Toward Mechanism-based Antioxidant Interventions

Lessons from Natural Antioxidants

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ABSTRACT: It is generally accepted that one of the major and important contributions to skin aging, skin disorders, and skin diseases results from reactive oxygen species. More than other tissues, the skin is exposed to numerous environmental chemical and physical agents, such as ultraviolet light, causing oxidative stress. Accelerated cutaneous UV-induced aging, photo aging, is only one of the harmful effects of continual oxygen radical production in the skin. Interestingly, our ELISA assays of 8-oxo-2′–deoxyguanosine in skin of young and old Balb/c mice showed that cumene hydroperoxide–induced accumulation of the biomarker of oxidative DNA damage in skin of 32-week-old mice occurred independently of their vitamin E status, while no accumultaion of oxo^{8}-dG was detectable in the skin of young animals. This suggests that vitamin E is not the major protector of skin against cumene hydroperoxide–induced oxidative stress. Production and accumulation of apoptotic cells is one of the characteristic features of skin damage by oxidative stress that, in the absence of effective scavenging by macrophages, dramatically enhances oxidative damage and inflammatory response. In our model experiments, we demonstrated that Cu-OOH induces significant oxidative stress in phospholipids of normal human epidermal keratinocytes (NHEK) whose characteristic feature is an early and profound oxidation of phosphatidylserine (PS), likely related to PS externalization. Since externalized PS is a signal for recognition of apoptotic cells by macrophage scavenger receptors, PS oxidation may be translatable into elimination of thus damaged NHEKs. Experiments are now underway to determine whether inhibition of PS oxidation by antioxidants may interfere with improtant signaling functions of oxidative stress in eliminating apoptotic cells.

KEYWORDS: vitamin E; natural antioxidants; prooxidant; signaling pathways

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Many theories have been advanced to account for the aging process. For example, the aging process has been attributed to molecular cross-linking, changes in immunologic function, damage by free radical reactions, senescence genes, and most recently, to telomere shortening. No single theory is generally accepted: "This remarkable process remains a mystery," and "it is doubtful that a single theory will explain all the mechanisms of aging."[1] Among more than 300 theories to explain the aging phenomenon, the free radical theory of aging, postulated first by D. Harman, is the most popular and widely tested, and is based on the chemical nature and ubiquitous presence of free radicals. Numerous studies emphasized the role of free radicals in DNA damage—both nuclear as well as mitochondrial—the oxidative stress they impose on cells, the role of antioxidants, the presence of autoantibodies, and their overall impact on the aging process.[2]

In the United States, the average life expectancy at birth rose from 69.7 years in 1960 to 75.7 years in 1994. Interestingly, since the 1960s the percentage of the population of the United States taking antioxidant supplements has increased to a value of 40–50% today. These data are in accord with beneficial effects expected from the growing use of antioxidant supplements since the 1960s to decrease disease and enhance life span and the widespread publicity about the ability of fruits and vegetables to decrease disease by depressing free radical–reaction damage.[3] This suggests that development of new mechanism-based antioxidant approaches combined with monitoring of oxidative stress/antioxidant status may be central to understanding the mechanisms of aging and developing novel optimized strategies for increased life span.

OPTIMIZING ANTIOXIDANT PROTECTION AGAINST OXIDATIVE STRESS

Redox Requirements

The intra- and extracellular regulation of relatively low reactive one- or two-electron reduction intermediates of molecular oxygen, such as superoxide and hydrogen peroxide, is successfully achieved by several specialized enzymes: different types of superoxide dismutases, catalase, and peroxidases (for a current review see Thiele *et al.*[4]). Other radical intermediates—hydroxyl, alkoxyl, and peroxyl radicals—are so reactive that they cannot be effectively controlled by these enzymatic mechanisms and require additional specialized systems for their management. Indeed, Mother Nature has created a unique network of small antioxidants that includes both lipid-soluble and water-soluble molecules. Notably, these molecules located in different compartments of cells closely interact with each other such that the overall antioxidant protection is very efficient. It is tempting to believe that supplementation with additional amounts of natural antioxidants or use of synthetic low molecular weight antioxidant molecules may offer relatively simple and effective ways to control oxidative stress. These expectations, however, should be based on detailed knowledge of antioxidant mechanisms and pathways that are essential for uninterrupted and physiologically justified antioxidant protection in specific intracellular environments.

