α-Tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation

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Abstract **α-Tocopheryl succinate (α-TOS), but not α-tocopherol, triggered apoptosis in Jurkat T cells. Apoptosis was induced by α-TOS in a time- and concentration-dependent mode, and signs of apoptosis were visible at concentrations of α-TOS as low as 30 μM, and within 3–5 h after addition of the ester. Employing a specific fluorogenic substrate, caspase-3 was found to be activated rapidly in response to α-TOS at 50 μM. We also found that Jurkat T cells challenged with α-TOS, when exposed to the lysosomotropic weak base acridine orange, showed decreased lysosomal uptake of the dye. This is suggestive of the involvement of lysosomal destabilisation in apoptosis of the cells. Apoptosis of Jurkat T cells induced with α-TOS also involved a drop in the mitochondrial membrane potential, although this phenomenon occurred after the initiation of lysosomal rupture. All apoptotic features observed with α-TOS were very similar to those found when cross-linking of the Fas receptor triggered apoptosis. These findings are consistent with the recent idea that vitamin E can contribute to elimination of malignant cells by the induction of apoptosis, and can be of (patho)physiological significance.**

Key words: Vitamin E; Caspase; Mitochondria; Lysosomal destabilisation; T lymphocyte; Apoptosis

1. Introduction

Apoptosis is a form of death in which a cell destroys itself in a regulated sequence of events without poisoning the neighbouring cells or by causing an inflammatory response [1]. A number of triggers have been reported to induce apoptosis, and these include both immunological and chemical stimuli [2]. Of the former, cross-linking of the Fas receptor has been studied intensively over the recent period. The generalised scheme is such that engagement of the Fas ligand results in recruitment of the proteolytic pro-caspase-8 (FLICE) to the death domain on the cytosolic side of the Fas receptor [3] followed by its activation that, in turn, leads to the activation of caspase-3 (CPP32). This protease is supposed to directly cleave the so-called death substrates, such as the lamins, α-fodrin or poly(ADP-ribose)polymerase, the consequences of which, in a still only partially understood way, are the irreversible cleavage of the genomic DNA and death of the cell [4].

One of the important features of apoptosis is that not all cell types respond to the various stimuli with the same sensitivity. Sometimes, transformed cell lines as well as malignant cells isolated from tumours are more sensitive to pro-apoptotic stimuli than normal cells. In line with this concept, there is a limited number of reports showing that certain forms of vitamin E, such as α-tocopheryl succinate (α-TOS) or Trolox (a water-soluble analogue of α-tocopherol) trigger or potentiate apoptotic cell death in several transformed cell lines, including a chicken reticuloendotheliosis virus-transformed cell line [5] and colon carcinoma cell lines [6].

Prompted by its potential (patho)physiological relevance, we studied whether different forms of vitamin E are capable of inducing programmed cell death in the apoptosis-sensitive Jurkat T (JT) lymphoma cell line. Here we show that α-tocopheryl succinate (α-TOS), but not α-tocopherol (α-TOH), triggers apoptosis in JT cells, and that this involves both caspase activation, lysosomal destabilisation, and changes in mitochondrial membrane potential.

2. Materials and methods

2.1. Cell culture

Jurkat T cells (line EG.1, European Collection of Animal Cell Cultures, Salisbury, UK) were grown in RPMI-1640 with 10% foetal calf serum (FCS), 2 mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C, in humidified air with 5% CO_2. Every 3 days, the rapidly growing cells were split with fresh medium to the density of about 0.5×10^6 cells/ml. For the experiments, cells were seeded in Falcon 6-well plates at 0.5×10^6 cells/ml in the complete medium, and treated as detailed below.

α-D-tocopheryl succinate (α-TOS; Sigma, St. Louis, MO, USA) or α-D-tocopherol (α-TOH; Sigma) was added to the cells as EtOH solution at concentrations of 0–50 μM so that the volume of EtOH added to the culture medium was $<0.5\%$ (v/v). The anti-human Fas/APO-1/CD95 monoclonal antibody (CH-11; Oncor, Gaithersburg, MD, USA, or Coulter-Immunotech Diagnostics, Hamburg, Germany) was added to the cells at 20 ng/ml. The control cultures, with no vitamin E or anti-Fas IgM added, were supplemented with the corresponding volume of the vehicle. Cells were exposed to α-TOS or α-TOH for up to 24 h and to anti-Fas IgM for 6 h.

