Mesothelin inhibits paclitaxel-induced apoptosis through the PI3K pathway

Ming-Cheng CHANG*, Chi-An CHEN*, Chang-Yao HSIEH*, Chien-Nan LEE*, Yi-Ning SU†, Yu-Hao HU* and Wen-Fang CHENG*†

*Department of Obstetrics and Gynecology, College of Medicine, National Taiwan University, Taipei, Taiwan, and †Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan

INTRODUCTION

Ovarian carcinoma has become a more and more important disease in recent years, because it has the highest mortality rate of all gynaecological malignancies [1,2]. Its overall 5-year survival rate is only 20–30%, because most cases (approx. 75%) are diagnosed at an advanced stage [3,4]. Conventional prognostic parameters cannot present a comprehensive picture of the tumour biology of ovarian cancer and are frequently interrelated. It has been demonstrated that mesothelin is expressed in the majority of ovarian cancers, but is absent from or expressed at low levels in normal tissues [5]. Hassan et al. [6] also report that the serum mesothelin levels correlate with tumour loads in patients with ovarian cancer and malignant mesothelioma. Mesothelin is documented to be a tumour-associated marker in epithelial ovarian carcinoma.

Mesothelin, originally found by the antibody CAK-1 on mesothelial cells, is a secreted protein anchored on the cell membrane by glycosylphosphatidylinositol linkage [7]. It has been identified as a potential new tumour antigen for mesotheliomas and ovarian cancers [8], and has been found to be overexpressed in cancers of the pancreas [9], stomach [10] and endometrium [11]. However, its exactly biological function remains uncertain.

A mesothelin-expressing ascitogenic malignant tumour model that demonstrates morphological features of intraperitoneal tumorigenesis has been created [12]. The tumour model (WF-3) also demonstrates relatively high proliferation and migration rates compared with the parental cell line (WF-0). Furthermore, the tumour expresses high levels of mesothelin, commonly observed in intraperitoneal tumours, such as malignant tumours and ovarian cancers. Therefore the WF-3 tumour model serves as an excellent pre-clinical tumour model to study clinically relevant issues, such as screening and evaluating the effectiveness of therapeutic intervention by monitoring mesothelin levels.

Paclitaxel is a frontline anti-neoplastic agent that is efficacious in the treatment of several malignancies, including ovarian, breast, lung and prostate cancers [13,14]. It acts through the stabilization of microtubules, cell-cycle arrest in G2/M-phase and the activation of pro-apoptotic signalling [15]. However, even though it is effective against various types of cancer, its effect is limited because of drug resistance. Several mechanisms of drug resistance in cancers have been posited, including overexpression of the multidrug-resistance gene [16], increased DNA repair [17] and suppression of apoptotic pathways [18].

The present study was designed to evaluate the possible biological function of mesothelin on the apoptotic effects of paclitaxel. The expression levels of mesothelin in ovarian cancer tissues are higher in paclitaxel-resistant than in paclitaxel-sensitive patients. It can reduce paclitaxel-induced cell death by mediating cell survival and can active both PI3K (phosphoinositide 3-kinase) and ERK (extracellular-signal-regulated kinase) 1/2 for enhancing MAPK (mitogen-activated protein kinase) activity. The anti-apoptotic ability was suppressed and the expression of Bcl-2 family in response to mesothelin was altered by inhibiting PI3K activity, but not by inhibiting MAPK activity. Thus mesothelin can inhibit paclitaxel-induced cell death mainly by involving PI3K signalling in the regulation of Bcl-2 family expression. Mesothelin is a potential target in reducing resistance to cytotoxic drugs.

Key words: apoptosis, cancer, mesothelin, ovarian cancer, paclitaxel, phosphoinositide 3-kinase (PI3K).

MATERIALS AND METHODS

Patients and specimens

A total of 30 patients with advanced-stage ovarian carcinoma who had undergone debulking surgery and six courses of...
experiments. Mesothelin-transfected WF-0 cells were utilized in the subsequent transcription–PCR and flow cytometric analysis. The original and for expression of the transfected mesothelin by RT (reverse transcription)–PCR and flow cytometric analysis. The original and mesothelin-transfected WF-0 cells were utilized in the subsequent experiments.

Real-time PCR
Mesothelin and G6PDH (glucose-6-phosphate dehydrogenase) RNA were first reverse-transcribed to cDNA. Real-time PCR was carried out using the LightCycler Real-Time detection system (Roche Diagnostics) according to the manufacturer’s protocol. 5'-CTATCCTCAACCCAGATGCGT-3' and 5'-GCA-CATCAGCTCGCTCA-3' were the primers used to detect mesothelin. Amplification was performed with 5 mM MgCl₂ and 50 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. The detection of G6PDH was carried out by the LightCycler h-G6PDH (human G6PDH) housekeeping gene set (Roche Applied Science) for 50 cycles of 95 °C for 10 s, 55 °C for 15 s and 72 °C for 15 s. Fluorescent signals generated during the informative log-linear phase were used to calculate the relative amount of template DNA as described previously [19].

