3-*O*-Methylfunicone, a metabolite produced by *Penicillium pinophilum*, modulates ERK1/2 activity, affecting cell motility of human mesothelioma cells

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Abstract

Objectives: 3-*O*-methylfunicone (OMF), a secondary metabolite produced by *Penicillium pinophilum*, affects cell proliferation and motility in a variety of human solid tumours. The aim of this study was to demonstrate whether OMF has the ability to arrest cell division and motility, in a human mesothelioma cell line. Malignant mesothelioma is an aggressive cancer that does not respond to standard therapies the cells of which are considered to be highly resistant to apoptosis.

Material and methods: Cell motility and invasion were measured using a modified Boyden chamber. Gene expression was examined by RT-PCR, while ERK1/2 was investigated by Western blot analysis. All experiments were also performed on primary cultures of mesothelial cells.

Results: The present study shows that OMF inhibited motility of the NCI mesothelioma cell line by modulating ERK signalling activity, and affected $\alpha V\beta 5$ integrin and MMP-2 expression, inducing marked downregulation at both mRNA and protein levels. Substantial downregulation of VEGF gene expression was also demonstrated. These effects were not observed in normal mesothelial cell cultures.

Conclusion: OMF may have potential as a naturally derived anti-tumour drug for treatment of mesothelioma.

Introduction

So called natural products, especially from plants and microorganisms, are a rich source drugs having biomedical and industrial applications (1). As a consequence of a competitive aptitude developed in many and diverse environments, microorganisms represent a considerable source of anti-tumour drugs. In particular, fungal species of the genus *Penicillium* produce a high number of biologically active extrolites that have been disclosed to have possible applications in cancer chemotherapy (2). 3-*O*-methylfunicone (OMF) is a secondary metabolite produced by the soil fungus *Penicillium pinophilum* (3) that has been found to antagonize growth of the plant pathogenic fungus *Rhizoctonia solani* (4). Previous studies carried out on cells of various tumour cell lines have demonstrated that OMF can induce the apoptotic pathway and inhibit cell motility (5–8).

Malignant mesothelioma (MM) is a highly aggressive tumour, which arises from mesothelium-lined surfaces, most often in the pleural cavities, but also of the peritoneum and pericardium. It is characterized by aggressive local spread into the pleura and the surrounding tissues, but has a low rate of distant metastasis (9–11).

Malignant invasion of tissues is a dynamic, complex and multi-step process in which proteolytic degradation of basement membranes and extracellular matrix (ECM) is an essential step favouring tumour cell migration (12). Metalloproteinases (MMPs) compose a family of zinc-dependent endopeptidases that degrade the ECM (13). In particular, gelatinases MMP2 and MMP9 are capable of degrading most of the individual ECM components that form the basement membrane. These gelatinases are therefore, essential for tumour cell migration, tissue invasion by tumour cells and metastasis (12). MMP2 expression has been reported to be characteristic of the pleural environment and has been suggested to be a predictive marker

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for poor prognosis (14–17). In addition, direct binding of MMP-2 matrix metalloprotease to $\alpha v\beta 3$ -integrins may be important in promoting localized ECM degradation and cell invasion (18).

Integrins, like MMPs, play an essential role in tumour angiogenesis, cell motility, metastasis and tissue remodeling and repair (19). Integrins are a large family of receptors that attach cells to the ECM, organize their cytoskeleton and cooperate with receptor protein kinases to regulate the fate of the cell (20). They are composed of non-covalently associated α and β subunits that heterodimerize to produce more than 20 different receptors for the extracellular matrix proteins. Integrins recognize positional cues encoded by the extracellular matrix and convert them into biochemical signals that control progression through G₁ phase of the cell cycle and promote either stable adhesion or migration in response to soluble growth factors and cytokines (21). These initial signalling events, known also as ‘outside in’ signalling, promote activation of Ras and Rho family GTPases, which in turn, influence activation of a number of intracellular signalling pathways. A second mode of integrin regulation, known as ‘inside out’ signalling, involves Ras-activated Raf-MEK-extracellular signal-regulated kinase (ERK) signalling pathway, as demonstrated for $\alpha 5\beta 1$ (22). Interestingly, it has also been demonstrated that pharmacological inhibition of MAP kinase kinase 1 (MEK1) activity leads to decreased expression of $\alpha 6\beta 3$ integrin in a number of human tumour cells (23). It has been reported that exposure to asbestos fibres is linked to development of both malignant (lung cancer, mesothelioma) and non-malignant (asbestosis) diseases, all of which involve dysregulation of cell proliferation (24). Such exposure induces protracted phosphorylation of mitogen-activated protein (MAP) kinases and ERK1/2, and increased kinase activity of ERK2 (25).