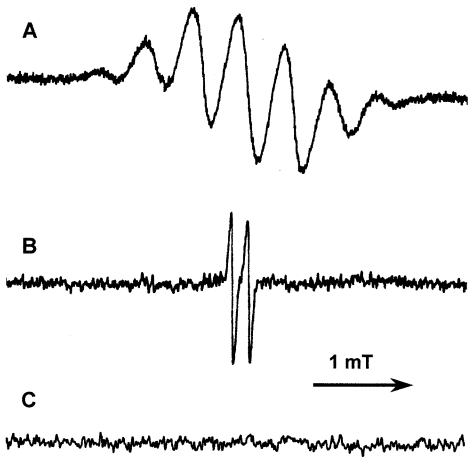

FIGURE 1. ESR spectra of α-tocopherol phenoxyl and ascorbate radicals produced upon UVB irradiation. α-Tocopherol suspension (0.1 mM) in SDS (100 mM) was irradiated with 290 nm light in the absence (**A**) or in the presence (**B**) of 0.5 mM ascorbate. Irradiation of ascorbate solution without α-tocopherol gave no ESR signal (**C**).

There are several critical requirements for a molecule that should be fulfilled in order to create a perfect and effective new antioxidant that include but are not limited to: (1) effective radical scavenging; (2) low reactivity of antioxidant radicals towards vital intracellular components; (3) low level of one-electron enzymatic metabolism of antioxidants; and (4) lack of interference with endogenous antioxidant networks, resulting in wasteful consumption of their resources. Vitamin E represents probably one of the best examples of a "perfect" lipid-soluble antioxidant in membranes and lipoproteins, where it can interact and be recycled by other lipid-soluble antioxidants (e.g., coenzyme Q)[5] and water-soluble antioxidants (e.g., vitamin C and thiols).[6] In addition, vitamin E can be regenerated from its radical by electron transport in mitochondria and endoplasmic reticulum.[7] Despite significant advantages that vitamin E's properties render to accomplish its role as interactive component of the antioxidant network, these same redox features of vitamin E may cause adverse effects under some circumstances. For example, it has been demonstrated that the vitamin E radical can directly attack lipids in lipoproteins and cause oxidative damage rather than antioxidant protection when the rate of oxidation-initiating radicals is high enough.[8] This prooxidant effect of vitamin E is only observable in the absence of other components of antioxidant network such as vitamin C or coenzyme Q, which can prevent unwanted direct interaction of the vitamin E radical with vital biomolecules.

Under some conditions, however, the recycling properties of vitamin E may cause adverse effects. Formation of the vitamin E radical, vitamin E phenoxyl radical, can be triggered by UVB light. We have demonstrated that UVB-induced vitamin E radicals can be reduced by vitamin C both in model systems and in skin.[9] As shown in FIGURE 1, UVB-induced vitamin E phenoxyl radical readily observable in the EPR

