α-Tocopheryl succinate, an agent with *in vivo* anti-tumour activity, induces apoptosis by causing lysosomal instability

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Certain vitamin E analogues, such as α-tocopheryl succinate (α-TOS), exhibit *in vivo* anti-tumour activity and, *in vitro*, induce apoptosis of cultured tumour cells. In the present study we report that these effects may be explained, at least in part, by destabilization of lysosomal membranes. α-TOS, but not α-tocopheryl acetate or α-tocopherol (α-TOH), induced early lysosomal destabilization followed by apoptosis. Similar effects were observed with β-TOS, whereas β-TOH was inactive. Cathepsin D-deficient cells were more resistant to α-TOS than their normal counterparts, and featured delayed caspase activation. Possible detergent and lysosomotropic effects of α- and β-TOS were suggested by their haemolytic activity in an *in vitro* test and their release of β-galactosidase from isolated lysosomes, whereas the non-succinylated analogues were inactive. The pro-apoptotic activity of α-TOS was pH-dependent, being greater at lower pH, typical of the interstitium of solid tumours. These findings indicate that lysosomal destabilization may partially or fully explain the induction of apoptosis in cultured cells by α-TOS and the mechanism whereby this agent exerts *in vivo* anti-tumour effects.

Key words: cathepsin D, lysosomes, pH, programmed cell death.

INTRODUCTION

Vitamin E is an essential micronutrient antioxidant [1]. Recent reports implicate the otherwise non-toxic succinyl ester of vitamin E, α-tocopheryl succinate (α-TOS), a redox-silent agent with a charged side group, as an apoptotic inducer in multiple transformed cell lines [2–4]. The underlying mechanism for the pro-apoptotic activity of α-TOS is not known at present, but it is clear that this activity requires the intact succinyl ester and that similar effects are exerted by analogues such as α-tocopheryl butyric ether [5,6]. Furthermore, cells capable of hydrolysing α-TOS, including intestinal epithelial cells [7] and hepatocytes [8], do not undergo apoptosis when exposed to the agent [9].

Current evidence suggests that α-TOS may induce apoptosis via signalling pathways shared by various pharmacological agents [3,4,10,11]. For example, α-TOS can induce cell death via the activator protein-1 (AP-1) pathway [2,12], which may explain its sensitizing effect on cancer cells to Fas killing [13]. α-TOS may also act by modulating the level of transforming growth factor-β (TGF-β) [14], by interfering with the cell cycle progression via inhibition of the transcriptional activity of E2F [15], and/or by enhancing the expression of the cell cycle check-point protein $p21^{Waf1/Cip1}$, probably via the CCAAT-enhancer-binding protein β-dependent pathway [16]. α-TOS has been shown to cause deregulation of the protein phosphatase-2A/protein kinase C (PKC) pathway [4], which can be important for maintaining the anti-apoptotic function of bcl-2 [4,17].

Although the above reports suggest several pathways through which α-TOS may transmit pro-apoptotic signals, it is by no means clear what the initial events in the process are. We and others have found that there is a requirement for the succinyl moiety of the agent to make vitamin E analogues pro-apoptotic, as neither α-tocopherol (α-TOH) nor α-tocopheryl acetate (α-TOA) induced apoptosis in a variety of cells [2,4,18,19]. Consistent with the emerging notion that lysosomes may be causally involved in initiation and/or progression of apoptosis [19–25], we previously reported that apoptosis induced by α-TOS involved lysosomal destabilization as a relatively early event [3]. Therefore the present investigations were aimed at determining whether lysosomal destabilization may be responsible for apoptosis initiated by α-TOS. In the present study we report evidence that α-TOS-induced apoptosis may be a consequence of lysosomal destabilization, probably due to the detergent-like activity of the agent. In addition, we find that the lytic and pro-apoptotic activity of α-TOS is increased at low pH, perhaps helping to explain the relative *in vivo* toxicity of this agent to solid tumours (which typically have low interstitial pH).

MATERIALS AND METHODS

Cell culture and treatment

Jurkat cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics. The cathepsin D-positive and -negative murine fibroblasts, prepared by gene targetting and immortalization of mouse fibroblasts [26], and the caspase-3-overexpressing MCF-7 cells [27] were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) FCS and antibiotics. For experiments, suspension cells were used at 0.5×10^6 cells/ml, while adherent cells were grown in dishes or on cover slips to 70–90% confluence. Cells were treated with

Abbreviations used: Ac, acetyl; AMC, aminomethylcoumarin; AP-1, activator protein-1; AO, Acridine Orange; CHS, cholesteryl hemisuccinate; FCS, fetal calf serum; HBSS, Hanks balanced salt solution; MSDH, *O*-methylserine dodecylamide hydrochloride; PKC, protein kinase C; pNA, *p*-nitroaniline; PS, phosphatidylserine; TGF-β, transforming growth factor-β; α-TOA, α-tocopheryl acetate; α-TOH, α-tocopherol; α-TOS, α-tocopheryl succinate; γ-T3, γ-tocotrienol.

