Effects of α-difluoromethylornithine on thrombospondin-1 production by human breast cancer cells

ANDREA MANNI[1], TERRY RAGER[2], SCOT R. KIMBALL[2], LEONARD S. JEFFERSON[2], SHARLENE WASHINGTON[1], XIN HU[1] and MICHAEL F. VERDERAME[1]

Departments of [1]Medicine and [2]Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, Hershey, PA, USA

Abstract. We have previously observed that inhibition of polyamine biosynthesis with α-difluoromethylornithine (DFMO) upregulates production of thrombospondin-1 (TSP-1), an extracellular matrix protein with potent anti-angiogenic and antimetastatic properties, by MDA-MB-435 human breast cancer cells in culture. The present experiments were designed to investigate the mechanisms by which DFMO regulates TSP-1 production in this system. ^{35}S-methionine pulse chase experiments indicated that DFMO administration increased TSP-1 synthesis by approximately 6-fold, while it slightly but significantly decreased protein half-life from 35 to 28 min. DFMO treatment increased steady state TSP-1 mRNA levels by 2-fold in MDA-MB-435 cells. TSP-1 promoter reporter studies indicated that this increase was largely due to activation of transcription. Analysis of distribution of TSP-1 mRNA levels between non-polysomal, subpolysomal and polysomal fractions in control and DFMO-treated cells suggested a major stimulatory effect of the drug on TSP-1 translation. A similar increase in TSP-1 transcription and translation in response to DFMO treatment was also observed *in vivo* in MDA-MB-435 breast cancer xenografts. Surprisingly however, we failed to detect an increase in TSP-1 protein as assessed by Western blot analysis. The reason for this unexpected finding is unknown but may be due to DFMO-induced stimulation of TSP-1 secretion into the systemic circulation, thus preventing its accumulation within the tumor.

Introduction

We have observed that inhibition of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine bio-synthesis, with α-difluoromethylornithine (DFMO) inhibits proliferation and *in vitro* invasiveness of breast cancer cells (1). More importantly, we have shown that DFMO administration to nude mice carrying MDA-MB-435 xenografts decreased by 80% the number of mice with pulmonary metastasis and by >90% the number of metastases per mouse (2). Furthermore, following intracardiac injection of GFP-tagged MDA-MB-435 cells, DFMO treatment significantly decreased the incidence of bone metastasis by 37% and the area of bone occupied by tumor by 65% (3). Our studies have revealed a complex interaction between ODC and multiple signal transduction pathways as well as cell cycle events which are likely related to the invasive and metastatic properties of breast cancer (3,4). Among the multiple effects of DFMO we have shown that induction of MAPK activation is instrumental in its anti-invasive action since the anti-invasive effects of the drug could be reversed with the MEK inhibitor PD98059 (5). Furthermore, we have shown that DFMO-induced MAPK activation is causally linked to upregulation of TSP-1 (5), an extracellular matrix glycoprotein with tumor suppressive and antimetastatic activity primarily resulting from its potent anti-angiogenic effect (6-9). Because of its possible role as mediator of the anti-metastatic effect of DFMO in breast cancer, we deemed it important to investigate the mechanisms of regulation of TSP-1 production by DFMO under both *in vitro* and *in vivo* conditions.

Materials and methods

Cell line and culture conditions. The MDA-MB-435 human breast cancer cell line was kindly provided by Dr Janet E. Price at the University of Texas, M.D. Anderson Cancer Center, Houston, TX. The cells were cultured in DMEM/F12 medium supplemented with 5% fetal bovine serum, 1% non-essential amino acids, 1 mM sodium pyruvate, and maintained at 37˚C with 5% CO_2 in a humidified atmosphere. The cells were passaged using 0.125% trypsin, 2 mM EDTA in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline.