2.2. Assessment of apoptosis

Apoptosis was assessed by means of visual evaluation in a light microscope of changes in the cytoarchitecture, such as budding membranes and pycnotic or fragmented nuclei. This was done either directly in a Rosenthal chamber, or after staining of the cells. Briefly, cells were fixed in 4% neutral-buffered formalin and stained with a 0.5% Giemsa solution (Merck, Dartmstadt, Germany) [7]. The annexin-V cell surface labelling method [8] was also used. For this, 1×10^6

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Abbreviations: AO, acridine orange; CCCP, carbonylcyanide-3-chlorphenylhydrazone; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; FACS, fluorescence-activated cell sorting; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; JT cells, Jurkat T cells; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, protein kinase C; α-TOH, α-tocopherol; α-TOS, α-tocopheryl succinate; $\Delta\Psi_m$, mitochondrial inner membrane potential

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cells were labelled on ice for 10–15 min in 0.1 ml incubation buffer containing 10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4, plus 20 μl annexin-V conjugated to fluorescein isothiocyanate (FITC; Boehringer Mannheim, Germany) and 50 μl of 50 μg/ml propidium iodide (PI; Sigma). The cells were washed, centrifuged, resuspended in the incubation buffer and analysed by fluorescence-activated cell sorting (FACS; Becton-Dickinson, San Jose, CA, USA). The data were evaluated using the CellQuest software. DNA laddering was performed as described in [9]. Briefly, genomic DNA was isolated [10] and then subjected to electrophoresis on a 2% agarose gel. The DNA bands were visualised by ethidium bromide staining and evaluated by comparison with a 100-base pair DNA ladder standard.

2.3. Measurement of caspase-3 activity

The method of Nicholson et al. [11] was used employing the fluorogenic substrate for caspase-3, Ac-DEVD-AMC (Pharmingen, San Diego, CA, USA). Briefly, $\approx 0.5 \times 10^6$ cells were pelleted and lysed with 150 μl of lysis buffer comprising 10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% Triton and 10 mM NaH_2PO_4. 100 μl of the cell lysate were combined with 1 ml of the reaction buffer consisting of 20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol and 20 μM Ac-DEVD-AMC, and incubated at 37°C for 2 h. The intensity of fluorescence of the liberated amino-4-methylcoumarin (AMC) was then measured ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 435$ nm).

2.4. Assessment of mitochondrial potential

Changes in inner mitochondrial membrane potential ($\Delta\Psi_m$) in JT cells following different treatments were assessed as reported elsewhere [12]. In brief, the cells were centrifuged, resuspended in PBS containing 0.5% FCS, and incubated for 15 min at 37°C with 40 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR, USA). Immediately before measuring in the flow cytometer, 1 μg/ml PI was added. As a positive control, cells were also incubated, prior to the $DiOC_6$ and PI addition, for 15–30 min with 50 nM carbonylcyanide-3-chlorphenylhydrazone (CCCP).

2.5. Lysosomal stability assessment

Cells in various stages were assessed for lysosomal stability using the acridine orange (AO) uptake method [13]. Washed cells ($\approx 0.5 \times 10^6$) were pelleted and resuspended in 2.5 ml complete culture medium with 10 mM HEPES supplemented with 5 μg/ml AO, incubated at 37°C for 15 min, washed, pelleted, and resuspended in 100 μl PBS for static cytometry and in 0.5 ml PBS for flow cytometry. Static cytometric evaluation of the AO-labelled cells was performed in a Nikon Microphot SA microscope equipped with a filter cube for green excitation and an extra 630-nm barrier filter, where 100 randomly selected cells were scored for red fluorescence intensity. The cells were also examined in an LSM 410 inverted confocal laser scanning microscope (Carl Zeiss). To evaluate randomly selected higher number of cells (10 000 per each run), the cells were subjected to assessment for AO fluorescence intensity using the FACS instrument fitted with the Lysis II software.

3. Results and discussion

To test whether vitamin E derivatives induce apoptosis in JT cells, we exposed the cells, seeded at a density below that which was found to trigger 'spontaneous' apoptosis [14], to increasing concentrations of α-TOS and α-TOH for 24 h. As shown in Fig. 1A, apoptosis occurred at concentrations of α-TOS as low as 30 μM, as assessed by visual evaluation of the Giemsa-stained cells, while no apoptosis was observed with α-TOH even at 50 μM. These observations were confirmed by assessment of genomic DNA fragmentation in α-TOS- and α-TOH-exposed cells (Fig. 1B). As a positive control, cross-linking of the Fas receptor with anti-Fas IgM was used which asserted features of apoptosis similar to those observed for α-TOS (cf. Fig. 1A, B).