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α-TOH, α-TOS, α-TOA, cholesteryl hemisuccinate (CHS), free succinate (all purchased from Sigma), γ-tocotrienol (γ-T3), β-TOH (both provided by Henkel, Dusseldorf, Germany), β-TOS (prepared as described below) or methoxy-α-TOH (a gift from Dr D. Liebler, University of Arizona, Tucson, AZ, U.S.A.).

For preparation of β-TOS, β-TOH was dissolved in acetic acid containing zinc powder and anhydrous sodium acetate in a catalytic amount, and mixed with succinyl anhydride. The mixture was brought to 130 °C, kept at this temperature under constant stirring for 6 h, poured into water and extracted with hexane containing diethyl ether. The β-TOS hexane layer was evaporated under vacuum, yielding a light-yellow viscous liquid. β-TOS was purified from this crude preparation to $> 98\%$ purity by silica gel column chromatography, and its authenticity and purity were verified by analytical HPLC and GLC–MS. MS analysis revealed a major molecular peak of m/z 516 and fragments corresponding to those of β-TOS having $> 95\%$ identity with the library spectrum of the compound.

Assessment of markers of apoptosis

Apoptosis was assessed essentially as described elsewhere [3,4,18], by staining cells with annexin V-FITC, which is based on its affinity for phosphatidylserine (PS) externalized in the early phases of apoptosis. Cells (0.5×10^6) were washed in PBS, resuspended in binding buffer (10 mM Hepes/NaOH, 140 mM NaCl and 25 mM $CaCl_2$, pH 7.4), incubated for 20 min at 20 °C with 2 μl of annexin V-FITC (PharMingen, Heidelberg, Germany), and analysed by flow cytometry (Becton Dickinson). In some experiments, the cytotoxicity of vitamin E analogues was estimated by quantification of detached cells.

Caspase activity was assessed as follows. Cells (10^6) were lysed in 100 μl of lysis buffer (10 mM Tris/HCl, 130 mM NaCl, 0.1% Triton X-100 and 10 mM NaH_2PO_4, pH 7.4), mixed with 900 μl of reaction buffer [20 mM Hepes, 10% (v/v) glycerol and 2 mM dithiothreitol, pH 7.4] and incubated for 2 h at 37 °C with the substrates for caspase-3, -6 or -9 (Ac-DEVD-pNA, Ac-VEID-pNA or Ac-LEHD-AMC respectively; 50 μM each; Calbiochem; where Ac, pNA and AMC stand for acetyl, *p*-nitroaniline and aminomethylcoumarin respectively). Fluorescence intensity of the liberated AMC was then measured at $\lambda_{ex} = 400$ nm and $\lambda_{em} = 505$ nm for Ac-LEHD-AMC, while absorbance at 405 nm of the liberated pNA was measured in the case of Ac-DEVD-pNA and Ac-VEID-pNA. Activation of caspase-3 was assessed by immunolabelling cells with an anti-caspase-3 IgG (PharMingen) preferentially recognizing the active form of the caspase, followed by staining with an FITC-conjugated secondary antibody and evaluation of the caspase-3-positive cells by flow cytometry.

Lysosomal destabilization assessment

Lysosomal stability analysis was performed by following the uptake of the weak base Acridine Orange (AO; Sigma) [4]. Following treatment with inducers of apoptosis, cells (0.5×10^6) were washed and resuspended in 2.5 ml of RPMI 1640 medium with 10 mM Hepes and 5 μg/ml AO, incubated at 37 °C for 15 min, washed, resuspended in PBS, and the red fluorescence was estimated by flow cytometry. The percentage of cells with low intensity of red fluorescence ('pale' cells) was used as a marker of the extent of lysosomal destabilization (impairment of AO uptake). For estimation of lysosomal leakage, cells were loaded with AO before treatment with the apoptosis inducer. In brief, cells were transferred to RPMI 1640 medium containing 10 mM Hepes, incubated at 37 °C for 15 min with 5 μg/ml AO, washed and resuspended in complete RPMI 1640 medium, and treated as indicated. AO leakage, due to lysosomal destabilization, was assessed by flow-cytometry measurement of the increase in the population of cells with high green fluorescence, reflecting re-localization of AO from lysosomes to the cytosol. As AO tends to spontaneously lose its localization under these conditions, treatments of cells were conducted for up to 2 h.

