α-Tocopheryl Succinate Inhibits Malignant Mesothelioma by Disrupting the Fibroblast Growth Factor Autocrine Loop

MECHANISM AND THE ROLE OF OXIDATIVE STRESS

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We have studied the potential effect against human malignant mesotheliomas (MM) of α-tocopheryl succinate (α-TOS), a redox-silent vitamin E analog with strong pro-apoptotic and anti-cancer activity. α-TOS at sub-apoptotic levels inhibited proliferation of MM cell lines, while being nontoxic to nonmalignant mesothelial cells. Because MM cells are typified by a highly meta-static phenotype, we investigated the effect of α-TOS on genes playing a major role in MM progression. Of these, α-TOS down regulated fibroblast growth factor (FGF)-1 and, in particular, FGF-2 on the transcriptional level in MM cells, and this was not observed in their nonmalignant counterparts. FGF-2 short interfering RNA suppressed proliferation of MM cells. Down-regulation of FGF-2 was likely because of inhibition of the egr-1 transcription activity that was decreased in MM cells via oxidative stress induced by α-TOS, as evidenced by EPR spectroscopy, whereas nonmalignant cells did not show this response. Treatment of MM cells with egr-1 short interfering RNA suppressed proliferation, which was overridden by exogenously added recombinant FGF-1 and, in particular, FGF-2. An analog of coenzyme Q targeted to mitochondria and superoxide dismutase overrode inhibition of MM cell proliferation by α-TOS as well as α-TOS-induced inhibition of egr-1-dependent transactivation. Finally, α-TOS significantly suppressed experimental MM in immunocompromised mice. Our data suggest that α-TOS suppresses MM cell proliferation by disrupting the FGF-FGF receptor autocrine signaling loop by generating oxidative stress and point to the agent as a selective drug against thus far fatal mesotheliomas.

Malignant mesothelioma (MM) is a highly aggressive tumor...
Vitamin E Succinate Disrupts FGF Autocrine

of these cytokines may control proliferation of MM cells via autocrine signaling (11, 12).

Because these inducers and mediators are essential for the process of formation and progression of MM, understanding the regulatory mechanisms of their expression is important, because they control pathways that may present a target for MM treatment (6, 13, 14). Conceivably, an ideal anti-cancer agent preventing progression of the metastatic disease would selectively down-regulate the expression of those cytokines that positively control tumor growth, while being nontoxic toward normal cells.

Recent data showed that analogs of vitamin E have potent anti-proliferative and pro-apoptotic effects on multiple cancer cell lines and inhibit cancer in pre-clinical models (15). These compounds are epimerized by α-tocopheryl succinate (α-TOS), a redox-silent compound that has been reported to suppress several types of neoplasia (16–19). The vitamin E analogs inhibit proliferation of cancer cells by several mechanisms, including inhibition of DNA synthesis, induction of apoptosis and cellular differentiation, and by affecting the protein kinase C and the MAP kinase pathways (19–24). More importantly, α-TOS exerts anti-proliferative/pro-apoptotic effects in malignant cell lines but is largely nontoxic toward normal cells and tissues (17, 23, 25). It has also been reported that α-TOS can modulate cytokine gene expression in cancer cells (26, 27). For example, α-TOS down-regulates FGFR-1 in MM cells, although the precise mechanism has not been resolved (28).

Because α-TOS is a potent inducer of apoptosis in the generally resistant MM cells (29) and inhibits MM in vivo (30), we investigated the effects of the vitamin E analog on expression of cytokines involved in control of cancer development and progression. We show here that α-TOS disrupts the FGF autocrine loop through suppression of egr-1 transcriptional activity in MM cells but not in their nonmalignant counterparts, further highlighting the potential of α-TOS as a therapeutic agent.