^{35}S-methionine pulse-chase experiment. MDA-MB-435 cells were plated in 6-well plates. Following overnight incubation, DFMO (1 mM) was added to half of the wells. After incubation for 72 h, the cells were cultured in methionine-free, cysteine-

Correspondence to: Dr Andrea Manni, Division of Endocrinology, H044, Hershey Medical Center, Pennsylvania State University College of Medicine, 500 University Drive, P.O. Box 850, Hershey, PA 17033, USA
E-mail: amanni@psu.edu

free, and serum-free DMEM for 30 min and were then pulsed with Trans[35]S-label (200 μCi/ml) (Amersham Biosciences) for 10 min. After washing, the cells were chased for 0, 30, 60, 90, and 120 min and harvested. Equal amounts of total proteins from control and DFMO-treated cells were immuno-precitated with an anti-human TSP-1 antibody. The immuno-precipitates were then fractionated by SDS-PAGE. The gels were dried, and the band of labeled TSP-1 was quantified with an imaging analyzer.

Quantitative real-time PCR. Total RNA was isolated from MDA-MB-435 breast cancer cells under the indicated experimental conditions. Total RNA quality for each sample was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and determined to be of high quality. Reverse transcription was carried out using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Oligo[(12-18)]-dT (Invitrogen) and random hexamer (Promega, Madison, WI) primers were used in the synthesis of cDNA and in the preparation of no-RT controls from each total RNA sample. The QuantiTect SYBR Green quantitative real-time PCR Kit (Qiagen, Valencia, CA) Q-RT-PCR was used to perform the real-time RT-PCR assays on the ABI7700 and the ABI7300 sequence detection system (Applied Biosystems, Foster City, CA). Absence of amplification product in the no-RT control reactions confirmed the absence of genomic DNA contamination in each sample, and a SYBR Green dissociation curve consisting of a single peak confirmed one amplification product for each reaction, which indicates adequate primer specificity. Thermal-cycling conditions as recommended by QuantiTect in the SYBR Green real-time PCR Kit protocol were followed (annealing temperature was 53˚C). Standard curve quantitation and normalization were performed using 18S rRNA as an endogenous control. TSP-1 forward primer sequence is 5'-TGG AAC TAT GGG CTT GAG AAA-3', and TSP-1 reverse primer sequence is 5'-CAC TGA TGC AAG CAC AGA AAA-3'. Primers for 18S rRNA were used as provided in the QuantmRNA Classic 18S Internal Standard Kit (Ambion, Inc., Austin, TX).

TSP-1 promoter reporter assay. MDA-MB-435 cells were plated in 12-well plates and were cotransfected the next day with 1.5 μg of TSP-1 promoter reporter luciferase plasmid (kindly provided by Dr S. Cohn at Northwestern University) and 100 ng of pRL-CMV (Promega, serving as transfection control), by Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection the cells were treated with or without 1.0 mM DFMO. After an additional 72 h the cells were harvested and luciferase activity was measured with the Dual Luciferase Assay (Promega).

Methylation specific PCR. Genomic DNA in the cells was isolated with Dneasy Tissue Kit (Qiagen). The DNA was modified by sodium bisulfite with the CpGenome DNA Modification Kit (Serologicals). Special primers for MSP-PCR were designed according to the instruction of the Modification kit. PCR was performed using modified DNA as template to distinguish methylated TSP-1 promoters from unmethylated ones.
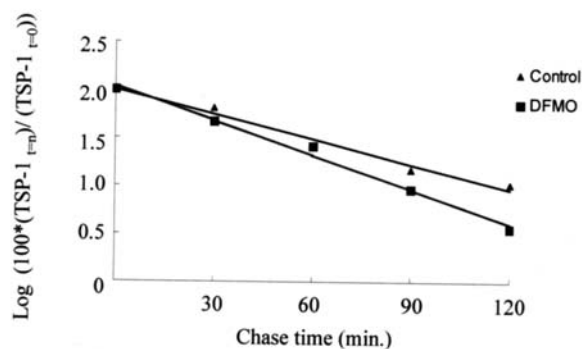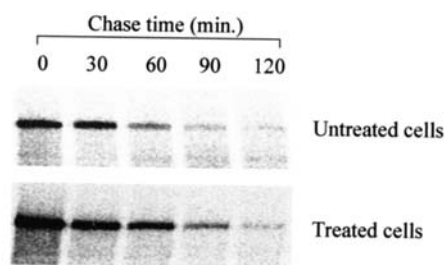

