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Tissue inhibitor of metalloproteinase-3 induces apoptosis in prostate cancer cells and confers increased sensitivity to paclitaxel

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ABSTRACT

Prostate cancer is the most common cancer and the second leading cause of cancer-related death in American men. To investigate the possible usefulness of tissue inhibitor of metalloproteinase-3 (TIMP-3) in prostate cancer gene therapy, we used an adenovirus expressing TIMP-3 to assess its role as an apoptosis trigger in highly metastatic prostate cancer cell lines PC-3 and DU-145. We showed that TIMP-3 alone induced apoptotic cell death which was triggered by mitochondrion-mediated caspase-3 activation. In combination treatment, we found that adenovirus-mediated expression of TIMP-3 greatly sensitised prostate cancer cells to chemotherapeutic drug paclitaxel, indicating a superadditive or synergistic effect of TIMP-3 and cytostatic treatment on prostate cancer cell death. The proper combination of adenovirus-mediated expression of TIMP-3 with conventional chemotherapeutic drug(s) could have potential benefits in treating highly metastatic prostate cancer.

1. Introduction

With an estimated 232,090 new cases and 30,350 deaths in 2005 in the United States of America (USA), prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in American men.[1] Surgical and hormonal therapies have shown beneficial effects only for early-stage, hormone-responsive disease. Metastatic prostate cancer inevitably progresses to an androgen unresponsive stage and is then essentially incurable.[2] Although chemotherapy is the primary treatment for most types of metastatic cancer, conventional chemotherapeutic agents are not effective for metastatic prostate cancer[3] and to date no overall survival benefit has been achieved in randomised trials.[4,5] Therefore, an effective therapeutic regimen for this stage of prostate cancer would tremendously improve patient survival.

The most effective cytotoxic therapies at the present time for treating hormone refractory prostate cancer seem to be combinations of estramustine phosphate with taxanes and etoposide.[6,7] The taxanes, docetaxel (Taxotere) and paclitaxel (Taxol), have received increased attention for their potential role in treating patients with hormone refractory prostate cancer due to their ability to induce cell death in prostate tumour cell lines. Taxanes preferentially bind to tubulin and promote the polymerisation of microtubules in the absence

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of microtubule-associated protein and guanosine triphosphate.[8]

Polymerised microtubules cannot be disassembled in the presence of taxanes. Thus, cells are arrested at the G2/M phase of the cell cycle, leading to apoptosis.[9] Paclitaxel has gained widespread acceptance as an active broad-spectrum antitumour agent, including its use in urological malignancies, particularly urothelial tract cancer and testicular cancer. In 2004, docetaxel became the first drug to prolong survival of patients with androgen-independent prostate cancer; however future studies are still needed.[10] These promising single-agent results have prompted the use of combination regimens including, in particular, cisplatin and paclitaxel.[11] However, high toxicities are frequently seen in combination chemotherapies and the effectiveness is still questionable.[12–14] Therefore, innovative approaches are needed to improve survival rates for the treatment of prostate cancer.

The tissue inhibitors of metalloproteinases (TIMPs) are a family of proteins consisting of four members (TIMPs-1 through-4) that block the extracellular matrix (ECM)-degrading activity of matrix metalloproteinases (MMPs).[15] TIMP-3 is a unique natural metalloproteinase inhibitor that plays a pro-apoptotic role through its ability to inhibit metalloproteinases that proteolytically cleave death receptors and their ligands from the cell surface. TIMP-3 has been shown to promote apoptosis in various cell types, including vascular smooth muscle cells[16] and a number of cancer cell lines.[17] Transfection of the human TIMP-3 gene into DLD colon carcinoma cells resulted in regaining of serum responsiveness, no tumour formation in nude mice, delayed cell cycle progression, and programmed cell death of the transfected cells.[18,19] Intratumoural injection of melanomas and squamous cell carcinomas in vivo with TIMP-3-expressing adenovirus inhibits tumour growth and results in reduced gelatinolytic activity, induction of apoptosis, and inhibition of tumour angiogenesis. Also, TIMP-3 is produced by melanoma cells in soluble form to the culture medium, where it exerts a cytotoxic effect on uninfected cells through a bystander mechanism.[20] Moreover, TIMP-3 has been identified as a putative tumour suppressor, the expression of which is specifically repressed during oncogenic transformation and inactivated by methylation in malignant tumours of various tissues, such as kidney, lung, colon, breast, brain, and pancreas.[21–25] These effects on tumours of different origin speak in favour of the potential of TIMP-3 for gene therapy. High levels of TIMP-3 are found to be associated with adjuvant endocrine therapy success and with successful tamoxifen treatment of patients with breast cancer.[26] TIMP-3 regulates neuronal sensitivity to doxorubicin-induced apoptosis in primary cortical neurons.[27] However, so far, no study has been performed on prostate cancer gene therapy using TIMP-3 in combination with chemotherapy.