EXPERIMENTAL PROCEDURES

Cell Culture—Human MM cell lines, MM-B1 (biphasic), Meso-2 (sarcomatoid), Iat-Mes and Iat-Mes2 (both epithelialid (31), and a nonmalignant mesothelial cell line, Met-5A (ATCC), were used in our studies. The cells were cultured in DMEM supplemented with 10% fetal calf serum (both from JRH Biosciences), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. All assays were performed when the cells reached 60–70% confluency.

Cell Proliferation Assay—Cell proliferation was determined by assessing DNA synthesis. An end point enzyme-linked immunoochrome assay (ELISA) colorimetric kit (Roche Applied Science) was used to determine cells in the S phase of the mitotic cycle, based on incorporation of 5-bromo-2-deoxyuridine (BrdUrd) into their DNA. Briefly, MM cells were seeded at 10⁴ cells per well in a 96-well plate, treated as specified, and incubated with 10 μM BrdUrd for 2 h at 37 °C. The cells were then fixed and denatured by a 30-min incubation with Fixdenat (Roche Applied Science), incubated for 90 min with an anti-BrdUrd antibody, washed, and incubated further with a substrate solution (tetramethylbenzidine). After 30 min, 1 μM H₂O₂ was added to each well to stop the reaction, and the absorbance was read at 450 nm using a plate reader. In some cases, cells were assessed for the expression of a proliferation-specific gene, the proliferating cell nuclear antigen by Western blotting, using an anti-proliferating cell nuclear antigen IgG (Santa Cruz Biotechnology). Where indicated, cells were exogenously supplemented with human recombinant (hr) FGF-1 or hrFGF-2 (both from Sigma), at 10 ng/ml 24 h prior to treatment.

Cell Cycle Assay—MM and Met-5A cells were plated at 10⁴ cells per well in 24-well plates. The cells were allowed to attach overnight and were then incubated for up to 3 days with α-TOS. The floating and attached cells were collected, washed with PBS, resuspended in buffer containing sodium citrate (1%), Triton X-100 (0.1%), RNase A (0.05 μg/ml), and propidium iodide (PI) at 5 μg/ml, and incubated in the dark for 30 min at 4 °C. The nuclear suspension was filtered through a 60-μm mesh and analyzed by flow cytometry.

Apoptosis Assessment—Apoptosis was quantified by the annexin V-fluorescein isothiocyanate (FITC) method, which detects phosphatidylserine externalization in the early phases of apoptosis (20). Briefly, cells were seeded at the density of 10⁵ cells per well in 24-well plates and treated with α-TOS after overnight recuperation. Floating and attached cells were collected, washed with PBS, resuspended in 0.1 ml of binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂, pH 7.4), incubated for 20 min at room temperature with 2 μl of annexin V-FITC supplemented with 10 μl of PI (10 μg/ml), and analyzed by flow cytometry (FACSCalibur; BD Biosciences) using channel 1 for annexin V-FITC binding and channel 2 for PI staining.

Detection of Reactive Oxygen Species (ROS)—Cellular ROS were detected indirectly by flow cytometry and directly by EPR spectroscopy, following treatment of cells with α-TOS as indicated in the figure legends. In some experiments, cells were pretreated for 1 h with 2 μM mitochondrially targeted coenzyme Q (mito-Q) (32) or co-incubated with superoxide dismutase (SOD; EC 1.15.1.1; Sigma S4636) at 750 μl/ml. For indirect evaluation, cells were treated with α-TOS and reacted with dihydrorhodaminefluorescein diacetate (DCF; Molecular Probes) for 30 min, and scored by flow cytometry for cells with high fluorescence, which was evaluated on the basis of an increase in mean fluorescence intensity. EPR spectroscopy analysis of ROS generation was based on the use of the radical trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Sigma). In brief, cells were plated in T25 flasks and maintained at 60–70% confluency (~5 × 10⁴ cells per flask). Cells were washed, overlaid with the PSS medium (33), and incubated with 50 μM α-TOS 5 min after addition of 10 mM DMPO. Analyses of DMPO adducts were performed with samples taken from the cell suspension as well as the cell-conditioned medium transferred into a quartz flat cell (Wilmad). The quartz cell was then placed into the cavity of the Bruker EMX bench-top spectrometer set at 293 K with the following spectrometer parameters: field strength 10 millitesla, microwave power 2 milliwatts, microwave frequency 100 kHz, modulation amplitude, 0.1 miltitesla, sweep time 83.9 s. The detection limit of the stable nitroxide (TEMPO) under identical conditions was ~50 nm.

