FEBS Letters 581 (2007) 4611–4615

α-Tocopheryl succinate inhibits angiogenesis by disrupting paracrine FGF2 signalling

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Received 11 June 2007; revised 2 August 2007; accepted 17 August 2007

Available online 31 August 2007

Edited by Michael R. Bubb

Abstract **Malignant mesothelioma (MM) cells enhanced proliferation of endothelial cells (ECs) as well as their angiogenesis in vitro by secretion of fibroblast growth factor-2 (FGF2). This effect was suppressed by pre-treating MM cells with α-tocopheryl succinate (α-TOS), which inhibited FGF2 secretion by inducing mitochondria-dependent generation of reactive oxygen species. The role of FGF2 was confirmed by its down-regulation by treating MM cells with siRNA, abolishing EC proliferation and wound healing enhancement afforded by MM cells. We conclude that α-TOS disrupts angiogenesis mediated by MM cells by inhibiting FGF2 paracrine signalling.**

Keywords: α-Tocopheryl succinate; FGF2; Endothelial cell; Angiogenesis; Paracrine signalling

1. Introduction

Tumour progression is dependent on formation of new blood vessels supplying cancer cells with oxygen and nutrients. Endothelial cells (ECs) are forced to re-enter the cell cycle during tumour progression to form new blood vessels [1,2]. Cancer cells secrete growth factors, including the vascular endothelial (VEGF) and fibroblast growth factors (FGF), that interact with corresponding receptors on ECs, promoting their proliferation [3]. As angiogenesis is essential for tumour progression, its suppression represents an intriguing anti-cancer strategy. Gene therapy, immunotherapy or chemotherapy have been a recent focus to inhibit angiogenesis [4–6].

We and others have shown that compounds like the mitocan α-tocopheryl succinate (α-TOS), a redox-silent analogue of vitamin E (VE) [7] and a potent inhibitor of cancer in pre-clinical models [8,9], also disrupts mitogenic signalling pathways [10,11]. These include the fibroblast growth factor-2 (FGF2) autocrine loop, by means of which cancer cells control their own proliferation [12]. We have shown that α-TOS suppressed expression of FGF2 by inhibiting the transcriptional activity of egr-1 [10] as well as down-regulating the cognate FGF2 receptor FGFR1, probably by impairing the activity of E2F1 [11].

As FGF2 is an important mitogen driving EC proliferation [12], we hypothesised that α-TOS could also suppress angiogenesis by inhibiting FGF2 expression in malignant mesothelioma (MM) cells, whereby disrupting the paracrine signalling pathway, as documented in this communication.

2. Materials and methods

2.1. Cell culture

Human MM cell lines, MM-B1, Meso-2, and Ist-Mes2 [13], and a non-malignant mesothelial cell line, Met-5A (ATCC), were cultured in DMEM supplemented with 10% FCS (both JRH Biosciences), 2 mM L-glutamine and antibiotics. Endothelial-like EAhy926 cells [14] were maintained in DMEM supplemented with HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine). The cells retain the properties of primary ECs, including expression of factor VIII and P-selectin [14], tube-forming activity and the propensity to persist in confluent cultures [4].

2.2. Cell proliferation

Cell proliferation was assessed using an ELISA kit (Roche) to determine cells in the S-phase, based on 5-bromo-2-deoxyuridine (BrdU) DNA incorporation. EAhy926 cells were seeded at 10^4 cells/well into a 96-well plate, treated, and incubated with 10 μM BrdU for 2 h at 37 °C. The cells were fixed and denatured with Fixdenat (Boehringer Mannheim), incubated with anti-BrdU IgG, and further incubated with tetramethylbenzidine. One mole of H_2SO_4 was then added to stop the reaction, and the absorbance was read at 450 nm.