In the present study, we report that OMF can affect cell proliferation and migration of human mesothelioma cells (NCI) by modulating the ERK signalling activity. Downregulation of $\alpha V\beta 5$ integrin and MMP-2 associated to ERK-reduced activity in NCI, together with its inactivity on normal cells, may indicate OMF as a possible anti-tumour drug of natural derivation to be used in mesothelioma therapy.

Materials and methods

OMF preparation

The OMF was extracted from liquid cultures of isolate LT4 of *Penicillium pinophilum*, as described previously (3). This strain is stored in the mycological collection of the Council for Research and Experimentation in Agricul-

ture, Scafati, Italy. For biological assays, the compound was dissolved in absolute ethanol at a concentration of 10 mg/ml.

Cell culture and treatments

NCI-H2452 human mesothelioma cells (NCI) were maintained in RPMI (Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37 °C, 5% CO₂. Primary cultures of mesothelial cells (Mes1) and mesothelioma cells (Mest) were isolated and developed from pleural biopsies from two patients who were cytologically, histologically and immunohistochemically confirmed as having non-malignant and malignant pleural mesotheliomas respectively. Tissue specimens were minced and incubated in growth medium with 1:1 composition of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% foetal calf serum (Gibco BRL, Grand Island, NY, USA), penicillin (0.1 mg/ml), streptomycin (0.1 mg/ml), epidermal growth factor (10 µg/ml), insulin (5 mg/ml) and hydrocortisone (0.2 mg/ml). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 14 days to achieve 75% confluence. Mes 1 cells displayed a highly flattened cell morphology composed of tightly packed non-overlapping cells, which covered the entire surface of the culture dish following confluence. In contrast, morphology of Mest cells resembled NCI.

Mes1 and Mest cells were analysed using RT-PCR for expression of carcinoembryonic antigen (CEA, negative marker), WT1, mesothelin and calretinin. Mesothelin and calretinin are proteins strongly expressed in mesothelial cells and mesotheliomas, whereas WT1 is mainly expressed in mesothelioma cells (26,27). Expression of mesothelin and calretinin in our primary cell culture confirmed mesothelial differentiation (data not shown); WT1 was strongly expressed in Mest cells. A preliminary time- and dose-response curve was constructed to determine concentration and the time at which OMF produced significant effects on cell morphology. Aliquots containing 4×10^5 cells were applied to six-well plates (35 mm diameter) with 2 ml DMEM, and were treated with 50 µg/ml OMF for 48 h. In addition, NCI, Mest and Mes1 cells were treated (or not) with 10 µl absolute ethanol. This treatment did not produce any effect on the cell lines (data not shown).

In kinase inhibitory experiments, cells were treated with MAPK/ERK kinase (MEK) inhibitor U0126 (10 µM) (Cell signaling, Danvers, MA, USA) for 2 h, and culture medium was then replaced with fresh U0126-free cell culture medium. RNA and proteins were extracted after 48 h

cell culture to assess modulation of α v, β 5, MMP2 and vascular endothelial growth factor (VEGF), by ERK1/2.

MTT cell proliferation assay

NCI, Mest and Mes1 cells numbering 3×10^3 were grown in microplates (96 wells, flat bottom) in a final volume of 100 μ l DMEM per well at 37 °C, 5% CO₂, and were treated with 25, 50 and 100 μ g/ml of OMF for 24, 48 and 72 h. Then, 10 μ l of MTT labelling reagent was added to each well (final concentration 0.5 mg/ml). After 4 h, 100 μ l of the solubilization solution was added to each well and plates were incubated overnight. Spectrophotometric absorbance was measured using a microplate (ELISA) reader at a wavelength of 600 nm.

Morphological analysis

Morphological features of NCI, Mest and Mes1 cells treated with OMF were defined by phase-contrast microscopy (Olympus CDK40) at 20 $\times$ magnification.

