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In vitro susceptibility to the pro-apoptotic effects of TIMP-3 gene delivery translates to greater in vivo efficacy versus gene delivery for TIMPs-1 or -2

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Summary Matrix metalloproteinases (MMPs) are essential for extracellular matrix (ECM) breakdown and repair, and have been implicated in the development of metastases. TIMP-3 was initially identified as a potent inhibitor of MMPs, however it also has several properties that are unique and not related to its ability to abrogate MMPs. We studied the effects of overexpression of tissue inhibitor of metalloproteinases-3 (TIMP-3) on lung cancer cells and explored the mechanisms involved in apoptosis-induction in susceptible cells and subsequently, the therapeutic effect in vivo. Overexpression of TIMP-3 resulted in apoptosis of A549 lung cancer cells and AdCMVTIMP3 up-regulated the expression of p53, Fas ligand, TNFR1 and TNFR2 on these cells. Adenoviral delivery of TIMP-3 gene inhibited the growth of pre-established A549 tumours in Balb/c nude mice, and was associated with a greater therapeutic effect than either TIMP-1 or -2 gene delivery. There was no evidence of increased hepatic toxicity following the delivery of TIMP-3 either from intra-tumoural or intravenous injection. Thus, at least in cells showing in vitro susceptibility, TIMP-3 gene therapy offers a therapeutic advantage over TIMPs 1 and 2. These findings establish the potential of adenoviral gene delivery of TIMP3 as a therapeutic agent for selected lung cancers.

1. Introduction

Lung cancer is responsible for more cancer-related deaths than cancers of colon, breast and prostate combined [1]. Recent advances in chemotherapeutic agents have had little

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impact on the overall 5-year survival, which is less than 15% for non-small cell lung cancer (NSCLC) [2]. The understanding of the molecular basis of cancer development and the mechanisms of the development of metastases has increased the potential targets for the development of effective anticancer agents. Gene-based therapies provide an ideal opportunity to exploit this knowledge as novel therapeutic agents for the management of various cancers.

Degradation of the extracellular matrix (ECM) occurs as cells change their form, migrate or proliferate. Controlled matrix destruction is considered a key event in both local invasion and the development of metastasis associated with tumour progression [3]. Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases that regulate the movement of cells within the ECM and also degrade all of its components. They have important roles in tumour development and in tumour cell survival [3,4]. MMPs are over-expressed in a number of cancers including lung cancer. This is associated with increased tumour aggressiveness and metastatic potential [3,4]. The intricate balance between net extracellular matrix deposition and degradation is controlled by a complex system of tightly regulated protease enzymes and their endogenous inhibitors, which includes the tissue inhibitors of metalloproteinases (TIMPs) [5]. It is postulated that inappropriate over-expression of MMPs or under-expression of TIMPs constitutes part of the pathogenic mechanism in cancer [6]. Therapies targeted to the processes that inhibit MMP proteolysis and ECM breakdown may specifically disrupt the growth of tumour without altering normal tissues. The increased local expression of specific TIMPs, which is achievable with gene delivery may prove a more efficient and effective mechanism for the inhibition of MMPs. Furthermore, it is now known that certain TIMPs possess additional properties which may have a beneficial effect in cancer therapy beyond MMP inhibition [7].

TIMP-3 has several features that distinguish it from the other TIMPs. It binds tightly to the ECM following secretion by the cell [8]. It inhibits vascular endothelial growth factor mediated angiogenesis by blocking the binding of VEGF to VEGF receptor-2, inhibiting downstream signalling and angiogenesis [9]. TIMP-3 also inhibits TNF-α-converting enzyme, a member of the ADAM family (a disintegrin and metalloproteinase). This may account for its ability to induce apoptosis [10]. It has been shown to induce apoptosis in a number of cancer cell lines [5,11,12]. It also inhibits shedding of ectodomains of cell surface receptors including syndecan-1 and -4, L-selectin and IL-6 receptors [10,13—15]. It is plausible that loss of TIMP-3 within tumours may abrogate normal apoptotic programs, enhance primary tumour growth and angiogenesis, invasiveness and metastasis and possibly therefore contribute to all stages of malignant progression [7]. Thus, TIMP-3 gene therapy may have significant advantages over other TIMPs.

