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Cellular mechanisms mediating the anti-invasive properties of the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) in human breast cancer cells

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Abstract

We have shown that inhibition of polyamine biosynthesis with α-difluoromethylornithine (DFMO) reduces *in vitro* invasiveness and metastatic capacity of MDA-MB-435 breast cancer cells. These experiments investigated the mechanisms mediating the anti-invasive properties of DFMO. DFMO did not affect phosphorylation of FAK or Akt, but increased ERK phosphorylation by approximately threefold. To test the biologic significance of this finding, we tested the effect of the MEK inhibitor PD98059 on *in vitro* invasiveness of MDA-MB-435 breast cancer cells, both in the absence and in the presence of the proinvasive peptide hepatocyte growth factor (HGF) as a chemoattractant. We observed that PD98059 treatment reversed the anti-invasive effect of DFMO under both experimental conditions. Next, we tested the influence of DFMO on the production of the prometastatic peptide osteopontin (OPN) and the anti-metastatic protein thrombospondin-1 (TSP-1). DFMO treatment, while not affecting OPN production, markedly increased the TSP-1 level in the conditioned media. This effect was abolished by putrescine administration, thus indicating the specificity of the DFMO action through the polyamine pathway. PD98059 completely blocked the stimulatory effect of DFMO on TSP-1 production, which supports a mediatory role for activation of the MAPK pathway in the upregulation of this anti-metastatic peptide by DFMO. In summary, our results show that the increase in ERK phosphorylation induced by DFMO plays a critical role in the anti-invasive action of the drug and in its ability to upregulate TSP-1 production.

Abbreviations: ODC – ornithine decarboxylase; DFMO – α-difluromethylornithine; ERK – extracellular signal-regulated kinase; MAPK – mitogen-activated protein kinase; MEK-1 – MAP/ERK kinase-1; OPN – osteopontin; TSP-1 – thrombospondin 1; HGF – hepatocyte growth factor; FAK – focal adhesion kinase

Introduction

Evidence in the literature indicates that polyamines (putrescine, spermidine and spermine) play an important role in multiple aspects of breast cancer biology, including hormone-dependent and independent growth [1, 2], as well as tumor progression [3]. We [4], and other investigators [5], have shown that elevated tumor levels of ODC activity, the first and rate-limiting enzyme in polyamine biosynthesis, are associated with reduced overall survival in women with localized disease. These data indicate that activation of the polyamine pathway may induce a more aggressive and metastatic breast cancer phenotype, since metastases represent the ultimate cause of death in virtually every woman dying of breast cancer.

To investigate the role played by polyamines in breast cancer metastasis, we have recently turned to the hormone-independent MDA-MB-435 human breast cancer cell line, which is highly invasive *in vitro* and metastatic when orthotopically implanted in the mammary fat pads of athymic nude mice [6, 7]. Using these experimental systems, we have reported that administration of DFMO, an irreversible inhibitor of ODC, significantly reduced *in vitro* invasiveness as well as the development of pulmonary metastasis in nude mice carrying tumor xenografts [6, 7].

The present experiments were designed to gain insight into the mechanisms mediating the anti-invasive action of DFMO *in vitro*. The specificity of the

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DFMO effect through the polyamine pathway was assessed by evaluating the reversibility of its effect by exogenous putrescine administration. We tested the influence of DFMO treatment on the activation of multiple signal transduction pathways in the absence and in the presence of OPN and HGF, two peptides shown to promote the invasive properties of MDA-MB-435 breast cancer cells [8–10]. Finally, we determined the effect of DFMO on the production of OPN and TSP-1, the latter being found to reduce the metastatic capacity of this cell line [11]. In the aggregate, our results point to the activation of the MAPK pathway as a mechanism mediating the anti-invasive effect of DFMO possibly through increased production of the anti-metastatic protein, TSP-1.