Fig. 2 shows the time dependence of apoptosis induced by 50 μM α-TOS in JT cells. The first signs of apoptosis, as documented by microscopic evaluation of the Giemsa-stained cells (A) and by DNA laddering (B), were evident as early as 3 h following the exposure of the cells to the ester. Apoptosis was also assessed by the annexin-V-FITC-labelling method (Fig. 2C), which is based on binding of annexin-V to phosphatidyl serine initially present in the inner leaflet of the cell membrane when exposed to the surface the cell enters apoptosis [8]. As shown also in Fig. 2B, C, apoptosis was evident in cells treated with 50 μM α-TOS for 24 h or with 20 ng/ml anti-Fas IgM for 6 h. Virtually no apoptosis was apparent in control cells and in cells exposed to 50 μM α-TOH for 24 h.

Another typical feature of apoptosis is the functional activation of caspases, a group of proteolytic enzymes that are involved in transmitting the early pro-apoptotic signals, such as engagement of the Fas receptor, to the later events that

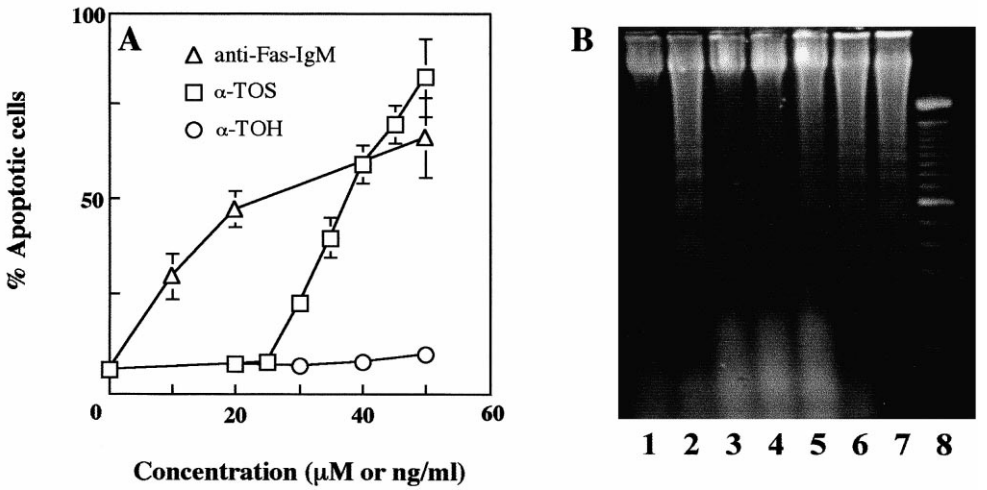

Fig. 1. Effect of increasing concentration of vitamin E on apoptosis in JT cells. JT cells were cultured at 0.5×10^5 per ml in complete RPMI for 24 h in the presence of increasing concentrations of α-TOS, α-TOH (μM) or anti-Fas IgM (ng/ml), and evaluated for apoptotic morphology using the Giemsa staining method (A), and for DNA laddering (B); lane 1, control; lane 2, 20 ng/ml anti-Fas for 6 h; lane 3, 50 μM α-TOH for 24 h; lanes 4–7, α-TOS for 24 h at 20, 30, 40 and 50 μM, respectively; lane 8, 100 bp DNA ladder standard. The data shown in A are average values ± S.D. ($n = 4$ for α-TOS and α-TOH, $n = 3$ for anti-Fas IgM), the images in B are typical representatives of three independent experiments.