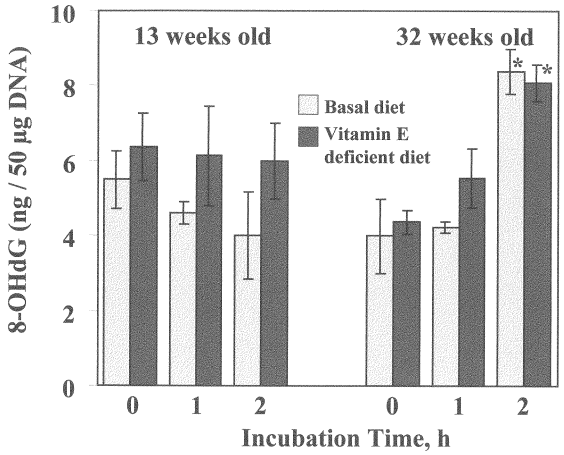

FIGURE 2. Levels of 8-oxo-2′-deoxyguanosine (oxo^8-dG) in skin of young and old Balb/c mice with different levels of vitamin E after exposure to cumene hydroperoxide. *Conditions:* Weaning Balb/c female mice were given vitamin E–deficient or –sufficient diets for 10 or 29 weeks. Levels of vitamin E in skin of mice given basal diet for 10 and 29 weeks were 98.5 ± 11.6 and 52.3 ± 7.9 pmol/mg, respectively. Levels of vitamin E in skin of mice given vitamin E–deficient diet for 10 and 29 weeks were 2.4 ± 0.7 and 0.62 ± 0.11 pmol/mg, respectively. *Treatment:* 100 µL cumene hydroperoxide (12 mmol/kg) were applied topically on the dorsal side. Mice were scarified after 1 or 2 hours postexposure. ELISA determination of oxo^8-dG was performed according to the manufacturer's instructions as described in the kit obtained from Genox Corporation (Baltimore, MD) after isolation of DNA using a chaotropic agent, NaI (obtained from Wako Chemical Co., Richmond, VA), as described by Helbock *et al.*[10] $P < 0.05$ vs. control.

spectra upon irradiation of vitamin E disappeared and was substituted by a typical doublet signal of vitamin C (ascorbate) radical. Given that UVB irradiation of vitamin C does not produce any signal in the EPR spectra in the absence of vitamin E, these data indicate that reduction of vitamin E phenoxyl radical was responsible for the spectral changes observed. This effect was also documented by UVB-induced generation of ascorbate radicals from endogenous vitamin C in mouse skin homogenates that was strictly dependent on the amount of vitamin E present in skin. Thus vitamin E that acts as a well-known protective skin antioxidant in the dark may also induce oxidative stress under UVB exposure. This suggests that under specific conditions, when skin is exposed to UVB irradiation, vitamin E may act as a photo-sensitizer catalytically consuming and destroying endogenous pools of vitamin C as a mechanism of wasteful vitamin E redox-recycling. Obviously, direct radical scavenging effects of vitamin E are also realized under the same conditions. As a result, the outcome of anti- versus prooxidant overall action of vitamin E will depend on specific conditions (i.e., intensity of UVB-irradiation and concentrations of vitamin E and vitamin C).

Interestingly, an accelerated cutaneous UV-induced aging (photoaging) is known as one of the harmful effects of continual oxidative stress in the skin. Interestingly, our ELISA assays of 8-oxo-2′-deoxyguanosine (oxo^8-dG) in skin of young and old

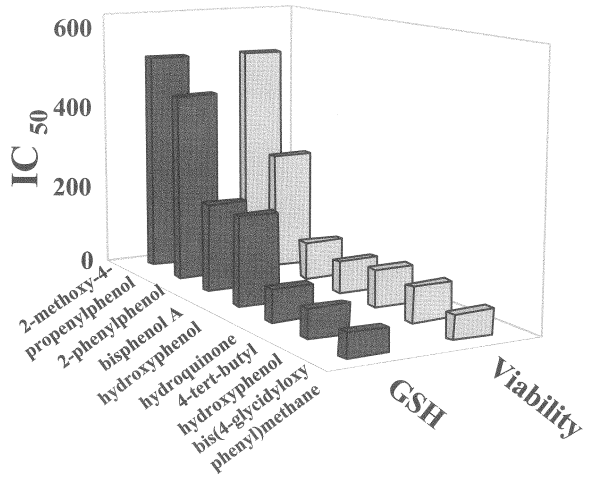

FIGURE 3. Comparison of cytotoxicity and GSH oxidation in normal human epidermal keratinocytes exposed to phenolic compounds. Normal human epidermal keratinocytes were incubated in phenol-free KGM-2 basal medium in the presence of seven phenolic compounds for 18 h at 37°C in 96-well plates. After the incubation, cells were washed twice with PBS, pH 7.4. GSH was measured using ThioGlo-1^{TM} (10 μM) as described by Kagan *et al.*[13] Cell viability was determined using 10% Alamar Blue, as described by Keane *et al.*[14]