Figure 1. Effect of DFMO on TSP-1 synthesis and half-life. Control and DFMO treated (48 h) MDA-MB-435 cells were pulsed for 10 min with [35]S (200 μCi/ml) and then chased for the indicated times. Please see Materials and methods for experimental details.

TSP-1 translational regulation. Non-polysomal, subpolysomal, and polysomal fractions were isolated by sucrose density centrifugation (20-47%) from control and DFMO treated (1 mM for 72 h) MDA-MB-435 breast cancer cells. Following reverse transcription of the RNA isolated from the different fractions, quantitative real-time PCR was performed to quantitate TSP-1 mRNA levels. The amount of TSP-1 mRNA in each fraction was expressed as percent of the total TSP-1 mRNA in the three fractions combined.

Western blot analysis. TSP-1 expression in cell lysates and conditioned media of MDA-MB-435 cells was assessed by Western blot analysis as previously reported (5).

Statistical analysis. Statistical significance was determined by two-tailed t-test. The difference in TSP-1 half-life between control and DFMO-treated cells was assessed by a linear regression model for comparing slopes.

Results

Regulation of TSP-1 production by DFMO in vitro. First, we performed a pulse-chase experiment with ^{35}S-methionine to determine the effects of DFMO on the synthesis and half-life of TSP-1. Using a 10 min pulse with ^{35}S-methionine, we observed that DFMO treatment increased TSP-1 synthesis by ~6-fold (Fig. 1, time 0). Of interest, the chase experiment, also depicted in Fig. 1, showed that DFMO modestly but significantly (p=0.018) reduced the half-life of TSP-1 from 35 to 28 min.

Next, we tested the transcriptional control of TSP-1 by DFMO. Using quantitative real-time PCR, we found that an increase in steady-state mRNA level only accounts for a ~2-fold induction of the protein by DFMO (Fig. 2). This
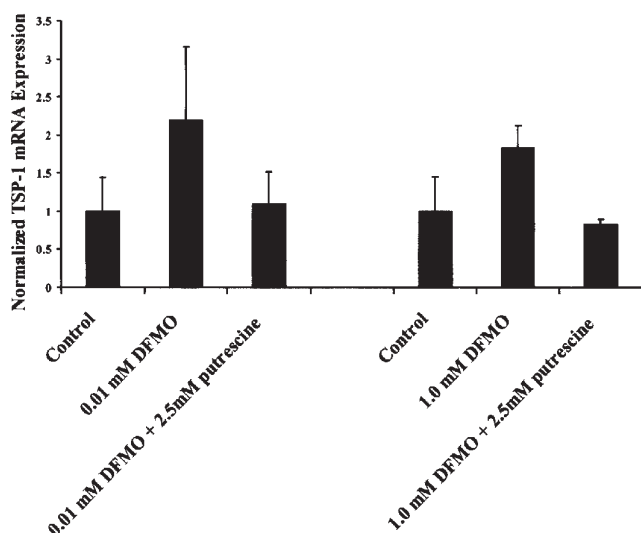
Figure 2. Effect of 48-h treatment with DFMO at two different concentrations (0.01 and 1.0 mM) with and without putrescine rescue on TSP-1 mRNA levels of MDA-MB-435 cells assessed by quantitative real-time PCR (see Materials and methods for experimental details). Data represent means ± SD. After 72 h of treatment, the stimulatory effect of DFMO on TSP-1 mRNA was reduced to 1.5-fold (data not shown). This reduction occurred despite a further increase in DFMO-induced stimulation of TSP-1 protein at 72 h compared to the 48-h time-point (see Fig. 3).
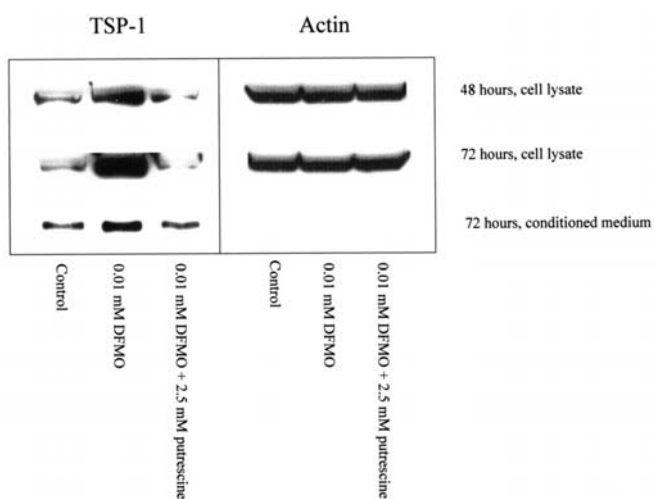

