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α-Tocopheryl succinate induces DR4 and DR5 expression by a p53-dependent route: Implication for sensitisation of resistant cancer cells to TRAIL apoptosis

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Abstract **We evaluated the ability of α-tocopheryl succinate (α-TOS) to sensitise TRAIL-resistant malignant mesothelioma (MM) cells to TRAIL-induced apoptosis. We show that α-TOS activates expression of DR4/DR5 in a p53-dependent manner and re-establishes sensitivity of resistant MM cells to TRAIL-mediated apoptosis, as documented in p53^{wt} MM cells but not in their p53^{null} counterparts. MM cells selected for TRAIL resistance expressed low cell surface levels of DR4 and DR5. Treatment with sub-lethal doses of α-TOS restored expression of DR4 and DR5. The ability of α-TOS to modulate expression of pro-apoptotic genes may play a role in sensitisation of tumour cells to immunological stimuli.**
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Keywords: Malignant mesothelioma; TRAIL; α-TOS; p53; DR5; DR4

1. Introduction

α-Tocopheryl succinate (α-TOS), an analogue of vitamin E, is a potent inducers of apoptosis and inhibits proliferation of several malignant cells [1–4]. α-TOS induces a variety of concentration-dependent cellular events. More specifically, it modulates signalling pathways in various in vitro models, in general in the 10–30 μM range, while its cytotoxic effect becomes prominent at higher concentrations [5]. Studies have shown that α-TOS induces cancer cells to undergo apoptosis via at least three pathways, i.e., the transforming growth factor-β (TGF-β), the c-jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK), and the tumour necrosis factor (TNF) signalling pathway [6–8]. Upregulation of Fas ligand expression was observed in Fas-insensitive human breast cancer cells followed by α-TOS treatment sensitising them to the immunological agent [8].

Among the TNF ligand members, TNF-related apoptosis-inducing ligand (TRAIL) has recently drawn considerable interest as a potential effective anti-tumour therapeutic agent, in particular since, in contrast to the Fas ligand toxic to normal cells, TRAIL appears largely selective for malignant cells [9,10]. TRAIL, present on the surface of immune cells as a type II membrane protein or secreted in a soluble form, mediates apoptosis in sensitive cells by binding to their cognate death receptors (DRs), DR4 and DR5 [11,12]. Although both DRs are widely expressed in human tissues, some cancer cells are insensitive to TRAIL-mediated killing [13–15].

Heterogeneous sensitivity of tumour cells to TRAIL-induced apoptosis has been observed in malignant mesothelioma (MM) [16], which may lead to a persistent growth of TRAIL-resistant cells, and may limit successful treatment of the neoplastic disease by TRAIL. Notably, MM is an aggressive and treatment-resistant tumour with currently only palliative cure [17].

Here, we investigated the ability of α-TOS to 'convert' TRAIL-resistant MM cells to TRAIL-responsive ones by inducing trans-activation of DR4 and DR5. In order to evaluate the involvement of p53, MM cell lines differing in their p53 status were used. α-TOS exerted differential effects on p53^{wt} and p53^{null} cells, and activated p53 that, in turn, induced expression of DRs, leading to increased sensitivity to TRAIL-induced apoptosis. Our data suggest that upregulation of cell surface DR expression induced by α-TOS may contribute to a shift in the anti- and pro-apoptotic signals in favour of the latter, triggering apoptotic signals.

2. Materials and methods

2.1. Reagents

α-Tocopheryl succinate (α-TOS), actinomycin D (ActD), and annexin V–FITC were purchased from Sigma (St. Louis, MO, USA). Soluble human recombinant TRAIL (hrTRAIL) was prepared as described elsewhere [18]. Anti-DR4 and anti-DR5 monoclonal IgGs were obtained from Alexis Biochemicals (Lausen, Switzerland). Anti-phospho-p53 (Ser-20) IgGs were from Cell Signalling Technology,

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Abbreviations: DR, death receptor; FLIP, FLICE inhibitory protein; MM, malignant mesothelioma; ActD, actinomycin D; Q-PCR, quantitative real-time PCR; siRNA, small interfering RNA; α-TOS, α-tocopheryl succinate; TR, TRAIL-resistant; TRAIL, TNF-related apoptosis-inducing ligand

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Inc. (Beverly, MA, USA). Anti-p53 (Ab-6) IgG was obtained from Calbiochem (San Diego, CA, USA). All primers for RT-PCR were obtained from Sigma Genosys (St. Louis, MO, USA). Foetal bovine serum was obtained from EuroClone (Paignton, UK).