Lysosomes were prepared from rat livers homogenized in 9 vol. of 0.33 M sucrose containing 2 mM Hepes and 5 mM $MgCl_2$ (pH 7.4) and centrifuged at 450 ***g*** for 2 min. The supernatant was centrifuged (3500 ***g*** for 10 min), and the resulting supernatant centrifuged again (10000 ***g*** for 10 min), yielding a lysosome-rich fraction. The fraction was incubated with the agents for the time specified, and β-galactosidase activity was assessed as published elsewhere [28], using 4-methylumbelliferyl-β-galactosidase (Sigma) as a substrate.

Haemolysis assay

Erythrocytes were prepared from fresh heparin-treated human blood by diluting 0.5 ml of whole blood in 40 ml of isotonic PBS (pH 7.4), followed by centrifugation at 5000 ***g*** for 10 min at 4 °C. The pellet was resuspended in 45 ml of Hanks balanced salt solution (HBSS), pH 7.4, and 1 ml of the erythrocyte suspension was incubated with vitamin E analogues (each at 50 μM) dissolved in DMSO. The samples were incubated for various periods at 37 °C, and centrifuged for 10 min at 5000 ***g***. Supernatant (100 μl) was diluted with 900 μl of water, and the absorbance at 414 nm was determined. For the effect of pH on erythrocyte haemolysis, the above protocol was used, except that erythrocytes were resuspended in HBSS adjusted to pH 6.3, 7.09 or 7.48, after which α-TOS was added at a final concentration of 25 μM.

Evaluation of the role of pH in pro-apoptotic activity of vitamin E analogues

To study the role of pH on apoptosis induction by vitamin E analogues, Jurkat cells and MCF-7 cells were transferred into the complete RPMI 1640 medium or Eagle's minimum essential medium respectively, whose pH values were adjusted by addition of 1 M Hepes to give pH values of 7.02, 6.56 or 6.2, or kept at the original pH of 7.4. The cells were then exposed to α-TOS or γ-T3 for 12 h (MCF-7 cells) or as shown (Jurkat cells) under standard culture conditions, and the extent of apoptosis was evaluated by counting the detached cells (MCF-7 cells) or by using the annexin V-binding method (Jurkat cells). The pH values did not change during the course of the incubations.

HPLC analysis of α-TOS and γ-T3

The levels of α-TOS and γ-T3 in cells were determined according to a method published elsewhere [29], following extraction of cells (2×10^6) treated with either vitamin E analogue with hexane/methanol (5:1, v/v).

RESULTS

Both α-TOH and β-TOH are redox-active, due to the labile hydrogen on the phenol ring, unlike the esterified α-TOA, α-TOS and β-TOS, of which the last two are negatively charged at physiological pH. As expected, exposure of Jurkat cells to α-TOS led to apoptosis induction, as well as lysosomal leakage. Figures 1(A)–1(C) show PS externalization (annexin V binding), activation of caspase-3, -6 and -9, and lysosomal destabilization in cells treated with α-TOS. Moreover, lysosomal leakage preceded both caspase activation and PS externalization, suggesting that