Cell migration assay

Cell migration assays were carried out in Boyden chambers under serum-free conditions. Polycarbonate filters, 10 μ m pore size, were coated with 5 μ g/ml fibronectin. After treatment with 50 μ g/ml OMF for 48 h, 2×10^5 NCI, Mest or Mes1 cells were trypsinized and placed in the upper compartment of Boyden chambers in serum-free medium, while in the lower compartment, FBS was introduced as the chemoattractant. Cells were allowed to migrate for 4 h at 37 °C in 5% CO₂. They were then fixed in ethanol and stained with haematoxylin, and 10 random fields/filter were counted at 200 $\times$ magnification (28).

Cell invasion assay

Cell invasion assays were carried out in Boyden chambers under serum-free conditions, as described previously (29). The 10 μ m pore size polycarbonate filters were coated with 5 μ g/ml fibronectin and then with 25 μ g/ml of matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After treatment with OMF 50 μ g/ml for 48 h, 2×10^5 NCI, Mest or Mes1, cells were trypsinized and placed in the upper compartment of the Boyden chamber in serum-free medium, and 10% FBS was introduced into the lower compartment as the chemoattractant. Cells were allowed to attach and spread for 24 h at 37 °C in 5% CO₂. Those on the upper surface of the filter were completely removed by wiping with a cotton swab, while those that had traversed the matrigel and attached to the lower surface of the filter were fixed in ethanol, stained with haematoxylin

and counted in 10 random fields/filter at 200 $\times$ magnification. In parallel, control cells were assessed for viability and counted using the trypan blue exclusion technique. Number of cells that had invaded was normalized to analyse the effects on cell viability.

Reverse transcription-polymerase chain reaction analysis

Total cell RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy) from NCI and Mes1 cells before and after treatment with 50 μ g/ml OMF. One hundred nanograms of total cell RNA was reverse-transcribed (Expand Reverse Transcriptase; Roche Diagnostics) into cDNA using random hexamer primers (Random hexamers; Roche Diagnostics) at 42 °C for 45 min, according to the manufacturer's instructions. Two microlitres of cDNA was amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 μ M dNTP and 2.5 U of Taq DNA polymerase (Roche Diagnostics) in a final volume of 50 μ l. For co-amplification of α v, β 5, MMP2 and VEGF with GAPDH or β -actin, PCR was carried out in the presence of 0.5 μ M sense and antisense α v, β 5, MMP2 and VEGF primers, and 0.05 μ M sense and antisense GAPDH or β -actin primers. Conditions and size of the products are shown in Table 1. The reaction was carried out in a DNA thermal cycler (Mastercycler gradient; Eppendorf, Milano, Italia). β -actin RT-PCR was performed on mRNA extracted at 24 h to confirm that the mRNA was suitable for RT-PCR analysis. PCR products were analysed by electrophoresis on 1.8% agarose gel in TBE. Densitometric analysis of ethidium bromide-stained agarose gel was carried out using NIH image V1.6 software. Ratio between the yield of each amplified product and that of co-amplified internal control, allowed a relative estimate of mRNA levels in the sample analysed. Internal control was a housekeeping gene whose PCR product did not overlap with the gene under investigation.

Protein extraction and western blotting analysis of α V, β 5, MMP-2, VEGF and p44/42

NCI and Mes1 cells were treated with 50 μ g/ml OMF for 24 (for VEGF analysis) and 48 h. Cells were scraped in 1 ml PBS, and the cell pellet was homogenized with 300 μ l ice-cold buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton, 1.5 mM MgCl₂ and 5 mM EGTA) supplemented with 20 mM sodium pyrophosphate, 40 μ g/ml aprotinin, 4 mM PMSF, 10 mM sodium orthovanadate and 25 mM NaF. Total extracts were cleared by centrifugation at 11 000 g for 30 min at 4 °C and assayed for protein content using Bradford's method. Fifty micrograms of protein from each cell lysate was separated by

Table 1. Human sense and antisense primer sequences and expected PCR products (bp)

