as cytochrome *c* and apoptosis-inducing factor. Reactive oxygen species are also produced by disrupted respiratory chain. MMP was measured by flow cytometric detection of fluorescence shift of a cationic dye, JC-1, from red to green according to the protocol outlined by the manufacturer (Molecular Probes). In brief, Jurkat T cells (10^6/ml) were labeled with JC-1 (1 μg/ml) in RPMI medium. After washing of cells from excess of stain, apoptosis was stimulated by anti-Fas. The time course of mitochondrial depolarization was measured using flow cytometry by a shift of green fluorescence (λ_{ex} = 485 ± 11 nm, λ_{em} = 530 ± 15 nm) to red fluorescence (λ_{ex} = 535 ± 17 nm, λ_{em} = 590 ± 17 nm) using 0.5 × 10^6 cells. Results are expressed as relative percentage of cells with red fluorescence, FL2, to total number of cells.

Annexin V staining of externalized PS

PS exposure was determined by flow cytometric detection of annexin V staining using a protocol outlined in the annexin V-FITC apoptosis detection kit. In brief, Jurkat T cells (1 × 10^6) exposed to anti-Fas, washed once with PBS, and resuspended in binding buffer were stained with annexin V and propidium iodide for 5 min at room temperature. After staining, cells were immediately analyzed using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA, U.S.A.) with simultaneous monitoring of green fluorescence (530 nm, 30 nm band-pass filter) for annexin V-FITC and red fluorescence (long-pass emission filter that transmits light of >650 nm) associated with propidium iodide. Ten thousand events were collected and analyzed using the LYSIS™ II software (Becton–Dickinson).

In the case of assaying recovery effects of reducing agent, dithiothreitol (DTT), on PS externalization induced by anti-Fas, PS externalization was measured after treatment of anti-Fas-induced cells with 10 m*M* DTT for 20 min at room temperature. Anti-Fas stimulation was carried out for 1 h to restrict limitation of energy source, ATP, availability.

Assay of aminophospholipid translocase (APT) activity

APT activity was measured using modifications of the methods of McIntyre and Sleight (26) and Williamson and co-authors (46). Jurkat T cells (1 × 10^6) treated with anti-Fas were centrifuged (400 *g*, 10 min) and washed once in incubation buffer (136 m*M* NaCl, 2.7 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* glucose, 10 m*M* HEPES, pH 7.5). Cell pellets were resuspended in incubation buffer (5 × 10^6 cells/ml), transferred to microfuge tube, and placed in ice water for 10 min. NBD-PS (ethanol solution) was added to cells (final concentration, 10 μ*M*) and incubated for 10 min at 4°C. Labeled cells were centrifuged and resuspended at the same density in incubation buffer. Cell suspensions were placed in a 28°C water bath to initiate internalization, and 65-μl aliquots of cell suspension were removed at various time intervals (1–20 min) and placed into 2.5 ml of incubation buffer including the reducing agent, sodium dithionite (10 m*M*). Fluorescence (λ_{ex} = 460 nm, λ_{em} = 534 nm) was then recorded (within 35 s). Samples from the last time point were also placed in incubation buffer without dithionite to obtain total fluorescence intensity (FL_{total}). Internalized fluorescence from NBD-PS at various times (FL_t) was normalized as a percentage of the total fluorescence by

the following equation: % Internalized = $(FL_t - FL_0)/(FL_{total} - FL_0) \times 100$.

Phagocytosis of Jurkat T cells by J774A.1 macrophages

Macrophage J774A.1 cells were used for phagocytosis assays. Before target cells were added, macrophages were seeded into an eight-well chamber slide (5×10^4 cells/well) and cultured overnight. Jurkat T cells were labeled with Cell Tracker Orange according to the protocol described by the manufacturer. After labeling, Jurkat T cells were washed and resuspended in serum-containing RPMI medium and apoptosis was induced by addition of anti-Fas (250 ng/ml). Fluorescently labeled target cells (5×10^5 cells/well) were cocultured with macrophages for 1 h at 37°C. After incubation, wells were washed three times with medium and three times with ice-cold PBS; well contents were fixed and stained with a fixing solution (2% paraformaldehyde in PBS containing 1 μg/ml Hoechst 33342) for 1 h at room temperature. The cells were examined under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742–95–12NBR) and analyzed using the MetaImaging Series™ software v. 4.6 (Universal Imaging Corp., Downingtown, PA, U.S.A.). A minimum of 500 macrophages was analyzed per experimental condition. Results are expressed as the percentage of the phagocytosis-positive macrophages that showed bond (side by side contact) or engulfed apoptotic target.

Statistics

The results are presented as means ± SD values from at least three experiments, and statistical analyses were performed by Student's *t* test or one-way ANOVA. The statistical significance of differences was set at $p < 0.05$.

RESULTS

Enrichment of Jurkat T cells with vitamin E and its depletion during anti-Fas-induced apoptosis

To enrich Jurkat T cells with vitamin E, we incubated them with different concentrations of vitamin E (0.25–50 μ*M*) for 24 h in cell culture medium. This resulted in a concentration-dependent increase of vitamin E content from 0.02 ± 0.01 to 2.2 ± 0.2 nmol of α-TOH/mg of protein (Fig. 1). Because vitamin E can be oxidized during its antioxidant radical scavenging function, we also performed measurements of vitamin E during anti-Fas-induced apoptosis. Our results showed that vitamin E, indeed, was depleted during anti-Fas-induced apoptosis in reverse proportion to its levels in cells such that 100, 47, 26, and 23% of its total content were consumed during 2 h of anti-Fas exposure in cells supplemented with 0.25, 2.5, 25, and 50 μ*M* vitamin E, respectively (Fig. 1). Importantly, in all cases (except for the lowest vitamin E concentration), depletion of vitamin E was not complete such that by the end of incubation its levels remained significantly higher than in non-supplemented control cells.