In the present study, we investigated the role of adenovirus-mediated TIMP-3 expression in apoptosis induction and showed that TIMP-3 alone induced apoptotic cell death in highly metastatic prostate cancer cell lines which was triggered by mitochondrion-mediated caspase-3 activation. In addition, we found that adenovirus-mediated expression of TIMP-3 resulted in increased sensitization of PC-3 prostate cancer cells to chemotherapeutic drug paclitaxel.

2. Materials and methods

2.1. Cell culture and infection procedure

PC-3 and DU-145, two androgen-independent, androgen receptor- and prostate-specific antigen-negative human prostate cancer cell lines established from the bone metastasis[28] and from brain metastasis,[29] respectively, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics in 37 °C CO_2 incubator. The recombinant adenoviral construct AdTIMP-3 under the control of the cytomegalovirus major immediate early promoter[16] was a generous gift from Dr. A.C. Newby, Department of Cardiology, University of Wales College of Medicine, U.K. The control adenovirus AdNull which contains a similar adenoviral backbone but not the transgene[30] was used as a mock-infection control. Recombinant adenovirus was obtained from virus-infected 293 cells, cesium chloride-banded, and titered using standard assays.[16] Infection was conducted by incubating the cells with the recombinant adenovirus at various multiplicities of infection (MOIs), expressed as plaque-forming unit (pfu)/cell in complete medium. The next day, cells were washed with PBS and further incubated in complete medium for the required period of time.

2.2. Western blot analysis

PC-3 cells plated in T75 flasks were infected with AdTIMP-3 at 0, 10, 30 or 100 pfu/cell for 48 h. Total cell lysates were prepared in SDS lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 1× complete protease inhibitor mixture). 100 μg protein was separated by 8–16% gradient SDS-polyacrylamide gel, and transferred to PVDF membrane (Bio-Rad, Hercules, CA) at 30 V for 2 h. The membrane was blocked with 5% non-fat milk in TBS-T, incubated with mouse anti-human TIMP-3 primary antibody (1 μg/ml, Chemicon, Temecula, CA) and immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) and visualised using the enhanced chemiluminescence system.

2.3. Immunocytochemistry

Cells grown on coverslips were infected for 72 h with AdTIMP-3 or mock-infected with AdNull at 100 pfu/cell, fixed with 3.7% buffered formaldehyde and stained for TIMP-3 or caspase-3 expression. Briefly, permeabilised cells were incubated for 2 h at 37 °C with mouse anti-human TIMP-3 (1 μg/ml, Chemicon) or rabbit anti-human caspase-3 (1 μg/ml, R& D Systems, Minneapolis, MN). For TIMP-3 detection, cells were incubated for 30 min each with rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) and alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes (Sigma, St. Louis, MO) and the alkaline phosphatase reaction was revealed using the Fast Red TR/Naphthol AS-MX substrate (Sigma). For caspase-3 detection, cells were detected using avidin-biotin-complex-alkaline phosphatase (ABC-AP) kit (VectorLabs, Burlingame, CA) followed by colour development with the Fast Red TR/Naphthol AS-MX substrate (Sigma). The cell nuclei were counterstained blue with hematoxylin.