To date, less work has been done to evaluate the therapeutic efficiency of TIMP-3 compared to TIMPs-1 and -2. Previous work with TIMP-2 gene delivery has shown therapeutic effects in animal models, whereas the effects of TIMP-1 are variable [16]. Further, not all cells lines are susceptible to the apoptosis-inducing effects of TIMP-3 [17]. No direct comparisons of in vivo delivery of TIMP family members have been reported. In the current study we determined the effects of overexpression of adenovirally delivered TIMP-3 in susceptible lung cancer cells on proliferation, apoptosis and cell death in vitro, in comparison to TIMPs-1 and -2. We then determined whether susceptibility to TIMP-3-induced apoptosis translated to therapeutic gains in vivo in over the effects seen to TIMPs-1 and 2.

2. Materials and methods

2.1. Adenoviral vectors

First generation adenoviral vectors containing the genes for either TIMP-1, -2 or -3, or the luciferase reporter gene each under the control of the cytomegalovirus promoter, have been described previously [18,19].

2.2. Cell lines and culture conditions

Cell lines were obtained from the American Type Culture Collection (ATCC). Human embryonic kidney cell line 293 were used in the amplification and titration of adenoviral vectors. The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM): F12 medium (50:50) supplemented with 10% foetal calf serum (FCS), penicillin and gentamycin, and maintained in a humidified atmosphere at 37 °C in 5% CO_2. A549 lung cancer cells were maintained in DMEM with bicarbonate, with similar antibiotics and under the same conditions. The lung cancer cells lines 1299, H1466, H322, 522, 2009 were maintained in RPMI medium supplemented with 10% FCS, penicillin and gentamycin, and maintained in a humidified atmosphere at 37 °C in 5% CO_2.

Viral particles were purified by caesium chloride gradient using standard techniques, then particle titres established using OD260 absorbance, where one OD corresponds to 1.1×10^{12} viral particles. Functional titres were determined by TCID50 in 293 cells.

2.3. Cell counting assays

The range of six lung cancer cell lines (1×10^5 cells ml^{-1}) were examined for Coxsackie and adenoviral receptor (CAR) expression by flow cytometry. Cells were infected with AdCMVTIMP1, AdCMVTIMP2, AdCMVTIMP3 or AdCMVLuc at 100 pfu cell^{-1} in 2% FCS medium, then after one hour switched to complete medium and incubated for 24, 48, 72 and 96 h. At these time points, viable adherent cells were counted using a standard haemocytometer and trypan blue exclusion. In parallel crystal violet staining measurements of viable cells were also carried out.

2.4. Bystander effect

2.4.1. Infected cells transfer

A549 cells were transduced with 100 pfu of adenoviral vectors-AdCMVLuc, AdCMVTIMP1, -2 and -3, as per the standard protocol. After 24 h the cells were detached, washed with PBS and suspended in serum free culture medium. The cells were mixed in different ratios with uninfected A549 cells and plated in six-well plates. The

number of viable cells was determined by manual counting at 96 h.

2.4.2. Conditioned media

The conditioned media was obtained from 1.5×10^6 A549 cells plated in T75 flasks and infected at 100 pfu cell^{-1} using the viruses described above. The cells were washed four times with PBS 24 h following infection and incubated in complete media. The media was collected 48 h later and transferred to A549 cells plated in six-well plates in either a 15% or 35% ratio. The number of viable cells was determined by manual counting at 96 h.

2.5. Flow cytometry of apoptosis

2.5.1. Annexin V staining

Cells infected as described were collected using standard trypsin and resuspended in media. Adherent cells were pooled with non-adherent cells. Cells were stained with Annexin V (Pharmingen CA, USA) and Propidium Iodide (PI) (Sigma) which are sensitive to early and late apoptosis, respectively, as previously reported [20]. Briefly, cells were washed once with Annexin binding buffer, then centrifuged ($300 \times g$ for 5 min) and the supernatant discarded. The cells were stained with 2 μl Annexin and gently resuspended in 50 μl Annexin buffer containing 0.5 mg/ml PI and data acquired immediately. Early apoptotic, (Annexin V positive, PI negative cells), late apoptotic (Annexin V positive, PI positive cells) and viable cells (Annexin V negative cells) were analysed using Cell Quest software (BD) on a FACSCalibur flow cytometer (BD).