Effects of vitamin E on anti-Fas-induced oxidative stress

Inhibition of phospholipid peroxidation We have previously reported that PS oxidation was induced during Fas-triggered apoptosis. In line with this, we found that PS was the only phospholipid oxidized during anti-Fas-induced apoptosis whereas other more abundant classes of phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, remained resistant to oxidation (data not shown). After 2 h of anti-Fas treatment, ~22 ± 2% of PnA-PS was lost due to oxidation ($p < 0.05$ versus control). Vitamin E protected PS against anti-Fas-induced peroxidation in a concentration-dependent manner

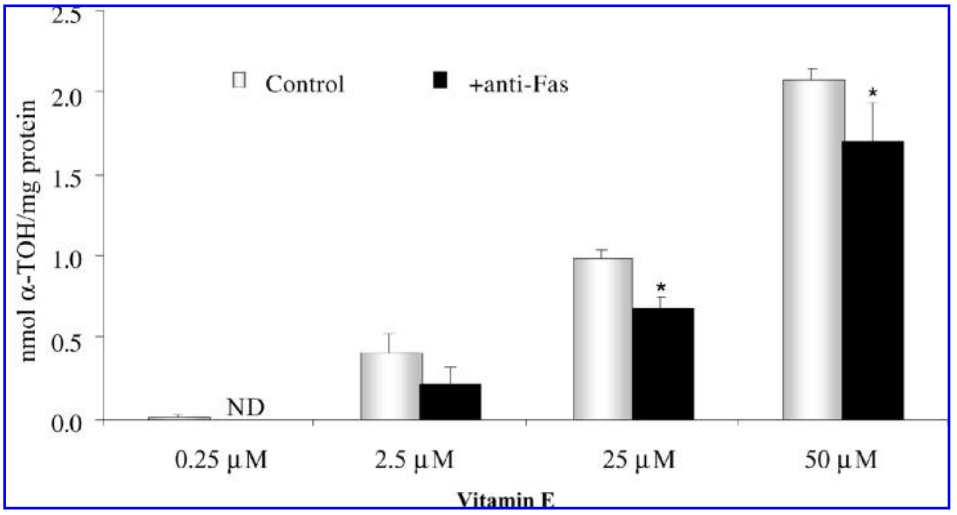

FIG. 1. Depletion of vitamin E during anti-Fas-induced apoptosis. Light columns correspond to controls (without anti-Fas stimulation); dark columns correspond to 2 h of anti-Fas exposure for each concentration of vitamin E. Data are expressed as means ± SD ($n = 3$). ND, nondetectable (<0.01 nmol of α-TOH/mg of protein). *$p < 0.05$, anti-Fas versus control.

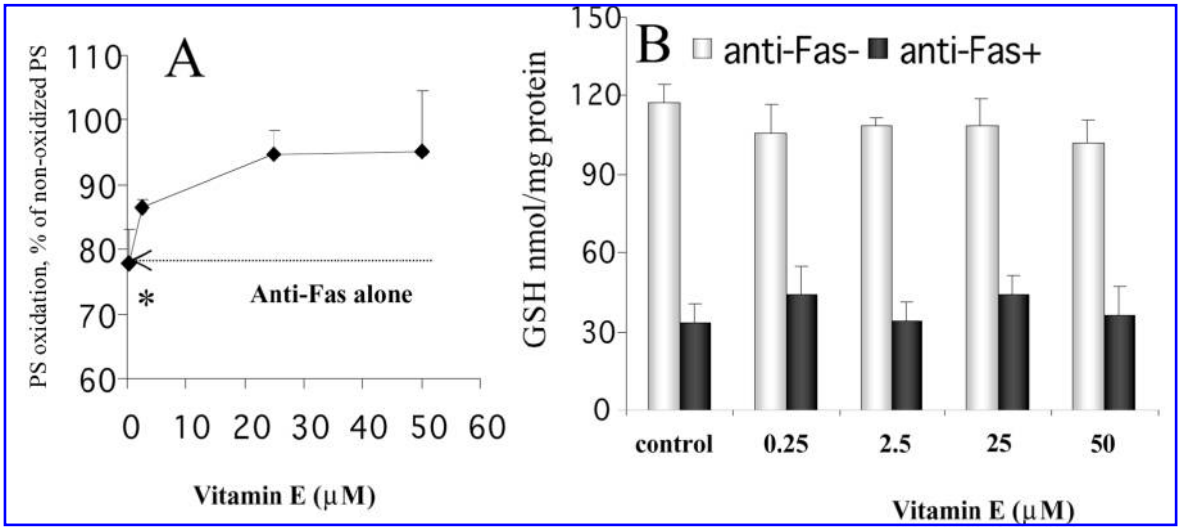

FIG. 2. Effects of vitamin E on anti-Fas-induced oxidation of PS and GSH. Anti-Fas-induced PnA-PS peroxidation was blocked by vitamin E at concentrations of 25–50 μ*M* (**A**). Anti-Fas-induced GSH depletion was not affected by vitamin E (**B**). Light columns correspond to GSH values without anti-Fas; dark columns correspond to 2 h of anti-Fas exposure for each concentration of vitamin E. Data are expressed as means ± SD ($n = 3$). *$p < 0.05$, anti-Fas versus control.