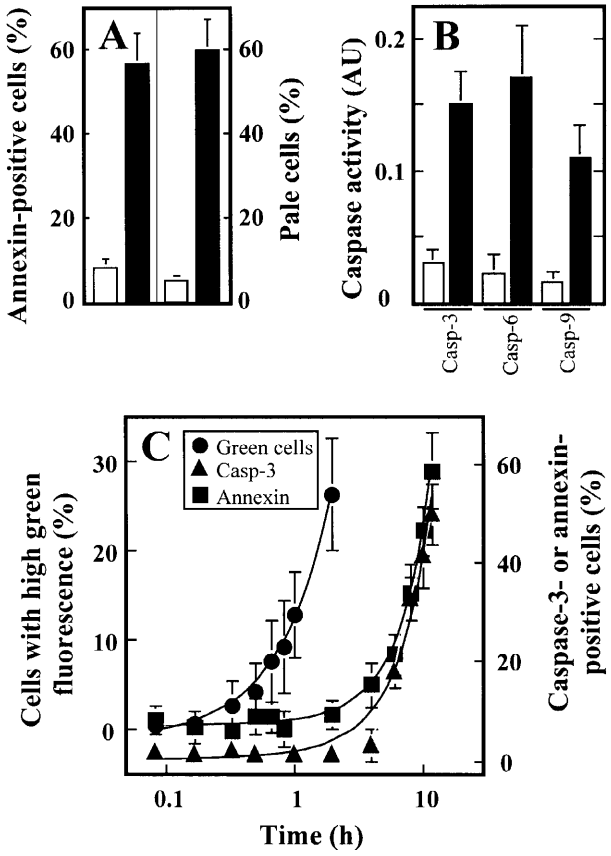

Figure 1 α-TOS induces apoptosis and destabilizes lysosomes

Jurkat cells were exposed to vehicle (control; white bars) or α-TOS (50 μM; black bars) for 12 h. The cells were assessed by flow cytometry for apoptosis (annexin V-FITC staining) and lysosomal destabilization (impairment of AO uptake) (**A**), and for the activity of caspase (Casp)-3, -6 and -9 (**B**). (**C**) The kinetics of lysosomal leakage (increase in the population of cells with high green fluorescence), caspase-3 activation (caspase-3-positive cells) and PS externalization (annexin V-positive cells) is shown for cells exposed to 50 μM α-TOS. The results are expressed as a fraction of the cells positive for the marker assessed. The data are presented as means ± S.D. ($n = 3$). AU, arbitrary units.

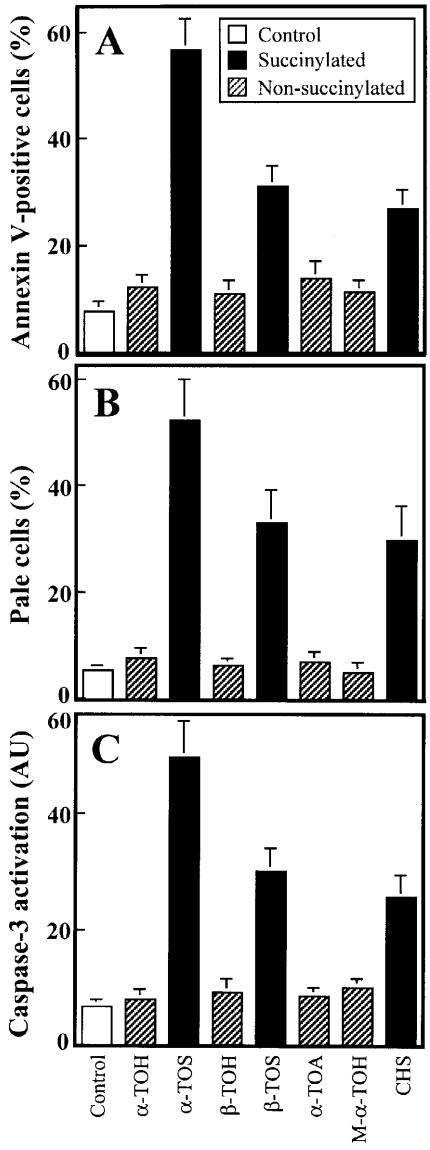

Figure 2 Apoptosis induction and lysosomal destabilization are dependent on the succinyl moiety of the agents

Jurkat cells were exposed to various vitamin E analogues or CHS (50 μM; for 12 h), and assessed for apoptosis (annexin V-FITC staining) (**A**), lysosomal destabilization (impairment of AO uptake) (**B**), and caspase-3 activation (**C**). The data are presented as means ± S.D. ($n = 3$). AU, arbitrary units; M-α-TOH, methoxy-α-TOH.

lysosomal destabilization may be an important early event in the pro-apoptotic machinery, consistent with and extending our previous findings [3].

In additional experiments, Jurkat cells were exposed to a variety of analogues of vitamin E, including α-TOH, α-TOS, α-TOA, β-TOH, β-TOS and methoxy-α-TOH, as well as to CHS and free succinate. Whereas α-TOH, β-TOH, α-TOA and methoxy-α-TOH had no effect on the cells, α-TOS, β-TOS and CHS induced apoptosis, caspase-3 activation and lysosomal destabilization (Figure 2). We observed no effect of free succinate at up to 1 mM, i.e. a 20-fold higher concentration than that of the vitamin E analogues used (results not shown). These results strongly support our previous observation suggesting that the succinyl moiety may be an absolute requirement for the apoptosis-inducing activity of α-TOS [4].