2.4. Detection of apoptosis

To detect apoptosis in TIMP-3-expressing cells, cells were infected with AdTIMP-3 or AdNull at 100 pfu/cell for 72 h and a terminal dUTP nick end labelling (TUNEL)-based apoptosis assay kit, TumorTACS (R& D Systems), was used following the manufacturer's instructions. Apoptosis was further confirmed using 7-amino-actinomycin D (7-AAD, Calbiochem, San Diego, CA) staining.[31] Briefly, cells seeded in chamber slides were infected with AdTIMP-3 at 100 pfu/cell or mock-infected for 72 h. The cells were fixed with 3.7% formaldehyde and stained with 7-AAD (Sigma) (20 μg/ml final concentration) for 30 min at room temperature followed by two washes with 0.1% Triton X-100, 3 min per wash. The cell nuclei were counterstained with DAPI and the slides were then mounted with Vectashield Mounting Medium (VectorLabs, Burlingame, CA) and visualised under fluorescence microscope.

2.5. Mitochondrial staining

PC-3 cells grown on coverslips were infected with AdTIMP-3 at different MOIs or AdNull (100 pfu/cell) for 72 h and stained with MitoTracker Red 580 (Invitrogen, Carlsbad, CA) for 30 min in growth medium at 37 °C. Cells were washed once with fresh medium, fixed in 3.7% formaldehyde in medium for 15 min at 37 °C, mounted and visualised under fluorescence microscope (as above).

2.6. Cytotoxicity assay

Cells seeded in 96-well plates at a density of 5×10^3 (PC-3) or 2×10^3 (DU-145) cells/well were infected with AdTIMP-3 or control virus at an MOI of 100 pfu/cell. Paclitaxel (10^{-5} M, Sigma) was added 24 h after initial viral infection. Vehicle (DMSO) was added to control cells. 72 h after drug treatment, cell viability was assessed using CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI). Plates were analysed in a Packard Spectracount microplate reader at a wavelength of 450 nm. Absorbance values were plotted assuming the survival of cells without drug treatment to be 100%. Results were expressed as mean ± SD. Significant differences between means were determined by ANOVA with Tukey multiple comparisons *post hoc* analysis using Prism software (Graphpad Software, San Diego, CA). *P* values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Expression of TIMP-3 in AdTIMP-3-infected prostate cancer cells

To confirm the expression of the transgene in infected cells, we first performed Western blot analysis on whole cell lysates prepared from adenovirus-infected PC-3 cells to analyse the expression of TIMP-3. Uninfected PC-3 cells did not express any detectable TIMP-3 protein. TIMP-3 immunoreactive bands at 29 and 27 kDa were detected in a dose-dependent manner in PC-3 cells infected with various concentrations of AdTIMP-3 (Fig. 1). These molecular weight species correspond to previously described glycosylated and unglycosylated forms of TIMP-3.[32]

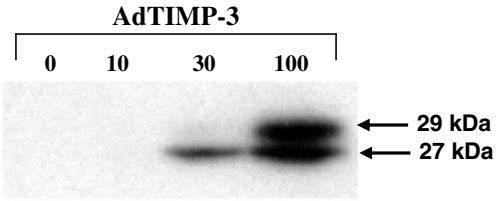

Fig. 1 – Adenovirus-mediated overexpression of TIMP-3 in prostate cancer cells. Cells were infected with different MOIs of AdTIMP-3 for 48 h. Whole cell lysates were prepared, subjected to Western blot analysis on SDS–PAGE, and immunoblotted with the antibody against TIMP-3. A dose-dependent expression of TIMP-3 was detected in PC-3 cells infected with AdTIMP-3 with two reactive bands representing glycosylated (29 kDa) and unglycosylated (27 kDa) expression products.