(Fig. 2A). At vitamin E concentrations of 25 and 50 μ*M*, PS oxidation was completely inhibited.

Anti-Fas-induced GSH depletion is not affected by vitamin E. Because vitamin E readily partitions into lipid domains of membranes, we were eager to assess the extent to which its protective effect was specific toward phospholipid oxidation. To this end, we studied the effects of vitamin E on oxidation of intracellular GSH in Jurkat T cells after anti-Fas induction. We found that the GSH content significantly decreased from 117 ± 7 nmol of GSH/mg of protein, its initial level in control cells, to 33 ± 7 nmol of GSH/mg of protein after anti-Fas (250 ng/ml) stimulation for 2 h at 37°C (Fig. 2B). Vitamin E enrichment of cells (at all different concentrations of vitamin E used) rendered no protection against GSH depletion. There were no significant changes of GSH content after 24 h of incubation of cells with different concentrations of vitamin E compared with control cells (Fig. 2B). The content of total protein sulfhydryl groups showed no changes after 2 h of anti-Fas exposure (116.1 ± 15.6 versus 116.6 ± 15.4 nmol of protein-SH/mg of protein before and after exposure, respectively).

Effects of vitamin E on anti-Fas-induced apoptosis

Nuclear morphology. Microscopic examination of nuclear morphology showed that, after exposure to anti-Fas, an increasing percentage of Jurkat T cells exhibited nuclear condensation and fragmentation—typical characteristics of apoptosis (62 ± 2% after 2 h) compared with control (9 ± 2%) (Fig. 3A). At all concentrations tested, vitamin E did not affect anti-Fas-triggered apoptosis as detected by the number of cells with typical nuclear morphology. Vitamin E alone did not induce apoptosis within the range of concentrations used.

Mitochondrial membrane potential. Because catalysis of PS oxidation is likely related to disruption of electron transport and release of cytochrome *c* from mitochondria into the cytosol, we further assessed whether vitamin E was able to exert stabilizing effects on mitochondria in anti-Fas-stimulated cells. MMP was measured by JC-1 staining. After 1 h of anti-Fas stimulation, MMP decreased in 32 ± 6% cells as compared with controls (5 ± 1%). Vitamin E did not have any effect on anti-Fas-induced changes of MMP (Fig. 3B). Vitamin E alone did not cause any changes of MMP.

PS externalization and APT activity. As PS externalization has been previously shown to depend on PS oxidation, we were interested to determine whether vitamin E was able to affect the PS signaling pathway. We used annexin V binding assay and found that anti-Fas stimulation markedly increased the number of cells with externalized PS to 68.3 ± 5.3% as compared with 8.1 ± 0.4% in control samples (Fig. 3C). Even the highest dose of vitamin E alone (50 μ*M*) did not induce any PS externalization (7.8 ± 0.2%). After anti-Fas exposure, vitamin E-enriched cells showed the same level of PS externalization as nonsupplemented cells.

Maintenance of PS asymmetric distribution across plasma membrane is largely due to uninterrupted functioning of APT, which is inhibited during apoptosis (45). Indeed, anti-Fas-triggered Jurkat T cells showed a six-fold lower APT activity as compared with nontreated cells (Fig. 3D). Vitamin E failed to shelter APT from anti-Fas-induced inactivation. In the absence of anti-Fas, vitamin E did not affect APT activity in Jurkat T cells (Fig. 3D). APT contains Cys residues sensitive to redox and electrophilic agents causing loss of enzymatic activity (7, 41). To determine the extent to which anti-Fas-induced APT inactivation was due to disulfide formation, we used a