Materials and methods

Reagents

Bacteria carrying an expression plasmid encoding a GST-human OPN fusion protein was the generous gift of Dr. Ann Chambers (London Regional Cancer Centre, London, Ontario, Canada). Recombinant human OPN was purified, as described [12]. DFMO was a generous gift from ILEX Oncology, San Antonio, Texas. HGF was purchased from Chemicon International, Temecula, California; the MEK inhibitor compound PD98059 was from Calbiochemi, La Jolla, California, and putrescine from Sigma Chemical Co., St. Louis, Missouri. The following antibodies (Ab) were used for Western analysis: FAK (#sc-932), PhosphoFAK (Tyr925) (#sc-11766), ERK2 (#sc-153), and Akt (#sc1619) abs were all obtained from Santa Cruz Biotechnology, Santa Cruz, California. Phospho FAK (Tyr 397) ab (#44-624) was purchased from Biosource, Camarillo, CA; PhosphoMAPK ab (V803A) was from Promega Corp., Madison, Wisconsin, and Phospho Akt ab (Ser473) (#9271S) from Cell Signaling Technology, Beverly, Massachusetts. The antibody against OPN (#MAB3057) was purchased from Chemicon International, Temecula, California, and the antibody against TSP-1 (#610916) was obtained from Transduction Laboratories, Lexington, Kentucky.

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/F-12 medium supplemented with 5% fetal bovine serum, 1% non-essential amino acids, 1.0 mM sodium pyruvate and maintained at 37°C with 5% CO_2 in a humidified atmosphere. Cells were passaged using 0.125% trypsin, 2 mM EDTA in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline.

Invasion assay

Matrigel basement membrane matrix (Becton Dickson, Bedford, Massachusetts) was dried onto 25 mm Transwell Polycarbonate Membranes (8.0 μm pore, Corning Costar Corp, Cambridge, Massachusetts) then reconstituted at 37 °C with serum-free DMEM/F-12 containing 0.1% BSA. One $\times 10^6$ cells resuspended in the same medium were added to each filter in the presence of the various treatments indicated in the figures. Chemoattractants in the lower chamber were either BSA, OPN, HGF, or HGF plus OPN as shown in the figures. Incubation was carried out under serum-free media conditions for 48 h in a humidified incubator at 37 °C in 5% CO_2/95% air. The cells, which had traversed the matrigel and spread to the lower surface of the filter, were stained with Diff-Quik (Dade Behring, Inc., Newark, Delaware). The filters were removed and mounted on microscope slides. Cells from 10 randomly selected fields/ filter were counted and averaged.

Western analysis

Cell lysates were prepared with extraction buffer (33 mM Sorenson's phosphate buffer pH 7.2, 1% NP-40, 200 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin-A, 2 μg/ml antipain, 1 mM Na_3VO_4, 1 mM NaP_2O_7, 10 mM NaF and 1 μM okadaic acid). Fifty μg of cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose and blotted with antibodies that recognize either the native or the phosphorylated forms of the various peptides. The signals were detected with ECL (Pierce Pico Super Signal) following incubation with horseradish peroxidase-conjugated secondary antibodies. A Coomassie blue staining was always performed to document equal protein loading.

Statistical analysis

Data from invasion assay experiments were analyzed using the mixed-effects linear model approach incorporating a random effect component for experiments conducted on different days and a fixed effect component to assess treatment differences. Statistical significance for differences between specific treatments was determined by post-hoc pairwise comparisons with *P*-values adjusted for multiple testing using the Tukey–Kramer method. Densitometric data were analyzed by calculating relative difference from control and then using the one-sample *t*-test to assess whether the mean relative difference was significantly different from 100%. All analyses were carried out using the SAS statistical software system, Version 8.1. The type-I error rate was set at 5% for determining statistical significance.

Results

Reversibility of the DFMO effect on in vitro *invasion by exogenous putrescine*

We have previously shown that DFMO treatment blocks the invasiveness of MDA-MB-435 breast cancer cells in matrigel [6]. Here, we tested the reversibility of this effect by exogenous putrescine to document the specificity of its action through the polyamine pathway. In these experiments, we evaluated the effect of DFMO with and without putrescine in the presence of OPN and/or HGF as chemoattractants. As can be seen in Figures 1a–c, OPN and HGF, either alone or in combination, significantly stimulated invasiveness of MDA-MB-435 cells, although to a modest degree. Under all experimental conditions, DFMO treatment markedly suppressed invasiveness to levels significantly below control. In all cases, exogenous putrescine administration completely reversed the anti-invasive effect of DFMO.