3.2. AdTIMP-3 induces apoptosis in prostate cancer cells via caspase activation

To investigate whether adenovirally expressed TIMP-3 itself could induce prostate cancer cell apoptosis, we performed double staining for apoptosis using *in situ* terminal dUTP nick end labeling (TUNEL) assay and for TIMP-3 expression using immunocytochemistry. *In situ* TUNEL analysis of AdTIMP-3-infected PC-3 and DU-145 cells revealed intense brown nuclear staining indicative of fragmented DNA, with extensive nuclear condensation (Fig. 2, brown signal) as compared to no staining at all in mock-infected cells. Expression of TIMP-3 was revealed by APAAP/Fast Red staining in AdTIMP-3-infected cells (Fig. 2, red signal). 7-AAD is a fluorescent DNA-binding agent, which, when used alone, can define the three cell populations (dead, apoptotic and live).[33] Therefore, apoptosis was further confirmed using 7-AAD staining. About 60% of AdTIMP-3-infected PC-3 or DU-145 cells were stained positive with 7-AAD, indicating cell death caused by AdTIMP-3 treatment (Fig. 3). These independent methods of measuring apoptosis provided convincing data showing the induction of apoptosis in PC-3 cells treated with AdTIMP-3. To characterise the intracellular downstream mechanism(s) activated by TIMP-3, we detected the effect of TIMP-3 overexpression on caspase-3 activity by immunocytochemistry. Our results showed the activation of caspase-3 48 h after infection with AdTIMP-3 in 40–50% of DU-145 cells as compared to no caspase-3 activity in mock-infected cells (Fig. 4).

One of the reasons for prostate cancer chemoresistance is the escaping of apoptosis. Impaired apoptosis is a significant impediment to cytotoxic therapy. Resistance to apoptosis can also augment the escape of tumour cells from surveillance by the immune system. Defects in the apoptosis-inducing pathways can eventually lead to expansion of a population of neoplastic cells and treatment might select more refractory clones.[34] Therefore, resistance to apoptosis constitutes an important clinical problem.[35] Thus, modulation of apoptosis