Balb/c mice showed that cumene hydroperoxide–induced accumulation of this biomaker of oxidative DNA damage in skin of 32-week old mice occurred independently of their vitamin E status (after keeping the animals on vitamin E–sufficient or vitamin E–deficient diets, respectively) while no accumulation of oxo^8-dG was detectable in the skin of young (13-week old) animals (FIG. 2).[10] This suggests that vitamin E is likely not the major protector of skin against cumene hydroperoxide–induced oxidative stress.

Vitamin E may exhibit its prooxidant activity only under some exquisite conditions. There are many examples of molecules that are good donors of electrons and hence extraordinary effective scavengers of oxidation-initiating radicals. In fact, many mono- and polyphenolic compounds have an appropriate electron-donating capacity to reduce peroxyl, alkoxyl, and hydroxyl radicals. Not all of them, however, may be good candidates for antioxidant protection. The major problem is that the products of their one-electron oxidation, the respective phenoxyl radicals, are sufficiently reactive to directly oxidize important targets in cells and biological fluids. Which intracellular constituents exactly are the targets that may suffer from oxidation by antioxidant phenoxyl radicals depends, to a large extent, on the redox potentials of phenoxyl radicals as well as on steric hindrance of the phenoxyl moiety by the surrounding groups. In the absence of vitamin C, intracellular thiols may be the most susceptible targets to attack by phenoxyl radicals. FIGURE 3 shows how different concentrations of environmentally and occupationally relevant phenolic compounds affect viability and levels of GSGH in cultures of normal human epidermal keratinocytes (NHEK). One can see that there is a strong correlation between the abil-