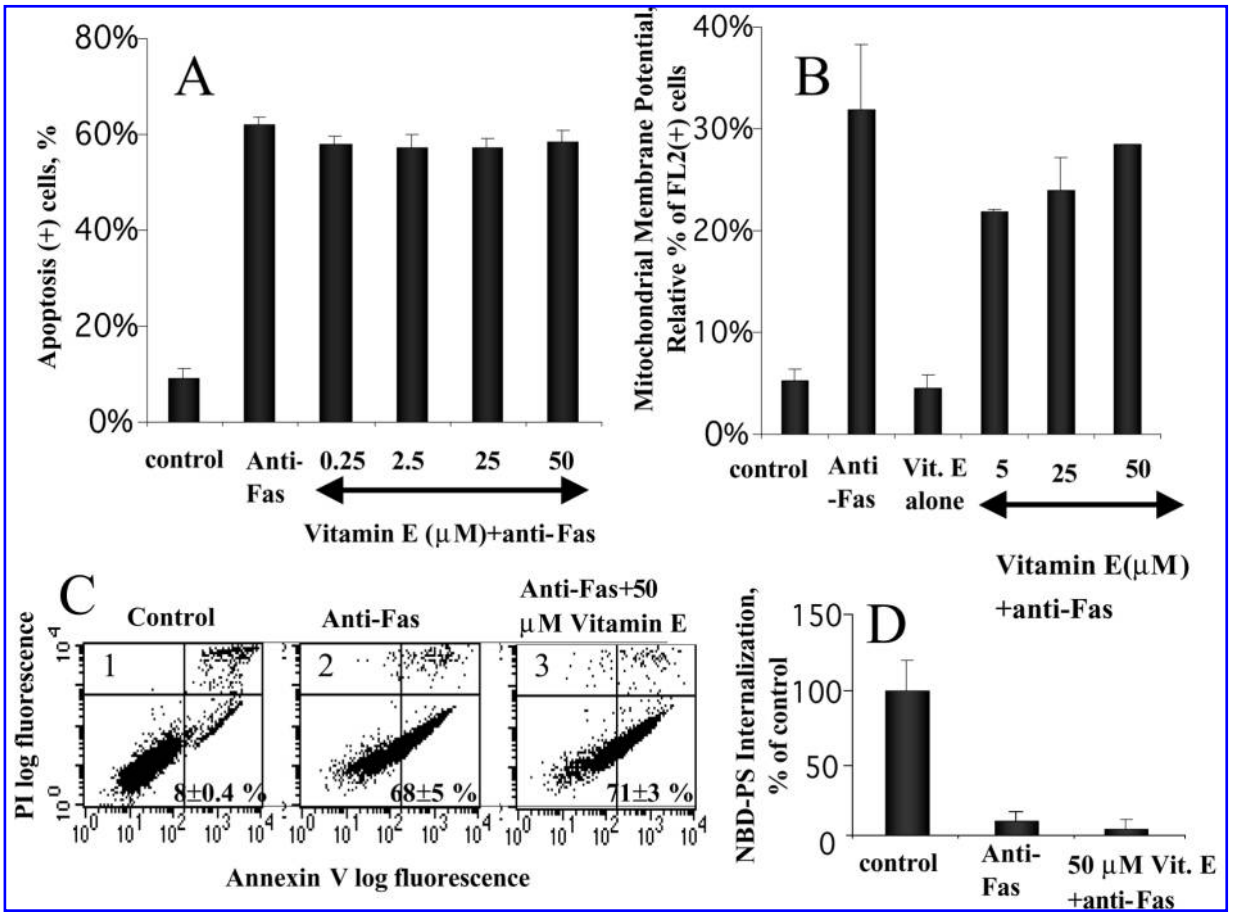

FIG. 3. Effects of vitamin E on anti-Fas-induced apoptosis. (**A**) Nuclear condensation and fragmentation. (**B**) Decrease of MMP. (**C**) PS externalization. (**D**) Inhibition of APT activity. Data are expressed as means ± SD ($n = 3$).

reducing reagent, DTT, on PS externalization and APT activity. To this end, Jurkat T cells were treated with DTT (10 m*M*, 20 min) after 1 h of anti-Fas induction, and then PS externalization and APT activity were measured. DTT was able to only partially reverse anti-Fas-induced PS externalization (by ~27%) (Fig. 4A). Interestingly, in cells challenged with anti-Fas in the presence of vitamin E, this effect of DTT was not observed. DTT was also able to slightly reconstitute anti-Fas-inhibited APT activity in Jurkat T cells, although the effect did not reach the level of statistical significance (Fig. 4B).

Phagocytosis of anti-Fas-triggered apoptotic Jurkat T cells enriched with vitamin E

As our previous work showed that some antioxidant enzymes (SOD plus catalase) as well as a lipid antioxidant, etoposide, affected phagocytosis of apoptotic cells by preventing PS oxidation (18, 42), we studied the effect of vitamin E enrichment on phagocytosis of anti-Fas-induced apoptotic cells using the J774A.1 murine macrophage cell line. Our results showed that within the range of concentrations tested, enrichment of control target cells with vitamin E did not result in any changes of phagocytosis, which remained at the level of 7–8% of phagocytosis-positive cells (Fig. 5). Anti-Fas treatment elicited a more than three-fold increase in the number of phagocytosis-positive macrophages (to ~24–26%). This effect was not changed by vitamin E enrichment of target cells within the range of vitamin E concentrations used.

DISCUSSION

The major errand of programmed cell death is to provide effective and harmless clearance of unwanted cells in the body. Although the details of sophisticated apoptotic machinery have not been deciphered, two principal pathways—intrinsic and extrinsic—based on primary activation of mitochondria and death receptors, respectively, have been identified (44). For anti-Fas-mediated apoptosis, prototypical of the extrinsic pathway, two different cascades have been described based on the level of caspase-8 activation by the death-inducing signaling complex (35). In type I cells, the death signal is propagated by a caspase cascade initiated by robust activation of caspase-8 followed by cleavage of caspase-3 and other caspases to finally complete the apoptotic program. In type II cells, in addition to initial triggering of caspase-8, mitochondria activation by truncated Bid plays a prominent role in final augmentation of executive caspases and realization of the apop-