Real Time mRNA Analysis—Relative quantification of mRNA expression was achieved using quantitative real time-PCR (Q-PCR). Briefly, this technique is based on the detection of a fluorescent signal that is proportional to the incorporation of the fluorescent dye SYBR-green during PCR amplification (Prism 7700 sequence detection system; Applied Biosystems). The expression of all genes of interest was related to that of the 18 S RNA control. Total RNA was extracted from MM cell cultures using Trizol (Invitrogen). To minimize potential genomic contamination, RNA samples were treated with RNase-free DNase (Promega) and purified using the RNeasy mini kit (Qiagen). Each assay was performed according to the manufacturer's protocol. First strand cDNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol. PCR primers were specifically designed for real time PCR. Particular attention was given to maintain a constant 60 °C annealing temperature of primer pairs, which is common for Q-PCR.

Primers used for primers was designed across intron/exon boundaries to detect genomic DNA contamination.

The primers used were as follows: FGF-1, 5'-GGG CTT TTA TAC GGC TCA CA-3', and 5'-GGG CAA CAA ACC AAT TCT TC-3'; FGF-2, 5'-GAC CCT CAC ATC AAG CTA CAA CT-3', and 5'-AAA GAA ACA CTC ATG AAC ACA-3'; VEGF, 5'-AGG CCA GAC CAT AGG AGA GA-3', and 5'-TTT CTC CGG CTT TGG TT-3'; TGF-β, 5'-GAG CCT GAC GCC GAC TAC TA-3', and 5'-TCG GAG CCT TGA TGA TGT GTT GA-3'.

RNA Interference (RNAi)—For RNAi, cells were seeded at a final density of 5 × 10⁴ cells per well in 12-well plates, cultured until ~50% confluency, and then treated with egr-1, FGF-1, or FGF-2 siRNA (all designed and synthesized by Prologo) as follows: siRNA (0.5 μg/ml) was combined with 100 μl of serum-free DMEM supplemented with 20 μl of Oligofectamine (Invitrogen) and left for 15 min at room temperature. The transfection mixture was added to cells, which were then left in the incubator for 24 h, after which they were overlaid with complete DMEM. 24–48 h later, the cells were used in experiments. Typically, 90–95% of treated cells showed significant down-regulation of the targeted genes as estimated by flow cytometric analysis (data not shown). Nonsilencing RNA was used as a negative control and FITC-tagged nonspecific RNA as a control for transfection efficacy (both Qiagen).

Analysis of FGF-1 and FGF-2 Protein—The FGF-1 and FGF-2 protein levels were assessed using an ELISA kit (R & D Systems) according to the manufacturer's instructions. In brief, cells were seeded in 24-well plates and allowed to reach 60–70% confluency. Following treatment, 100 μl of cell-conditioned medium was transferred to the ELISA 96-well plate, mixed with 100 μl of the assay diluent, and incubated for 2 h at

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of apoptosis was comparable irrespective of the cell phenotype. MM and nonmalignant mesothelial cells (Met-5A) were seeded in 24-well plates and allowed to reach 30–70% confluence, and treated for 24 h with \( \alpha \)-TOS at the concentrations shown, and the level of apoptosis was assessed by the annexin V-binding assay. Data shown represent mean values \( \pm \) S.D. (\( n = 3 \)).