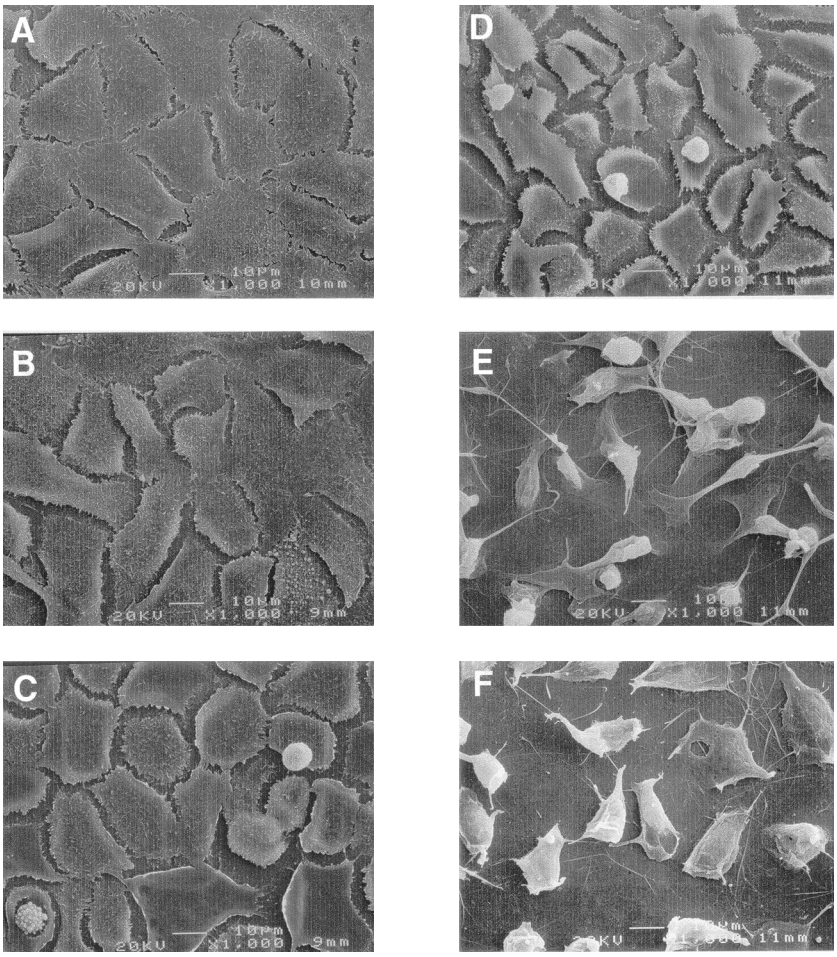

FIGURE 4. Scanning electron microphotographs of normal human epidermal keratinocytes exposed to bisphenol. Keratinocyte separation from monolayers in tissue cell culture, loss of keratinocytes, and altered cell surface homogeneity seem to be suitable for characterizing differences in keratinocyte morphology after bisphenol exposure. The signs of flattening keratinocyte cell surface, reduction of the size, and blurring of the cell borders were observed after exposure of keratinocyte cell cultures to bisphenol (**D–F**). Overall, confident signs of cell shrinking were evident after exposure to 200 μM bisphenol. Altered cell integrity was clearly visible 18 h after 300–500 μM bisphenol exposure (**E–F**). *Conditions:* Normal human epidermal keratinocytes were incubated in phenol-free KGM-2 basal medium in the absence and in the presence of 50–500 μM bisphenol for 18 h at 37°C in chamber slides. After the incubation, cells were washed twice with PBS, pH 7.4, and scanning microscopy was performed. (**A**) control cells; (**B**) after incubation with 50 μM of bisphenol; (**C**) after incubation with 100 μM of bisphenol; (**D**) after incubation with 200 μM of bisphenol; (**E**) after incubation with 300 μM of bisphenol; (**F**) after incubation with 500 μM of bisphenol.

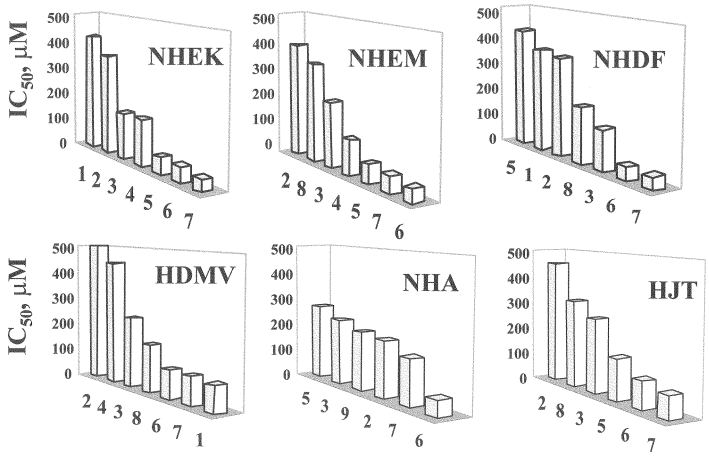

FIGURE 5. Effectiveness of glutathione (GSH) oxidation in different human cell lines exposed to phenolic compounds. Human cells (normal human keratinocytes, NHEK; normal human melanocytes, NHEM; normal human fibroblasts, NHDF; normal human astrocytes, NHA; normal human dermal microvascular cells, NHMV; and human Jurkat cells, HJT) were incubated in phenol-free KGM-2 basal medium in the presence of phenolic compounds for 18 h at 37°C in 96-well plates. After the incubation, cells were washed twice with Na-phosphate buffer, pH 7.4. GSH was measured using ThioGlo-1^{TM} (10 μM) as described by Kagan *et al.*[13] *Columns:* (1) 2-methoxy-4-propenylphenol; (2) 2-phenylphenol; (3) bis(4-hydroxyphenyl)dimethylmethane (4) hydroxyphenol; (5) 1,4-benzenediol; (6) 4-*tert*-butylhydroxyphenol; (7) bis(4-glycidyloxyphenyl)methane; (8) 4-*tert*-butylphenol; (9) phenol.