Figure 3. Effect of 48- and 72-h treatment with DFMO with and without putrescine rescue on TSP-1 protein expression in the cell lysates and conditioned media of MDA-MB-435 cells determined by Western blot analysis. This is part of the same experiment depicted in the left panel of Fig. 2 (testing the effect of 0.01 mM DFMO).
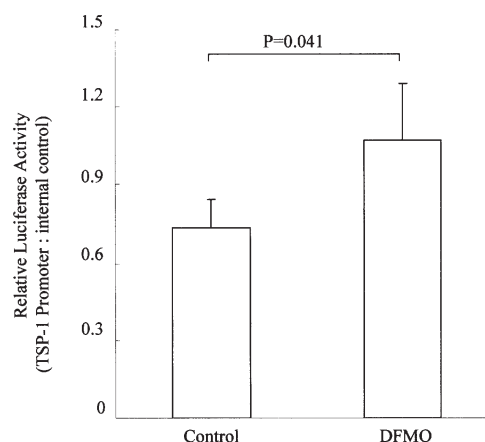

Figure 4. Effect of DFMO on transcription of TSP-1 in MDA-MB-435 cells assessed by TSP-1 promoter reporter assay. See Materials and methods for experimental details. Bars represent means (n=3) ± SD.
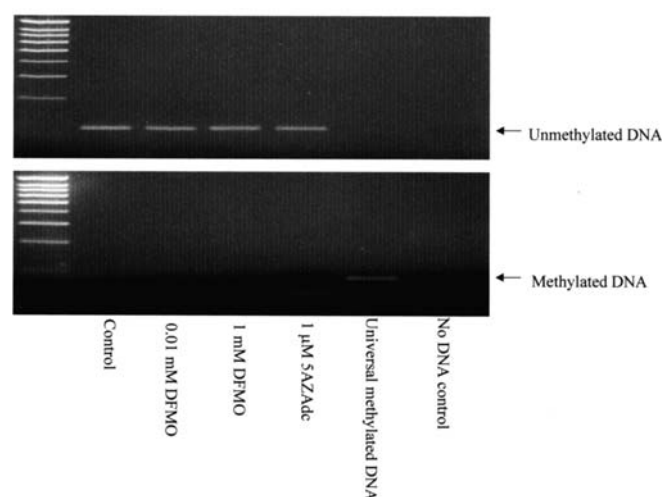

Figure 5. TSP-1 promoter methylation status of MDA-MB-435 cells untreated and treated with DFMO (0.01 and 1.0 mM or 1 μM 5AZAdc (an inhibitor of methyltransferase activity). MSP-PCR was performed as described in Materials and methods.

effect of DFMO was completely reversed by exogenous putrescine administration, thus attesting to its specificity through the polyamine pathway. In contrast, DFMO had a much stronger stimulatory effect on TSP-1 protein, simultaneously tested in the same experiment (Fig. 3). The effect of DFMO on TSP-1 protein was also entirely reversed by the addition of putrescine. TSP-1 promoter-reporter assays demonstrated that the increase in message was, at least in part, due to induction of transcriptional activation by DFMO (Fig. 4). Based on evidence provided in neuroblastoma cells that TSP-1 transcription can be affected by changes in promoter methylation (6), we tested this possible level of regulation by methylation-specific PCR. As can be seen in Fig. 5, DFMO did not affect the degree of methylation of the TSP-1 promoter, which was actually totally unmethylated under control conditions.