Generation of quantitative data was based on the number of cycles needed for amplification-generated fluorescence to reach a specific CP (crossing point) value as described previously [19]. CP values were derived directly from co-ordinates of points where the threshold line crossed fluorescent plots obtained after noise filtering [20]. The relative expression ratio of a target gene was calculated based on corresponding real-time PCR efficiency and the CP value deviation of an unknown sample compared with a control sample (normal ovarian tissues). The equations to calculate mesothelin expression level in each sample were:

Relative expression level of mesothelin = \(2^{-\Delta\Delta CP}\)

\(\Delta CP = CP_{\text{target}} - CP_{\text{housekeeping}}\)

\(\Delta\Delta CP = \Delta CP_{\text{sample}} - \Delta CP_{\text{calibrator}}\)

Cell culture and transfection
The low-mesothelin-expressed WF-0 cells generated from C57BL/6 murine peritoneal cells have been described previously [12]. To generate pcDNA3-mesothelin, mesothelin was first amplified with PCR using murine WF-0 cell cDNA as the template and 5'-CCGGGTTACAGATGGGGAAGCCTGAGTCA-3' and 5'-CCGGGATCCCGGGACATGCGCTGTTTT-3' as primers. The amplified product was then cloned into the XbaI/BamH1 sites of pcDNA3 vector (Invitrogen Life Technologies). The transfection of mesothelin was performed using Lipofectamine™ 2000 reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions.

For selecting stable clones, hygromycin (700 μg/ml) was added to the culture medium 48 h after transfection. The hygromycin-resistant clones were individually picked, expanded and assayed for expression of the transfected mesothelin by RT (reverse transcription)–PCR and flow cytometric analysis. The original and mesothelin-transfected WF-0 cells were utilized in the subsequent experiments.

The human ovarian cancer cell line OVCAR-3 was obtained from the A.T.C.C. (Manassas, VA, U.S.A.). siRNA (short interfering RNA) for mesothelin was designed and synthesized using the software 2 and Silencer™ siRNA construction kit from Ambion, according to the manufacturer’s instructions. The sequence of the double-stranded RNA employed to block mesothelin expression in the experiments was 5'-AGAAGAGA-GGCCUCGCUAUCUC-3' (sense) and 5'-GAGAUAGACGAGACCUCUAUCUU-3' (antisense). GAPDH (gluceraldehyde-3-phosphate dehydrogenase) siRNA (siGAPDH) (Ambion) was used as the control siRNA. RT–PCR was used to assess the inhibition of mesothelin expression following transfection of human OVCAR cells with mesothelin siRNA (siMSLN). Briefly, OVCAR cells were grown and transfected with 50 pM siRNA using 12 μg of Lipofectamine 2000™ transfection reagent (4:1) in a total of 2 ml of serum-free RPMI 1640 medium. After incubation at 37 °C, 5% CO₂ for 6 h, 2 ml of RPMI 1640 medium containing 20% normal growth medium was added. Samples were then prepared and analysed for RT–PCR.

Cytotoxicity assay of WF-0 cells treated under serum-deprivation conditions
Recombinant mouse mesothelin protein was purchased from Abnova. Its purity was determined by SDS/PAGE (10% gel) (results not shown). Cytotoxicity and cell survival of WF-0 cells were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay with some modifications [21]. Briefly, the WF-0 cells were plated in 96-well microtitre plates overnight. The cells were treated under serum-deprived conditions for 24 h, after which recombinant mesothelin was added into the medium for another for 24 h. During the final 4 h of incubation, 30 μl of MTT (0.5 mg/ml) was added to each well. At the end of the experiment, the culture medium was removed and the precipitated dye was solubilized in DMSO. The absorbance of each well was determined using a microplate reader at 570 nm.

Proliferative assays of WF-0 cells
Cell proliferation was determined by the measurement of BrdU (bromodeoxyuridine) incorporation using a cell proliferation ELISA kit (Roche Molecular Biochemicals) as described previously [22,23]. Briefly, WF-0 cells were plated in 96-well microtitre plates overnight. The cells were washed with serum-free medium and fed with fresh medium containing 100 nM recombinant mesothelin for 48 or 72 h. At 4 h before analysis, BrdU labelling solution was added. The cells were then treated with fix solution at room temperature (23–27°C) and incubated with an anti-BrdU antibody conjugated with horseradish peroxidase. A substrate solution was then added into each well, and the absorbance of each well was determined using a microplate reader at 470 nm.

Detection and quantification of apoptotic WF-0 cells by Hoechst staining
To evaluate and quantify the apoptotic WF-0 and the other mesothelin-transfectant cells, the Hoechst staining method was performed as described previously [24]. The cells were seeded on the chamber slides in serum-free medium overnight and then treated with 100 nM mesothelin or 0.5 μM paclitaxel, or combined mesothelin and paclitaxel. After overnight incubation, the cells were then fixed with ice-cold 75% methanol/25% acetic acid solution at room temperature. The Hoechst 33258 (Sigma)/PBS solution (0.5 μg/ml) was then added. The cells

© The Authors. Journal compilation © 2009 Biochemical Society
were kept in the dark at room temperature for 10 min. Cells with typical apoptotic nuclear morphology such as nuclear shrinkage, fragmentation and condensation were defined as apoptotic. The apoptotic scores were counted from five random fields (for a total of 500 cells counted) for each sample, and the percentage of apoptotic cells was calculated as the number of apoptotic cells/number of total cells counted.