2.3. Detection of reactive oxygen species (ROS)

Following treatment, cells were reacted with dihydrodichlorofluorescein diacetate (DCF; Molecular Probes) and scored by flow cytometry for cells with high fluorescence as described [10]. In some experiments, cells were pre-treated for 1 h with 2 μM mitochondrially targeted coenzyme Q (MitoQ) [15].

2.4. Transwell experiments

Co-culture experiments were performed using the clear Transwell supports (0.4 μm pore, 6.5 mm diameter) in a 24-well configuration (Corning). EAhy926 cells were seeded (5×10^5/well) in the 12 central wells, overlaid with 0.6 ml of media, and allowed to adhere. Inserts were seeded with MM cells (10^5/insert), first in the outer wells of the plate where they were pre-treated with 25 μM α-TOS for 12 h. The inserts were then transferred to the central wells containing ECs and overlaid with 0.1 ml media. Proliferation of the EAhy926 cells was assessed 12 h later.

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Abbreviations: DCF, dihydrodichlorofluorescein diacetate; EC, endothelial cell; FGF2, fibroblast growth factor-2; MM, malignant mesothelioma; NS, non-silencing; RNAi, RNA interference; ROS, reactive oxygen species; siRNA, short interfering RNA; α-TOS, α-tocopheryl succinate; VE, vitamin E

0014-5793/$32.00
doi:10.1016/j.febslet.2007.08.051

2.5. Analysis of FGF1 and FGF2 protein

FGF1 and FGF2 protein levels were assessed using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Cells were seeded in 24-well plates and allowed to reach 60–70% confluence. Following treatment, 100 μl of cell-conditioned medium was transferred to the ELISA plate, mixed with 100 μl of the diluent and incubated at room temperature. After washing, each well was supplemented with the FGF conjugate, which was followed by incubation at room temperature with the substrate solution. Absorbance at 450 nm was read. The system was calibrated using hrFGF1 or hrFGF2.

2.6. RNA interference (RNAi)

Cells were seeded at 5×10^4/well in 12-well plates, allowed to reach ~50% confluence and exposed to FGF1 or FGF2 short interfering RNA (siRNA) (Proligo) as follows: siRNA (0.5 μg/ml) was combined with 100 μl 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. The cells were used in experiments 48 h later. Typically, 90–95% of treated cells showed significant down-regulation of the target genes as estimated by flow cytometric analysis (data not shown). Non-silencing RNA was used as a negative control and FITC-tagged non-specific RNA as a control for transfection efficacy (both Qiagen).

2.7. Wound healing experiments

EAhy926 cells were seeded in 35-mm Petri dishes and allowed to reach complete confluency. The monolayer was 'wounded' by removal of cells, generating a denuded region ~0.5 mm wide. Re-growth in the presence of α-TOS was assessed by following the time-dependent narrowing of the denuded region using a microscope equipped with a grid in the eyepiece, and the healing rate was expressed in μm/h.

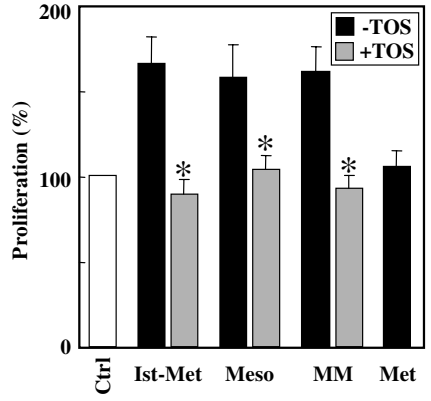

Fig. 1. α-TOS suppresses MM-mediated EC proliferation. EAhy926 cells were seeded in 24-well plates and left to reach 50% confluency. The cells were then allowed to proliferate following addition of 1 ml media conditioned with Ist-Mes2, Meso-2, MM-BI, MCF-7 or Met5A cells pre-treated with α-TOS (25 μM, 24 h) for 12 h, at which stage their proliferative rate was assessed as specified in Section 2.