2.2. Cell culture

The wild-type p53 ($p53^{wt}$) MM-B1, Meso-2, and Ist-Mes human MM cell lines [19] and the p53-deficient ($p53^{null}$) REN human MM cells were used. REN cells were a generous gift from Steven Albelda, University of Pennsylvania Medical Centre, Philadelphia, PA, USA. Cells were cultured in the RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS.

2.3. Apoptosis detection

Apoptosis was quantified using the annexin V–FITC method, which detects phosphatidyl serine (PS) externalised in the early phases of apoptosis [20]. Briefly, cells were plated at 10^5 per well in 24-well plates. After an overnight incubation, cells were treated with α-TOS (30 μM) or hrTRAIL (10 ng/ml) alone or in combination. Floating and attached cells were collected, washed twice with PBS, re-suspended in 0.1 ml binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4), incubated for 20 min at room temperature with 2 μl annexin V–FITC, supplemented with 10 μl of propidium iodide (PI) (10 μg/ml), and analysed by flow cytometry (FACScalibur), using channel 1 for annexin V–FITC binding and channel 2 for PI staining.

2.4. Selection of TRAIL-resistant MM cells

Selection of TRAIL-resistant cells was performed as previously described [21]. The $p53^{wt}$ MM-B1 cells were seeded in a 6-well plate at the concentration of 2×10^5 cells/well (60% of confluence) and treated with 200 ng/ml TRAIL for 24 h resulting in ~30–40% cell death. The apoptotic cells were removed and surviving cells were fed every day for 5–6 weeks with culture medium containing TRAIL (200 ng/ml) until they reached 70–80% confluence. The resulting cells were then cultured in the presence of TRAIL (10 ng/ml) and subsequently examined for expression of apoptosis modulators including DR4, DR5, FLIP and caspases, as well as for their sensitivity to TRAIL-induced cell death by the annexin V–FITC assay.

2.5. Western blot analysis

Cells were treated as indicated and lysed in a buffer containing 250 mM NaCl, 25 mM Tris–HCl (pH 7.5), 5 mM EDTA (pH 8), 1% Nonidet P-40, and a cocktail of protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethyl-sulfonyl fluoride and 2 μg/ml proteinin) and stored at −80 °C until analysis. Protein level was quantified using the Bradford assay (Sigma). Protein samples (80 μg per lane) were boiled for 5 min and resolved using 12.5% SDS–PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked (PBS containing 0.1% Tween and 5% skimmed milk) for 1 h, and incubated overnight with anti-DR4, anti-DR5, anti-p21, anti-p53 or anti-phospho-p53 (ser-20) IgG. After incubation with an HRP-conjugated secondary IgG (Amersham, London UK), the blots were developed using the ECL kit (Pierce, Rockford, IL, USA). Protein loading was corrected for using anti-β-actin IgG.

2.6. Small interfering RNA (siRNA) transfections

siRNA duplex oligonucleotides, sense 5′-GCA UGA ACC GGA GGC CCA-3′-dTdT, anti-sense 5′-AUG GGC CUC CGG UUC AUG C-3′-dTdT, corresponding to the target sequence CGG CAU GAA CCG GAG GCC CAU of p53, were used to inhibit p53 protein expression. As a negative control, a non-silencing (NS) RNA was used: sense 5′-UUC UCC GAA CGU GUC ACG U-3′-dTdT, anti-sense 5′-ACG UGA CAC GUU CGG AGA A-3′-dTdT (all siRNAs from Qiagen, Hilden, Germany). The oligonucleotide pairs were dissolved in the suspension buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) and annealed by incubation for 2 min at 95 °C. MM-B1 cells were seeded into 6-well plates at 10^5 per well and transfected with the oligonucleotides according to the manufacturer's instructions. Briefly, 10 μl of siRNA (5 μg) was dissolved in 100 μl of the cell culture medium and supplemented with 15 μl of RNAiFect (Qiagen). The solution was incubated for 30 min at room temperature and added to each well containing 1.9 ml of medium. Cells were incubated for 6 h prior to the addition of 2 ml of fresh medium. After 48 h of incubation, p53 expression was evaluated by Western blotting.