Detection of active caspases 3 and 7 by flow cytometric analysis
Detection of activities of caspases 3 and 7 in WF-0 and WF-0 mesothelin-transfectant cells was determined using a Vybrant® FAM Caspase-3 and -7 Assay Kit (Molecular Probes) according to the manufacturer’s instructions [25,26]. Briefly, PBS- or recombinant mesothelin-treated WF-0 cells were resuspended. FLICA™ (fluorescent inhibitor of caspases) solution was then added. The WF-0 cells were incubated under 5 % CO₂ in a light-protected environment for 60 min at 37 °C and analysed by flow cytometry (FACScan; Becton Dickinson).

RNA isolation and RT–PCR
Total RNA from the original WF-0 and various mesothelin-transfected WF-0 cell lines was isolated by the TRIzol® RNA isolation kit following the manufacturer’s instructions (Invitrogen Life Technologies). Reverse-transcribed cDNA products were amplified by PCR with primers specific for mesothelin and GAPDH. The primers used were 5'-TTG-TGCCCAGCTTCTTTCCCTCA-3' and 5'-CTCATCAACGACGCTGACGCG-3'. The GAPDH forward primer was 5'-ACCCAGAGACTGTTGATGG-3', and the reverse primer was 5'-TGCTGTACGCAAATTCGTTG3'. The amplification products were separated by 1% agarose gel electrophoresis and visualized after staining with ethidium bromide.

Immunoprecipitation and immunoblotting
The cells were seeded and serum-free medium was replaced overnight, followed by treatment with various concentrations of mesothelin for 24 h. The cells were lysed in the immunoprecipitation assay buffer and analysed as described previously [8]. The protein extracts were quantified using the BCA Protein Assay Kit (Pierce). Then, 50 μg of each cell lysate was resolved by SDS/PAGE (12% gel), transferred on to a PVDF/nylon membrane (Millipore), and probed with antibodies specific to Bcl-2, Bax, Bak, Mcl-1, ERK, phospho-ERK or Akt (Upstate Biotechnology) or β-actin or phospho-Akt (Ser473) (Chemicon International). The membrane was then probed with either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody. The specific bands were visualized by an ECL® (enhanced chemiluminescence) Western blotting system (GE Healthcare).

In the PI3K subunit p85 phosphorylation experiments, immunoprecipitation was performed as described in [8]. Briefly, WF-0 cells were treated with various concentrations of mesothelin for 1 h and lysed with immunoprecipitation assay buffer. Samples of 500 μg of cell extracts were incubated with 1 μg of anti-p85 antibody (clone 11E6; Upstate Biotechnology) overnight at 4 °C. The immunocomplex was precipitated with 50 μl of Protein A–Sepharose (GE Healthcare) for 1 h at 4 °C. The precipitated beads were resolved by SDS/PAGE (8% gel), transferred on to PVDF/nylon membranes (Millipore), and probed with an antibody specific to phosphotyrosine residues (clone 4G10; Upstate Biotechnology). Total p85 expression was considered as the internal control. The membrane was then probed with horseradish peroxidase-conjugated goat anti-rabbit antibody. The specific bands were visualized by an ECL® Western blot system.

Statistical analyses
Statistical analyses were carried out using SPSS version 6.0 for Windows, which involved a one-way ANOVA and the
were performed in triplicate and repeated at least twice. Results are means ± S.E.M.

induced cell death, WF-0 cells were pre-treated with mesothelin to block a step specific to the apoptotic signalling cascades. Mesothelin protected cells from paclitaxel-induced cell death induced by serum-deprivation. Mesothelin signalling was likely to block DNA synthesis and proliferation even though it could prevent apoptosis (Figure 2B). These indicated that mesothelin did not affect DNA synthesis to enhance cell viability.

The correlation between mesothelin and cell viability interrupted by serum withdrawal was initially investigated. Cell viability was determined by MTT assays, even at low concentrations of mesothelin (10 nM) (Figure 2A). Mesothelin did not stimulate or inhibit cell proliferation even in the extended incubation period of 72 h (10 nM) (Figure 2A). Mesothelin did not affect cell viability.

**RESULTS**

**Paclitaxel-resistant ovarian carcinoma expressed higher levels of mesothelin**

To validate the correlation between mesothelin expression and patients who were resistant to chemotherapy, mesothelin expressions were analysed comparatively from specimens of ovarian carcinoma. A total of 30 patients, including 15 paclitaxel-resistant and 15 paclitaxel-sensitive patients, were included. The representative real-time PCR curves of mesothelin in each sample in the paclitaxel-sensitive and -resistant groups are shown in Figure 1(A). The mean CP value change of mesothelin in the paclitaxel-resistant group was significantly lower than that in the paclitaxel-sensitive group (26.9 ± 0.4 compared with 34.3 ± 0.7; P < 0.001, one-way ANOVA) (Figure 1B). Expression levels of mesothelin were significantly higher in the paclitaxel-resistant group than in the paclitaxel-sensitive group. The related expressions of mesothelin in patients with chemo-sensitive and -resistant parameters were calibrated further. The related mesothelin expression levels in the paclitaxel-resistant group were significantly higher (26.19 ± 3.3) than those in the paclitaxel-sensitive patients (2.87 ± 0.51) (Figure 1C).