ity of these phenolic compounds to deplete GSH and their cytotoxicity. Morphological features of cytotoxicity of phenolic compounds to keratinocytes exemplified by the effects of bisphenol on NHEK are shown on FIGURE 4. This reveals gradual keratinocyte separation from monolayers in tissue cell culture, altered cell surface homogeneity, and finally damage and loss of keratinocytes after exposure to bisphenol.

It should be noted that different types of peroxidase activity in NHEK (e.g., peroxidase activity of prostagladin synthase) can catalyze one-electron oxidation of phenolics and generate their phenoxyl radicals. Moreover, not only keratinocytes but also other types of cells in normal human skin, such as melanocytes, fibroblasts, astrocytes, dermal microvascular cells, as well as Jurkat cells, display a similar similarity between depletion of GSH and loss of viability upon exposure to different phenolic compounds (FIGS. 5 and 6). Since vitamin C is not usually present in culture growth media, it is not present at any significant levels in cultured cells. This implies that protection of cultured cells from toxic effects of phenoxyl radicals does not involve their reduction due to interaction with vitamin C. In the presence of vitamin C, one can envision that generation of phenoxyl radicals may be causative to depletion of vitamin C that will precede oxidation of GSH and protein thiols. In any case, formation of reactive phenoxyl radicals by oxidative stress or as a result of enzymatic one-electron metabolism may be associated with further enhancement of oxidative

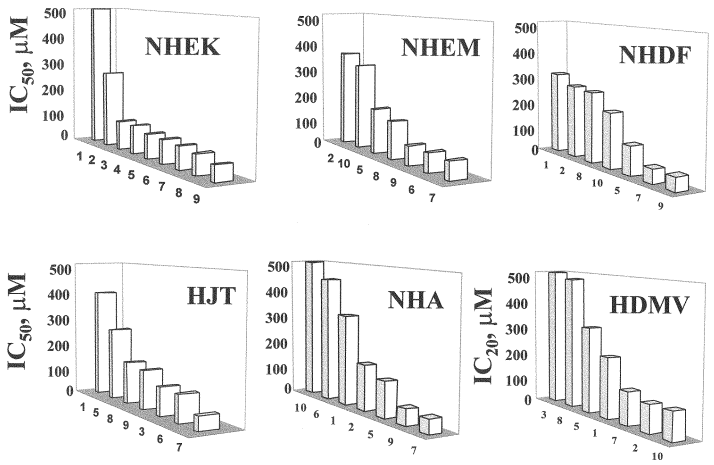

FIGURE 6. Cytotoxicity of phenolic compounds to different human cell lines. Human cells (normal human keratinocytes, NHEK; normal human melanocytes, NHEM; normal human fibroblasts, NHDF; normal human astrocytes, NHA; normal human dermal microvascular cells, NHMV; and human Jurkat cells, HJT) were incubated in phenol-free KGM-2 basal medium in the presence of phenolic compounds for 18 h at 37°C in 96-well plates. After incubation, cells were washed twice with Na-phosphate buffer, pH 7.4. Cell viability was determined using 10% Alamar Blue as described by Keane *et al.*[14] *Columns:* (1) 2-methoxy-4-propenylphenol; (2) 2-phenylphenol; (3) bis(4-hydroxyphenyl)dimethylmethane; (4) hydroxyphenol; (5) 1,4-benzenediol; (6) 4-*tert*-butylhydroxyphenol; (7) bis(4-glycidyloxyphenyl)methane; (8) 4-*tert*-butylphenol; (9) phenol; (10) 4-allyl-2-methoxyphenol.

stress rather than protective effects of phenolic compounds if their redox characteristics are compatible with direct oxidation of essential intracellular constituents.