DFMO effects on cell signaling

These experiments were designed to gain insight into the cellular mechanisms mediating the anti-invasive properties of DFMO. The effect of DFMO on the activation status of different signaling proteins in the absence and in the presence of HGF and OPN was determined by Western analysis using antibodies which recognize the phosphorylated (i.e., active) form of the peptides. As can be seen in Figure 2, FAK was found to be activated under basal conditions (e.g., after 24 h step down in serum-free media) using two phospho-specific antibodies against tyrosine 397 and 925. None of the treatments modified this level of basic activity. HGF markedly stimulated Akt phosphorylation but its effect was not influenced by DFMO treatment. DFMO, on the other hand, seemed to induce an increase in ERK phosphorylation (Figure 2). To quantitate the effect of DFMO, we performed densitometric analysis of six replicate experiments. As can be seen in Figure 3, DFMO treatment increased phosphorylation of ERK-1 and ERK-2 by approximately threefold, although only the effect on ERK-2 phosphorylation reached statistical significance ($P < 0.03$). When the densitometric data on phospho-ERK-1 and -ERK-2 were combined, the stimulatory effect of DFMO on total phospho-ERK levels was statistically significant ($P < 0.03$).

Effect of PD98059 on in vitro *invasion*

To investigate the physiologic relevance of ERK activation by DFMO, we examined the influence of the MEK inhibitor PD98059 on the anti-invasive action of DFMO. These experiments were conducted both in the presence (Figure 4a) and in the absence (Figure 4b) of HGF as a chemoattractant. As can be seen in Figure 4a, in agreement with the experiments shown in Figure 1, HGF significantly stimulated invasion, although to a modest degree ($P < 0.01$). Administration of PD98059 did not effect this action of HGF. In contrast, DFMO treatment significantly reduced invasiveness ($P < 0.01$), in agreement with our previous data. Remarkably, treatment with PD98059 reversed the DFMO effect ($P = 0.001$ vs. HGF + DFMO) and restored invasiveness to a level which was not significantly different from either control or HGF treatment alone (Figure 4a). In the

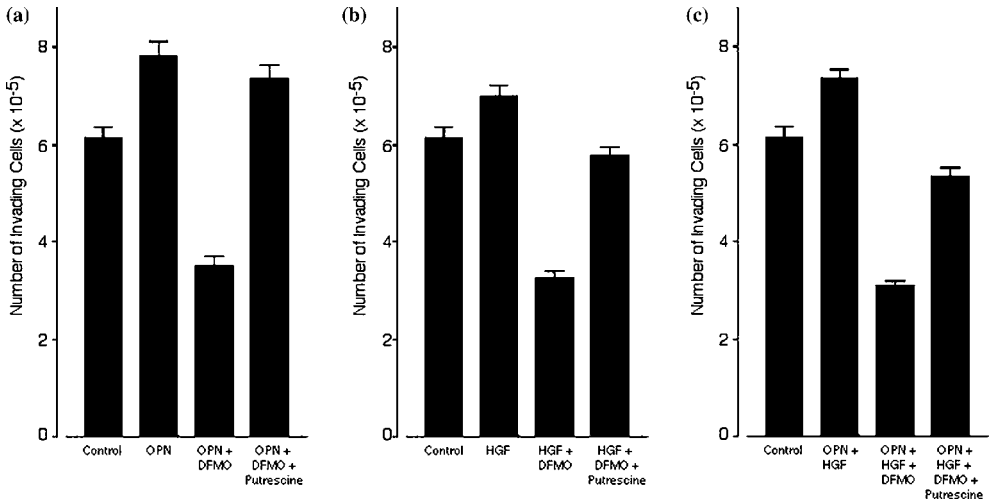

Figure 1. Reversibility by exogenous putrescine of DFMO effect on invasiveness in matrigel of MDA-MB-435 cells. Following 48 h pretreatment in regular medium with DFMO (1 mM) with and without putrescine (2.5 mM), MDA-MB-435 cells were plated in the matrigel assay under serum-free media conditions in the presence of 0.1% BSA plus OPN (50 μg/ml) (a), HGF (20 ng ml) (b) or OPN plus HGF (c) as chemoattractants in the lower chamber. 0.1% BSA alone was used as chemoattractant in control cells. Two filters per experimental conditions were used. Cells from 10 randomly selected fields per filter were counted and averaged. Bars represent means ± SEM. Statistical analysis (ANOVA): Control was significantly different from OPN ($P < 0.001$), HGF ($P < 0.05$), and OPN plus HGF ($P < 0.001$). OPN + DFMO, HGF + DFMO, and OPN + HGF + DFMO were all significantly different ($P < 0.001$) from the rest of the groups within their respective panels.