2.7. RT-PCR and quantitative real-time mRNA analysis

Total RNA was isolated from 10^6 MM cells before and after treatment with 30 μM α-TOS using Trizol (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. The first-strand cDNA was synthesized using the GeneAmp RNA PCR kit (Perkin–Elmer Life Sciences, Boston, MA, USA). PCR analyses were performed in the final volume of 20 μl of buffer containing 1 μl of the retro-transcription product, dNTPs (150 μM each), $MgCl_2$ (2 mM), 1 U of Taq Gold polymerase (Roche Molecular Biochemicals, Basel, Switzerland), and each primer at 1 μM. The house-keeping gene β-actin was used as a loading control.

Relative quantification of mRNA expression was achieved by quantitative real time-PCR (Q-PCR). The technique is based on the detection of a fluorescent signal produced by incorporation of the fluorescent dye SYBR-green (DyNamo™HS, Finnzymes, Espoo, Finland) during PCR amplification (Chromo 4tm Detector, MJ Research, Waltham, MA, USA). The sequences of the primers were published elsewhere [22]. Expression of DR4 and DR5 was normalized to β-actin. The increase of DR mRNA in α-TOS-treated cells with respect to the control (untreated cells) was determined using the formula: $X = 2^{-\Delta\Delta c_t}$; $\Delta\Delta c_t = \delta E - \delta C$; $\delta E = n°$ cycles of DR gene − $n°$ cycles of house-keeping gene in the treated cells; $\delta C = n°$ cycles of DR gene $-n°$ cycles of house-keeping gene in the control cells.

2.8. Flow cytometric detection of DR expression

Expression of DR4 and DR5 was evaluated by flow cytometry before and after treatment with α-TOS (30 μM). MM cells were seeded 24 h before the treatment in 6-well plates at 3×10^5 per well. After 16 h of incubation, floating and attached cells were collected, washed twice with PBS, incubated at 4 °C with antibodies against DR4 and DR5 followed by a secondary FITC-conjugated IgG, and then analysed by flow cytometry. Cytoplasm expression of TRAIL receptors was assessed after cell permeabilisation. Briefly, cells were fixed in 4% formaldehyde in PBS for 30 min, washed, permeabilised with a saponine solution (0.2% saponine in PBS plus 1% FCS) for 30 min, incubated with the antibodies as described above, and assessed by flow cytometry.

2.9. Immunocytochemistry

Parental MM-B1 cells and their TRAIL-resistant (TR-1) counterparts were placed overnight in 35-mm dishes on poly-L-lysine-coated glass cover-slips. After 6 h of incubation with α-TOS (30 μM), the cells were washed 2-times with PBS, fixed with 4% formaldehyde in PBS, and incubated with (permeabilised cells) or without (intact cells) a saponine solution (0.05% saponine and 2% FCS in PBS). Cells were then incubated with mouse anti-human phospho-p53 (Ser-20), DR4 or DR5 IgG for 1 h at room temperature. FITC-conjugated, anti-mouse secondary IgG was added. The cover-slips were mounted on glass slides with VectaShield plus DAPI (Vector Laboratories, Burlingame, CA, USA) and inspected in a confocal microscope (BioRad, MRC 1024, Hercules, CA, USA).

2.10. Transcriptional inhibition and cytotoxicity analysis

For the inhibition experiments the MM cells were incubated with actinomycin D (ActD) which is generally used as an inhibitor of transcription [23].