If, as the results above suggest, lysosomal destabilization is an early (perhaps initiating) event in apoptosis, this raises the question of which released lysosomal enzymes might be involved. In this regard, previous work suggests that the lysosomal enzyme cathepsin D could play a role [30–32]. In order to study this, we used immortalized fibroblasts prepared from a cathepsin D-deficient mouse [26] and exposed them to α-TOS. Table 1 shows that cathepsin $D^{+/+}$ cells were significantly more susceptible to the agent compared with their cathepsin $D^{-/-}$ counterparts, as demonstrated by PS externalization, lysosomal destabilization and caspase-3 activation.

The observed early lysosomal leakage caused by α-TOS might imply a lysosomal membrane-destabilizing activity of this agent. α-TOS is amphipathic, having both hydrophobic and hydrophilic ends connected by an aliphatic chain, and could, thus, act as a detergent. We tested the possible detergent activity of α-TOS in two systems. First, erythrocytes were exposed to various vitamin E analogues. Of these, the succinyl-containing α-TOS and β-TOS caused erythrocyte haemolysis, whereas α-TOH, α-TOA and β-TOH were inactive (Figure 3A). Data in Figure 3(B) show that at the lowest pH tested, the haemolytic activity of α-TOS was the highest. Because the pK_a of α-TOS is 5.64, a greater proportion

Table 1 Cathepsin D plays a role in α-TOS-induced apoptosis

Apoptosis was assessed using the annexin V method, lysosomal destabilization was estimated by scoring 'pale' cells following staining with AO, and caspase-3 activation was assessed using the antibody specifically recognizing the activated form of the protease. The data are presented as means ± S.D. ($n = 3$).

Marker	Cathepsin $D^{+/+}$ cells	Cathepsin $D^{-/-}$ cells
Apoptosis (%)	34.2 ± 7.2	17.2 ± 3.9
Lysosomal destabilization (%)	38.3 ± 5.4	20.3 ± 3.8
Caspase-3 activation (arbitrary units)	76.4 ± 14.8	37.8 ± 5.6

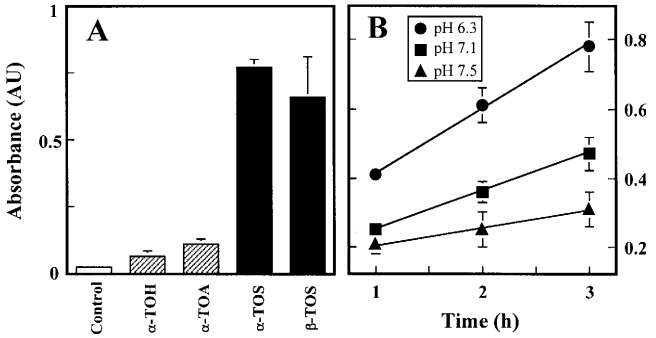

Figure 3 Haemolysis of erythrocytes is specific for vitamin E succinate and is pH-dependent

Isolated erythrocytes were incubated for 2 h at pH 7.4 with different vitamin E analogues at 50 μM (**A**), or for the indicated periods of time at different pH values with 25 μM α-TOS (**B**), and their integrity was assessed by spectrophotometric evaluation of absorbance at 414 nm. The data are presented as means ± S.D. ($n = 3$). AU, arbitrary units.

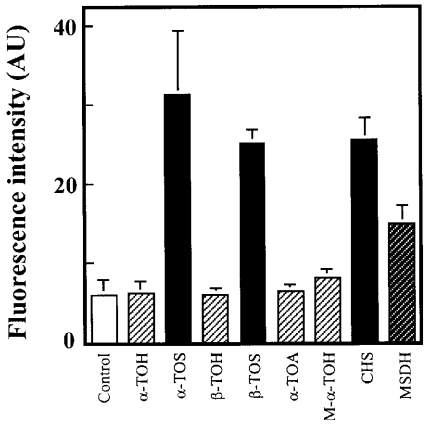

Figure 4 α-TOS and β-TOS destabilize isolated lysosomes

Lysosomes were prepared from rat liver, supplemented with vitamin E analogues or MSDH (at 50 μM), incubated for 30 min at 37 °C, and the supernatant was assessed for the activity of β-galactosidase. The data are presented as means ± S.D. ($n = 3$). AU, arbitrary units.