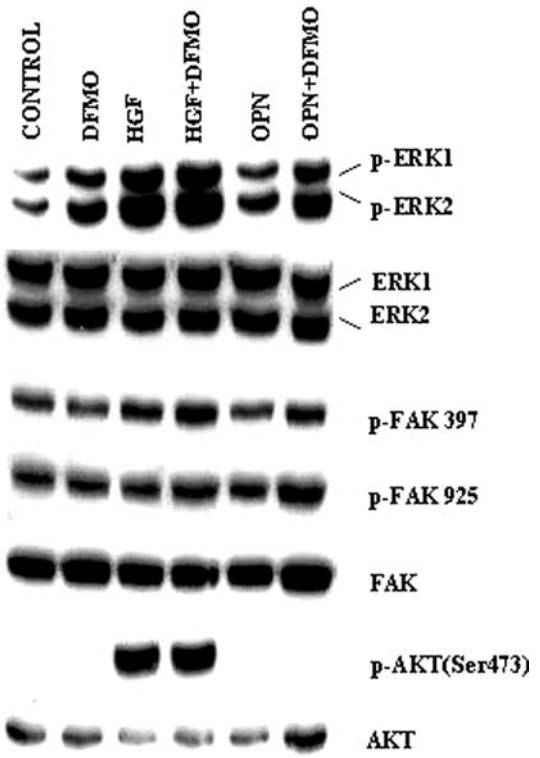

Figure 2. DFMO effect on cell signaling. MDA-MB-435 breast cancer cells were cultured in regular medium for 24 h in the absence or presence of DFMO (1 mM). The cells were then stepped down to serum-free OPTI-MEM containing the same treatments for an additional 24 h prior to addition of HGF (40 ng/ml), OPN 50 μg/ml), or vehicle for 15 min. The cells were then harvested and Western blots of cell lysates were performed (see Materials and methods) using antibodies that recognize either the native or the phosphorylated forms of the indicated peptides.

absence of HGF as a chemoattractant (Figure 4b), treatment with PD98059 alone reduced invasiveness by approximately 25% ($P < 0.03$), while it significantly reversed the anti-invasive effect of DFMO ($P < 0.001$ vs. DFMO alone), although invasion was still significantly reduced compared to control ($P < 0.01$) (Figure 4b). In parallel experiments, we observed that treatment with PD98059 (50 μM) for 1 h completely blocked ERK phosphorylation, both in the absence and in the presence of HGF ± DFMO (data not shown). Therefore, in the aggregate, these results indicate that the upregulation of ERK phosphorylation by DFMO is instrumental in mediating the anti-invasive effect of this compound.

DFMO effects on OPN and TSP-1 production

OPN and TSP-1 have been shown to exert a major positive and negative influence, respectively, on the invasive properties of MDA-MB-435 breast cancer cells [8, 9, 11]. Therefore, we deemed it important to test the effect of DFMO on the production of these peptides by these breast cancer cells. The results of these experiments are shown in Figure 5. DFMO treatment did not affect the level of OPN in the conditioned media of treated cells (Figure 5a). In contrast, it induced a marked increase in the level of TSP-1 (Figure 5b). The effect of DFMO was reversed by exogenous putrescine administration which reduced the amount of TSP-1 present in the conditioned medium to that detected under control conditions (Figure 5b).

Next, we investigated whether there was a relation between the activation of the MAPK pathway and induction of TSP-1 production by DFMO. As can be seen in Figure 6, DFMO treatment was again found to markedly increase TSP-1 level in the conditioned medium. Administration of the MEK inhibitor PD98059 completely blocked this stimulatory effect of DFMO. This finding indicates that activation of the MAPK pathway by DFMO plays a mediatory role in the upregulation of TSP-1 production by the drug.