Requirements Related to Essential Signaling Pathways

When cellular production of reactive radical intermediates overwhelms the capacity of its antioxidant network, damage to cellular macromolecules such as lipids, protein, and DNA may ensue. As a result "oxidative stress" may contribute to the pathogenesis of a number of human diseases. Recent studies, however, have also implicated alterations in cellular redox state, production of reactive oxygen species as well as organic radical intermediates, and resulting oxidative modifications of proteins in normal physiological signaling (e.g., by growth factors, cytokines) (for a review, see paper of Thannickal and Fanburg[11]). Moreover, phospholipid oxidation products can also serve as signaling molecules with potent physiologic effects.[12] It seems that blockade of these fundamental signaling pathways by antioxidants may interfere with the fulfillment of their physiologic functions. Therefore, design of new potent antioxidants should take into consideration these important consequences that potent antioxidants may exert via their interference into signaling pathways.

Production and accumulation of apoptotic cells is one of characteristic features of tissue damage by oxidative stress that in the absence of effective scavenging by macrophages dramatically enhances oxidative damage and inflammatory response.

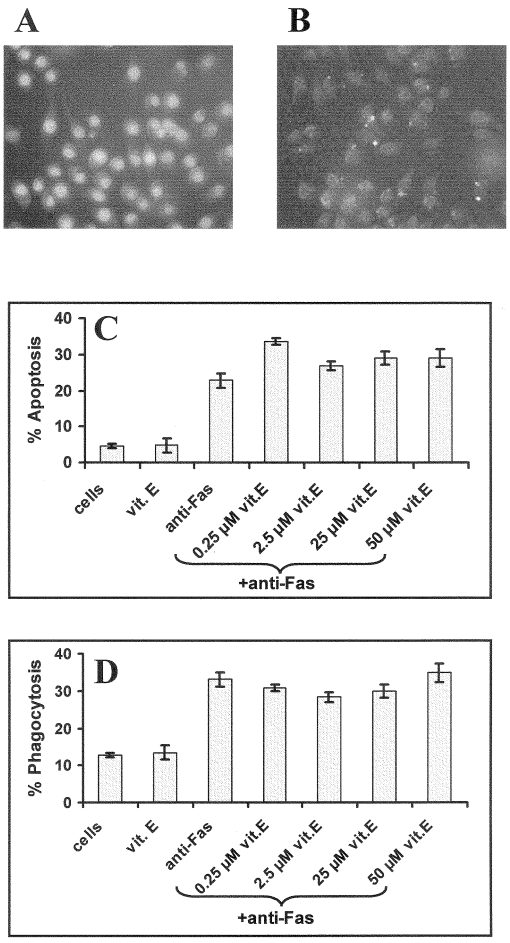

FIGURE 7. Vitamin E does not affect phagocytosis of apoptotic Jurkat cells. Typical fluorescence microphotographs showing phagocytosis of control Jurkat cells by J774A.1 macrophages (**A**) and Fas-triggered apoptotic Jurkat cells (**B**). Quantitative results on Fas-triggered apoptosis and phagocytosis of apoptotic cells are shown in panels **C** and **D**, respectively. Exposure to anti-Fas agonistic antibody induced apoptosis in 33 ± 2% of Jurkat cells in the absence of vitamin E. Pretreatment with vitamin E did not affect the yield of Fas-induced apoptosis (**C**). Phagocytosis of apoptotic Jurkat cells by J774A.1 macrophages (25 ± 3%) was not significantly affected by vitamin E in the concentration range 0.25–50 µM (**D**). Apoptosis was induced in Jurkat cells by anti-Fas agonistic antibody (250 ng/10^6 cells) after preincubation of the cells with different concentrations of vitamin E (0.25–50µM) for 48 h. J774A.1 macrophage cell line was used for phagocytosis assay. Target Jurkat cells (typically 30×10^6) were labeled with 0.5 mM Cell Tracker Green^{TM} (Molecular Probes, Eugene OR) in serum-free medium for 15 min at 37°C and cultured at 10^6 cells/mL in the presence of anti-Fas agonistic antibody. After labeling, cells were washed and resuspended in serum-containing RPMI medium. Fluorescently labeled cells were added to macrophages