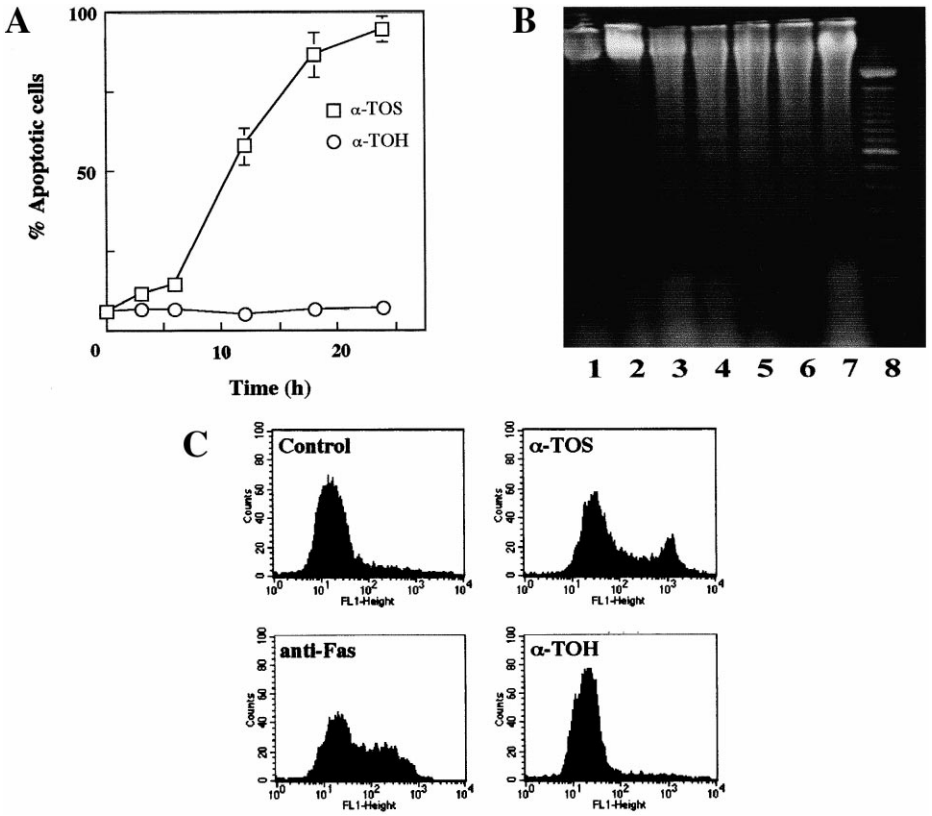

Fig. 2. Time-dependent induction of apoptosis in JT cells exposed to α-TOS. The cells were seeded at 0.5×10^6 per ml in complete RPMI and supplemented with 50 μM α-TOS, 50 μM α-TOH or 20 ng/ml anti-Fas IgM at different time points so that the end point for all treatments was simultaneous. Cells were analysed for apoptosis by visual microscope evaluation after Giemsa staining (A), DNA laddering (B) (lane 1, control; lane 2–7, 50 μM α-TOS for 3, 6, 9, 12, 18 and 24 h, respectively; lane 8, 100 bp DNA ladder standard), and annexin-V-FITC binding (C). Images shown in B and C are representative examples of at least three independent experiments, data in A are mean values ± S.D. ($n=4$).

include cleavage of the so-called death substrates, i.e. proteins associated directly with the stabilisation and/or repair of the genomic DNA. While the function of some of the known caspases has not been clearly established yet [15], activation of caspase-3 appears to be crucial in cells undergoing apoptosis [16]. Consistent with this notion, we observed activation of caspase-3 as early as 3 h after the exposure of JT cells to 50 μM α-TOS. The activity of the protease was maximal at about 12 h, and this was then followed by its slow decline (Fig. 3). As expected, substantial caspase-3 activity was observed when the cells were exposed to 20 ng/ml anti-Fas IgM for 6 h, but no caspase-3 activity was apparent upon treatment with 50 μM α-TOH for 24 h. The observation that α-TOS-induced apoptosis in JT cells involves caspase-3 activation is consistent with a recent report according to which several anti-cancer drugs induced apoptosis in cancer cells by activating caspase-3 [17].

Apoptosis induced with anti-Fas IgM or by oxidative stress involves changes in the mitochondrial membrane potential ($\Delta\Psi_m$) [18,19]. To see whether this is also the case for vitamin E-induced apoptosis in JT cells, we exposed the cells to the stimulus and assessed $\Delta\Psi_m$ using the fluorogenic dye $DiOC_6$. As shown in Fig. 4, significant $\Delta\Psi_m$ dissipation was observed upon exposure of the cells to 50 μM α-TOS for 12 h, but not to α-TOH, and the change was accompanied by a significant increase in the population of apoptotic cells. Similar results were achieved when the cells were treated with anti-Fas IgM. The protonophore CCCP was used as a positive control in these experiments as it brings down $\Delta\Psi_m$ (cf. Fig. 4).