**Mesothelin does not affect DNA synthesis to enhance cell viability**

The correlation between mesothelin and cell viability interrupted by serum withdrawal was initially investigated. Cell viability was maintained for 72 h under serum-starved conditions, as measured using MTT assays, even at low concentrations of mesothelin (10 nM) (Figure 2A). Mesothelin did not stimulate or inhibit cell proliferation even in the extended incubation period of 72 h (Figure 2B). These indicated that mesothelin did not affect DNA synthesis and proliferation even though it could prevent apoptosis induced by serum-deprivation. Mesothelin signalling was likely to block a step specific to the apoptotic signalling cascades.

**Mesothelin protected cells from paclitaxel-induced cell death**

To investigate whether mesothelin could protect from paclitaxel-induced cell death, WF-0 cells were pre-treated with mesothelin followed by paclitaxel as described in the Materials and methods section. Mesothelin suppressed paclitaxel-induced cell death in a dose-dependent manner (Figure 3A). The apoptotic percentages of WF-0 cells treated with 0.5 μM paclitaxel was 67.4 ± 15.6%. When WF-0 cells were pre-treated with a gradient dosage of mesothelin followed by paclitaxel, the apoptotic percentages decreased significantly, from 57.5 ± 6.2% (10 nM mesothelin), then 48.2 ± 12.1% (33 nM mesothelin), to 37.1 ± 4.7% (100 nM mesothelin).

The preventive effects of mesothelin on the apoptotic phenomenon triggered by paclitaxel were quantified. Representative apoptotic WF-0 cells (chromatin-condensed cells) under various conditions stained with Hoechst 33258 are shown in Figure 3(B). The apoptotic WF-0 cells were commonly seen when exposed to 0.5 μM paclitaxel. In contrast, there were significantly lower percentages of apoptotic WF-0 cells when pre-treated with 100 nM of mesothelin compared with those without mesothelin pre-treatment (Figure 3B).

To characterize further the possible anti-apoptotic pathway generated by mesothelin, activation of caspases 3 and 7 were investigated. Cells treated with paclitaxel and/or mesothelin were analysed by measuring cleavage of a fluorogenic substrate, Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin). Paclitaxel-induced caspase activity was significantly abolished by mesothelin co-treatment (Figure 3C). Thus mesothelin prevented the apoptotic activity of paclitaxel, possibly by inhibiting the activation of caspases 3 and 7.

The effects of down-regulation of endogenous mesothelin on paclitaxel-induced apoptosis were investigated further. OVCAR cells were transfected with siMSLN and expression levels of mesothelin siRNA transfectants, such as OVCAR/siMSLN-c12, OVCAR/siMSLN-c13 and OVCAR/siMSLN-c14, were first confirmed by RT–PCR (Figure 3D). Characterization of apoptotic phenomena by cell morphology analysis are shown in Figure 3(E). Knockdown of mesothelin expression by siMSLN induction increased intracellular chromatin condensation and cell apoptosis induced by paclitaxel.

Quantification of apoptotic cells are shown in Figure 3(F). The blockage of endogenous mesothelin in the siMSLN RNA-transfected OVCAR transfectants showed significantly higher numbers of apoptotic cells when treated with paclitaxel (Figure 3F). Approx. 60–75% of OVCAR-3 (from three independent clones: c12, 77.45 ± 5.61%; c13, 61.51 ± 3.16%; and c14, 74.67 ± 7.94%) cells were chromatin-condensed by

**Figure 2** Mesothelin does not affect DNA synthesis to enhance cell viability

(A) Protective effects of mesothelin on serum-withdrawal-induced cytotoxicity in WF-0 cells. The percentages of viable WF-0 cells decreased significantly when serum was withdrawn over 24 h, which mesothelin rescued in a dose-dependent manner. (B) Cell proliferative activity determined by BrdU incorporation assay. Mesothelin did not promote the proliferation of WF-0 cells. All assays were performed in triplicate and repeated at least twice. Results are means ± S.E.M.
Mesothelin inhibits paclitaxel-induced apoptosis

Figure 3 Mesothelin alters paclitaxel-induced apoptosis phenomena

(A) The percentages of apoptotic cells in Hoechst assays. WF-0 cells were pre-treated with various concentrations of mesothelin and incubated with 0.5 μM paclitaxel for 24 h. Results are means ± S.E.M. * P < 0.05. (B) Representative images of Hoechst assays. Arrows indicate the apoptotic cell morphology. B1, PBS; B2, mesothelin (100 nM); B3, paclitaxel (0.5 μM); B4, mesothelin (100 nM)+paclitaxel (0.5 μM). (C) Mesothelin suppressed caspase 3 and 7 activation induced by paclitaxel. C1, PBS; C2, mesothelin (100 nM); C3, paclitaxel (0.5 μM); C4, mesothelin (100 nM)+paclitaxel (0.5 μM). The M value indicates the median value of total cells stained with caspase 3 and 7 fluorescent substrate. (D) Mesothelin expression levels in siMSLN-transfected OVCAR derivatives (confirmed by RT–PCR analysis). (E) Representative images of Hoechst assays. Arrows indicate the apoptotic cells. E1, OVCAR; E2, OVCAR (mock); E3, OVCAR/siMSLN-c12; E4, OVCAR/siMSLN-c13; E5, OVCAR/siMSLN-c14. E6–E10 indicate the same groups as described in E1–E5, but treated with 0.5 μM paclitaxel. (F) The percentages of apoptosis in siMSLN-transfected OVCAR derivatives treated with or without paclitaxel in Hoechst assays. Results are means±S.E.M. for three independent experiments. * P<0.05. (G) Blockage of mesothelin expression reversed caspase 3 and 7 activation induced by paclitaxel. G1, OVCAR treated with 0.5 μM paclitaxel; G2, OVCAR (mock) treated with 0.5 μM paclitaxel; G3, OVCAR/siMSLN-c12 treated with 0.5 μM paclitaxel; G4, OVCAR/siMSLN-c13 treated with 0.5 μM paclitaxel; G5, OVCAR/siMSLN-c14 treated with 0.5 μM paclitaxel. The M value indicates the median value of total cells stained with caspase 3 and 7 fluorescent substrate.