MM cells were plated in 96-well flat-bottom tissue culture plates at 10^4 per well. The cells were incubated overnight with ActD at final concentration of 5 μM dissolved in DMSO (0.5%) or DMSO alone (0.5%). To remove unincorporated ActD, the treated cells were washed three times with RPMI-1640 and then incubated for 24 h with α-TOS and TRAIL at 30 μM and 10 ng/ml, respectively, alone or in combination. DR4 and DR5 gene expression was evaluated by RT-PCR performed as described above. Cytotoxicity was determined by using the MTT assay [24]. Briefly, following cell treatment, 10 μl of MTT (5 mg/ml in PBS) was added, and after incubation for 4 h at 37 °C, the medium was removed and combined with 200 μl of 1% SDS. Absorbance was read at 550 nm using an ELISA plate reader with control absorbance set at 100%.

2.11. Statistical analysis

All experiments were conducted at least three times, and data are shown as means ± S.D. Significance was evaluated by the ANOVA repeated measure test. Data were considered statistically significant at $P < 0.05$.

3. Results and discussion

The p53 protein is a key component of the cellular 'emergency-response' mechanism [25,26]. The most studied function of p53 is its role as a transcription factor that can activate transcription of an ever-increasing number of target genes [27,28]. A recent paper reported that p53 can also act as an antioxidant protein [29]. A variety of stress-associated signals activate p53 that induces growth arrest or apoptosis, thereby eliminating damaged and potentially dangerous cells [30]. Here, we assessed the ability of α-TOS to induce transcriptional activation of the pro-apoptotic genes DR4 and DR5 by a p53-dependent route. Exposure of $p53^{wt}$ MM cells to sub-toxic doses of α-TOS induced expression and activation of p53. The level of p53 phosphorylated on Ser-20 increased in a dose-dependent manner (Fig. 1A and B). Activation of p53 was associated with expression of DR4 and DR5 as revealed by Q-PCR. Notably, such expression of DRs was not observed in the $p53^{null}$ REN cells and was abrogated in $p53^{wt}$ MM cells when p53 expression was partially silenced by siRNA (Fig. 2A). The flow cytometric analysis, performed on both intact and permeabilised cells, and the Western blot assay revealed that sub-lethal doses of α-TOS increased expression of the DR4 and DR5 protein, which was not observed in MM cells lacking normal expression of p53 (Table 1, Fig. 2B).

DR5 and, more so, DR4 play a critical role in mediating TRAIL-induced apoptosis. It has been recently reported that siRNA targeting of DR5 was ineffective at blocking TRAIL-induced apoptosis, whereas siRNA-mediated knock-down of DR4 conferred protection against TRAIL-induced cell killing [31]. Further, MM cells exert heterogeneous sensitivity to TRAIL-induced cell death, even though both DR4 and DR5 are endogenously expressed on their cell surface [16]. By prolonged exposure to TRAIL, we generated TRAIL-resistant MM cells (TR-1). The TRAIL-resistant population was