It has been reported recently that apoptosis of lymphoid cells triggered with anti-Fas IgM, oxidative stress and growth factor starvation, i.e. unrelated stimuli, involves the destabilisation of lysosomes [9] and that the 'acidic compartment' is of importance for cells during apoptosis [20]. To investigate whether such lysosomal rupture would also occur in apoptosis induced in JT cells by their exposure to vitamin E, we stained the cells with AO after treatment with increasing concentrations of α-TOS or α-TOH at a set point of time, or with α-TOS or α-TOH at 50 μM for increasing periods of time. A short-term exposure of cells to AO results in a high concentration of the lysosomotropic weak base in lysosomes, unless they are destabilised. Hence, upon lysosomal 'rupture' or 'leakage', AO, normally localised in the acidic vacuolar apparatus as shown by strong granular, red fluorescence, translocates to the cytoplasm, and the cells appear 'pale' with a significantly reduced number of intact lysosomes [9]. Fig. 5 shows that lysosomes were destabilised in JT cells treated with α-TOS or with anti-Fas IgM. This is illustrated by the imaging of both apoptotic and non-apoptotic cells by fluorescence confocal microscopy (A) and by flow cytometry (B–D). As with the apoptotic markers (see above), lysosomal destabilisation in JT cells with α-TOS was similar to that with anti-Fas IgM, while no destabilisation was seen with α-TOH. The time dependence of lysosomal destabilisation resembled that

of the apoptotic markers in cells exposed to α-TOS, although, at earlier time points (e.g. at 3 h) only few lysosomes appeared to be affected (cf. Fig. 5C). It is plausible to suggest, similarly as in [9] or [21], that only a relatively small fraction of lysosomes needs to become leaky in order to release sufficient amounts of lytic enzymes to activate the pro-apoptotic cascade of events. Cathepsin D is one of the proteases that relocalises upon lysosomal disruption and that could activate the caspase pathway, as has been suggested [7,9,21,22]. Recently, however, it was shown that the cysteine proteases cathepsin B and L had the capacity to activate caspase-3 following lysosomal permeabilisation [23].

It is an intriguing question why all the effects observed here were induced with α-TOS but not with the non-esterified, redox-active form of the vitamin. It has been reported that α-TOS, but not α-TOH or α-tocopheryl acetate, inhibited adhesion of monocytes to endothelial cells by inhibiting activation of the nuclear factor-κB [24]. Similar observations have been reported elsewhere [5], and the authors argued against the involvement of the redox function of the vitamin. Similarly, Azzi and associates [25] suggested that α-TOH-induced inhibition of smooth muscle cell proliferation was unrelated to the redox function of the vitamin, and was receptor-mediated. Whether the redox properties of vitamin E derivatives are important for their role in apoptosis induction is unclear at the moment. However, a recent report has shown that in a colon cancer cell line, low levels of reactive species, presumably involved in proliferation of the cells, were depleted upon treatment with Trolox, a redox-active, water-soluble α-TOH analogue, and this resulted in massive apoptosis [6].

While we believe that the redox function of α-TOS can play a role in JT cell apoptosis (vitamin E can exert both pro- and antioxidant activities [26]), it is also possible that inhibitory activity of vitamin E on protein kinase C (PKC), an important factor regulating cell proliferation, can be involved in vitamin E-induced apoptosis. This assumption is based on reports that show vitamin E to inhibit various functions in

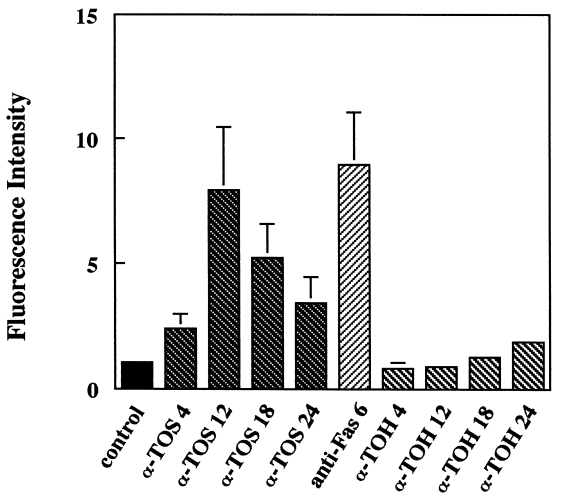

Fig. 3. Apoptosis in JT cells induced with α-TOS involves caspase-3 activation. Cells, seeded at 0.5×10^6 per ml, were cultured in complete RPMI in the presence of 50 μM α-TOS, 50 μM α-TOH or 20 ng/ml anti-Fas IgM for different time periods, and analysed for caspase-3 activity using the fluorogenic substrate Ac-DEVD-AMC as detailed in Section 2. The numbers at the individual additives at the *x*-axis indicate the time of exposure in h. The data shown are mean values ± S.D. ($n=3$).