Mesothelin inhibits paclitaxel when endogenous mesothelin synthesis was blocked. However, there were <6% cell apoptosis in both parental and control siRNA (mock) transfectant groups (parental OVCAR, 5.67 ± 0.24%; mock 5.91 ± 0.37%). The caspase activity was also significantly increased in these siMSLN RNA-transfected OVCAR transfectants treated with paclitaxel when compared with the mock-transfected OVCAR cells (Figure 3G). These suggested the correlation between paclitaxel resistance and mesothelin expression in vitro. Although endogenous mesothelin might eliminate the chemotherapeutic effect of paclitaxel treatment, blocking mesothelin expression could strongly reverse the chemotherapeutic sensitivity. Mesothelin might play an important role in chemotherapeutic resistance to paclitaxel treatment.

To investigate whether endogenous mesothelin could suppress the apoptotic activity of paclitaxel, various WF-0 cell clones constitutively expressing mesothelin were generated. The expression level of the stable mesothelin-transfectants WF-0/M1, WF-0/M2, and WF-0/M3 cell clones were confirmed by RT–PCR. The mesothelin-transfected WF-0 cell clones (WF-0/M1, WF-0/M2 and WF-0/M3) expressed approx. 2-fold higher levels of mesothelin than the parental WF-0 cells (Figure 4A). Representative apoptotic cells by Hoechst staining in each group are shown in Figure 4B. When treated with paclitaxel, the numbers of mesothelin transfectants were significantly lower than those in the parental WF-0 cells (for 24 h: WF-0, 17.5 ± 2.4%; WF-0/M1, 12.4 ± 3.7%; WF-0/M2, 3.4 ± 1.06%
Figure 4 Anti-apoptotic effects and expression levels of apoptosis-related molecules of mesothelin in WF-0 cells and its derivatives against paclitaxel

(A) Mesothelin expression levels in various mesothelin-transfected WF-0 derivatives. The transfectants (WF-0/M1, WF-0/M2, and WF-0/M3) expressed more mesothelin than the parental WF-0 cells. (B) Representative images of Hoechst assays. Arrows indicate the apoptotic cell morphology. B1, WF-0; B2, WF-0/M1; B3, WF-0/M2; B4, WF-0/M3 cells all treated without paclitaxel for 24 h. B5, WF-0; B6, WF-0/M1; B7, WF-0/M2; B8, WF-0/M3 cells treated with 0.5 μM paclitaxel for 24 h. (C) Apoptotic percentages of Hoechst assays in WF-0 and WF-0-derived transfectants treated with paclitaxel for 24 h. Results are means ± S.E.M. *P < 0.05. (D) Mesothelin suppressed caspase 3 and 7 activation induced by paclitaxel. D1, WF-0; D2, WF-0/M1; D3, WF-0/M2; D4, WF-0/M3 cells treated without paclitaxel for 24 h; D5, WF-0; D6, WF-0/M1; D7, WF-0/M2; D8, WF-0/M3 cells treated with 0.5 μM paclitaxel for 24 h. The M value indicates the median value of total cells stained with caspase 3 and 7 fluorescent substrate. (E) Expression levels of apoptosis-related molecules in mesothelin-treated WF-0 cells. (F) Expression levels of apoptosis-related molecules in mesothelin-overexpressed transfectants.

WF-0/M1, 2.2 ± 2.6%; for 48 h: WF-0, 54.6 ± 5.64%; WF-0/M1, 41.5 ± 8.6%; WF-0/M2, 14.7 ± 2.1%; WF-0/M3, 7.7 ± 1.7%; P < 0.05, one-way ANOVA) (Fig. 4C). The activation of caspases 3 and 7 was lower in the mesothelin-transfectants than that in the original WF-0 cells when treated with paclitaxel (Figure 4D).

The results revealed that mesothelin played an important role in resistance to paclitaxel-induced apoptosis in both human ovarian cancer cells and murine WF-0 cells.

Mesothelin influenced the expression of molecules in Bcl-2 family

To investigate whether mesothelin resists paclitaxel-induced apoptosis by regulating Bcl-2 family proteins, WF-0 cells were exposed to various concentrations of mesothelin, treated by paclitaxel and analysed by Western blotting as described in the Materials and methods section. The expression levels of anti-apoptotic molecules, including Bcl-2 and Mcl-1 proteins, increased by mesothelin stimulation in a dose-dependent manner (3–4-fold compared with the controls) (Figure 4E). The levels of the other pro-apoptotic molecules in the Bcl-2 family, such as Bax and Bak, did not change in WF-0 cells after mesothelin stimulation, even after treatment with 100 nM of mesothelin.