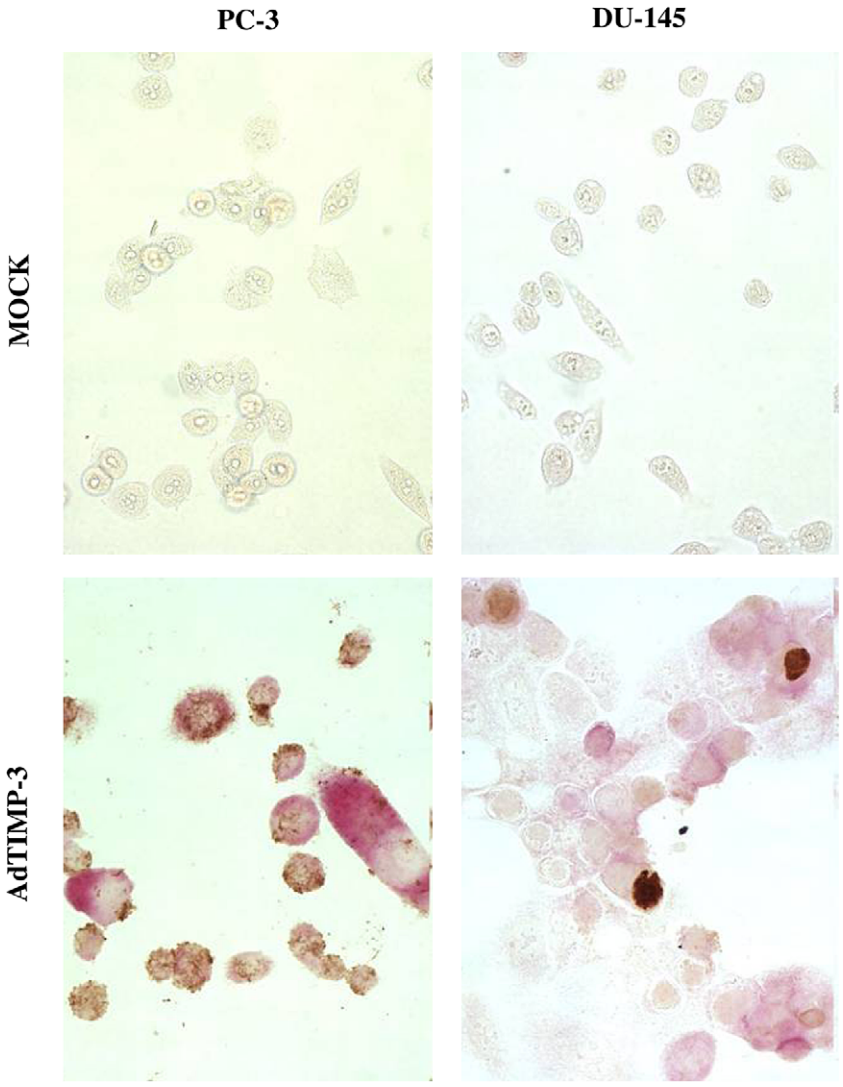

Fig. 2 – Induction of apoptosis in prostate cancer cells. Cells infected with 100 pfu/cell of AdTIMP-3 or AdNull for 72 h were fixed and double-stained for apoptosis using *in situ* terminal dUTP nick end labeling (TUNEL) assay and for TIMP-3 expression using APAAP-Fast Red staining system as described in Materials and methods. Note the brown apoptotic signals in the nucleus and the red signals in the cytoplasm showing TIMP-3 expression in AdTIMP-3-infected cells as compared to mock-infected cells (×100).

at the molecular level might ultimately lead to new therapeutic approaches.

3.3. *AdTIMP-3 induces mitochondrial fragmentation*

Expression of TIMP-3 in tumour cells leads to apoptosis, possibly due to stabilisation of TNF-α receptors on the membrane.[18] Ahonen et al. found that in melanoma cells, TIMP-3 promotes apoptosis through stabilisation of distinct death receptors and activation of their apoptotic signaling cascade through caspase-8.[36] As a cross-talk between the death receptor-mediated and mitochondrion-mediated apoptotic pathways, activation of caspase-8 can initiate mitochondrial activation, resulting in mitochondrial membrane depolarisation, release of cytochrome *c* into the cytosol, and subsequent activation of caspase-9 and effector caspases (caspase-3, -6, and -7).[37] Bond et al. showed that TIMP-3 overexpression induces an apoptotic pathway via a Fas-associated death domain-dependent mechanism which involves mitochondrial activation in rat smooth muscle cells.[38]

In our study, mitochondrial activation was documented in PC-3 prostate cancer cells by staining with the fluorescent dye MitoTracker Red 580 followed by fluorescence microscopy. We found that when PC-3 cells were exposed to AdTIMP-3 at different doses (from 10 to 100 pfu/cell) for 72 h, the typical reticular mitochondria found in healthy cells were disintegrated into small, fragmented and sometimes spherical organelles in a dose-dependent manner (Fig. 5). Mitochondrial condensation was also noted as shown by increased density of mitochondrial staining. Mitochondrial fragmentation has been described in connection with many modes of apoptosis.[39]