Gene	Sense and antisense sequences	Conditions	bp
α V	5'-TAA AGG CAG ATG GCA AAG GAG T-3' 5'-CAG TGG AAT GGA AAC GAT GAG C-3'	30 cycles at 95 °C for 30 s 62 °C for 40 s, 72 °C for 40 s	510
β 5	5'-CAG CCC CGG CTA CCT GGG CAC-3' 5'-CTG GCA CAG GAG AAG TTG TCG CAC-3'	29 cycles at 94 °C for 60 s 62 °C for 60 s, 72 °C for 60 s	200
MMP2	5'-TGA CGG TAA GGA CGG ACT C-3' 5'-TGG AAG CGG ATT GGA AAC T-3'	33 cycles at 94 °C for 60 s 57 °C for 60 s, 72 °C for 60 s	342
VEGF	5'-CCA TGA ACT TTC TGC TGT CTT-3' 5'-TCG ATC GTT CTG TAT CAG TCT-3'	34 cycles at 94 °C for 30 s 60 °C for 30 s, 72 °C for 60 s	516, 648, 730 (Multiplex)
Calretinin	5'-CATACTACGGATGTTTGACTT-3' 5'-TCACGCTCTCTGAGTCTGG-3'	33 cycles at 95 °C for 60 s 56 °C for 60 s, 72 °C for 60 s	427
Mesothelin	5'-AACGGCTACCTGGTCCTAG-3' 5'-TTTACTGAGCGCGAGTTCTC-3'	35 cycles at 95 °C for 60 s 58 °C for 60 s, 72 °C for 60 s	226
WT1	5'-CTCTTGACGGTCGGCATCT-3' 5'-CAGCTGGAGTTTGGTCATG-3'	33 cycles at 95 °C for 60 s 56 °C for 60 s, 72 °C for 60 s	471
CEA	5'-ACAGTCTCTGCGGAGCTGC-3' 5'-TAGGTCCCGTTATTATTGGCG-3'	35 cycles at 95 °C for 60 s 58 °C for 60 s, 72 °C for 60 s	476
GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3' 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'		306
β -actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'		661

Figure 1. Effect of OMF treatment on cell proliferation (panels a and b, MTT assay) and on cell morphology of NCI (panel c–e) and Mes1 cells (panels f–h), by phase-contrast microscopy. Panels a and b: NCI and Mes1 cells were treated with various concentrations of OMF for 24, 48, and 72 h. Data are presented as mean $\pm$ SD of the results from three independent experiments. Cell viability was determined using the MTT assay. Panel C: untreated NCI cells (24 h). Panels d and e: 50 μ g/ml OMF-treated NCI cells for 48 and 72 h respectively. Panel F: untreated Mes1 cells (24 h). Panels g and h: 50 μ g/ml OMF-treated Mes1 cells for 48 and 72 h respectively. Magnification: 20 $\times$.

10% SDS-PAGE and transferred on to nitrocellulose membranes. Filters were then stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. In addition, protein normalization was verified by densitometric analysis of bands. Blots were blocked overnight with 5% non-fat dry milk, then incubated with anti- α V (Chemicon International, Temecula, CA, USA), anti- β 5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) rabbit polyclonal antibodies, anti-MMP-2, anti-VEGF (A-20: sc-152) mouse monoclonal antibodies (Santa Cruz Biotechnology), anti-p44/42 (ERK1/2) rabbit polyclonal antibody (Cell Signaling) and anti-tubulin mouse monoclonal antibody (Santa Cruz Biotechnology) 1:200 in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 8) for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, filters were incubated with 1:2500 peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin for 1 h at 22 °C. They were extensively washed and finally analysed using the ECL system (Amersham, Otelfingen, Switzerland).

Statistical analysis

Each experiment was performed at least three times. Results are expressed as mean $\pm$ standard deviation. Student's *t*-test was used to determine statistical differences between mean values, and $P < 0.01$ was considered to be a significant difference.

Results

Effect of OMF on proliferation and motility of mesothelioma cells

We have shown previously that OMF can affect cell population growth of different tumour cell lines and induce apoptosis (6–8). In the present study, we investigated the effect of OMF on proliferation of NCI cells derived from human mesothelioma. As non-tumour cell counterpart, we used Mes1 cells, as described in the Materials and methods section, above. Cells were treated with 25, 50 and 100 μ g/ml of OMF for 24, 48 and 72 h, and their viability was determined using the MTT assay. OMF displayed a strong time- as well as dose-dependent cell proliferation inhibition (Fig. 1a). In particular, cells treated with 50 μ g/ml OMF for 48 h showed the best results in terms of population growth inhibition and minor cell-shape changes, whereas prolonged treatment (72 h) or higher OMF concentration (100 μ g/ml) had increasing cell detachment from the substratum, with modified morphology and cell rounding (Fig. 1d,e). In contrast, Mes1 cell lines treated with 25, 50 and 100 μ g/ml of OMF for 24, 48 and 72 h

showed the inability of the molecule to affect either proliferation or cell morphology (Fig. 1b,g,h), indicating that the toxic effect was more specific to mesothelioma cells. In view of the above-mentioned results, to investigate further the biochemical and molecular mechanism affecting cell proliferation, we decided to work with an OMF concentration of 50 μ g/ml for 48 h.