The expression levels of both Bcl-2 and Mcl-1 proteins also significantly increased in mesothelin-overexpressed WF-0 transfectants. Interestingly, more endogenous mesothelin introduced caused lower expression of the pro-apoptotic protein Bax (Figure 4F).

These results indicate that the anti-apoptotic proteins Bcl-2 and Mcl-1 were involved in the anti-apoptotic effect of mesothelin in inhibiting paclitaxel-induced apoptosis. Endogenous mesothelin not only enhanced the expression of the anti-apoptotic proteins Bcl-2 and Mcl-1, but also reduced the expression of the pro-apoptotic protein Bax.

Mesothelin inhibited apoptotic signalling through both PI3K/Akt and MAPK/ERK pathways

Exogenous mesothelin-treated WF-0 cells showed tyrosine phosphorylation of the regulatory subunit of PI3K, p85, as determined by immunoprecipitation with an anti-p85 antibody, followed by Western blotting (Figure 5A). Mesothelin-overexpressed WF-0 transfectants also revealed more phosphorylation of the p85 subunit of PI3K than did the original WF-0 cells (Figure 5B). Moreover, ERK 1/2, the downstream target of MAPK signalling, was also phosphorylated after exogenous mesothelin stimulation (Figure 5C). Mesothelin-overexpressed WF-0 transfectants also revealed more phosphorylation of ERK 1/2 than did the original WF-0 cells (Figure 5D). These results indicate that mesothelin could turn on the machinery of survival signals via at least two possible routes: the PI3K cascade and the MAPK network.

To assess the role of PI3K or MAPK in the anti-apoptosis of mesothelin against paclitaxel, specific inhibitors were tested.
Mesothelin inhibits paclitaxel-induced apoptosis

Figure 5  Mesothelin induces rapid phosphorylation of PI3K and MAPK

Induction of tyrosine phosphorylation of the p85 subunit of PI3K was by exogenous mesothelin. (A) Protein expression levels after mesothelin treatment in WF-0 cells. Upper panel: cell lysates were immunoprecipitated with an anti-p85 antibody and detected with the anti-phosphotyrosine antibody. Lower panel: cell lysates were detected by the anti-p85 antibody. (B) Protein expression levels of mesothelin transfectants. Upper panel: cell lysates were immunoprecipitated with an anti-p85 antibody and detected with the anti-phosphotyrosine antibody. Lower panel: cell lysates were detected by the anti-p85 antibody. (C) Induction of tyrosine phosphorylation of ERK1/2 by exogenous mesothelin. Upper panel: cell lysates were detected with the anti-phospho-ERK1/2 antibody. Lower panel: cell lysates were detected by the anti-ERK1/2 antibody. Exogenous mesothelin induced ERK1/2 tyrosine phosphorylation even at low concentrations (10 nM). (D) Enhancement of ERK1/2 tyrosine phosphorylation by endogenous mesothelin in WF-0 and mesothelin transfectants. Upper panel: cell lysates were detected with the anti-phospho-ERK1/2 antibody. Lower panel: cell lysates were detected by the anti-ERK1/2 antibody.

to block mesothelin-induced cell protection. Chemical inhibitors specific to the signal transduction pathways, including LY294002 and PD098059, were given to WF-0 cells. Mesothelin induced both PI3K (Figure 6A) and MAPK (Figure 6B) activities within 30 min. The stimulation of PI3K or MAPK phosphorylation by mesothelin could be blocked by either inhibitor, as detected by the decreased phosphorylated form of Akt (Figure 6A, upper panel) or ERK proteins (Figure 6B, upper panel). However, the total intracellular protein expression of Akt and ERK was not altered during mesothelin treatment (Figures 6A and 6B, lower panels).

The induction of mRNA expression, including bcl-2 and mcl-1, was eliminated by the blockage of PI3K signalling, but not MAPK signalling (Figure 6C). Furthermore, only LY294002 (PI3K inhibitor), but not PD098059 (MAPK inhibitor), significantly reduced mesothelin-induced Bcl-2 and Mcl-1 protein expression levels (Figure 6D). Blockage of PI3K signalling suppressed >60% of induced anti-apoptotic protein Bcl-2 and Mcl-1 expression (for Bcl-2 expression, 3.75 ± 0.28-fold in the mesothelin group compared with 0.26 ± 0.04-fold in LY294002 combined with the mesothelin group; for Mcl-1 expression, 1.72 ± 0.24-fold in the mesothelin group compared with 0.37 ± 0.03-fold in LY294002 combined with the mesothelin group) (Figure 6E).

Representative images of Hoechst staining are shown in Figure 6(F). As in previous results, paclitaxel induced >40% of apoptosis in WF-0 cells, and mesothelin rescued the apoptotic effect to <10% (42.7 ± 7.7% for paclitaxel-treated group, 6.7 ± 2.6% for mesothelin in the paclitaxel-treated group; P < 0.01, one-way ANOVA) (Figure 6G). The blockage of PI3K abolished the anti-apoptotic effect by mesothelin treatment (6.7 ± 2.6% for mesothelin in the paclitaxel-treated group, 44.3 ± 6.7% for pre-treated LY294002 followed by mesothelin in the paclitaxel-treated group; P < 0.01, one-way ANOVA). Moreover, WF-0 cells pre-treated with the MAPK inhibitor did not alter the anti-apoptotic effect enhanced by mesothelin (6.7 ± 2.6% for mesothelin in the paclitaxel-treated group, 8.3 ± 2.1% for pre-treated PD098059 followed by mesothelin in the paclitaxel-treated group; P > 0.05, one-way ANOVA).