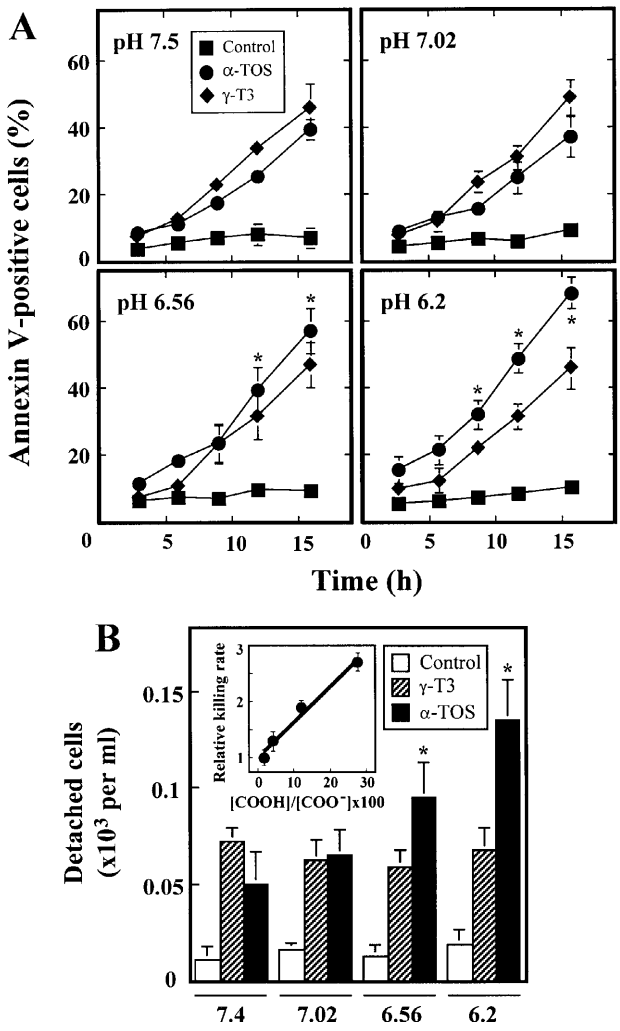

Figure 5 α-TOS is more pro-apoptotic at acidic pH

Jurkat cells (0.5×10^6 cells/ml) and MCF-7 cells (60–70% confluent) were transferred to medium with pH values (adjusted by the addition of Hepes solution) as shown. Following exposure to the vehicle (control), α-TOS (50 μM) or γ-T3 (25 μM) for 12 h (MCF-7 cells) or for the times shown (Jurkat cells), the extent of apoptosis was estimated by annexin V-FITC staining (**A**; Jurkat cells) or by counting detached cells (**B**; MCF-7 cells). The inset in (**B**) shows the relative rate of toxicity of α-TOS to MCF-7 cells, expressed as a ratio of the detached cells at individual pH values, as a function of the ratio between the deprotonated and protonated form of the agent, calculated using the Henderson–Hasselbalch equation, $pH = pK_a + \log([COO^-]/[COOH])$, where $pK_a = 5.64$, $[COO^-]$ is the concentration of the charged (deprotonated) form of α-TOS and [COOH] is the concentration of its uncharged (protonated) form. The data are presented as means ± S.D. ($n = 3$). *The extent of apoptosis at pH 7.02 and 7.5 is significantly different ($P < 0.05$) from the extent of apoptosis at pH 6.2 and 6.56.

would be uncharged at lower pH and, consequently, enter the cells more readily than at neutral pH.

The possibility that α-TOS might directly destabilize lysosomes was tested using isolated lysosomes. As an indicator of lysosomal leakage/rupture, we employed the lysosomal enzyme β-galactosidase. As shown in Figure 4, exposure of isolated lysosomes to either α-TOS or β-TOS, but not α-TOH, β-TOH or α-TOA, led to lysosomal leakage as reflected by the increased release of β-galactosidase. The fact that similar effects were caused by the lysosomotropic detergent *O*-methylserine dodecylamide hydrochloride (MSDH) and by CHS provides further evidence that the lysosome destabilizing effect of these vitamin E analogues depends on the presence of the succinyl moiety.

Given the fact that the detergent-like activity of α-TOS is pH-dependent (see Figure 3B), we studied whether pH may also play a role in apoptosis induction by the agent in intact cells, using Jurkat cells and MCF-7 cells. As shown in Figure 5, apoptosis induced in both cell lines by α-TOS was indirectly proportional

Table 2 α-TOS is taken up faster at lower pH

Jurkat cells (0.5×10^6/ml) were exposed to 50 μM α-TOS or γ-T3 for the time period indicated, harvested, washed, and the amount of the vitamin E analogue associated with the cells was assessed by HPLC. Cellular levels of α-TOS and γ-T3 are expressed as nmol/mg of cell protein. The data are presented as means ± S.D. ($n = 3$).