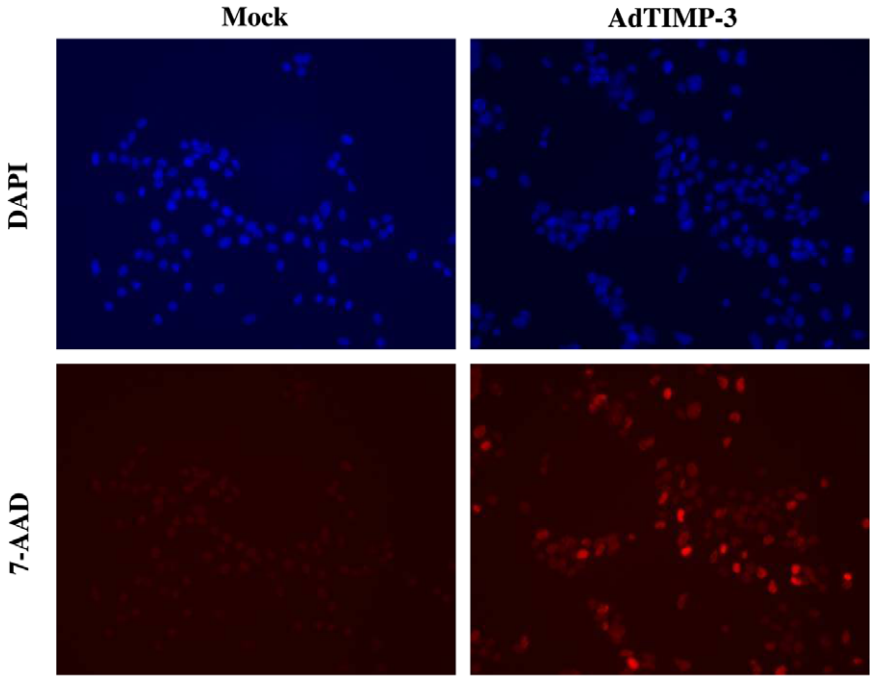

Fig. 3 – 7-AAD staining of prostate cancer cells. Cells infected with 100 pfu/cell of AdTIMP-3 or AdNull for 72 h were fixed and stained with 7-AAD as described in Materials and methods. The cell nuclei were counterstained with DAPI. Apoptotic cells in AdTIMP-3-infected cells were stained red with 7-AAD as compared to mock-infected cells (×100).

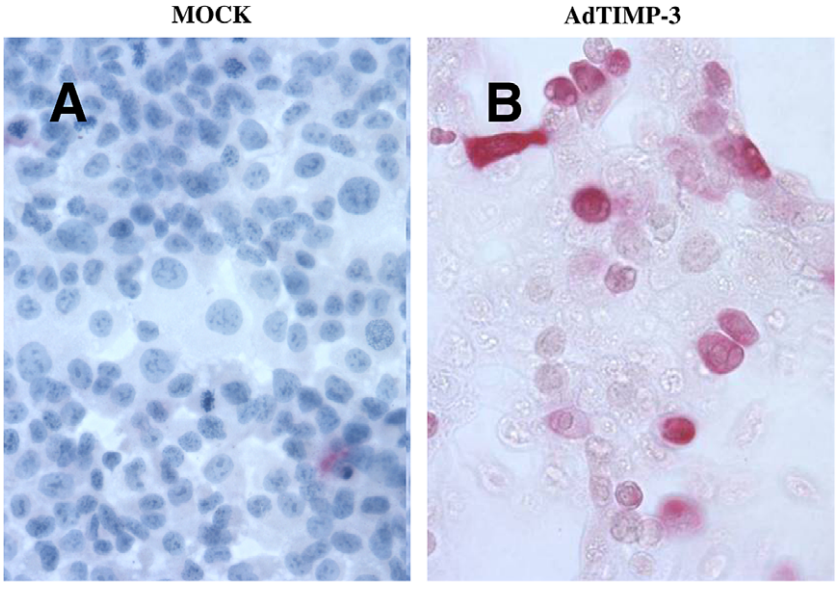

Fig. 4 – Activation of caspase-3 in prostate cancer cells. Cells infected with AdTIMP-3 or AdNull (100 pfu/cell) for 72 h were fixed, analysed for active caspase-3 by immunocytochemistry and developed with ABC-AP-Fast Red system. The cell nuclei were counterstained blue with hematoxylin. Active caspase-3 was stained red in AdTIMP-3-infected cells as compared to mock-infected cells (×100).

3.4. *Synergistic toxicity of AdTIMP-3 and cytostatic treatment*

Finally, we studied whether infection with AdTIMP-3 acts synergistically with paclitaxel to enhance tumour cell death. To determine the optimal dose for combination treatment, various MOIs of AdTIMP-3 (10–10,000 pfu/cell) were used to infect PC-3 and DU-145 cells. At doses higher than 1 000 pfu/cell, AdTIMP-3 showed extensive cytotoxicity on cells after 72 h infection; while the control virus AdNull had little effect on cell survival with nearly 90% of cells still viable at the same concentrations and the same time point (data not shown). Therefore, 100 pfu/cell was chosen for synergistic toxicity study. Our results showed that infection of PC-3 and DU-145