The activation of caspases 3 and 7 was also inhibited by blocking PI3K cascades, but not by blocking MAPK (Figure 6H). Inhibition of PI3K cascades increased the apoptotic cells even with mesothelin pre-treatment (Figure 6H). These results indicated that mesothelin enhanced at least two signal transduction pathways, PI3K/Akt and MAPK activation, to contribute to the anti-apoptotic pathway. The PI3K/Akt pathway was the main contributing pathway to the mesothelin-mediated anti-apoptotic effect.

DISCUSSION

Mesothelin, a differentiation antigen of mesothelial cells, is very abundant in normal mesothelial cells that line the serous membranes of pleural, pericardial and peritoneal spaces, and regulate the traffic of molecules into and out of the peritoneal cavity. Its exact function remains unclear, although it can be utilized as a new marker for detecting ovarian epithelial cancers [27,28]. The mesothelin receptor has not yet been identified. Clarifying the mesothelin receptor is an important issue to address the biological activity in the mesothelin-mediated signalling network.

However, elevation of mesothelin levels is also found in other types of malignancies, such as pancreatic cancer [29,30], biliary...
M.-C. Chang and others

Figure 6 PI3K is involved in mesothelin-regulated apoptosis

(A) De-phosphorylation of Akt in PI3K inhibitor LY294002-pre-treated WF-0 cells. Cell lysates were obtained and used for Western blot analysis with either anti-phospho-Akt (upper panel) or anti-Akt (lower panel) antibodies. (B) De-phosphorylation of ERK in MAPK inhibitor PD098059-treated WF-0 cells. Cell lysates were obtained and used for Western blot analysis by either anti-phospho-ERK (upper panel) or anti-ERK (lower panel) antibodies. (C) Mesothelin-stimulated Bcl-2 family expression was affected by the PI3K inhibitor, but not by the MAPK inhibitor, as determined by RT–PCR. (D) Mesothelin-stimulated Bcl-2 family expression was affected by the PI3K inhibitor, but not by the MAPK inhibitor, as determined by Western blotting. (E) Quantification of Bcl-2 and Mcl-1 protein expression. Protein expression of WF-0 cells affected by mesothelin was detected by Western blotting. Relative intensities were quantified from immunoblotting by a densitometer and the fold of induction was determined by at least three individual experiments. (F) Representative images of Hoechst assays. Arrows indicate the apoptotic cells. F1, PBS; F2, PD098059 only; F3, LY294002 only; F4, paclitaxel; F5, mesothelin+LY294002+paclitaxel; F6, mesothelin+PD098059+paclitaxel. (G) Percentages of apoptotic cells in Hoechst assays. Mesothelin-erased paclitaxel-induced apoptosis was rescued by the PI3K (LY294002), but not the MAPK (PD098059), inhibitor. Results are means±S.E.M. for three independent experiments. *P<0.01. (H) Inhibition of caspase 3 and 7 activation by mesothelin was reversed by blockage of PI3K, but not MAPK. H1, PBS; H2, mesothelin+PD098059; H3, mesothelin+LY294002; H4, paclitaxel; H5, mesothelin+LY294002+paclitaxel; H6, mesothelin+PD098059+paclitaxel. The M value indicates the median value of total cells stained with caspase 3 and 7 fluorescent substrate.

carcinomas [31] and mesotheliomas [32]. Large comprehensive studies are needed to survey whether mesothelin can be a specific marker or predictor for disease severity in ovarian cancer. The identification of the function of mesothelin will enhance its clinical applications in ovarian cancer prognosis and chemotherapeutic responses. The mesothelin receptor can be a potential therapeutic target in the future.

The signal transduction pathways induced by mesothelin resulting in cell survival can be elucidated using an in vitro WF-0 cell model. Paclitaxel can induce cell apoptosis, as confirmed by morphological changes, and paclitaxel-induced apoptosis can be rescued by mesothelin (Figures 3A and 3B). The most important cell survival signalling pathways, PI3K/Akt and MAPK/ERK, have both been investigated in the present study. Akt activation is a key player in the PI3K downstream cascade by growth factor stimulation [33]. The ability of activated Akt to promote survival is seen in fibroblasts [34] and PC12 phaeochromocytoma cells [35]. A number of different growth factors can rapidly activate Akt via PI3K activation, such as PDGF (platelet-derived growth factor), EGF (epidermal growth factor), bFGF (basic fibroblast growth factor), insulin and IGF (insulin-like growth factor) 1 [36].

In the present study, mesothelin also induced Akt phosphorylation rapidly under PI3K activation. However, in the human breast cancer model, mesothelin can prevent anoikis and promote cell growth by suppressing Bim induction, albeit dependent on the activation of the ERK signalling cascades [37]. These different conclusions may be due to the different cell cultures and observed targets. Mesothelin may have many undefined functions and mediate tumour progression via many as yet unknown molecular mechanisms. Thus further study of mesothelin is needed.