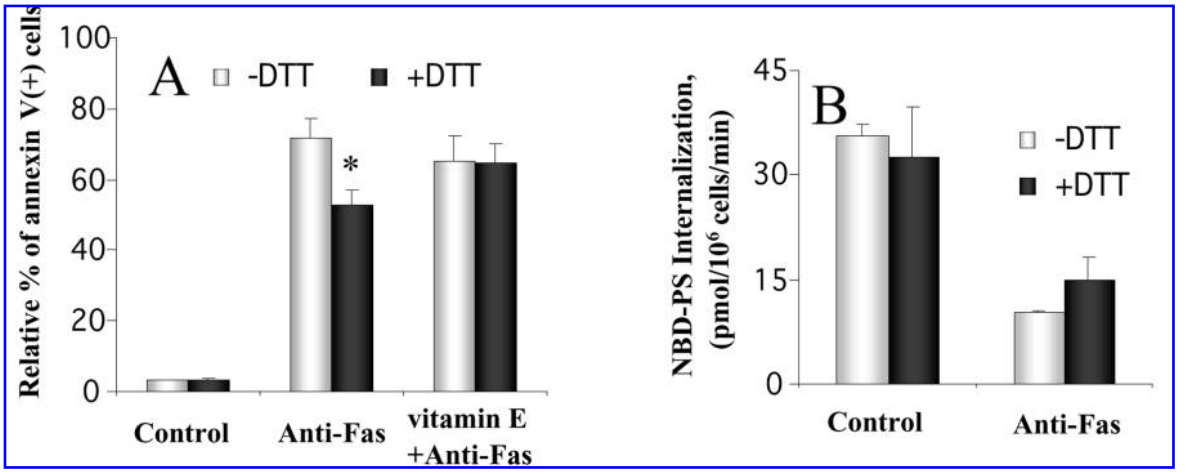

FIG. 4. Effects of a reducing agent, DTT, on PS externalization and APT activity. DTT only slightly decreased anti-Fas-induced PS externalization (**A**) and slightly reconstituted anti-Fas-inhibited APT activity (**B**) in Jurkat T cells. Data are expressed as means ± SD (n = 3–6). *$p < 0.01$, anti-Fas + DTT versus anti-Fas alone.

totic program (19, 35). Accordingly, the degree of mitochondrial involvement may vary in these apoptotically different types of cell death mechanisms. This is essential because disrupted mitochondrial electron transport is the major source of reactive oxygen species that may affect redox-dependent signaling pathways of apoptosis (4, 49)

We have previously reported that phospholipid oxidation, particularly oxidative modification of PS, may be involved in PS-dependent apoptotic signaling, as well as in recognition and clearance of apoptotic cells by macrophages. More specifically, PS externalization and/or redox-sensitive APT activity are likely to be affected by PS oxidation. Further, PS oxidation products may act as independent "eat me" signals or enhancers of known signals to aid tethering and recognition of apoptotic cells by phagocytes. Others and we have shown that oxidized epitopes on apoptotic cells are perceived as "eat me" signals by phagocytic receptors such as CD36, LOX-1, etc. (6, 17, 29, 33, 34). Moreover, nonapoptotic cells enriched with oxidized PS are phagocytosed more effectively than those enriched with only nonoxidized PS (17). Further, pharmacologic concentrations of antioxidant enzymes (SOD plus catalase), as well as a lipid antioxidant drug, etoposide, were able to abrogate phagocytic clearance of apoptotic cells via inhibition of PS oxidation (17, 42).

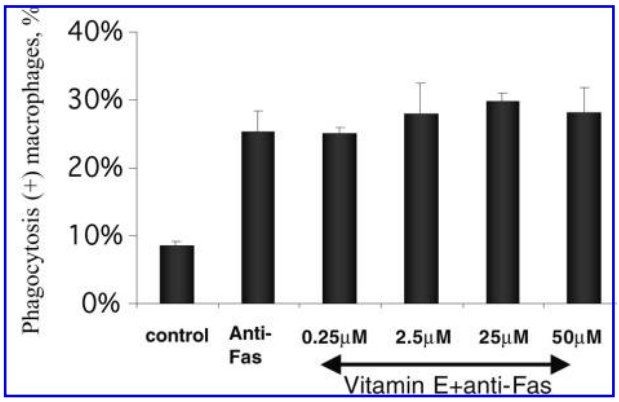

FIG. 5. Phagocytosis of anti-Fas-triggered apoptotic Jurkat T cells enriched with vitamin E by J774A.1 macrophages. Macrophages that had side-by-side connection with target cells (binding) and/or internalized target cells (engulfment) were considered as phagocytosis-positive. Data are presented as means ± SD (n = 3–5).

Taken together, these results suggest that PS oxidation may be essential for the regulation of one of the basic functions—clearance of apoptotic cells—whose antioxidant interruption may have severe consequences, particularly in critical conditions such as inflammation, whose aggravation may be catastrophic. Because dietary supplementation of the population at large with relatively high doses of vitamin E is becoming a common practice in addition to its specific utilization for chemopreventive and therapeutic goals (1, 3, 28, 30, 31, 38), it is imperative to determine its potential role in the regulation of apoptosis and phagocytosis of apoptotic cells.

The proposal that vitamin E operates on a "no interference with apoptosis/phagocytosis" principle appeals to common sense. One way to investigate this is by directly interrogating relationships between phospholipid oxidation and PS signaling pathways during apoptosis induced in cell culture model systems. Cells grown in cultures without any supplementation with vitamin E experience its deficiency (20). Therefore, we utilized incubation of Jurkat T cells with different concentrations of vitamin E (from 0.25 to 50 μM) that are relatively close to its concentrations in human plasma (12–42 μM) (40). This resulted in the enrichment of Jurkat T cells with vitamin E to levels from 0.02 to 2.2 nmol/mg of protein, *i.e.*, a broad range covering its content from approximately fivefold lower to fivefold higher than commonly found in human lymphocytes *in vivo* (21). We chose anti-Fas monoclonal antibody as a well-defined inducer to trigger extrinsic type II apoptosis by a nonoxidant compound to avoid trivial and nonspecific effects of abundant lipid oxidation readily preventable by vitamin E (27, 39).