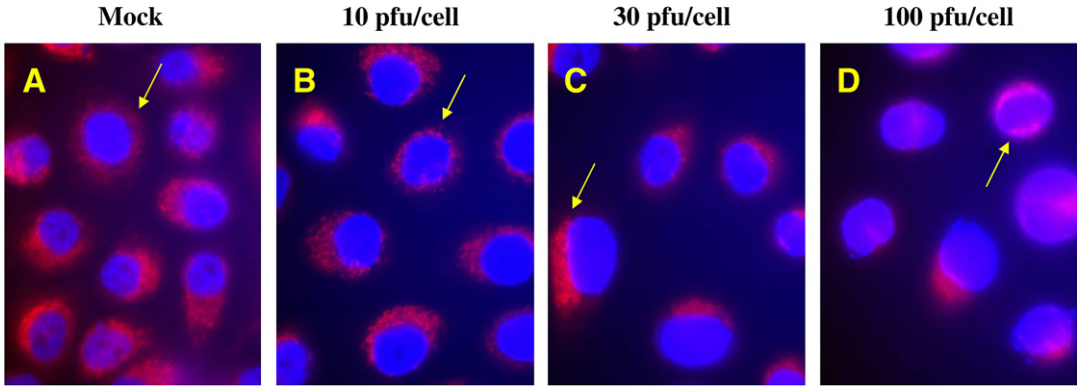

Fig. 5 – Conversion of the mitochondrial morphology during AdTIMP-3-induced apoptosis. PC-3 cells were infected with AdTIMP-3 or mock-infected, stained with MitoTracker Red 580 and visualised by fluorescence microscopy. The cell nuclei were counterstained blue with DAPI. The reticular mitochondrial morphology characteristic of healthy cells seen in mock-infected cells (A) was converted into the punctiform phenotype in AdTIMP-3-infected cells (B through D). Note the gradual change of mitochondria from reticular to punctiform morphology (arrow) in AdTIMP-3-infected cells with increasing AdTIMP-3 concentrations. B: 10 pfu/cell; C: 30 pfu/cell; D: 100 pfu/cell (×400).

cells with AdTIMP-3 significantly sensitised these cells to paclitaxel treatment. The combined cytotoxicity in the paclitaxel + AdTIMP-3 group was much higher than the paclitaxel only, paclitaxel + control virus or the AdTIMP-3 only groups ($P < 0.01$) (Fig. 6), indicating a superadditive or synergistic effect of TIMP-3 and cytostatic treatment on prostate cancer cell death.

In conclusion, our present study demonstrated that TIMP-3 plays an important role in the induction of apoptosis in prostate cancer cells, and the proper combination of adenovirus-mediated expression of TIMP-3 with conventional chemotherapeutic drug(s) could have potential benefits in treating highly metastatic prostate cancer. The *in vivo* usefulness of this combination for prostate cancer needs to be further investigated.

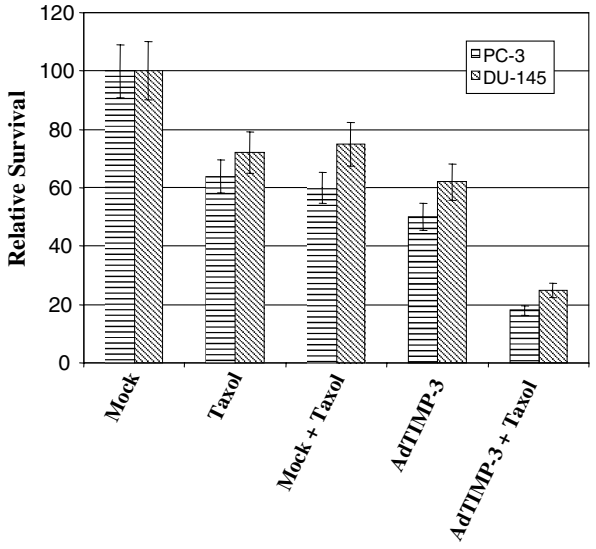

Fig. 6 – AdTIMP-3-mediated sensitisation of prostate cancer cells to paclitaxel. Cells were infected with AdTIMP-3 or AdNull at 100 pfu/cell and treated with paclitaxel (Taxol) (10^{-5} M) for 72 h. Cell viability was assessed using CellTiter-Glo luminescent cell viability assay kit. Note the much reduced cell survival rate in the AdTIMP-3 + Taxol group than the Taxol only, control virus + Taxol or the AdTIMP-3 only groups ($P < 0.01$).

Conflict of interest statement

None declared.

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