We have reported previously that decreased cell proliferation is also associated with inhibition of cell motility in breast cancer cells (7). To investigate if this was also the case in OMF-treated NCI cells, a chemotaxis assay was performed. As shown in Fig. 2a, using the chemoattractant FBS (10%), untreated NCI cells were induced to migrate from the upper to the lower compartment of the Boyden chamber through the fibronectin-coated filter. In contrast, chemotactic response of NCI cells in the presence of FBS was inhibited when cells were treated with 50 μ g/ml OMF for 48 h. Cell migration inhibition in comparison with untreated cells was 67%. The same treatment did not modify motility of Mes1 cells (Fig. 2a). Cell invasion, as measured using a modified Boyden chamber with a matrigel-coated filter, was also reduced (41%) after 48 h treatment

Figure 2. Effect of OMF treatment on chemotactic migration (panel a) and invasion (panel b) of NCI and Mes1 cells. Panel a: control and OMF-treated cells were left to migrate in the presence of FBS. Data are reported as percentage of basal random migration in the presence of the chemoattractant. Panel b: control and OMF-treated cells were plated on to a matrigel modified Boyden chamber. Cells were allowed to attach and spread for 24 h. Only cells that had passed through the matrigel were stained and counted. Average number of cells per field is expressed as a percentage of control after normalizing for cell number. Results are reported as mean value of three different experiments.

Figure 3. Effect of OMF treatment on cell morphology (panel a and b), proliferation (panel c, MTT assay), chemotactic migration (panel d) and invasion (panel e) of Mest cells.

Panel a: untreated Mest cells (48 h). Panel b: 50 µg/ml OMF-treated Mest cells for 48 h. Magnification: 20x. Data are presented as mean ± SD of results from three independent experiments. Viability was determined using MTT assay. Panel d: control and OMF-treated cells were left to migrate in presence of FBS. Data are reported as percentage of basal random migration in the presence of the chemoattractant. Panel e: control and OMF-treated cells were plated on to a matrigel modified Boyden chamber. Cells were allowed to attach and spread for 24 h. Only cells that had passed through the matrigel were stained and counted. Average number of cells per field is expressed as percentage of control after normalizing for cell number. Results are reported as mean value of three different experiments. *Significantly different compared to control ($P < 0.01$).

with OMF on NCI compared with OMF-untreated NCI cells. In contrast, Mes1 cells were unable to invade matrigel-coated filters (Fig. 2b).

To strengthen the results obtained till now, we investigated the effect of OMF on the growth, migration and invasion of a primary cultures of mesothelioma cells (Mest), isolated from biopsy of malignant pleural mesotheliomas, as described in the Materials and methods. Mest cells were treated with 50 µg/ml of OMF for 48 h to evaluate the effect of the molecule. OMF affected Mest cell growth, migration and invasion as well as NCI (Fig. 3). In particular, a strong cell growth inhibition (52%) was observed when Mest cells were treated with OMF compared with OMF-untreated Mest cells (Fig. 3c). Migration and invasion of Mest cells were also reduced (42% and 34% respectively) after OMF treatment (Fig. 3d,e respectively).

Modulation of αV and $\beta 5$ integrin subunits in OMF-treated NCI cells

To investigate the mechanism of NCI-inhibited motility, we analysed mRNA levels of αV and $\beta 5$ integrin subunits using RT-PCR. OMF treatment produced marked

decrease in both αV and $\beta 5$ gene expression in NCI cells, while no such effect was observed in Mes1 cells (Fig. 4a,d). Western blot analysis confirmed reduction of αV and $\beta 5$ integrin protein levels in OMF-treated NCI cells (Fig. 4c,f).

Reduced expression of MMP-2 by OMF treatment in NCI cells

The effect of OMF on expression of MMP-2 in NCI and Mes1 cells was studied using RT-PCR and western blot analysis. As shown in Fig. 5, MMP-2 expression clearly decreased after 48 h of OMF treatment in NCI cells, while the same treatment did not affect MMP2 expression in Mes1 cells.