MAPK/ERK is a critical mediator of the cell proliferative effect of these pathways [38]. This is consistent with previously published data demonstrating that inhibition of these pathways...
eliminate the growth of many types of cancer cell lines in vitro [39]. Regulating the Bcl-2 family member is also considered as one of the anti-apoptotic mechanisms of MAPK/ERK, which is reportedly capable of inducing Bcl-2 [40]. Thus both MAPK/ERK and PI3K/Akt signalling pathways transduced by mesothelin are potential candidates to converge on Bcl-2 family members, which can act upstream of caspase 3. In the present study, blockade of MAPK cascade activation does not affect either the anti-apoptotic activity or Bcl-2 family expression of mesothelin against paclitaxel. This suggests that Bcl-2 expression and the anti-apoptotic effect may not be dependent on MAPK/ERK activation, and that the activated MAPK/ERK cascades may play other important roles in mesothelin-enhanced tumour progression, even invasion.

Apoptosis is a cascade of events involving the activation of many genes and the synthesis of proteins. Paclitaxel cytotoxicity involves signalling that is distinct from DNA-damaging drugs, but common to all microtubule-active drugs with the specific hallmark of Bcl-2 hyperphosphorylation [41,42]. Paclitaxel treatment itself can modulate the expression of Bcl-2 family members and cause post-translational modification of Bcl-2 molecules. Several studies have shown that phosphorylated Bcl-2 does not homodimerize with Bax, resulting in increased levels of free Bax, a situation favourable to apoptosis [43], whereas phosphorylated Bcl-2 may remain complexed with Bax in paclitaxel-treated cells [44]. Studies have shown that Bcl-XL can be down-regulated [45], whereas Bak [45,46] and Bax [47] can be up-regulated by paclitaxel.

In addition, Bcl-2 phosphorylation can also be induced by paclitaxel, a phenomenon generally believed to promote paclitaxel-initiated apoptosis. Previous studies have also observed that both the anti-apoptotic proteins Bcl-2 and Mcl-1 can be up-regulated upon recombinant mesothelin stimulation. Interestingly, endogenous mesothelin can suppress further the expression of the pro-apoptotic protein Bax, as shown in the present study. Endogenous mesothelin may express more quantized protein than those added into the medium. The overdosed mesothelin not only increases both Bcl-2 and Mcl-1 expression, but also inhibits the pro-apoptotic protein Bax to enhance the apoptotic effects.

Paclitaxel in combination with other platinum agents is used as the standard chemotherapeutic regimen in patients with advanced ovarian cancer. Although it can activate MAPK in vitro, there is no significant evidence available from patients treated with paclitaxel. The clinical use of paclitaxel in treating solid malignancies has been investigated for several types of cancers that have developed an intrinsic resistance to the drug. For instance, the high expression of P-glycoprotein is regarded as resistance to paclitaxel [48], which is seen in patients with advanced colorectal tumours [49,50]. Understanding the detailed mechanism of paclitaxel resistance will improve the therapeutic response of cancer patients clinically.

Paclitaxel-resistant ovarian cancer patients have higher mesothelin levels in their cancer tissues. The results of the present study imply that mesothelin expression may be related with chemotherapeutic effects in cancer patients. Bera and Pastan [51] found that the function of mesothelin is not essential for growth or reproduction in knockout mice. Paclitaxel resistance is indeed a complex biological interaction characterized by a continuum of changes in gene expression. We hypothesize that mesothelin may alter the cytotoxicity effects enhanced by chemotherapeutic drugs, and thereby maintain cancer cell survival through a similar mechanism. The results of the present study also show that higher mesothelin expression occurs in chemoresistant rather than in chemosensitive populations (Figures 1A and 1B). Mesothelin overexpression leads to cancer cell growth advantages and greater viability in the tumour micro-environment of ovarian cancer during chemotherapy. Other mechanisms may also exist, although the major mechanisms of resistance identified thus far involve reduced drug uptake, increased drug efflux, increased repair of platinum–DNA adducts, increased tolerance of DNA damage and increased levels of intracellular thiols such as glutathione and metallothionein [5.6,52–55]. These possibilities should be confirmed further by more experiments. The results of the present study are also the first clinical-based evidence that correlates chemotherapeutic-resistance effects and mesothelin expression in ovarian epithelial cancer patients.

AUTHOR CONTRIBUTION

Wen-Fang Cheng was the key principal investigator. He designed the experiments and wrote the paper with input from all of the other authors. Chi-An Chen, Chang-Yao Hsieh and Wen-Fang Cheng provided clinical specimens and correlated the clinical parameters. Ming-Cheng Chang participated in all of the experimental procedures in the study, including the design and performance of the experiments. Chi-An Chen and Chien-Nan Lee participated in the interpretation of experimental results and discussion. Chang-Yao Hsieh also advised on and evaluated the apoptosis-related analyses. Yi-Ning Su offered technical support for the experiments and helped with the interpretation and discussion of the results. RNA isolation and RT–PCR experiments were performed by Yu-Hao Hu, who was also the research assistant of the project.

FUNDING

This work was supported in part by the Department of Medical Research of National Taiwan University Hospital and by the National Science Committee of Taiwan [grant number 97-2314-B-002-064-MY3] to C.-A.C.

REFERENCES

8 Chen, R. H., Chang, M. C., Su, Y. H., Tsai, Y. T. and Kuo, M. L. (1999) Interleukin-6 inhibits transforming growth factor-β-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. J. Biol. Chem. 274, 23013–23019

© The Authors Journal compilation © 2009 Biochemical Society