![Fig. 1. \( \alpha \)-TOS induces apoptosis in mesothelioma cells in a dose-dependent manner and is selective for malignant cells. MM and nonmalignant mesothelial cells (Met-5A) were seeded in 24-well plates and allowed to reach 30–70% confluence, and treated for 24 h with \( \alpha \)-TOS at the concentrations shown, and the level of apoptosis was assessed by the annexin V-binding assay. Data shown represent mean values \( \pm \) S.D. (\( n = 3 \)).](Image 103x627 to 262x738)

**RESULTS**

**\( \alpha \)-TOS Is Selectively Toxic to MM Cells**—\( \alpha \)-TOS suppresses several types of cancer in pre-clinical models (16–19, 27), although the reasons for this effect are not completely understood. Here we studied the effect of \( \alpha \)-TOS on several MM cell lines of different phenotypic origin and on their nonmalignant counterparts. \( \alpha \)-TOS consistently induced apoptosis in all MM cell lines in a concentration-dependent manner, and the extent of apoptosis was comparable irrespective of the cell phenotype (Fig. 1). In all cases, doses of \( >30 \) \( \mu M \) were required for apoptosis induction, and such doses are achievable in vivo (17). In contrast, and important to the therapeutic potential of \( \alpha \)-TOS, the vitamin E analog was nontoxic to Met-5A cells (Fig. 1).

We next tested the effect of \( \alpha \)-TOS on cell proliferation. The cells were treated with the agent at 10–50 \( \mu M \) for up to 72 h. As shown in Fig. 2, significant inhibition of cell proliferation was observed in MM cells treated for more than 24 h with \( \alpha \)-TOS at concentrations between 10 and 50 \( \mu M \). It is noteworthy that the vitamin E analog inhibited proliferation at levels where it did not induce apoptosis, pointing to effects that are apoptosis-independent. Suppression of cellular proliferation by the vitamin E analog suggests that it may inhibit cell cycle progression (Fig. 3). Indeed, treatment of Meso-2 MM cells with a sub-apoptotic concentration of \( \alpha \)-TOS resulted in accumulation of cells in G1 phase at the expense of cells in the S-phase and, to a lesser extent, cells in G2 phase (Fig. 3B). Cell cycle arrest was comparable in the other MM cell lines tested (data not shown). Cell cycle analysis also revealed a low number of cells accumulating in sub-G0 (data not shown), supporting the idea that \( \alpha \)-TOS exhibits anti-proliferative activity in addition to induction of apoptosis. Again, little effect was observed in Met-5A cells (Fig. 3A).