The modest effect of DFMO on transcription compared to the robust induction of protein synthesis raised the possibility that a significant component of the DFMO effect may be at the translational level. As can be seen in Fig. 6, DFMO administration caused a significant shift (~4-fold) in TSP-1 mRNA distribution from the sub-polysomal to the polysomal fractions, consistent with a major translational effect of the drug. In the aggregate, these results indicate that DFMO increased TSP-1 production by MDA-MB-435 breast cancer cells *in vitro* both transcriptionally and translationally, although the latter effect seems to predominate.
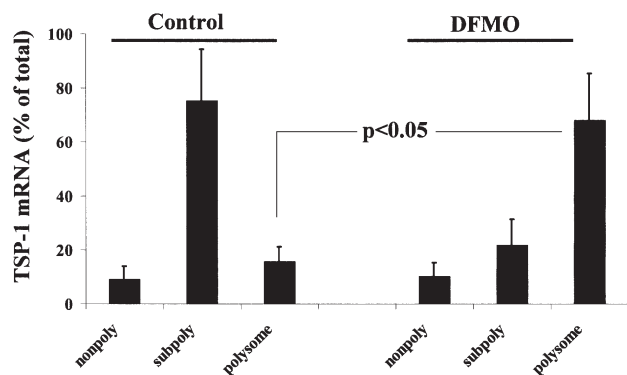
Figure 6. Effect of DFMO (1 mM for 72 h) on translational regulation of TSP-1 (see Materials and Methods for experimental details). Bars represent means ± SD from 5 control and 5 DFMO treated samples, each run in quadruplicates.

Regulation of TSP-1 production by DFMO in vivo. In these experiments, we utilized tumors from control and chronically treated nude mice carrying MDA-MB-435 xenografts. These mice are part of experiments recently reported by us (2). As can be seen in Fig. 7, DFMO treatment caused a significant increase in both transcription and translation of TSP-1. However, despite these concordant effects, we were unable to detect an increase in TSP-1 protein expression in the tumors of DFMO-treated mice by Western analysis (data not shown).

Discussion

Evidence from our and other laboratories has indicated that activation of the polyamine pathway plays an important role in several aspects of breast cancer biology ranging from tumorigenesis to cell proliferation and tumor progression to a more aggressive and hormone-independent phenotype (reviewed in ref. 7). Of clinical relevance, increased ODC activity in primary tumors has been found to be associated with adverse prognostic features (8,9). More importantly, we (10) and subsequently other investigators (9) have shown that high tumor ODC activity is an independent, adverse prognostic factor for disease-free and overall survival in patients with localized breast cancer. This finding suggests that increased ODC activity may be important in the development of metastasis and may be therefore an attractive target for adjuvant therapy.