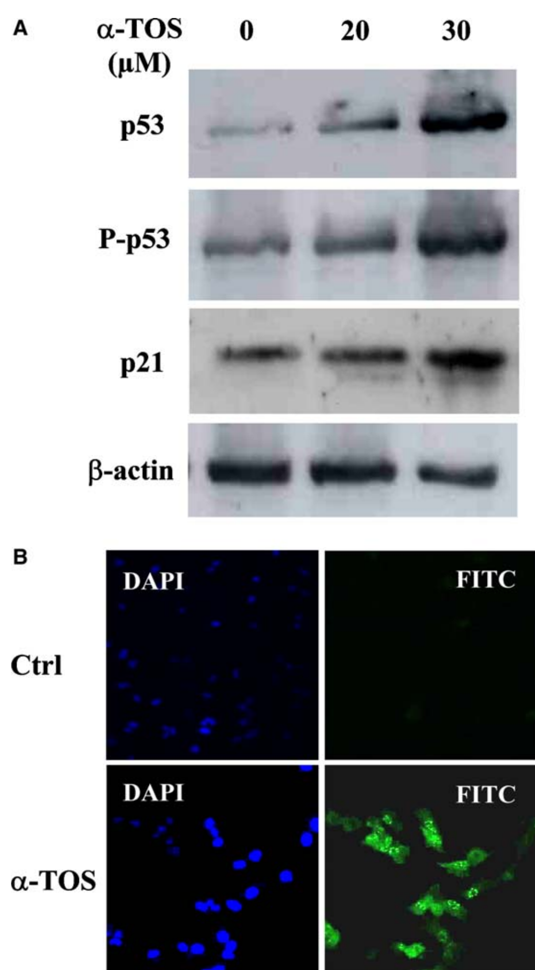

Fig. 1. Effect of α-TOS exposure on the level of p53. (A) MM-B1 cells (as representative of $p53^{wt}$ MM cells), were incubated with α-TOS at sub-apoptotic doses (20 and 30 μM) for 24 h. Cell lysates were subjected to Western blotting using p53, phospho-p53 (Ser-20), p21 and β-actin antibodies. (B) MM-B1 cells were grown on cover-slips, and treated for 6 h with α-TOS (30 μM). Cells were then incubated with anti-phospho-p53 (Ser-20) IgG, and after incubation with FITC-conjugated secondary IgG, the cover-slips were mounted on glass slides with Vectashield plus DAPI and observed in a confocal microscope. Results shown are representative of three independent experiments.

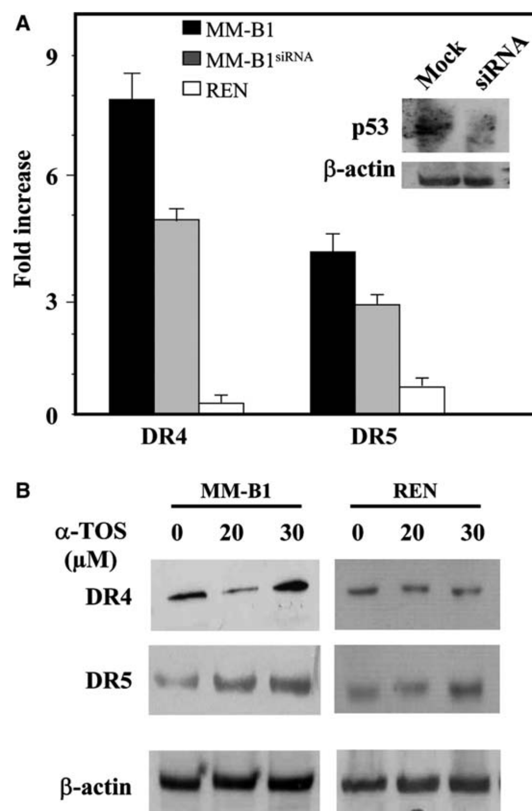

Fig. 2. Effect of α-TOS treatment on the level of TRAIL death receptors in MM-B1 and REN cells. (A) DR4 and DR5 were evaluated in $p53^{wt}$ MM-B1, $MM\text{-}B1^{siRNA}$ (p53 siRNA was used to inhibit p53 protein expression), and $p53^{null}$ REN cells as mRNA expression by Q-PCR before and after treatment with 30 μM α-TOS for 4 h. (B) DR4 and DR5 protein expression in $p53^{wt}$ MM-B1 and $p53^{null}$ REN cells before and after treatment with α-TOS (0, 20, and 30 μM) for 24 h. β-Actin was used as a loading control. Results are representative of three independent experiments.

Table 1
Evaluation of cytoplasmic and cell surface expression of TRAIL death receptors before and after α-TOS treatment in MM-B1 and REN cells

Cell type	Intact				Permeabilised			
	DR4		DR5		DR4		DR5	
	Ctrl	α-TOS	Ctrl	α-TOS	Ctrl	α-TOS	Ctrl	α-TOS
MM-B1	1.9 ± 0.2	2.2 ± 0.3	8.1 ± 0.4	10.1 ± 0.5*	3.4 ± 0.1	5.2 ± 0.2*	15.0 ± 0.6	17.1 ± 0.4*
REN	1.8 ± 0.3	1.8 ± 0.2	8.4 ± 0.6	8.6 ± 0.4	1.9 ± 0.1	2.2 ± 0.2	10.4 ± 0.4	11.3 ± 0.3