		α-TOS (nmol/mg of protein)				γ-T3 (nmol/mg of protein)			
Time (h)	pH ...	7.4	7.02	6.56	6.2	7.4	7.02	6.56	6.2
1		2.6 ± 1.6	2.9 ± 1.8	3.7 ± 2.2	4.6 ± 2.6	3.1 ± 1.6	3.5 ± 2.2	2.6 ± 1.4	2.4 ± 1.9
4		4.2 ± 3.1	4.5 ± 2.9	5.8 ± 3.4	6.7 ± 3.2	5.6 ± 3.4	6.4 ± 2.8	4.3 ± 2.1	5.1 ± 3.2
12		7.1 ± 4.7	8.2 ± 4.3	8.8 ± 5.1	8.9 ± 4.2	8.9 ± 4.5	11.2 ± 5.1	7.9 ± 3.5	10.1 ± 4.4

to the pH level, being most pronounced at the lowest pH. Control cells exposed to this range of pH with no addition showed very little spontaneous apoptosis. Importantly, both Jurkat cells and MCF-7 cells underwent pH-independent apoptosis when exposed to γ-T3, a pro-apoptotic analogue of vitamin E that cannot be charged. Thus, as was the case for erythrocyte haemolysis, α-TOS acts more efficiently at lower pH where it is more likely to be uncharged, facilitating its entry into cells. This is supported by the inset in Figure 5 showing direct proportionality between the toxicity of α-TOS to MCF-7 cells at the pH used and the proportion of the agent in the protonated form. For example, there is an approx. 25–30-fold higher amount of α-TOS in the uncharged, bioavailable form at pH 6.2 than at neutral pH, where almost 99 % of the vitamin E analogue is in the deprotonated form.

To see if the higher efficacy of α-TOS at lower pH is due to its greater bioavailability, we assessed the kinetics of pH-dependent uptake of the agent by Jurkat cells. As shown in Table 2, more α-TOS associated with cells faster at acidic pH than at neutral pH. There was no discernible difference in the uptake of γ-T3 at the pH assessed.

DISCUSSION

Some analogues of vitamin E possess biological activity unrelated to their anti-oxidant capacity. Of these, the redox-silent semi-synthetic α-TOS has been shown to induce apoptosis in a variety of malignant cell types [2–4,9,12–14,33], and this toxicity may be relatively specific for malignant cells [4,9]. Multiple normal cell types, including cardiac myocytes, hepatocytes, fibroblasts and intestinal epithelial cells, appear resistant to α-TOS [7–9], whereas their function is often compromised by established anti-tumour agents. Moreover, in experimental animals with human colon cancer xenografts, α-TOS suppresses tumour growth [4,34], and a recent report shows that it promotes dormancy of lung cancer xenografts [35].

The exact mechanism by which α-TOS induces apoptosis is not known. Several pathways, which may be operational in pro-apoptotic signalling of α-TOS, have been suggested. One possible route in α-TOS-induced apoptosis involves the mitogen-activated protein kinase and c-Jun pathway, whereby the agent might regulate expression of AP-1-controlled genes [2,12,13,36]. In other reports, a pro-apoptotic activity of α-TOS was linked to its effect on the cell cycle by inhibiting the activity of the transcription factor E2F [15], or to its modulation of the TGF-β pathway [14]. α-TOS has also been found to have inhibitory effects on PKC [4]. However, the activity of PKC is also suppressed by α-TOH [4], which is not capable of apoptosis induction [2,4].

In spite of these reports, it is still not clear by which mechanism α-TOS initiates the cascade of apoptotic signalling, but several reports suggest that this may involve mitochondrial and lysosomal destabilization [3,4,10]. As lysosomal rupture has been recently suggested as an early event in apoptosis, we investigated whether lysosomal destabilization may play a role in apoptosis initiation by α-TOS. In the present study we show that, indeed, lysosomal rupture may be an important, early event in apoptosis induced by α-TOS, based on the following lines of evidence: (1) α-TOS-initiated apoptosis involves lysosomal destabilization, and this precedes both caspase-3 activation and PS externalization; (2) lysosomal destabilization and apoptosis induction were observed in analogues of α-TOS containing the succinyl moiety; (3) immortalized fibroblasts deficient in a lysosomal protease were resistant to α-TOS-induced apoptosis, which was paralleled by low caspase-3 activation and suppressed lysosomal destabilization; and (4) α-TOS caused rupture of isolated lysosomes.