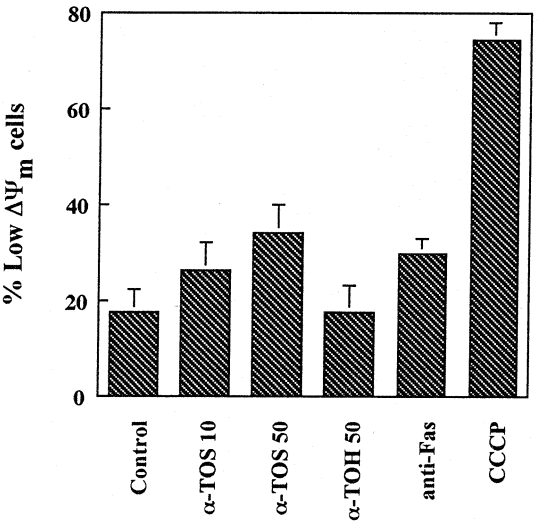

Fig. 4. Apoptosis in JT cells involves changes in the mitochondrial inner membrane potential. The cells were grown at 0.5×10^6 per ml in complete RPMI and treated for 12 h with vitamin E derivatives at 10 or 50 μM, or for 6 h with anti-Fas IgM at 20 ng/ml. Cells exposed to 50 nM CCCP were used as positive control. The cells were then labelled with $DiOC_6$ and PI and $\Delta\Psi_m$ dissipation estimated by flow cytometry as detailed in Section 2. The bar graph shows average values ± S.D. ($n=3$–5) of the percentage of the $\Delta\Psi_m$ low cells.

smooth muscle, endothelial and monocytic cells associated with proliferation/inflammation by inhibiting PKC [25,27,28]. Interestingly, Qiang et al. [5] have shown that in an avian reticuloendotheliosis virus-transformed T-cell line, α-TOS induced apoptosis via transformed growth factor-β signalling. This led to activation of c-fos and c-jun, and subsequently increased binding of the activator protein-1 (that can result in an inactivation of PKC [25]), and downregulation of the protooncogene c-myc. Interestingly, an apoptotic pathway induced by anti-Fas IgM, independent of caspases, that involves the Daxx adapter protein and leads to activation of transcription factors, such as c-jun, has been recently described [29]. Notwithstanding, it should be stressed that the involvement of PKC inactivation in apoptosis has been established (see e.g. [30–32]). Studies are under way in order to establish links between α-TOS-induced apoptosis in JT cells and the early and later signals as shown in this communication and by others, viz. the activation of caspases, and destabilisation of lysosomes due to changes in their membrane permeability (see e.g. [19,33,34]).

To conclude, we document in this study that: (i) α-TOS, but not α-TOH, induces apoptosis in JT cells as documented by three independent methods; (ii) α-TOS-induced apoptosis involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation; and (iii) the features shown for α-TOS-triggered apoptosis in JT cells closely resemble those observed for apoptosis induced by anti-Fas IgM. Hence, our results are consistent with the idea that certain compounds of potential pharmacological relevance, as shown here for α-tocopheryl succinate, can induce apoptotic cell death in transformed cells. Studies are under way to establish whether α-TOS and other derivatives of vitamin E can induce apoptosis also in other cell lines and in cells isolated from tumour biopsies, and to elucidate the molecular mechanism of such vitamin E-induced apoptosis.