Expectedly, we found that vitamin E was able to inhibit anti-Fas-induced PS oxidation such that at concentrations normally present in T lymphocytes no PS oxidation was detectable. Surprisingly, however, this potent inhibitory effect on PS oxidation was not accompanied by any vitamin E-dependent changes of several biomarkers of apoptosis tested, including PS externalization, as well as phagocytosis of apoptotic Jurkat T cells by J774A.1 macrophages. These results are in noticeable contrast with our previous findings that a hindered phenolic lipid antioxidant, etoposide, and a combination of antioxidant enzymes (SOD plus catalase) were both able to effectively block PS oxidation and phagocytosis of apoptotic cells proportionally to their ability to inhibit PS oxidation (18). Our earlier work has also reported that in addition to PS oxidation, there are effective alternative pathways for PS externalization, likely realized through redox modifications of APT (8). In particular, nitric oxide-mediated complete inhibition of PS oxidation induced in HL-60 cells by an azo initiator of peroxyl radicals, AMVN [2,2′-azobis(2,4-dimethylisovaleronitrile)], was not accompanied by any changes in PS externalization, likely due to nitric oxide-mediated enhancement of APT inhibition.

Our current results also showed that GSH content and APT activity dramatically decreased in anti-Fas-challenged Jurkat T cells and vitamin E enrichment did not exert any protective effects. This suggests that, in this case as well, redox modifications of APT might be involved in vitamin E-insensitive APT redox modification. This may explain, at least in part, the differences between significant effects of SOD plus catalase and the lack of effect of vitamin E, which did not protect APT against redox modification.

To determine the extent to which redox modifications contributed to PS externalization and APT inhibition, we treated anti-Fas-induced cells with a disulfide reducing agent, DTT. To avoid apoptosis-associated restrictions in availability of energy source, ATP, a short-term anti-Fas induction (1 h) was carried out in glucose containing PBS followed by treatment with DTT. This resulted in ~27% protection against maximal anti-Fas-induced PS externalization. Vitamin E supplementation essentially abolished the protective effects of DTT on PS externalization. Under the same conditions, anti-Fas-inhibited APT was slightly reconstituted (although the effect did not reach the level of statistical significance). Our measurements of protein SH groups did not elicit any measurable anti-Fas-induced changes in their content. Taken together, these data suggest that the redox modifications are not likely the major contributors to APT inhibition and PS externalization during anti-Fas-induced apoptosis.

Because in our experiments cells were pretreated with vitamin E for 24 h, it is possible that nonantioxidant vitamin E-dependent signaling pathways could be operable in Jurkat T cells, making them more vulnerable to extrinsic proapoptotic stimuli. Although it is difficult to specifically identify possible routes of vitamin E action, it is worth mentioning that there are several reported mechanisms through which vitamin E can participate in the regulation of apoptosis, including changes in the mitogen-activated protein kinase signal transduction pathways (32) and H_2O_2/lipid peroxidation-induced adhesion molecules (43), and inhibition of production of chemokines and inflammatory cytokines (47). In addition, vitamin E can inhibit the activity of the transcription factors nuclear factor-κB and AP-1 and block expression of CD95L (Fas ligand), and thus prevent T cell activation-induced cell death (23). Thus, it does not seem unlikely that expression of some proteins required for phagocytosis of apoptotic cells may be affected by vitamin E supplementation.

Perspectives

It should be emphasized that our results are relevant to extrinsic type II anti-Fas-induced apoptosis in Jurkat T cells (35). It is quite possible that intrinsic apoptotic pathways, which rely on mitochondria-activated redox mechanisms to a much greater extent, are more vulnerable to antioxidant vitamin E interventions. Further, vitamin E is a generic name for a family of several relevant but distinctive molecules (different isomers of tocopherols and tocotrienols) that may have not only different chemical reactivities, but also different physiologic effects (48). Therefore, thorough further studies of different members of vitamin E and their effects on intrinsic PS-dependent signaling pathways are necessary before any conclusions on the lack of vitamin E action on apoptosis and phagocytic clearance of apoptotic cells are made.

ACKNOWLEDGMENTS

This work was supported by NIH grants HL64145–01A1, HL70755, and GM 64610.

ABBREVIATIONS

APT, aminophospholipid translocase; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; FBS, fetal bovine serum; GSH, L-γ-glutamyl-L-cysteinyl-glycine (glutathione); H_2O_2, hydrogen peroxide; hSA, human serum albumin; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1; MMP, mitochondrial membrane potential; NBD-PS, 1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphoserine; PBS, phosphate-buffered saline; PnA, *cis*-parinaric acid; PS, phosphatidylserine; SOD, superoxide dismutase; α-TOH, α-tocopherol.

REFERENCES

1. Aghdassi E, Wendland BE, Steinhart AH, Wolman SL, Jeejeebhoy K, and Allard JP. Antioxidant vitamin supplementation in Crohn's disease decreases oxidative stress. A randomized controlled trial. *Am J Gastroenterol* 98: 348–353, 2003.
2. Arroyo A, Modriansky M, Serinkan FB, Bello RI, Matsura T, Jiang J, Tyurin VA, Tyurina YY, Fadeel B, and Kagan VE. NADPH oxidase-dependent oxidation and externalization of phosphatidylserine during apoptosis in Me2SO-