The notion that destabilization of the acidic compartment, late endosomes and lysosomes may be involved in apoptosis initiation and/or amplification is not new, although it has been rather neglected. One reason for this may be that a number of studies on the role of caspases in apoptosis employed caspase inhibitors, some of which inhibit cathepsins as well [37]. In fact, a number of inducers of apoptosis also cause lysosomal rupture, including the redox-cycling quinone naphthazarin [31,38,39], hydrogen peroxide [40], atractyloside [41], the lysosomotropic detergent MSDH [42], and the lipid second messenger sphingosine [25]. Consistent with the crucial role of lysosomal rupture in the initiation of apoptosis, fibroblasts from a mucolipidosis II patient (characterized by mis-sorting of lysosomal enzymes) are resistant to apoptotic stimuli, including naphthazarin, MSDH and staurosporine [43].

The structural features shared by succinyl analogues of vitamin E comprise a hydrophobic tail, essential for membrane docking of the agents, and a hydrophilic end. Therefore these agents have features that qualify them as detergents for lipid structures, including biological membranes. α-TOS can exist in solution in two forms, the deprotonated, charged species, and its protonated, uncharged counterpart. Of the two, the latter can enter cells by free diffusion. Therefore the bioavailability of α-TOS is affected by the ratio of its two forms, which is largely dependent on the pH of the environment. According to the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log([\mathrm{COO}^-]/[\mathrm{COOH}])$$

where $\mathrm{p}K_a$ is the dissociation constant of α-TOS (approx. 5.6), [COOH] is the concentration of the uncharged form and [COO^-] is the concentration of the charged form, almost 99 % of the total α-TOS will be charged at neutral pH, while the proportion of its uncharged form will increase some 25-fold at pH 6.2. Consistent with the importance of these dissociation constants, α-TOS was more pro-apoptotic to T-lymphoma and breast cancer cells at

acidic pH (see Figure 5). The role of pH in α-TOS uptake and ensuing pro-apoptotic signalling is further supported by data showing that γ-T3, a vitamin E analogue with high pro-apoptotic activity that cannot be charged [44], induced apoptosis in a pH-independent way (see Figure 5).

Malignant cells, compared with normal cells, rely heavily on glycolysis, which leads to generation of lactic acid, and solid tumours typically have an interstitial pH of 6.3–6.6 [45–50]. The idea that weak acids with cytotoxic activity may be efficient anti-tumour agents has been invoked recently by Kozin et al. [47]. The authors performed experiments in which they injected mice with breast cancer xenografts with glucose to promote glycolysis. This resulted in a fall in tumour interstitial pH by approx. 0.3 unit, which significantly enhanced the anti-cancer efficacy of the weak acid chlorambucil, an inducer of apoptosis with a pK_a of 5.8. This enhanced drug activity due to increased partitioning of the agent into cells/cell membranes at more acidic pH. Therefore it is possible that the acidic environment of tumours could be beneficial for developing cancer treatment strategies based on the enhanced bioavailability of pro-apoptotic agents which are weak acids, i.e. with pK_a values of less than 6.5, a principle that has not been appreciated thus far [51]. We believe that our data shown in the present study, combined with the fact that α-TOS has a pK_a of 5.64, clearly support α-TOS as a candidate anti-cancer agent of potential practical importance, and may help explain the relative selectivity of this vitamin E analogue for malignant cells.

α-TOS has been shown to associate avidly with lipoproteins [52]. Therefore it can be expected to rapidly incorporate into circulating lipoproteins which would carry it throughout the bloodstream, including the microvasculature of solid tumours. Its level in tissues will be dependent largely on the amount of lipoproteins. However, regardless of the absolute levels, the bioavailable, freely diffusible form of α-TOS can be expected to be significantly higher in the acidic environment of the tumour interstitium. Moreover, it can be envisaged that the efficacy of the agent against tumours could be manipulated by synthesizing analogues of α-TOS with even more optimal pK_a values, and we are currently investigating this intriguing possibility.

We conclude that α-TOS, an agent with anti-neoplastic activity [34], induces apoptosis due to its lysosomal membrane-destabilizing activity, which is an early event in the pro-apoptotic signalling of the agent. This does not rule out the possibility that other pathways, such as the PKC [4], c-Jun [2,12] or TGF-β [14] signalling pathways, are parallel to or amplify the lysosomal destabilizing activity of the vitamin E analogue. Notwithstanding, because α-TOS is a weak acid with a pK_a of 5.64, it is possible that it acts as a potentially selective and effective anti-tumour drug at the acidic pH of tumour interstitium, a potentially powerful anti-cancer strategy that has yet to be explored [47,50,52].

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