**\( \alpha \)-TOS Selectively Suppresses Expression of FGF-1 and FGF-2**—Inhibition of cell cycle progression and proliferation by \( \alpha \)-TOS is indicative of modulation by the agent of expression of genes that are involved in these processes. Therefore, we treated the cells with \( \alpha \)-TOS at sub-apoptotic levels and assessed for the expression of several genes that have an important role in tumor growth, namely FGF-1 (acidic FGF), FGF-2 (basic FGF), TGF-\( \beta \), and VEGF. Overall, the MM and nonmalignant mesothelial cells express the individual mRNAs, with the exception of Ist-Mes2, where no VEGF mRNA was detected (Fig. 4). Although the relative levels of mRNA for TGF-\( \beta \) and VEGF did not differ substantially, significant differences in the expression of FGFs were observed. FGF-2 mRNA was expressed \( \sim 2-3 \) times more than FGF-1 mRNA in all cell lines. Notably, Meso-2 cells expressed the highest levels of FGF-2 mRNA, whereas these cells expressed less FGF-1 mRNA. This pattern was similar for other MM cell lines. The nonmalignant Met-5A cells expressed relatively low levels of FGF-2 mRNA. When challenged with \( \alpha \)-TOS at 10 or 20 \( \mu M \), there was no significant effect on the level of expression of TGF-\( \beta \) and VEGF mRNA. However, FGF-1 and FGF-2 mRNA was differentially regulated by the vitamin E analog (Fig. 5). In general, the pro-vitamin had an effect on both FGF-1 and FGF-2, particularly in Meso-2 and MM-B1 cells, whereas FGF-2 mRNA was consistently depressed to an increased extent. Finally, \( \alpha \)-TOS...
confluency. The cells were then treated with (were seeded in 24-well plates and left overnight to reach B
Meso-2 cells as detailed under "Experimental Procedures." All data are Q-PCR. The data are expressed relative to the level of FGF-2 mRNA in
mean values
sion of TGF-
immunostaining the cells for FGF-1 and FGF-2. We also ob-
the effect was more pronounced for FGF-2 as documented by
secretion followed a similar trend to the corresponding mRNA,
i.e.
secretion was suppressed in
protein level of egr-1 in MM and Met-5A cells and found no
difference. We first determined the effect of the pro-vitamin on the
expression of FGF-2 (36, 37) and possibly FGF-1 expression
Fig. 9 shows analysis of
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mito-Q suppressed significantly radical accumulation in
DCF fluorescence. Moreover, pre-loading Meso-2 cells with
SOD both abrogated the EPR detection of ROS and suppressed
mito-Q is linked to
selectivity of inhibition of proliferation by α-TOS is linked to
suppression of egr-1 trans-activation efficacy.
Selective Down-regulation of FGF-2 by α-TOS and ROS Gen-
eration—That inhibition of FGF-1 and FGF-2 mRNA expres-
sion is selective for MM cells strongly suggests a clinical po-
tential of α-TOS. We were therefore interested in the
mechanism underlying this selectivity. One possibility was
that the nonmalignant cells hydrolyze the pro-vitamin to
α-TOH, because this has been reported for several nonmalign-
ant cell types, including hepatocytes and cardiomyocytes (25).
Fig. 9 shows analysis of α-TOS and α-TOH in Met-5A and
Meso-2 cells incubated with α-TOS. Although there was a 5–10-
fold increase in α-TOH levels, this represents only a minor
portion of the total intracellular α-TOS (50–100 ng/mg protein),
indicating that hydrolysis of α-TOS is not responsible for the
selectivity of the drug for MM cells.
We then investigated generation of radicals by the two cell
lines in the presence of added α-TOS. Both the indirect flow
cytometric assay, using the probe DCF, and the direct radical
trapping consistently indicated ROS accumulation in α-TOS-
treated Meso-2 but not Met-5A cells (Fig. 10). These data sup-
port the idea that Meso-2 cells respond to α-TOS by rapid
accumulation of ROS. It is likely that superoxide is formed in
Meso-2 cells exposed to α-TOS, because inclusion of exogenous
SOD both abrogated the EPR detection of ROS and suppressed
DCF fluorescence. Moreover, pre-loading Meso-2 cells with
mito-Q suppressed significantly radical accumulation in
Meso-2 cells. Mito-Q is a coenzyme Q analog specifically tar-
got to mitochondria because of the attachment of a trimeth-
ylphosphonium group (32). That mito-Q preferentially associ-
ates with mitochondria in mito-Q-exposed cells as well in
exerted no effect on either FGF-1 or FGF-2 mRNAs in the
nonmalignant Met-5A cells.
To test whether suppression of the level of mRNA translates
into a comparable change in protein expression, we studied
FGF-1 and FGF-2 secretion from MM and Met-5A cells in the
presence of α-TOS (Fig. 6). Inhibition of FGF-1 and FGF-2 secretion
followed a similar trend to the corresponding mRNA, i.e. with no significant inhibition of FGF-1 and FGF-2 protein
in Met-5A cells, whereas protein secretion was suppressed in
MM-BI and, to an increased extent, in Meso-2 cells. Overall,
the effect was more pronounced for FGF-2 as documented by
immunostaining the cells for FGF-1 and FGF-2. We also ob-
served less fluorescence in the pericellular regions of the MM
cells treated with α-TOS compared with controls, whereas no
difference in immunostaining was observed for Met-5A cells
(data not shown).