2.6. Intracellular components of apoptosis

2.6.1. Staining of apoptotic cells with Mab to active Caspases

Cells were prepared as previously described with viral and non-viral controls. CaspACE FITC-VAD-FMK (Promega, WI, USA) 10 μl was added to 900 μl cell sample and incubated at 37 °C for 15 min. Cells were washed with 1 ml 0.5% bovine serum albumin (Sigma) in Isoton II (Coulter), centrifuged ($300 \times g$ for 5 min) and gently resuspended in 50 μl of 5 μg ml^{-1} PI, then analysed using Cell Quest software (BD) on a FACSCalibur flow cytometer (BD).

2.6.2. Bax/Bcl2 staining

Samples were washed with calcium and magnesium-free PBS, supernatant discarded and 200 μl of membrane permeabilising solution (0.5% Triton X-100 (Sigma), 0.2 μg/ml $Na_2EDTA{\cdot}2H_2O$ (APS Chemicals, NSW, Australia), 1% bovine serum albumin in PBS) added to the cell pellet. Tubes were mixed, and incubated at room temperature, in the dark, for 15 min. Mabs to Bax (5 μl) (Oncogene Science, NY, USA) and Bcl-2 (10 μl) (Dako, Denmark) were added for 15 min at room temperature, in the dark. Irrelevant, conjugated antibodies of the same isotypes were used as negative controls. For Bcl-2 analysis of unwashed, unfixed cells was carried out immediately by flow cytometry. For Bax, cells were washed and 5 μl PE-conjugated rat-anti-mouse Mab (RAM) (BD Biosciences, Sydney, Australia) was added for a further 15 min prior to washing with 0.5% bovine serum albumin in Isoton II and list mode data acquired using CellQuest software.

2.7. Surface markers involved in apoptosis

2.7.1. Death receptors Fas and TNF-R1 expression and TNF-R2 expression

Cell surface proteins that mediate apoptosis were stained using indirect flow cytometric techniques. Aliquots of 1×10^5 cells were washed using 1 ml 0.5% bovine serum albumin in Isoton II, centrifuged ($300 \times g$ for 5 min), and gently resuspended in 50 μl of wash buffer. Mabs to Fas (5 μl) (Pharmingen, CA, USA), TNF-R1 (5 μl) and TNF-R2 (5 μl) (HyCult Biotechnology) were added for 15 min at room temperature and irrelevant, conjugated antibodies of the same isotypes were used as controls. Samples for TNF-R1 and TNF-R2 were washed and resuspended with 5 μl Streptavidin PE Mab (BD Biosciences) for 15 min. Cells were washed and data acquired by flow cytometry immediately using CellQuest software.

2.7.2. Death receptor ligand

Aliquots of cells were washed as above and gently resuspended in 50 μl of wash buffer. Mab to Fas ligand (2 μl) (Pharmingen) was added for 15 min at room temperature and irrelevant conjugated antibodies of the same isotype were used as control. The samples were washed and resuspended with 5 μl Streptavidin PE Mab for 15 min. Cells were washed and data acquired immediately.

2.8. Intracellular p53 in lung cancer cells

To detect the presence of p53 in the uninfected cell lines we used a PE labelled anti-p53 antibody (Pharmingen, CA, USA) with an isotype control. Aliquots of cells were added to a membrane permeabilising solution (0.5% Triton-X-100, 0.2 μg/ml $Na_2EDTA{\cdot}2H_2O$, 1% BSA in PBS). The samples were mixed and left at room temperature, in the dark, for 15 min. Then 5 μl anti-p53 was added for 15 min at room temperature. Analysis of unwashed, unfixed cells was carried out immediately by flow cytometry and list mode data acquired using CellQuest software.