differentiated HL-60 cells. Role in phagocytic clearance. *J Biol Chem* 277: 49965–49975, 2002.
3. Brockes C, Buchli C, Locher R, Koch J, and Vetter W. Vitamin E prevents extensive lipid peroxidation in patients with hypertension. *Br J Biomed Sci* 60: 5–8, 2003.
4. Cadenas E and Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 29: 222–230, 2000.
5. Chalvardjian A and Rudnicki E. Determination of lipid phosphorus in the nanomolar range. *Anal Biochem* 36: 225–226, 1970.
6. Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, Dennis EA, and Witztum JL. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A* 96: 6353–6358, 1999.
7. Connor J and Schroit AJ. Transbilayer movement of phosphatidylserine in erythrocytes: inhibition of transport and preferential labeling of a 31,000-dalton protein by sulfhydryl reactive reagents. *Biochemistry* 27: 848–851, 1988.
8. Fabisiak JP, Tyurin VA, Tyurina YY, Sedlov A, Lazo JS, and Kagan VE. Nitric oxide dissociates lipid oxidation from apoptosis and phosphatidylserine externalization during oxidative stress. *Biochemistry* 39: 127–138, 2000.
9. Fadok VA and Chimini G. The phagocytosis of apoptotic cells. *Semin Immunol* 13: 365–372, 2001.
10. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, and Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148: 2207–2216, 1992.
11. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, and Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405: 85–90, 2000.
12. Folch J, Lees M, and Sloan-Stanley GH. A simple method for isolation and purification of total lipids from animal tissue. *J Biol Chem* 226: 497–509, 1957.
13. Fukasawa M, Adachi H, Hirota K, Tsujimoto M, Arai H, and Inoue K. SRB1, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells. *Exp Cell Res* 222: 246–250, 1996.
14. Henson PM, Bratton DL, and Fadok VA. Apoptotic cell removal. *Curr Biol* 11: R795–R805, 2001.
15. Kagan VE, Ritov VB, Tyurina YY, and Tyurin VA. Sensitive and specific fluorescent probing of oxidative stress in different classes of membrane phospholipids in live cells using metabolically integrated *cis*-parinaric acid. *Methods Mol Biol* 108: 71–87, 1998.
16. Kagan VE, Kuzmenko AI, Tyurina YY, Shvedova AA, Matsura T, and Yalowich JC. Pro-oxidant and antioxidant mechanisms of etoposide in HL-60 cells: role of myeloperoxidase. *Cancer Res* 61: 7777–7784, 2001
17. Kagan VE, Gleiss B, Tyurina YY, Tyurin VA, Elenstrom-Magnusson C, Liu SX, Serinkan FB, Arroyo A, Chandra J, Orrenius S, and Fadeel B. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J Immunol* 169: 487–499, 2002.
18. Kagan VE, Borisenko GG, Serinkan BF, Tyurina YY, Tyurin VA, Jiang J, Liu SX, Shvedova AA, Fabisiak JP, Uthaisang W, and Fadeel B. Appetizing rancidity of apoptotic cells for macrophages: oxidation/externalization/recognition of phosphatidylserine. *Am J Physiol Lung Cell Mol Physiol* 285: L1–L17, 2003.
19. Krammer PH. CD95's deadly mission in the immune system. *Nature* 407: 789–795, 2000.
20. Leist M, Raab B, Maurer S, Rosick U, and Brigelius-Flohe R. Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide-induced genotoxicity. *Free Radic Biol Med* 21: 297–306, 1996.
21. Lenton KJ, Therriault H, Cantin AM, Fulop T, Payette H, and Wagner JR. Direct correlation of glutathione and ascorbate and their dependence on age and season in human lymphocytes. *Am J Clin Nutr* 71: 1194–1200, 2000.
22. Lilja H, Laurell CB, and Jeppsson JO. Characterization of the predominant basic protein in human seminal plasma, one cleavage product of the major seminal vesicle protein. *Scand J Clin Lab Invest* 44: 439–446, 1984.
23. Li-Weber M, Weigand MA, Giaisi M, Suss D, Treiber MK, Baumann S, Ritsou E, Breitkreutz R, and Krammer PH. Vitamin E inhibits CD95 ligand expression and protects T cells from activation-induced cell death. *J Clin Invest* 110: 681–690, 2002.
24. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, and Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182: 1545–1556, 1995.
25. Matsura T, Serinkan BF, Jiang J, and Kagan VE. Phosphatidylserine peroxidation/externalization during staurosporine-induced apoptosis in HL-60 cells. *FEBS Lett* 524: 25–30, 2002.
26. McIntyre JC and Sleight RG. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry* 30: 11819–11827, 1991.
27. Miki M, Tamai H, Mino M, Yamamoto Y, and Niki E. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by alpha-tocopherol. *Arch Biochem Biophys* 258: 373–380, 1987.
28. Nathens AB, Neff MJ, Jurkovich GJ, Klotz P, Farver K, Ruzinski JT, Radella F, Garcia I, and Maier RV. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* 236: 814–822, 2002.
29. Oka K, Sawamura T, Kikuta K, Itokawa S, Kume N, Kita T, and Masaki T. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A* 95: 9535–9540, 1998.
30. Pace A, Savarese A, Picardo M, Maresca V, Pacetti U, Del Monte G, Biroccio A, Leonetti C, Jandolo B, Cognetti F, and Bove L. Neuroprotective effect of vitamin E supplementation in patients treated with cisplatin chemotherapy. *J Clin Oncol* 21: 927–931, 2003.