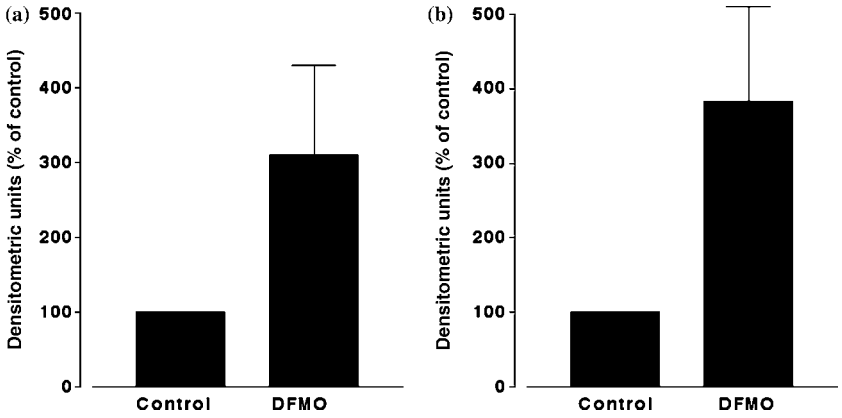

Figure 3. Densitometric quantitation of the effect of DFMO on ERK-1 (a) and ERK-2 (b) phosphorylation. MDA-MB-435 cells were cultured in the absence (control) or in the presence of DFMO (1 mM) for 48 h prior to cell harvesting. The cell lysates were subjected to Western analysis using the anti-phospho-ERK antibody. The blots were then stripped and reprobed with the antibody against native ERK. The bands were quantitated using a densitometer (Molecular Dynamics, Sunnyvale, California) and Bio-Rad Quantity One Software and the ratio of the phospho- ERK/ERK band intensity was calculated. The data (expressed as percent of control) represent the mean ± SEM of six replicate experiments.

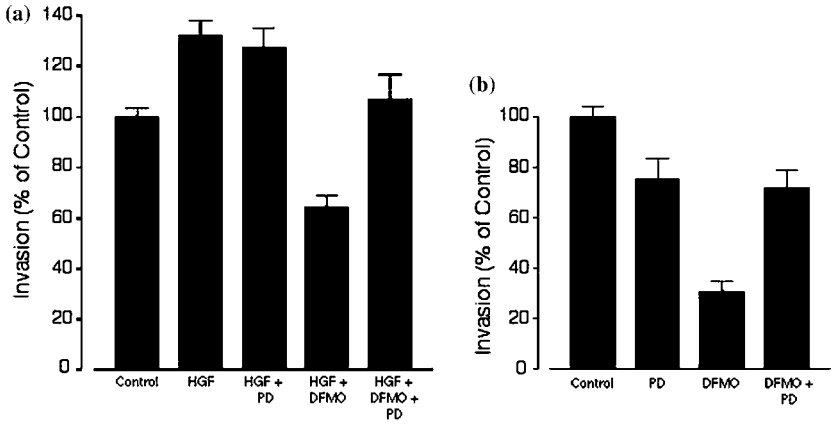

Figure 4. Effect of PD98059 (PD) on DFMO-induced suppression of invasiveness in the presence (a) or in the absence (b) of HGF (40 ng/ml) as a chemoattractant. MDA-MB-435 cells were cultured in regular medium in the absence and in the presence of DFMO (1 mM) for 48 h prior to plating in the matrigel assay described in Materials and methods. PD98059 treatment (50 μM) was added 1 h prior to plating in the matrigel assay (time found by us to be sufficient to block ERK phosphorylation) and continued for the duration of the experiment. Data are expressed as percent of control. Bars represent means $\pm$ SEM. The number of replicate experiments is five in (a) and four in panel (b). For each experiment, two filters per experimental condition were used. Cells from 10 randomly selected fields per filter were counted and averaged. See text for statistically significant differences.

Discussion

According to the American Cancer Society, approximately 200,000 new cases of breast cancer have been diagnosed in the US in 2003. Although adjuvant hormone treatment and chemotherapy have significantly improved survival [13], close to 50,000 American women die yearly from breast cancer as a result of metastasis. Therefore, there is an urgent need to develop specifically targeted treatments aimed at blocking the metastatic process, particularly in the case of hormone-independent tumors which are more frequent than hormone-dependent ones (two-thirds at the time of initial diagnosis) and are more aggressive.