α-TOS Suppresses FGF-1 and FGF-2 Expression by Inhibit-
ing egr-1 Trans-activation—Because the inhibition of FGF-1
and FGF-2 occurred at a transcriptional level, we studied the
effect of α-TOS on the transcription factor egr-1 that controls
expression of FGF-2 (36, 37) and possibly FGF-1 expression
(38). We first determined the effect of the pro-vitamin on the
protein level of egr-1 in MM and Met-5A cells and found no
difference (data not shown). Next, we studied the effect of
α-TOS on egr-1 trans-activation. Thus, Met-5A and Meso-2
cells were transiently transfected with a plasmid harboring the
egr-1 promoter, and luciferase activity was assessed as a sur-
rrogate for egr-1 trans-activation in the presence of α-TOS (Fig.
7). Both doses of α-TOS employed suppressed luciferase activity
when compared with corresponding controls, whereas no
effect was observed in Met-5A cells.

To obtain data that support a role for egr-1 trans-activation
in FGF-1/FGF-2 expression and in proliferation, we used the
RNAi approach. Thus, Meso-2 cells were challenged with
egr-1, FGF-1, or FGF-2 siRNA, and proliferation was as-
sessed. Treatment with the three different siRNAs sup-
pressed proliferation of the cells, although the effect of egr-1
siRNA was most prominent, suggesting redundancy when
only one of the two FGFs was knocked down. However, this
effect appeared to be more pronounced for FGF-2. Inhibition
of proliferation of siRNA-treated cells was overcome, at least
partially, when the cells were supplemented with endogenous
hrFGF-1 or, more significantly, with hrFGF-2. Next, we as-
sessed the egr-1 siRNA-transfected cells for secretion of
FGF-1 and FGF-2 (Fig. 8D). Overall, egr-1 knock-down also
resulted in lower secretion of FGF-1 and FGF-2, again with a
greater effect for FGF-2. These data strongly suggest that
selectivity of inhibition of proliferation by α-TOS is linked to
suppression of egr-1 trans-activation efficacy.
animals fed mito-Q has been well documented (32, 39). It has also been shown that minutes upon uptake, the originally semiquinone form of mito-Q is reduced by the mitochondrial electron redox chain, acquiring a high activity to suppress ROS-dependent apoptosis (32, 40–42). Therefore, these data suggest that mitochondria are the source and/or the target for ROS generated by MM cells in response to added /H9251-TOS.

We next studied the effect of mito-Q on the inhibition of egr-1 trans-activation in MM cells exposed to /H9251-TOS (Fig. 11). Addition of either mito-Q or SOD overcame /H9251-TOS-inhibited egr-1-dependent trans-activation in cells transfected with egr-1-lux (Fig. 11A). In addition, mito-Q abrogated the down-regulation of FGF-2 by the added vitamin E analog in Meso-2 cells (Fig. 11B). Taken together with the data confirming generation of ROS upon /H9251-TOS treatment, our findings support the notion that ROS are important mediators of the effect of /H9251-TOS on the expression of FGF-2, which is regulated by egr-1. These data also provide evidence that resistance of nonmalignant cells to the toxic effects of /H9251-TOS may be due to their lower capacity to generate ROS and/or their more efficient anti-oxidant system.

/H9251-TOS Suppresses Mesotheliomas in Vivo—We have observed recently that /H9251-TOS extended the life span of immunocompromised mice with experimental human peritoneal mesotheliomas (30). Here we investigated whether /H9251-TOS retains this propensity also in the case of subcutaneously placed MM cell-derived xenografts in athymic mice. Fig. 12 shows that intraperitoneally administered /H9251-TOS exerted a strong and highly significant effect, with ~90–95% inhibition of tumor growth over the 15-day period of treatment, further pointing to the potential of the vitamin E analog as an anti-mesothelioma agent.