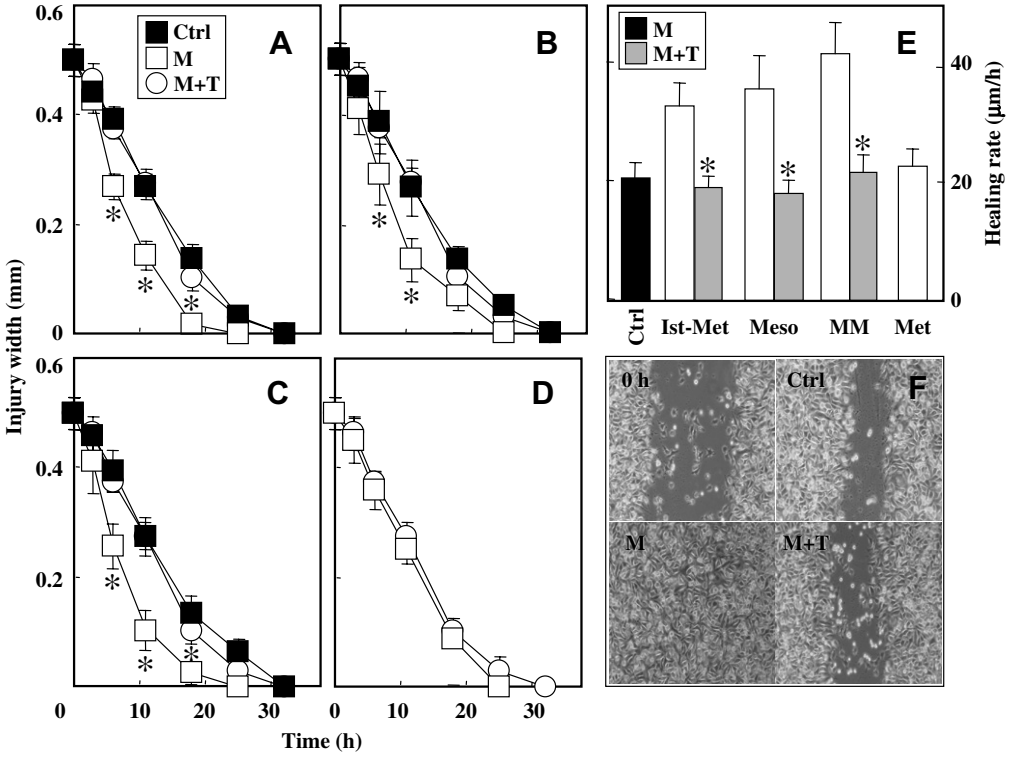

Fig. 2. α-TOS suppresses cancer cell-mediated angiogenesis in vitro. EAhy926 cells were seeded in 35-mm Petri dishes and left to reach complete confluence. The EC monolayer was 'injured' by denudation of central region of the endothelium (~0.5 mm wide cell-free zone). The cells were then allowed to re-grow in the absence (Ctrl) or presence of conditioned media from Ist-Mes2 (A), Meso-2 (B), MM-BI (C) or Met-5A cells (D) pre-treated (M+T) or not (M) with 25 μM α-TOS for 24 h prior to media withdrawal. Kinetics of injury healing was assessed by measuring the width of the denuded zone in a phase contrast microscope fitted with a grid in the objective. Panel E documents the 'healing rate' (in μm/h) for EAhy926 in the absence and presence of conditioned media from cancer cells obtained by performing linear regression on the re-growth curves in panels A–D. Panel F shows EAhy926 cells at times 0 and 18 h following injury in the absence (Ctrl) and presence of conditioned media from Ist-Mes2 cells pre-treated (M+T) or not (M) with α-TOS.

2.8. Statistics

The data shown are mean values ± S.D. derived from at least three independent experiments. Statistical differences were calculated using the Student's *t*-test, and differences were considered significantly different at $P < 0.05$.