Effect of OMF on angiogenesis: VEGF gene expression

The VEGF is a potent inducer of angiogenesis and its critical role in tumour-derived angiogenesis is well established (30). To investigate the effect of OMF on VEGF expression in NCI, cells were treated with 50 µg/ml OMF for 48 h. As shown in Fig. 6, OMF clearly induced a

Figure 4. RT-PCR analysis using specific primers for αV and $\beta 5$ mRNA expression (Panels a and d) and western blot analysis (Panels c and f). Panels a and d: Lane 1, mRNA from untreated Mes1 cells; lane 2, mRNA from Mes1 treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h; lane 3, mRNA from untreated NCI cells; lane 4, mRNA from NCI treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h. M, 100 bp ladder MW-marker (Roche Diagnostics). Panels c and f: Lane 1, untreated Mes1 cells; lane 2, Mes1 treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h; lane 3, untreated NCI cells; lane 4, NCI treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h. Panels b and e: Measurements of RT-PCR band intensities of αV and $\beta 5$ relative to GAPDH. Data shown are representative of three different experiments $\pm$ SD. *Significantly different compared to control ($P < 0.01$).

Figure 5. RT-PCR analysis using specific primers for MMP2 mRNA expression (Panel a) and western blot analysis (Panel c). Panel a: Lane 1, mRNA from untreated Mes1 cells; lane 2, mRNA from Mes1 treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h; lane 3, mRNA from untreated NCI cells; lane 4, mRNA from NCI treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h. M, 100 bp ladder MW-marker (Roche Diagnostics). Panel c: Lane 1, untreated Mes1 cells; lane 2, Mes1 treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h; lane 3, untreated NCI cells; lane 4, NCI treated with 50 $\mu\text{g}/\text{ml}$ OMF for 48 h. Panel b: Measurements of RT-PCR band intensities of MMP2 relative to β -actin. Data shown are representative of three different experiments $\pm$ SD. *Significantly different compared to control ($P < 0.01$).

marked decrease in all three VEGF isoforms (VEGF₁₂₁, 165, 189) (31). VEGF protein levels were also significantly reduced as confirmed using western blot analysis. Conversely, Mes1 cells expressed VEGF₁₂₁ only weakly.

ERK1/2 signal transduction pathway mediates integrin-, MMP2- and VEGF-reduced OMF

To determine the mechanisms underlying integrin, MMP2 or VEGF regulation in response to OMF treatment, we attempted to identify the molecular pathway involved. Protracted phosphorylation of ERK1/2 and increased kinase activity of ERK2 have been previously reported in rat pleural mesothelial (RPM) cells exposed to asbestos, thus suggesting a possible target in OMF-induced effects on NCI cells (25). Figure 7 shows reduced ERK1/2 activities in OMF-treated NCI cells at 24 and 48 h, as evaluated using western blot assay. This reduction was comparable with that obtained when NCI cells were treated with a specific MAPK/ERK kinase (MEK) inhibitor U0126 (32) (Fig. 7). The same OMF treatment did not modify ERK activity in Mes1 cells. To better investigate involvement of this signalling pathway in OMF-treated NCI cells, we examined expression of $\alpha V\beta 5$ integrin, MMP2 and VEGF, in the presence or absence of U0126. NCI cells were treated for 2 h with 10 μM U0126. As shown in Fig. 8, this treatment was found to inhibit downstream targets of ERK1/2. These results were similar to that obtained in the presence of OMF, thus suggesting that this molecule might affect cell

Figure 6. RT-PCR analysis using specific primers for VEGF mRNA expression (Panel a) and western blot analysis (Panel c). Panel a: Lane 1, mRNA from untreated Mes1 cells; lane 2, mRNA from Mes1 treated with 50 µg/ml OMF for 48 h; lane 3, mRNA from untreated NCI cells; lane 4, mRNA from NCI treated with 50 µg/ml OMF for 48 h. M, 100 bp ladder MW-marker (Roche Diagnostics). Panel c: Lane 1, untreated Mes1 cells; lane 2, Mes1 treated with 50 µg/ml OMF for 48 h; lane 3, untreated NCI cells; lane 4, NCI treated with 50 µg/ml OMF for 48 h. Panel b: Measurements of RT-PCR band intensities of VEGF relative to GAPDH. Data shown are representative of three different experiments $\pm$ SD. *Significantly different compared to control ($P < 0.01$).