Expression of DR4 and DR5 was evaluated by flow cytometry before (Ctrl) and after treatment with α-TOS (30 μM, 16 h) in $p53^{wt}$ MM-B1 and $p53^{null}$ REN cells. The values represent fluorescence intensity of the receptors normalized for a blank incubated with an irrelevant primary antibody. Data are expressed as means ± S.D. from three independent experiments and statistical differences between controls versus α-TOS treatment are marked with the ∗ symbol, $P < 0.05$.

deficient in expression of DR4, whereas relatively low expression of DR5 was observed when compared to their parental counterparts (Fig. 3A, top panel). Interestingly, exposure to α-TOS (30 μM) significantly enhanced both cytosolic and cell surface expression of both DR4 and DR5 in TRAIL-resistant cells as evaluated by cytometry (Fig. 3A, lower panel) and confocal microscopy (Fig. 3B).

Elevated expression of the TRAIL DRs resulted in a synergistic and cooperative α-TOS/TRAIL effect, which was observed only in MM cells and in selected TRAIL-resistant MM cells (TR-1) with functional p53 (Fig. 4A). Thus, p53-dependent up-regulation of TRAIL's DRs is required for sensitisation of MM cells to TRAIL-induced apoptosis. It has been demonstrated that activation of p53 is a prerequisite for restoration of TRAIL sensitivity in colon carcinomas [32], and p53-dependent upregulation of DR4 and DR5 expression has been observed [33,34]. Moreover, it has been reported that p53 directly regulates transcription of the DR4 gene via an intronic sequence-specific p53 binding-site [35].

To investigate whether the synergistic and cooperative α-TOS/TRAIL effect was related to the transcriptional activation of the TRAIL's DR genes as a response to α-TOS treatment,