2.9. Inhibitory experiments

Each of the blocking experiments was carried out in a similar manner. The media was changed to 2% FCS 1 h prior to the addition of the inhibitory agent. For the UV light experiments the cells were exposed to UV light for 20 min prior to the addition of the blocking agent. The Anti Fas clone, ZB-4, (Upstate cell signalling solutions, NY, USA) is a neutralising monoclonal antibody to the Fas receptor, was used in a concentration of 500 ηg/ml, Z-VAD-FMK (Sigma—Aldrich Inc., MS, USA) the caspase inhibitor was used at a final concentration of 50 μM/ml and the p53 inhibitor-Pifithrin-α (Sigma—Aldrich Inc.) was used at a concentration of 20 μM/ml. The cells were transduced by the addition of 100 μl of viral infection media (at 100 pfu cell^{-1} as previously) 1 h after the inhibitory agents were added. Analysis by flow cytometry using Annexin V and PI stain-

ing as previously described, and cell counts were carried out.

2.10. Growth of lung cancer xenografts in nude Balb/c mice

All experiments with mice were performed in accordance with institutional animal care guidelines and approval of the animal ethics board. Balb/c nude mice (4—6-week-old) were used in all experiments. For in vivo experiments 1×10^6 A549 cells were injected subcutaneously into the flanks of mice and tumours established a minimum volume of 50—100 mm^3. Tumour volume was calculated using the equation, volume = tumour length $\times$ (tumour width)^2/2. We injected tumours with 1×10^9 pfu of adenovirus in 50 μl PBS and control tumours with 50 μl PBS. Two injections on consecutive days were given. Tumour volumes were measured twice weekly using callipers. For TUNEL staining and immunohistochemical analysis of angiogenesis, excised tumours from euthanised animals were immediately fixed in 10% formaldehyde overnight, and then embedded in paraffin.

To assess the potential liver toxicity that may develop as a consequence of adenoviral delivery of vectors we injected the tail veins of Balb/c nude mice with 5×10^9 pfu of adenovirus in 100 μl PBS. The animals were euthanised 72 h later; the livers were examined directly using H + E staining and analysis of liver enzymes (ALT and AST).

2.11. In situ TUNEL staining

ApopTag Apoptosis Detection kit (CHEMICON International Inc.) was used to detect DNA fragmentation in apoptotic cells in vivo. Briefly, after deparaffinisation, tissues were digested with proteinase K (20 μg/ml) and endogenous peroxidase inactivated with 3% hydrogen peroxide. Subsequently, a mixture of Digoxenin-dUTP and terminal deoxynucleotidyl transferase enzyme was added, and samples incubated for 1 h at 37 °C. Anti-digoxigenin conjugate was added; colour developed with peroxidase substrate and counterstained with methyl green (0.5%, w/v, methyl green in 0.1 M sodium Acetate, pH 4.0). Specimens were mounted under glass coverslips. TUNEL positive cells were visualised by direct microscopy.

2.12. Immunohistochemistry of tumours

The target antigen retrieval solution (S-1699, DakoCytomation Pty Ltd., NSW, Australia) was used to maximise the antibody reaction. Factor VIII-R Ag (Cell Marque, AR, USA) and Anti CD-31 (Abcam, Cambridge, UK) were used to detect the presence of blood vessels within the tumour sections. The fluorescent secondary antibodies used were Alexa Flour 488, goat anti-rabbit and goat anti-mouse, respectively (Molecular Probes, Eugene, OR, USA). Sections of tumour were prepared on polysine slides using standard re-hydration techniques and then placed in preheated target retrieval solution according to the manufacturer's instructions. The slides were washed and then blocked in 10% goat serum for 1 h at room temperature. The primary antibody applied to the selected tissues, Factor VIII at 1 in 100 dilution and Anti CD-31 at 1 in 10 dilution at 4 °C overnight. The following morning the tissues were washed and then blocked in 10% goat serum for 1 h at room temperature. The secondary antibodies were applied at 1 in 1000 dilution for 1.5 h at room temperature in the dark. They were then washed again five times for 5 min each time in PBS. Nuclei were counterstained with 4′,6-diamidine-2′-phenylinodole dihydrochloride (DAPI (Roche, Mannheim, Germany) at 1 μg/ml dilution was applied for 3—4 min, and then the slides were washed once for 5 min in PBS. Photographs of the slides were taken at the Detmold Family Trust Cell Imaging Centre at the Hanson Institute using the Biorad Radiance 2100 Confocal Microscope.