3. Results and discussion

We have reported that α-TOS suppressed expression of cytokines, such as FGF1, FGF2 and TGFβ, but not VEGF, in MM cells, whereby impairing their autocrine-dependent proliferative signalling pathways [10]. Since cytokines like FGF2 are potent mediators of paracrine angiogenic signalling [12] and since angiogenesis is essential for progression of MM [16], we studied whether α-TOS also suppresses angiogenesis by impairing secretion of FGF1 and FGF2 by MM cells.

Since angiogenic cytokines are soluble proteins secreted by cancer cells, we first investigated whether media from MM cultures stimulate proliferation of ECs. Fig. 1 shows that the level of proliferation of EAhy926 cells increased by 50–60% when ECs were supplemented with conditioned media from MM cell, but not from the non-malignant mesothelial Met-5A cells. The stimulatory effect was abolished when the MM cells were pre-treated with sub-apoptotic levels of α-TOS.

We next studied the effect of conditioned media on an in vitro angiogenesis model, which is based on wound healing. Following removal of a stripe of confluent EC cultures, re-growth was followed on the basis of 'closing the gap', and the rate of wound healing was expressed in μm/h. Fig. 2 shows that conditioned media from MM cell cultures enhanced the would-healing process by 60–90%, from ~20 μm/h to 35–40 μm/h. No effect of media from Met-5A cell cultures was observed. Media from MM cells pre-treated with sub-apoptotic doses of α-TOS failed to enhance angiogenesis in vitro.

The above data suggest presence of soluble compounds, secreted by MM cells, that enhance EC proliferation and wound healing. We therefore subjected ECs to co-culture with MM and Met-5A cells in Transwell plates. Fig. 3 documents that presence of the MM cells enhanced proliferation of ECs in the co-cultures by ~50%. This was partially abolished when MM cells were pre-treated with α-TOS. Met-5A cells did not

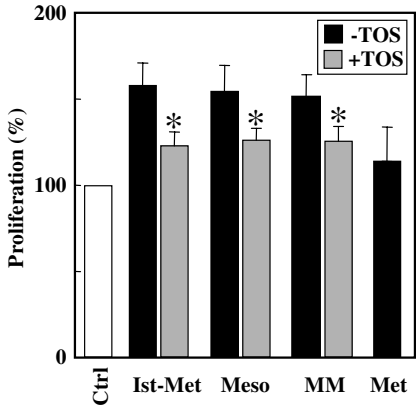

Fig. 3. Co-culture of endothelial cells with cancer cells stimulates their growth, and this is suppressed by α-TOS. EAhy926 cells were seeded at ~5×10^5/well in the bottom compartments of the 24-well Transwell plate. Untreated cancer cells or cells pre-treated with α-TOS (12 h, 25 μM) were seeded in the upper compartment of the corresponding wells of the Transwell plate (~10^5 per well), and the proliferative status of ECs was assessed 12 h later using the BrdU assay.