We have extensively tested the antimetastatic effect of DFMO in MDA-MB-435 cells and have shown that administration of this inhibitor of ODC markedly reduces both pulmonary and bone metastasis (1-3). In this system, DFMO-induced MAPK activation has been shown by us to be causally linked to its anti-invasive effect and to its ability to stimulate the extracellular matrix protein thrombospondin-1 (5). Several lines of evidence indicate that TSP-1 may have a metastasis-suppressive effect in breast cancer. First, TSP-1 production has been shown to be higher in the tumorigenic but not metastatic MCF-7 and T47D cell lines compared to the metastatic MDA-MB-435 line (11). Secondly, induction of TSP-1 overexpression in MDA-MB-435 cells has been shown to reduce their metastatic capacity (12). Thirdly, TSP-1 expression in ductal carcinoma *in situ* was found to be correlated with markers of better differentiation and features associated with lower probability of invasive carcinoma (13). Therefore, it is possible that induction of TSP-1 by DFMO may be at least one of the mechanisms by which the drug reduces the invasive and metastatic capacity of MDA-MB-435 breast cancer cells. This hypothesis will need to be rigorously tested by silencing TSP-1 expression and determining the influence of this manipulation on the metastatic phenotype of breast cancer cells in the absence and in the presence of DFMO treatment.
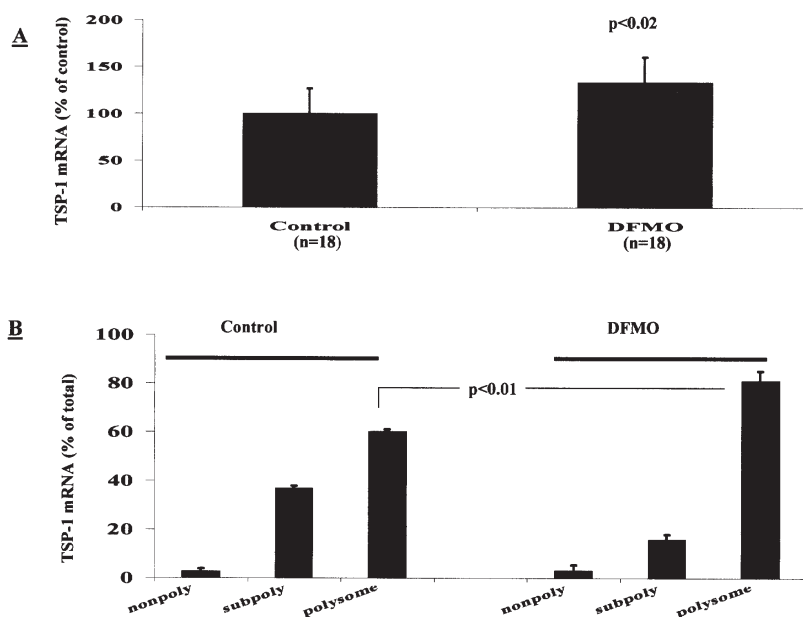

Figure 7. A, Effect of DFMO treatment (2% in drinking H_2O for 6-8 weeks) on TSP-1 mRNA levels *in vivo* assessed by Q-RTPCR. TSP-1 mRNA levels were normalized to 18S RNA expression. B, Effect of DFMO on TSP-1 translation *in vivo*. Non-polysomal, subpolysomal, and polysomal fractions were isolated from control and DFMO-treated tumor xenografts. TSP-1 mRNA levels in each fraction were measured and expressed as described in the legend to Fig. 6. Bars represent means ± SD of quadruplicate determinations from one control and two DFMO-treated tumors.

The present experiments were designed to test in detail the levels of regulation of TSP-1 by DFMO in MDA-MB-435 cells, both *in vitro* and *in vivo*. In agreement with our Western blot analysis data reported herein (Fig. 3) and previously (5), the ^{35}S-methionine labeling study showed that DFMO increased TSP-1 production *in vitro* by 6-fold. The effect of the drug appears to be mostly translational although an increase in transcription was also observed. Despite similar effects on transcription and translation *in vivo*, we failed to identify an increase in TSP-1 protein expression following chronic DFMO treatment. This is in agreement with the lack of immunohistochemical detection of an increase in TSP-1 protein in the same tumors recently reported by us (2). The reason for this unexpected finding is at present unknown. It is possible that DFMO may increase the secretion of TSP-1, possibly into the systemic circulation, thus preventing its accumulation within the tumor. This possibility is suggested by the decrease in half-life of the TSP-1 by DFMO observed in the pulse-chase experiments, although this finding could also reflect increased degradation.

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