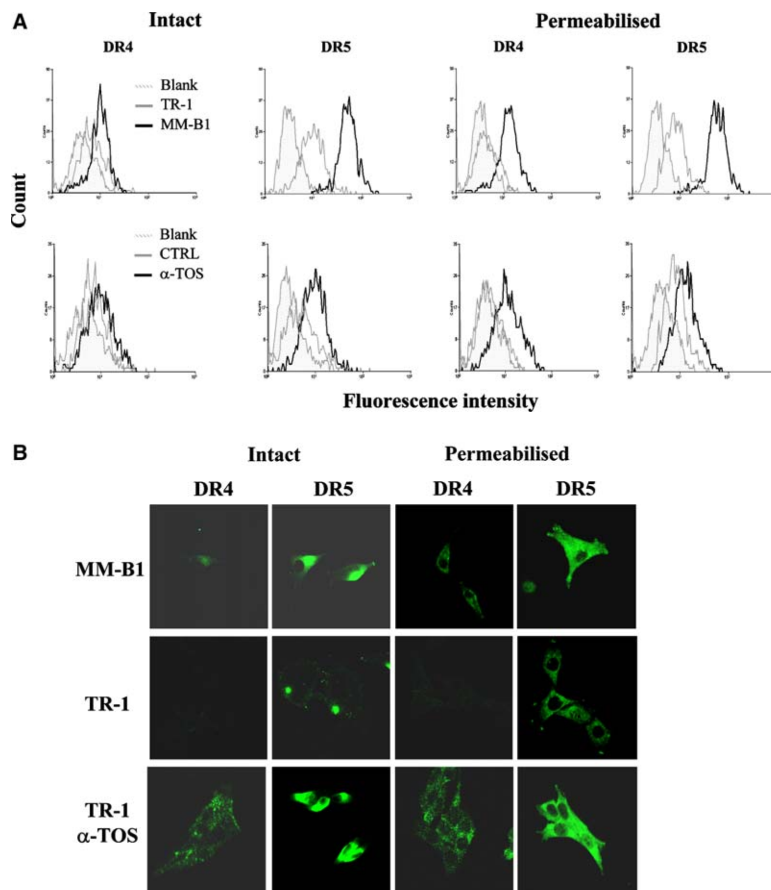

Fig. 3. Expression of DR4 and DR5 in selected TRAIL-resistant cells and their parental counterpart before and after α-TOS exposure. (A) Expression of DR4 and DR5 in parental $p53^{wt}$ MM-B1 and selected TRAIL-resistant (TR-1) cells (top panel). DR4 and DR5 expression in selected TRAIL-resistant (TR-1) cells before and after α-TOS (30 μM) exposure (bottom panel). The cells were or were not permeablised (intact cells) and then labelled with anti-DR4 or anti-DR5 antibody followed by labelling with FITC-conjugated antibody and analysed by flow cytometry (A), and confocal microscopy (B). Images representative of three independent experiments are shown.