Using a xenograft model of hormone-independent human breast cancer, we have recently shown that targeting the polyamine pathway may be an effective approach for reducing metastasis [6, 7]. We found that inhibition of ODC activity with DFMO significantly reduced the development of pulmonary metastasis in nude mice orthotopically implanted with MDA-MB-435 breast cancer cells.

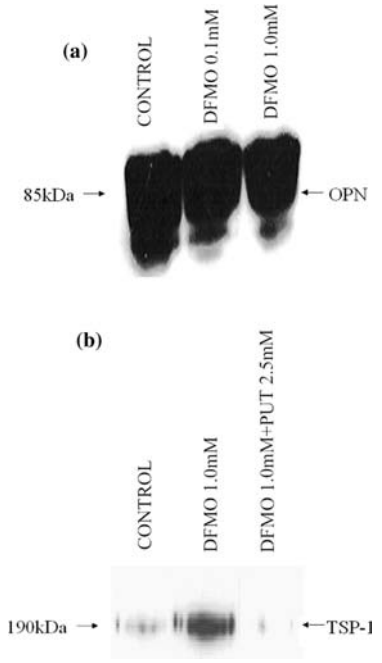

Figure 5. DFMO effect on OPN and TSP-1 production. MDA-MB-435 breast cancer cells were cultured in regular medium for 48 h in the absence (control) or in the presence of the indicated treatments. The cells were then stepped down to serum-free OPTI-MEM containing the same treatments for 24 h prior to collection of the conditioned media. The media were concentrated using Centricon-30 concentrators as per manufacturer's protocol. Following volume adjustment based upon the number of cells in the corresponding plates, the media were run on 12% SDS-PAGE, transferred to nitrocellulose and blotted with antibodies directed against OPN (a) or TSP-1 (b). Several replicate experiments gave similar results.

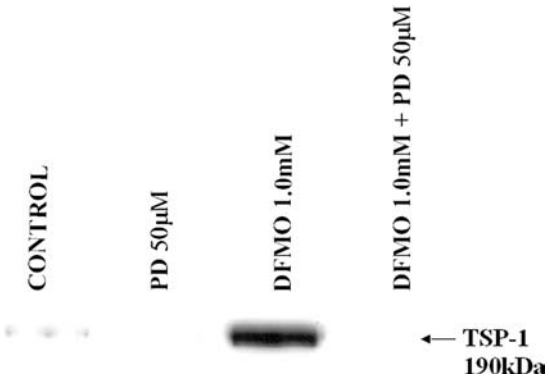

Figure 6. Effect of PD98059 (PD) on DFMO-induced stimulation of TSP-1 production. MDA-MB-435 breast cancer cells were cultured for 48 h in regular medium in the absence and in the presence of DFMO. The cells were then stepped down to serum-free OPTI-MEM containing the same treatments for 48 h. PD98059 was added 1 h prior to transition to serum-free media conditions and continued till the end of the experiment. The conditioned media were collected and processed as described in the legend to Figure 5 and subjected to Western analysis using an antibody against TSP-1.

The present experiments were designed to gain insight into the cellular mechanisms possibly responsible for the anti-metastatic effect of DFMO. Our results clearly show that DFMO treatment activates the MAPK pathway (as indicated by increased ERK phosphorylation), and that this activation mediates the anti-invasive action of DFMO, since the suppressive effect of the drug on invasion can be reversed by the MEK inhibitor compound PD98059.