DISCUSSION

In this communication we present data pointing to a novel mode of action of /H9251-TOS, a redox-silent vitamin E analog, relevant to its anti-tumor activity. Here we show that the pro-vitamin (i) selectively inhibits proliferation in MM cells; (ii) suppresses expression of FGF-1 and, in particular, FGF-2 in MM cells; (iii) causes high level of generation of ROS in MM cells, thereby inhibiting egr-1-dependent FGF-1/-2 trans-activation; and (iv) suppresses mesotheliomas in vivo. These major

FIG. 6. /H9251-TOS suppresses secretion of FGF-1 and FGF-2 protein in MM cells. Met-5A (A and B), MM-B1 (C and D), and Meso-2 cells (E and F) were seeded in 24-well plates, allowed to reach ~50% confluency, and treated for 24, 48, and 72 h with /H9251-TOS at 10 or 20 μM. The level of secreted FGF-1 (A, C, and E) and FGF-2 protein (B, D, and F) was assessed by ELISA and is expressed as total amount of protein in the medium per well. Data shown represent mean values ± S.D. (n = 3).

FIG. 7. /H9251-TOS suppresses the egr-1 transcription activity in malignant but not nonmalignant mesothelioma cells. Met-5A and Meso-2 cells were seeded in 24-well plates and allowed to reach ~50% confluency, after which they were transiently transfected with a plasmid harboring the egr-1 promoter and the luciferase reporter. The cells were either left untreated or exposed for 24 h to 10 or 20 μM /H9251-TOS. Luciferase activity was then assessed by a standard assay and expressed as relative luminescence compared with the relevant control cells. Data shown represent mean values ± S.D. (n = 3). * indicates data significantly different from controls (p < 0.05).
points of this communication epitomize an intriguing activity of α-TOS, by which it suppresses the FGF-FGFR autocrine signaling that results in decreased proliferation of MM cells, an effect that may translates into inhibition of mesothelioma progression. Our data with immunocompromised mice strongly support the idea that α-TOS is active against tumor progression and highlights its therapeutic potential of this vitamin E analog.

We and others (17, 21, 25, 26) have reported over the last few years anti-proliferative and pro-apoptotic activity of α-TOS and other analogs of vitamin E. The anti-proliferative/pro-apoptotic activity of these compounds is based on their structure; the molecule comprises three domains, the hydrophobic (phytyl chain), the signaling (α-tocopheryl moiety), and the functional domain (succinyl moiety in case of α-TOS) (24, 43). The major signaling pathway underlying the anti-proliferative/pro-apoptotic activity of α-TOS includes translocation of the pro-vitamin into the cell where it associates with subcellular struc-
mice were injected subcutaneously with Isth-Met2 cells (2 × 10⁶ cells per mouse). The animals were left until solid tumors were established (~100 mm³), after which they were treated with intraperitoneal injections of 100 μl of 200 mM α-TOS in Me₂SO or the vehicle every 2nd day. Tumor volume was estimated by micro-calipers and expressed as relative increase over the volume at the onset of treatment. The data shown represent mean values ± S.E. (n = 10). * indicates data significantly different from controls (p < 0.05).

Fig. 12. α-TOS suppresses mesothelioma tumors in vivo. Nude mice were injected subcutaneously with Isth-Met2 cells (2 × 10⁶ cells per mouse). The animals were left until solid tumors were established (~100 mm³), after which they were treated with intraperitoneal injections of 100 μl of 200 mM α-TOS in Me₂SO or the vehicle every 2nd day. Tumor volume was estimated by micro-calipers and expressed as relative increase over the volume at the onset of treatment. The data shown represent mean values ± S.E. (n = 10). * indicates data significantly different from controls (p < 0.05).