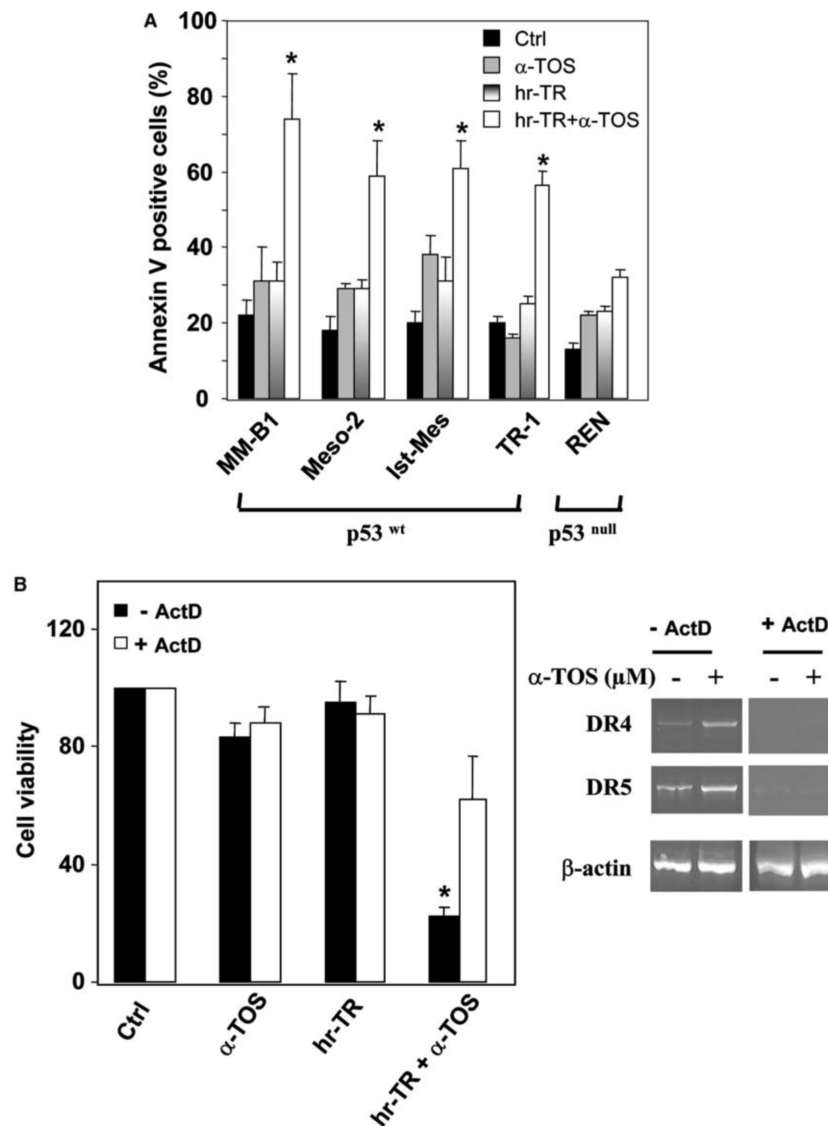

Fig. 4. (A) Cytotoxic effect of hrTRAIL and α-TOS alone and their combination in $p53^{wt}/p53^{null}$ MM cell lines. MM cells were seeded into 24-well tissue culture plates (10^5 per well) and treated for 24 h with hrTRAIL (10 ng/ml) and α-TOS (30 μM) alone or in combination. Apoptosis induction was evaluated by annexin-V–FITC. (B) Cytotoxic effect of hrTRAIL and α-TOS alone and their combination in $p53^{wt}$ MM cells incubated with and without actinomycin D (ActD, right panel). MM cells were plated in 96-well flat-bottom tissue culture plates at 10^4 per well. The cells were incubated overnight with ActD at final concentration of 5 μM (dissolved in DMSO) or DMSO alone. Unincorporated ActD was removed by washing the cells with RPMI-1640, and the cells were then incubated for 24 h with α-TOS (30 μM) and TRAIL (10 ng/ml) alone or in combination. DR4 and DR5 mRNA transcripts were evaluated by RT-PCR performed as described under Section 2 (left panel). Cytotoxicity was determined using the MTT assay. *The combined effect was significantly greater than the effects of individual agents, $P < 0.05$. Results are expressed as means ± S.D. from three independent experiments.

$p53^{wt}$ MM cells were pre-treated with the transcriptional inhibitor actinomycin D (5 μM), then incubated with α-TOS and TRAIL alone or in combination. DR4 and DR5 gene expression and cytotoxicity were evaluated. As shown in Fig. 4B (left panel), α-TOS-exposed cells pre-treated with actinomycin D failed to accumulate TRAIL's DR transcripts, whereas levels in untreated cells were increased over the control for both DR4 and DR5 at 4 h of α-TOS treatment. Thus, inhibition of TRAIL's DR gene expression suppresses the cooperative TRAIL/α-TOS effect as observed in Fig. 4B (right panel).

Apoptosis induced by death receptors can be modulated at several levels. Intracellular anti-apoptotic molecules can block the apoptotic signalling pathway or divert them towards alternative responses. Such molecules include the cellular FLICE-like inhibitory protein (c-FLIP), which competes with caspase-8 for binding to FADD [36], or XIAP, cIAP-1 and cIAP-2, that directly inhibit caspase activity. A role of FLICE inhibitory protein (FLIP) in inhibiting TRAIL-induced cell death has been previously observed in MM cells [15]. Upregulation of TRAIL death receptors by α-TOS may contribute to a shift in the anti- and pro-apoptotic signals in favour of the latter, triggering apoptotic signals, which may then be amplified by the intrinsic pathway. Kinetics of analysis of TRAIL-induced signalling revealed a transient activation of caspase-8, which resulted in induction, albeit low, of apoptosis. Caspase-8 activation was less pronounced in the presence of

TRAIL plus α-TOS. Under this setting, activation of the mitochondria-dependent apoptotic pathway, including Bid cleavage, cytochrome *c* cytosolic mobilisation and, finally, caspase-9 activation, was observed [16]. Bid cleavage may lead to mitochondrial translocation of Bax, as shown for α-TOS in other cancer models [37,38]. Thus, the elevation of p53 in response to α-TOS could facilitate TRAIL-induced apoptosis by inducing transcription of TRAIL death receptors, whose engagement by the ligand causes activation of caspase-8, releasing both Bid and Bax from their sequestration by Bcl-x_L, promoting mitochondrial-dependent apoptosis.

In the present study, we demonstrate the ability of α-TOS, a redox-silent analogue of vitamin E, to enhance or restore TRAIL sensitivity by upregulating its death receptors in MM cells. We show that α-TOS upregulated DR4 and DR5 in $p53^{wt}$ MM cells but not in MM cells lacking functional p53 and that the effect was more evident in TRAIL-resistant MM cells deficient in DR4 and DR5 as compared to the parental $p53^{wt}$ MM cells.

Reversal of TRAIL resistance in cancers like mesothelioma by α-TOS appears to be a viable strategy and is expected to impact on the future on anti-cancer therapeutic approaches, in particular taking into consideration strong anti-mesothelioma activity of the vitamin E analogue [39,40] and the current lack of other than palliative treatment of MM [17].

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