2.13. Statistics

Statistical significance was determined by Students *t*-test compared with viral control. Figures are shown with mean $\pm$ S.D. Significance was accepted at $p < 0.05$.

3. Results

3.1. Inhibition of tumour cell growth by overexpression of TIMP-3 delivered via adenovirus

We detected the presence of TIMP-3 in the cell matrix fraction of A549 lung cancer cells following infection with AdCMVTIMP3 by Western blot analysis [5], confirming the localisation of TIMP-3 to the matrix of these cells. TIMP-3 was not detected in the cell matrix fractions of either uninfected controls or cells infected with AdCMVLuc, AdCMVTIMP-1 or -2. We detected the presence of TIMP-1 and -2 in conditioned media from cells transduced by these viruses, thus confirming the functionality of all elements. We then characterised the cell lines with regard to the levels of expression of coxsackie and adenoviral receptor (CAR; Fig. 1a), transducibility with an Ad vector carrying the luciferase reporter gene (AdCMVLuc; Fig. 1b) and some of the cellular markers known to be involved in the apoptotic process to see if these parameters would correlate to TIMP-3 susceptibility. The cell lines showed that there were differences in the surface marker expression of Fas, (Fig. 1c), whereas TNF-R1 and the death receptor ligand Fas ligand expression was detectable on less than 1% of cells for all lines (not shown). The levels of intracellular p53 expression in each of the cell lines examined varied between each of the cell lines examined (Fig. 1d). To examine the effects of TIMP-3 on the growth of lung cancer cells we studied the adenoviral delivery of TIMP-3 on the viability of the lung cancer cell lines compared to AdCMVLuc and AdCMVTIMP-1 and -2. Using manual cell counts and crystal violet staining there was a significant decrease in cell numbers in the A549 cell line following infection with AdCMVTIMP-3 at 24 h until 96 h (when all cells infected by AdCMVTIMP-3 were dead). There was no significant effect of adenovirally delivered AdCMVTIMP-1 or AdCMVTIMP-2 on the growth of A549 cells. This reduction in absolute cell numbers was not detected in the other cell lines examined (Fig. 2(a—f)). No consistent correlation to growth inhibition and susceptibility to AdCMVTIMP-3 as determined by baseline levels of CAR expression, viral transducibility, TNFR1, Fas or Fas ligand expression was seen.