Vitamin E Succinate Disrupts FGF Autocrine

Our major interest was to understand the selectivity with which α-TOS suppresses expression of FGF-1 and, in particular, FGF-2 in MM cells. The promoter of the FGF-2 gene contains an egr-1-binding site (57, 58). A link between egr-1 and FGF-1 is not well understood, although recent data suggest that there may be some degree of mutual regulation (38, 54). Both the FGF-2 mRNA and protein are depressed significantly more by α-TOS in MM cells than is FGF-1, which is consistent with the notion that FGF-2 plays an important role in MM progression and the clinical outcome of the pathology (6, 9). Several lines of evidence stipulate a role for egr-1 in a-α-TOS-induced FGF-2 and probably FGF-1 down-regulation. This follows from experiments in which α-TOS inhibited trans-activation of the lux gene under egr-1 control and from experiments where the pro-vitamin suppressed FGF-1 and FGF-2 secretion combined with the finding that egr-1 RNAi suppressed FGF-1 and FGF-2 secretion by MM cells. That these effects were more pronounced for FGF-2 than FGF-1 is consistent with the notion that FGF-2 has been associated with tumor promotion (59–61).

An intriguing finding is that these effects were observed in malignant cells, although their nonmalignant counterparts were not affected. The possibility that Met-5A cells were resistant to the toxic effects of α-TOS because they may hydrolyze it to its nontoxic vitamin form was ruled out because the concentration of intracellular α-TOS remained above toxicity levels in both cell lines. Such resistance to α-TOS was reported for cells like hepatocytes (62) or cardiomyocytes (63); moreover, it was stipulated that for such cells α-TOS is a rich source of vitamin E, rendering them more resistant to toxic insults (62, 64). We found that at least one major reason for resistance of nonmalignant mesothelial cells to α-TOS is because of their low level of accumulation of ROS (consistent with only marginally increased intracellular levels of α-TOH, cf. Fig. 9), whereas the malignant cells responded by generation of substantial ROS levels. This effect translated to inhibition of egr-1 trans-activation and secretion of FGF-2, because it was overridden in both cases by addition of SOD or mito-Q. The latter also suggests that mitochondria of the malignant cells are the source and/or target of ROS, although this needs more clarification. It is cannot be excluded at this stage that ROS are generated by other systems solely as a result of leakage from the mitochondrial electron transport chain, including the plasma membrane NADPH oxidase, the nonphagocytic gp91phox-like oxidase, xanthine oxidase, nitric-oxide synthase, phospholipase A₂, or lipoxigenases (65–68). That ROS generation readily occurs following exposure of cancer cells to α-TOS has been reported before (42, 44, 69, 70). Moreover, it has been documented that a failure to respond to α-TOS by generation of ROS may render the cells resistant to the drug (69, 70).

The novel finding here is that ROS are probably responsible for the egr-1-mediated effect of α-TOS on FGF-1 and FGF-2 expression. Reports on the effect of oxidative stress on egr-1 are controversial. Although it has been suggested that hydrogen peroxide suppressed the transcriptional activity of egr-1 (71), others propose its role in cell survival following exposure to oxidative stress (72, 73). To complicate matters even further, a recent report (52) suggested up-regulation of egr-1 by hydrogen peroxide and showed inhibition of cell proliferation under identical conditions. It is thus possible that egr-1 is a component of a system that fine-tunes cellular responses to various levels of oxidative stress. Nevertheless, our data clearly suggest selective inhibition of egr-1 trans-activation by ROS generated by MM cells following exposure to α-TOS, which is based on its reversal and on reversal in expression of the egr-1-controlled FGF-2 by SOD and mito-Q.

Taken together, we propose that α-TOS, a redox-silent vitamin E analog with strong anti-cancer activity, selectively suppresses egr-1-dependent trans-activation of FGF-2, an important autocrine signaling molecule. By virtue of this, the pro-vitamin disrupts the FGF-FGFR autocrine signaling loop while effi-

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Vitamin E Succinate Disrupts FGFR Autocrine

ciently suppressing proliferation of malignant mesothelioma cells. Because MM is currently a fatal type of neoplasia and because a-TOS, epitomizing a new group of anti-cancer agents (25), suppresses proliferation of MM cells, the vitamin E analog is a promising anti-mesothelioma agent.

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α-Tocopheryl Succinate Inhibits Malignant Mesothelioma by Disrupting the Fibroblast Growth Factor Autocrine Loop: MECHANISM AND THE ROLE OF OXIDATIVE STRESS

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