While oxidative stress has been implicated as an initiator of apoptosis in at least some of these conditions, generation of reactive oxygen species and subsequent oxidative stress has also been demonstrated to be a component of a final common pathway of apoptotic execution via mitochondrial dysfunction. It is well established that mitochondrial permeability transition and departure of cytochrome *c*, the hallmarks of apoptosis, are associated with excessive generation of radical intermediates and oxidative stress. What is not known is whether there is any specialized mechanistic role(s) for ROS and oxidative stress in guiding or directing apoptotic pathways and outcomes.

Importantly, apoptotic cell death is not accompanied by inflammatory response due to safe engulfment and digestion of damaged cells by macrophages. This critical function of macrophages is mediated by the specific interactions of their cognate receptor(s) with an anionic phospholipid, phosphatidylserine (PS), appearing on the surface of cells undergoing apoptosis. Normally, most cells maintain asymmetric distribution of phospholipids across plasma membrane such that aminophospholipids—PS and phosphatidylethanolamine (PE)—are mainly confined to the inner leaflet of plasma membrane. This is accomplished by a specialized enzymatic system, aminophospholipid translocase (APT). Apoptotic cells lose the ability to sustain this vital asymmetry and allow for externalization of PS (and PE) from the inner leaflet of plasma membrane to its outer surface, due to inhibition of APT and activation of "scramblase," an enzymatic activity responsible for bidirectional nonspecific translocation of phospholipids.

We have recently established that apoptosis induced by various stimuli in a number of different cells is accompanied by selective oxidation of PS that was significantly more pronounced than oxidation of other more abundant phospholipids (PE, phosphatidylcholine). We further demonstrated that PS oxidation is involved in the mechanisms responsible for PS externalization in apoptotic cells and its subsequent recognition by macrophage receptors. Therefore, antioxidants may possess dramatic effects on PS externalization and hence macrophage recognition/phagocytosis of apoptotic cells. This is because inhibition of PS oxidation may result in failure of its externalization and interaction with macrophages, hence producing an exuberant inflammatory response. Therefore, it is critical to determine whether various antioxidants are capable of inhibiting PS oxidation during execution of apoptotic program in cells.

In a series of preliminary experiments, we studied the ability of vitamin E to affect phagocytosis of apoptotic Jurkat cells (in which apoptosis was induced by anti-Fas agonistic antibody) by a macrophage cell line, J.774A.1. We found that in the concentration range from 5 to 50 μM, vitamin E did not cause any changes in the effectiveness of phagocytosis (FIG. 7). This suggests that vitamin E is not likely to in-

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pretreated with 1 mg/mL Hoechst 33342 fluorescent dye at a ratio of 10:1, and the mixture was incubated for 1 h at 37°C. Phagocytosis was evaluated by two-color fluorescence analysis (Hoechst 33342 and Cell Tracker Green™) using a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742-95-12NRB) and analyzed using MetaImaging Series™ software version 4.6 (Universal Imaging Corp., Downingtown, PA). At least 300 macrophages/experimental condition were counted. Phagocytosis data are reported as percent phagocytes positive for uptake.

terfere with the apoptotic signaling pathway dependent on PS externalization. In separate experiments with Jurkat cells, however, we established that a combination of catalase/SOD was able to inhibit both oxidation of PS and phagocytosis of Fas-triggered Jurkat cells. Therefore, it is imperative that antioxidants are tested as potential inhibitors of oxidation reactions of specific molecules that may be important for vital signaling pathways.

ACKNOWLEDGMENT

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