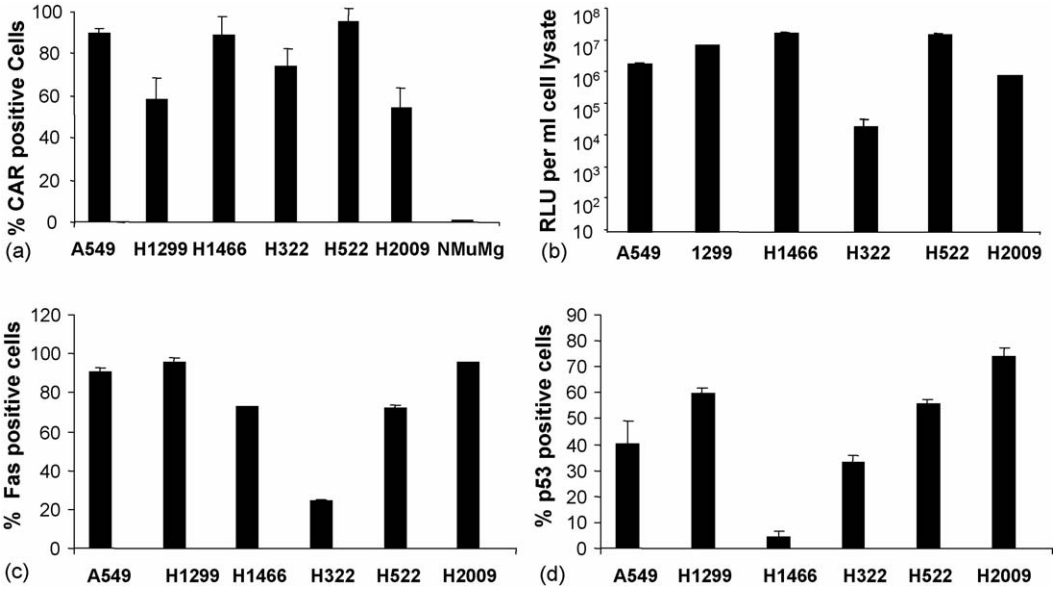

Fig. 1 Cell line characterisation. (a) CAR expression of each of the six cell lines employed determined by flow cytometry. NMuMG is a CAR-negative murine line used as a control. (b) Comparison of transgene expression (relative light units, RLU) in the lines after infection with AdCMVLuc. (c) Fas and (d) intracellular p53 expression determined by flow cytometry.

3.2. Effects of AdCMVTIMP3 on apoptosis in A549 cells

The viability of cells transduced with adenoviral vectors for TIMP1, 2 and 3 was determined by flow cytometry using Annexin V and PI staining. The delivery of AdCMVTIMP3 initiated the apoptotic process in A549 cells within 24 h. At 66 h following infection 65.1 ± 4.2% of A549 cells were positive for Annexin V and PI indicating that these cells are undergoing apoptosis, while 18.6 ± 0.7% of control cells (uninfected cells) and 17.8 ± 5.7% of cells infected with AdCMVLuc (control virus) demonstrated positive staining.

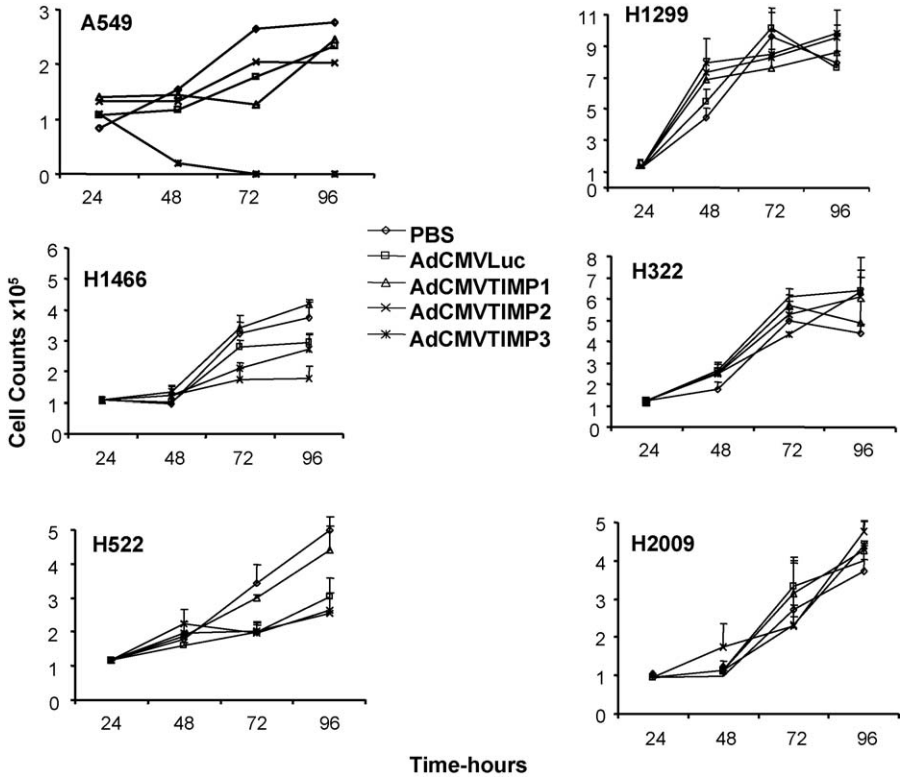

Fig. 2 (a—f) The effect of adenoviral delivery of TIMPs on absolute cell numbers on lung cancer cell lines over 96 h compared to PBS and AdCMVLuc viral control.