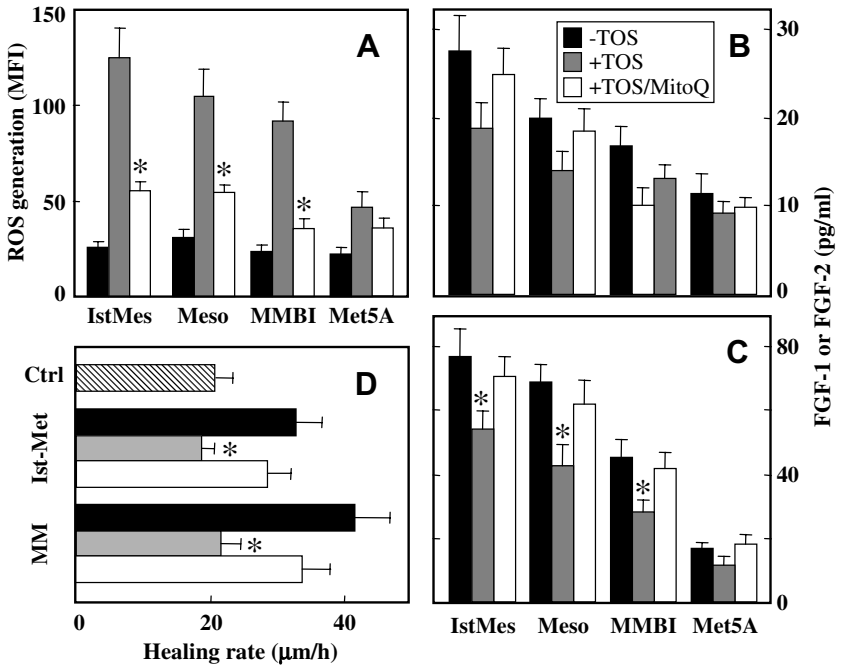

Fig. 4. α-TOS suppresses FGF2 secretion by cancer cells due to oxidative stress. Ist-Mes2, Meso-2, MM-BI, and Met-5A cells were seeded in 24-well plates and exposed to 25 μM α-TOS for 3 (A) or 24 h (B, C), following a 30-min pre-treatment, as shown, with 2 μM MitoQ. The cell cultures were then assessed for generation of ROS using the DCF assay (A) and for FGF1 (B) and FGF2 (C) protein as detailed in Section 2. Panel D reveals wound healing of EAhy926 cultures following, as shown, addition of conditioned media from Ist-Mes2 or MM-BI cells treated for 24 h with α-TOS following 30-min pre-treatment with 2 μM MitoQ.

stimulate proliferation of ECs in the co-culture experiments. We next tested the effect of hrFGF1 and hrFGF2 on ECs and found very little effect of FGF1, while FGF2 enhanced EC proliferation by 50% and wound healing by more than 100% (data not shown).

Recent data document that α-TOS suppresses expression of FGF1 and FGF2 by causing oxidative stress in MM cells, resulting in generation of mitochondria-derived superoxide [10]. This is confirmed in Fig. 4, showing that accumulation of ROS in MM cells as a response to α-TOS was suppressed by pre-treatment with MitoQ. Met-5A cells did not respond to the VE analogue by ROS accumulation, which may explain their lack of FGF1/2 down-regulation upon stimulation with α-TOS. MitoQ also restored the levels of secreted FGF1 and FGF2 by MM cells, suppressed by α-TOS, and inhibited angiogenesis in vitro promoted by MM cell-conditioned media and reversed the inhibitory effect of α-TOS on the wound healing-promoting activity of MM cell-conditioned media.

To confirm the role of FGF2 paracrine signalling on EC proliferation and angiogenesis in vitro, we treated MM cells with FGF2 siRNA. Fig. 5A reveals that this resulted in much lower secretion of FGF2 by the cells, similarly as observed for FGF1 using FGF1 siRNA. Media from FGF2 siRNA (but not FGF1 siRNA)-treated cells suppressed the enhancing effect of MM cell-conditioned media on EC proliferation and angiogenesis in vitro.

In this communication we report on a novel activity of an anti-cancer agent, α-TOS, which is presented by its anti-angiogenic effect due to disruption of paracrine signalling. This work follows our previous paper showing suppression of FGF2 expression by α-TOS, whereby inhibiting the autocrine proliferative signalling of MM cells [10]. The VE analogue exerted its effect by promoting generation of ROS by the cells, which resulted in inhibition of the activity of the transcription factor egr-1 that controls expression of FGF2 [10,17].