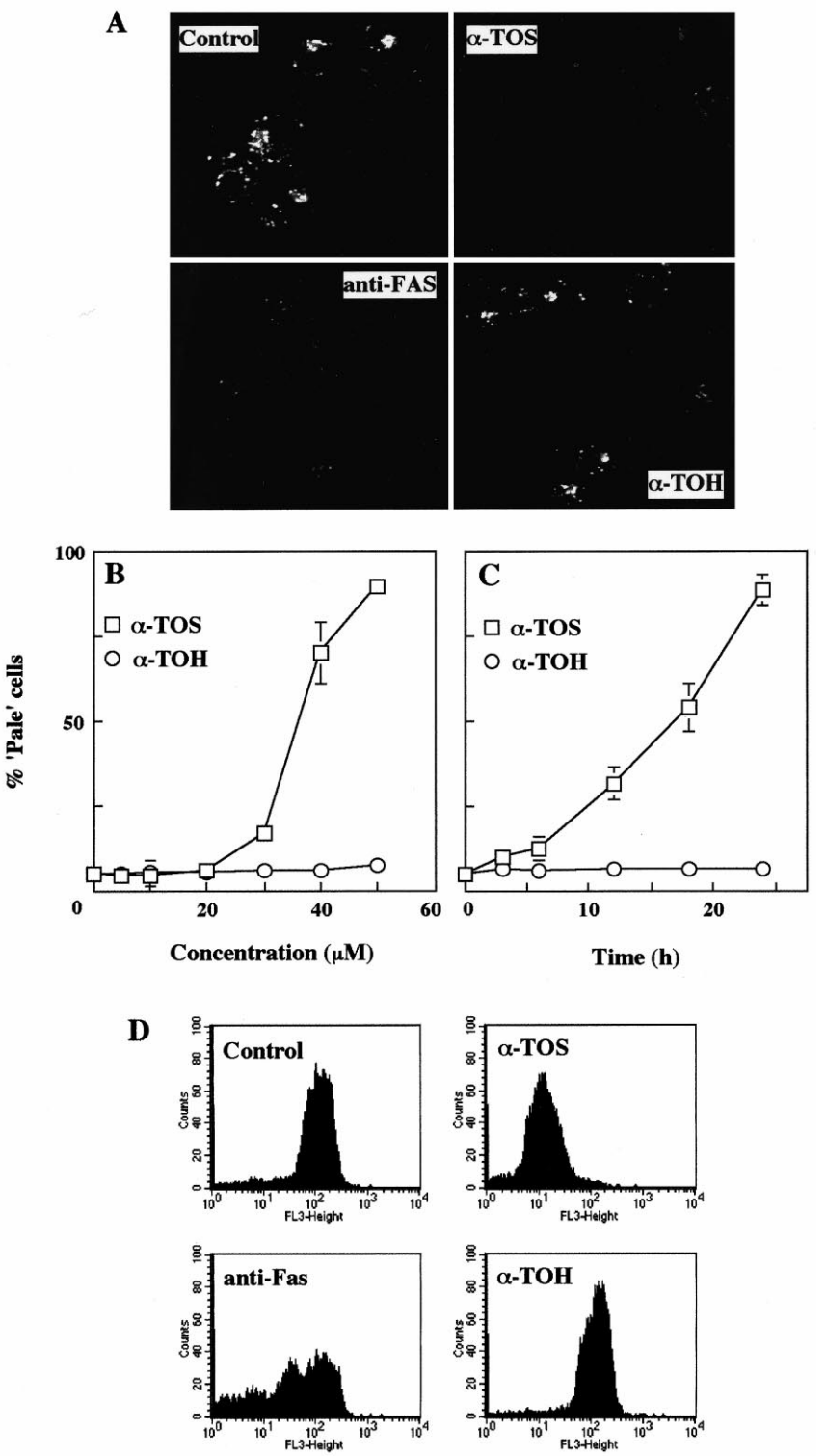

Fig. 5. Apoptosis in JT cells induced with α-TOS involves lysosomal destabilisation. The cells, seeded at 0.5×10^6 per ml complete RPMI, were treated with α-TOS, α-TOH or anti-Fas IgM at concentrations and for periods indicated at the individual figures, harvested, and incubated with AO as detailed in Section 2. After the incubation (within 15 min), the cells were analysed for red fluorescence using the confocal microscope (A: exposure to 50 μM α-TOS or α-TOH for 24 h, or to 20 ng/ml anti-Fas IgM for 6 h) or flow cytometry (B: increasing concentrations of α-TOS or α-TOH for 24 h; C: different time periods of treatment with 50 μM α-TOS or α-TOH). The images shown in A are representative examples of at least two independent experiments, the data in B and C are mean values ± S.D. ($n=3$). Images of the flow cytometer scans representing selected samples, as indicated by the text at the individual graphs, are shown in D.

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