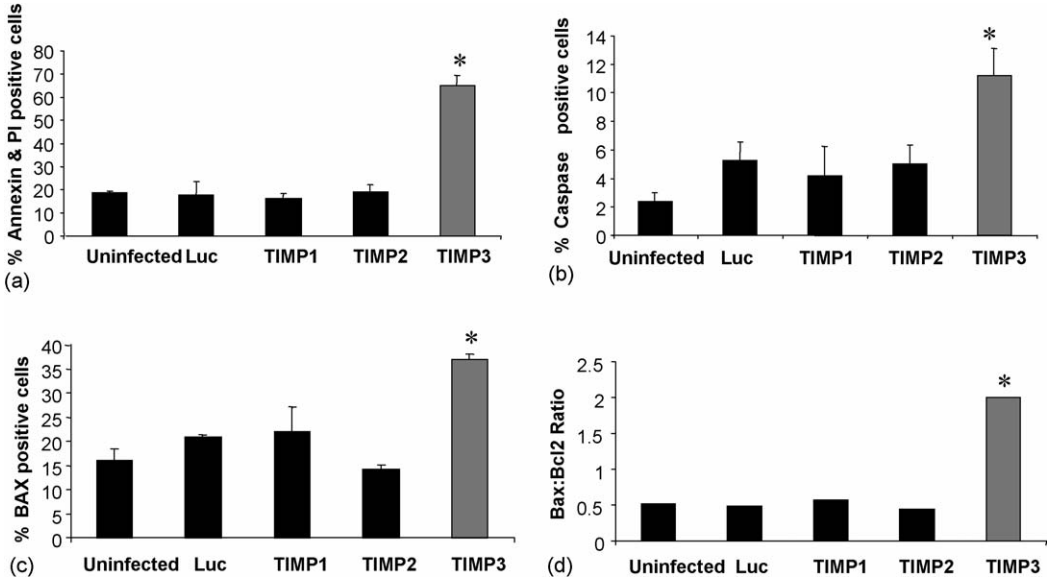

Fig. 3 The effect of adenoviral delivery of AdCMVTIMP-3 compared to AdCMVTIMP-1, -2 and control virus, AdCMVLuc on apoptosis in A549 lung cancer cells. (a) Annexin V and PI flow cytometry results at 66 h post transduction, (b) caspase staining of A549 cells following transduction with TIMPs 1—3 abd AdCMVLuc, (c) changes to Bax expression following transduction with AdCMVTIMP-3 and (d) effect of AdCMVTIMP-3 delivery on the Bax/Bcl2 ratio. $^{*}p < 0.05$.

Cells treated with AdCMVTIMP-1 or AdCMVTIMP-2 did not demonstrate any increase in apoptosis compared to uninfected cells (Fig. 3a).

A549 cells treated with TIMP-3 demonstrate caspase elevation in $11.2 \pm 1.6\%$ of the cells, while only $2.39 \pm 1.9\%$ of uninfected controls and $5.4 \pm 1.2\%$ of AdCMVLuc infected cells showed caspase elevation. Again, infection with AdCMVTIMP-1 and AdCMVTIMP-2 did not differ significantly from the uninfected cells (Fig. 3b). All cell lines represented in Fig. 1 were also assessed for apoptosis by Annexin V and PI staining. Of these other cell lines only the H1466 line showed a consistent increase in Annexin V and PI, however, as noted this did not translate to a consistent net cell killing effect.

3.3. Bax and Bcl2 expression

Previous studies have shown good correlation between Western blot (the ''gold standard'') and flow cytometry for the detection of these apoptosis related proteins [21]. The elevation of Bax following transduction with AdCMVTIMP3 was consistent with the increased level of apoptosis detected in the A549 cells. In the uninfected cells there were $15.95 \pm 2.4\%$ Bax positive cells, while the TIMP-3 treated cells had $36.95 \pm 1.06\%$ positive cells for Bax. AdCMVLuc, AdCMVTIMP1 and AdCMVTIMP2 demonstrated $21 \pm 0.28\%$, $22.05 \pm 5.02\%$ and $14.14 \pm 1.06\%$, respectively (Fig. 3c). The change in the ratio of the balance between pro-apoptotic Bax and anti-apoptotic Bcl2 indicates increased apoptosis. The balance changed from 0.5 in uninfected cells and 0.49 in cells infected with AdCMVLuc to 2.0 in cells infected with AdCMVTIMP3 (Fig. 3d). This is consistent with activation of the caspase apoptotic pathway and a consequent change in the regulators of this pathway from the anti-apoptotic Bcl2 to the pro-apoptotic Bax.