As FGF2 is an important angiogenic factor [3,12], we expected that down-regulation by α-TOS of the cytokine in cancer cells may result in inhibition of EC proliferation. We observed this and, moreover, it appears that the effect is due to generation of ROS by the VE analogue in MM cells. This is documented using MitoQ that is known to inhibit oxidative stress by accumulating in the mitochondrial inner membrane, its redox-active head-group reaching the coenzyme Q binding site(s) in complex II [18]. We have recently documented that ROS are generated by cancer cells in response to α-TOS due to displacement of coenzyme Q from complex II by the VE analogue [19]. This is likely the reason for the toxicity of the agent, including modulation of the transcriptional activity [10,11], and this can also explain the novel activity of α-TOS reported here.

Malignant mesothelioma is an exceedingly difficult type of cancer, resisting established treatments. Novel strategies are being sought, including immunotherapy and gene therapy [20]. Inhibition of angiogenesis appears to be a viable mode of mesothelioma suppression, and small anti-angiogenic agents are currently trialed [20]. Our work suggests that α-TOS is of substantial promise to be used as anti-mesothelioma drug, since it suppresses MM by at least several independent mechanisms. These include direct induction of apoptosis in MM cells [21], inhibition of MM cell proliferation by disrupting the paracrine signalling pathway [10,11], and suppression of the angiogenic paracrine FGF2 signalling reported here.

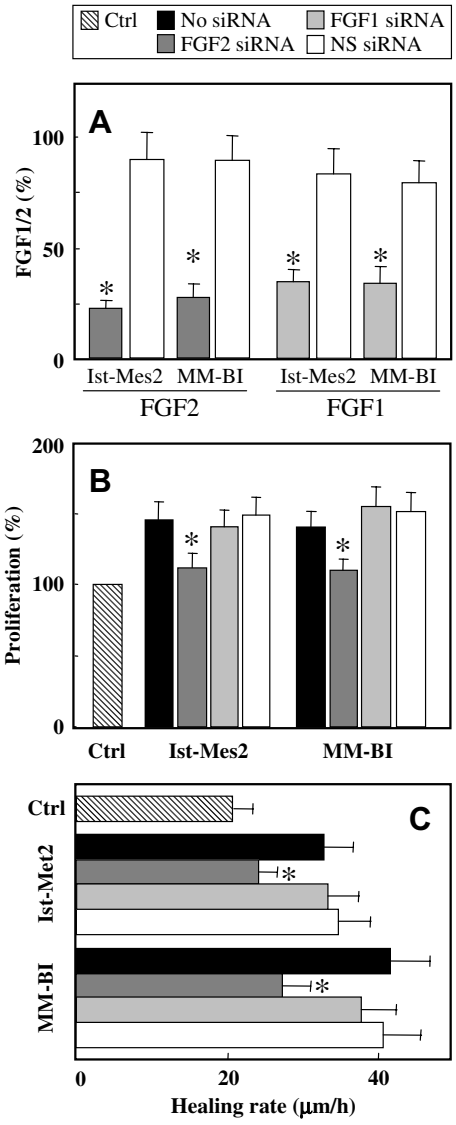

Fig. 5. Down-regulation of FGF2 suppresses the angiogenesis stimulatory effect of cancer cells. Ist-Mes2 or MM-BI cells were treated, as shown, for 48 h with FGF2 or FGF1 siRNA as well as non-silencing siRNA, and assessed for the level of FGF2 and FGF1 (with FGF levels of control cells set at 100%) (A). EAhy926 cells were seeded so that they acquired 50% confluency (B), or in 35-mm Petri dishes, allowed to reach confluence and subjected to injury as detailed in Section 2 (C). The effect of media conditioned with Ist-Mes2 and Meso-2 pre-treated with the siRNA as shown on proliferation (B) and wound healing (C) was assessed.

Acknowledgements: The authors thank Prof. R. Smith for donation of MitoQ. This work was supported in part by Grants from the Australian Research Council, the Queensland Cancer Fund and the Grant Agency of the Czech Republic as well as the Grant Agency of the Academy of Sciences of the Czech Republic and the Institutional Research Concept AV0Z50520701.

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