31. Prasad KN, Cole WC, Kumar B, and Che Prasad K. Pros and cons of antioxidant use during radiation therapy. *Cancer Treat Rev* 28: 79–91, 2002.
32. Qin F, Shite J, and Liang CS. Antioxidants attenuate myocyte apoptosis and improve cardiac function in CHF: association with changes in the MAPK pathways. *Am J Physiol Heart Circ Physiol* 285: H822–H832, 2003.
33. Sambrano GR, Parthasarathy S, and Steinberg, D. Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low density lipoprotein. *Proc Natl Acad Sci U S A* 91: 3265–3269, 1994.
34. Savill J, Hogg N, Ren Y, and Haslett C. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest* 90: 1513–1522, 1992.
35. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, and Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 17: 1675–1687, 1998.
36. Shvedova AA, Kommineni C, Jeffries BA, Castranova V, Tyurina YY, Tyurin VA, Serbinova EA, Fabisiak JP, and Kagan VE. Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes. *J Invest Dermatol* 114: 354–364, 2000.
37. Sies H, Stahl W, and Sundquist AR. Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Ann N Y Acad Sci* 669: 7–20, 1992.
38. Steinberg FM and Chait A. Antioxidant vitamin supplementation and lipid peroxidation in smokers. *Am J Clin Nutr* 68: 319–327, 1998.
39. Tamai H, Miki M, and Mino M. Hemolysis and membrane lipid changes induced by xanthine oxidase in vitamin E deficient red cells. *J Free Radic Biol Med* 2: 49–56, 1986.
40. Tietz NW (Ed). *Clinical Guide to Laboratory Tests*. Philadelphia, PA: W.B. Saunders Company, 1995, p. 644.
41. Truong HT, Daleke DL, and Huestis WH. Dithiothreitol stimulates the activity of the plasma membrane aminophospholipid translocator. *Biochim Biophys Acta* 1150: 57–62, 1993.
42. Tyurina YY, Serinkan FB, Tyurin VA, Kini V, Yalowich JC, Schroit AJ, Fadeel B, and Kagan VE. Lipid antioxidant, etoposide, prevents phosphatidylserine oxidation and externalization during hydrogen peroxide-induced apoptosis: dissociation of phosphatidylserine-dependent clearance from other features of apoptosis. *J Biol Chem* 279: 6056–6064, 2004.
43. van Dam B, van Hinsbergh VW, Stehouwer CD, Versteilen A, Dekker H, Buytenhek R, Princen HM, and Schalkwijk CG. Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells and decreases soluble cell adhesion molecules in healthy subjects. *Cardiovasc Res* 57: 563–571, 2003.
44. Wajant H. The Fas signaling pathway: more than a paradigm. *Science* 296: 1635–1636, 2002.
45. Williamson P and Schlegel RA. Transbilayer phospholipid movement and the clearance of apoptotic cells. *Biochim Biophys Acta* 1585: 53–63, 2002.
46. Williamson P, Bevers EM, Smeets EF, Comfurius P, Schlegel RA, and Zwaal RFA. Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. *Biochemistry* 34: 10448–10455, 1995.
47. Wu D, Koga T, Martin KR, and Meydani M. Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes. *Atherosclerosis* 147: 297–307, 1999.
48. Yoshida Y, Niki E, and Noguchi N. Comparative study on the action of tocopherols and tocotrienols as antioxidant: chemical and physical effects. *Chem Phys Lipids* 123: 63–75, 2003.
49. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, and Kroemer G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 182: 367–377, 1995.

Address reprint requests to:
V.E. Kagan
Department of Environmental and Occupational Health
University of Pittsburgh
Pittsburgh, PA 15260

E-mail: kagan@pitt.edu

Received for publication October 31, 2003; accepted November 10, 2003.

This article has been cited by:

1. Jeffrey Atkinson, Thad Harroun, Stephen R. Wassall, William Stillwell, John Katsaras. 2010. The location and behavior of #-tocopherol in membranes. *Molecular Nutrition & Food Research* **54**:5, 641-651. [CrossRef]
2. Yongsheng Wang, Candace Panagabko, Jeffrey Atkinson. 2010. Synthesis of #-tocohexaenol (#-T6) a fluorescent, oxidatively sensitive polyene analogue of #-tocopherol. *Bioorganic & Medicinal Chemistry* **18**:2, 777-786. [CrossRef]
3. Hirofumi Fujita, Daisuke Shiva, Toshihiko Utsumi, Tetsuya Ogino, Tomohiro Ogawa, Koichi Abe, Tatsuji Yasuda, Kozo Utsumi, Junzo Sasaki. 2010. #-Tocopheryl succinate induces rapid and reversible phosphatidylserine externalization in histiocytic lymphoma through the caspase-independent pathway. *Molecular and Cellular Biochemistry* **333**:1-2, 137-149. [CrossRef]
4. Mirjana #uki#, Milica Ninkovi#, Marina Jovanovi#. 2008. Oxidative Stress - Clinical Diagnostic Significance. *Journal of Medical Biochemistry* **27**:4, 409-425. [CrossRef]
5. Jeffrey Atkinson, Raquel F. Epand, Richard M. Epand. 2008. Tocopherols and tocotrienols in membranes: A critical review. *Free Radical Biology and Medicine* **44**:5, 739-764. [CrossRef]
6. Valerian E. Kagan, Grigory G. Borisenko, Yulia Y. Tyurina, Vladimir A. Tyurin, Jianfei Jiang, Alla I. Potapovich, Vidisha Kini, Andrew A. Amoscato, Yasu Fujii. 2004. Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. *Free Radical Biology and Medicine* **37**:12, 1963-1985. [CrossRef]
7. Valerian E. Kagan , Peter J. Quinn . 2004. Toward Oxidative Lipidomics of Cell Signaling. *Antioxidants & Redox Signaling* **6**:2, 199-202. [Abstract] [PDF] [PDF Plus]