Figure 7. Western blot analysis showing phosphorylation (ERK-p) (top) and protein (ERK) (bottom) levels of ERK1/2 kinase in NCI and Mes1 cells treated with 50 µg/ml OMF for 24 and 48 h. Lane 1, untreated NCI cells; lane 2, NCI treated with 50 µg/ml OMF for 24 h; lane 3, NCI treated with 50 µg/ml OMF for 48 h; lane 4, NCI treated with 10 µM UO126; lane 5, untreated Mes1 cells; lane 6, Mes1 treated with 50 µg/ml OMF for 24 h; lane 7, Mes1 treated with 50 µg/ml OMF for 48 h.

proliferation and migration of NCI cells by modulating ERK.

Discussion

In this study, we demonstrated that OMF inhibits mesothelioma tissue invasion by modulating the three fundamental steps that have a role in malignant tumour cell invasion of normal tissue, degradation of the extracellular

Figure 8. RT-PCR analysis using specific primers for α V (panel a), β 5 (panel b), MMP2 (panel c) and VEGF mRNA expression (panel d). Lane 1, mRNA from untreated NCI cells; lane 2, mRNA from NCI treated with 50 µg/ml OMF for 48 h; lane 3, mRNA from NCI treated with 10 µM UO126. M, 100 bp ladder MW-marker (Roche Diagnostics).

matrix, cell migration and proliferation. When NCI cells were treated with OMF, a strong reduction in cell proliferation was observed to be associated with reduced motility and invasiveness. The same results were obtained when primary cultures of mesothelioma cells were used. In addition, OMF treatment induced marked downregulation of α V β 5 integrin and MMP2 expression. These results are consistent with the fact that MMP2, the most abundant gelatinase, plays an important role in MM tumour growth and metastasis, and that agents reducing MMP synthesis and/or activity should play a role in management of MM (17). Similarly, the effects on integrin expression are of interest because the latter have been reported to play an important part in acquisition of migratory, invasive, or metastatic properties by human tumour cells (33). It has been reported that integrins are required for TGF- α - and VEGF-mediated angiogenesis (34). Antibodies that target α V β 5 blocked angiogenesis induced by VEGF in both the rabbit corneal eye pocket and chick chorioallantoic membrane (CAM) assay (35). It has been reported that VEGF upregulation plays an important role in mesothelial cell transformation. Higher levels of VEGF have been observed in serum of mesothelioma patients compared to serum of normal subjects (36). The effect of OMF on VEGF regulation is therefore, very interesting. Molecules that are able to counteract VEGF production, such as OMF, might be promising candidates in management of mesothelioma, either alone or in combination therapy with other chemotherapeutics. To stimulate

neovascularization essential for tumour growth and metastasis, VEGF may also act in a functional autocrine loop capable of directly stimulating proliferation of malignant mesothelioma cells (37). Interestingly, all the effects reported in this study were not observed when normal primary mesothelial cells were treated with OMF, indicating that the molecule may work by specifically affecting mesothelioma tumour cells only. In summary, OMF seems to be able to elicit a double effect on mesothelioma cells, that is, it blocks formation of new vessels through VEGF downregulation and at the same time, negatively regulates cell proliferation by modulating the autocrine loop.

Despite the extensive literature on effects of OMF in human cancer cells, no data have been reported on the intracellular signalling pathways that regulate expression of OMF-modulated genes in cancer cells. ERK-1/2 belongs to the mitogen-activated protein kinase (MAPK) family and are crucial in control of cell population growth, differentiation and survival. Upon stimulation, they rapidly translocate to the nucleus and trigger regulation of downstream molecules. In this study, we demonstrate that OMF selectively inhibits ERK1/2 activity in a mesothelioma cell line. In addition, we provide evidence that expression of the genes downstream of ERK regulation (for α V- and β 5 integrins, MMP2 and VEGF) is reduced when specific MAPK/ERK kinase (MEK) inhibitor U0126 is used. These data indicate that OMF can influence the ERK signalling pathway by regulating expression of genes associated with invasion and metastasis of mesothelioma tumour cells. To our knowledge, this is the first report that shows a possible molecular mechanism triggering OMF effects.

Mesothelioma is largely unresponsive to conventional chemotherapy or radiotherapy, and surgical treatment (extrapleuralpneumonectomy or pleurectomy) has not shown a significant survival advantage over supportive treatment (38). Because of its ability to counteract ERK1/2 activity and, consequently, affect cell proliferation and motility, OMF might be a promising chemotherapeutic compound able to play a significant role in treatment of human mesothelioma. Further studies on *in-vivo* models may further elucidate therapeutic effects of OMF.

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