The MAPK cascade plays a central regulatory role in multiple signaling pathways, and therefore, modification of its activity differentially affects several downstream events in a cell context specific fashion. For instance, depending upon the stimulus and the cell type, activation of the MAPK pathway can either lead to proliferation or differentiation[14, 15]. The connection between the polyamine and the MAPK pathways is just beginning to evolve and remains incompletely defined. Ray et al. [16] reported that treatment of intestinal epithelial cells with DFMO activated ERK-1 and especially ERK-2, concomitantly with induction of cell cycle arrest along with stimulation of p53 and cyclin-dependent kinase inhibitory proteins. However, a cause–effect relationship between activation of the MAPK pathway and inhibition of proliferation was not established. Recently, Chen et al. [17] showed that polyamine depletion in melanoma cells with the combination of DFMO and an *S*-adenosylmethionine decarboxylase inhibitor led to potent activation of ERK-1 and -2 which was associated with cell cycle arrest in G1 and G2-M but not apoptosis. Interestingly, these authors observed that treatment of melanoma cells with a polyamine analogue also induced activation of ERK-1 and -2 which was causally linked to protection from apoptosis in some, but not all melanoma cells [17]. Our results, showing activation of ERK-1 and -2 by DFMO in breast cancer cells, are in agreement with the above reports. Furthermore, we demonstrate that this activation is causally linked to the anti-invasive action of DFMO, since inhibition of ERK phosphorylation with PD98059 reversed the effect of DFMO (Figure 4). Our results also indicate that the role of the MAPK pathway in invasiveness in our experimental system depends upon the specific cellular context. Although PD98059 exerted a proinvasive effect in the presence of DFMO (Figure 4), it inhibited invasion under control conditions (Figure 4b) or had no effect in the presence of HGF (Figure 4a).

At present, these findings are difficult to reconcile with our previous report that induction of ODC overexpression in the normal but immortalized MCF10A human breast epithelial cell line leads to increased ERK-1 and -2 activation, although only in the presence of EGF or NDF[18]. Clearly, the link between polyamines and the MAPK pathway is complex and likely to vary, depending upon the cell type and the specific stimulus to which the cells are exposed.

TSP-1 is a 450-kd extracellular matrix glycoprotein which has been found to differentially affect angiogenesis in a cell context specific manner. In most cases, TSP-1 exerts a potent antiangiogenic effect which is associated with a tumor suppressive function and antimetastatic activity [11, 19–22]. This association is not surprising given the critical role of angiogenesis in tumor growth and development of metastasis. In colon cancer patients, on the other hand, high circulating levels of TSP-1 have been found to be associated with increased angiogenesis [23], thus indicating that in some tumors TSP-1 may not have a tumor inhibitory effect. In MDA-MB-435 breast cancer cells, TSP-1 appears to have both an antiangiogenic and antimetastatic effect. A 15-fold increase in TSP-1 expression has been found in a non-metastatic isogenic MDA-MB-435 cell line derived by serial dilution cloning of the parent line [24]. In addition, induction of TSP-1 overexpression in MDA-MB-435 cells has resulted in inhibition of primary tumor growth as well as pulmonary metastasis [11].

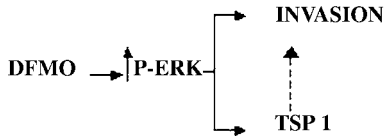

Figure 7. Diagram summarizing our results which show a mediatory role of ERK activation in the anti-invasive action of DFMO. While increased ERK phosphorylation also mediates stimulation of TSP-1 production by DFMO, the direct role of TSP-1 in suppressing invasion in our system remains to be demonstrated (dotted line).

We show here that DFMO treatment causes a marked increase in TSP-1 production by MDA-MB-435 cells. We also demonstrate that this increase is mediated by DFMO-induced ERK activation since it could be blocked by the MEK inhibitor, PD98059. It is tempting to postulate that the increase in TSP-1 mediates the anti-invasive action of DFMO. However, it will be necessary to determine whether the effect of DFMO is blocked when TSP-1 production or action is inhibited before the increase in TSP-1 can be causally linked to the anti-invasive effect of DFMO.

To the best of our knowledge, this is the first report describing at least one of the mechanisms (summarized in Figure 7) by which antipolyamine therapy may reduce the invasiveness and, possibly, the metastatic properties of hormone-independent breast cancer cells. Certainly, additional mechanisms are likely to play an important role. In addition to ERK-1 and -2, DFMO administration has been shown to activate the stress activated protein kinases, JUN-K and p38 [16, 17]. We have observed similar findings in MDA-MB-435 (unpublished observations). However, their role, if any, in mediating the anti-invasive effects of DFMO is unknown at present.

Acknowledgement

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