3.4. Cell surface recognition of apoptosis

3.4.1. Death receptor and ligand expression

Fas, TNF-R1 and TNF-R2 are members of the TNF superfamily of receptors. Specific ligand binding activates death receptors and subsequently, the downstream signalling that causes cell death. Multiple caspase pathways are recruited by activated TNF receptors [22]. TNF-R1 has been shown to direct its signalling toward either transcription or apoptosis [23]. Although TNF-R2 does not have a death receptor domain, it may also have a role in apoptosis through TNF-R1 [24,25] activation. The expression of TNF-R1 on cells infected with AdCMVTIMP3 was $3.64 \pm 0.67\%$, compared with $0.21 \pm 0.15\%$ in uninfected cells and $0.9 \pm 0.5\%$ in cells infected with AdCMVLuc. These levels are low but there is a significant increase ($p < 0.004$) following AdCMVTIMP3 infection (Fig. 4a). The expression of Fas ligand following AdCMVTIMP3 infection was $4.66 \pm 1.07\%$. In uninfected cells the expression was $0.62 \pm 0.21\%$, AdCMVLuc $1.31 \pm 0.43\%$, AdCMVTIMP1 $0.71 \pm 0.22\%$ and AdCMVTIMP2 $2.08 \pm 0.35\%$. There is a small but significant increase in the expression of Fas ligand following delivery of TIMP-3 to the A549 cells ($p < 0.004$) (Fig. 4b). Expression of TNF-R2 on TIMP-3 infected cells was $3.41 \pm 0.37\%$, compared with uninfected controls $1.04 \pm 0.05\%$ and AdCMVLuc viral controls $1.54 \pm 0.27\%$. As anticipated this expression is low but detectable (Fig. 4c). Metalloproteinases have significant role in the regulation of the cytoxicity of Fas ligand [26]. Inhibition of MMPs via TIMPs would be expected to have significant effects on the activation of the TNFR family and influence the regulation of diverse cell systems including those involved in cell proliferation [27]. A549 cells are known to have functional p53 [28]. We assessed the impact of AdCMVTIMP-3 transduction on p53 levels as detected by immunohistochem-

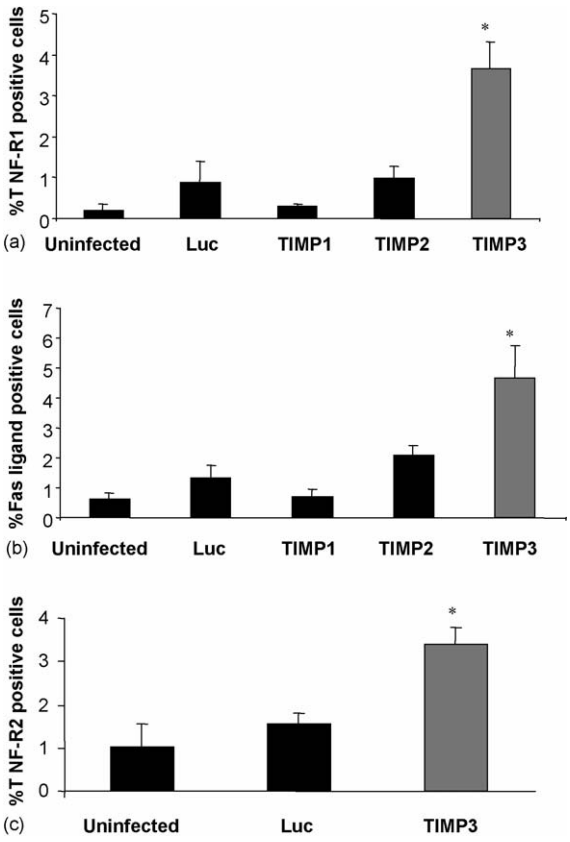

Fig. 4 The changes in surface receptor expression on A549 cells following the delivery of AdMCVTIMP-3 compared to AdCMVTIMP-1, -2 and control virus. (a) TNF-R1, (b) Fas ligand and (c) TNF-R2. $^{*}p < 0.05$.

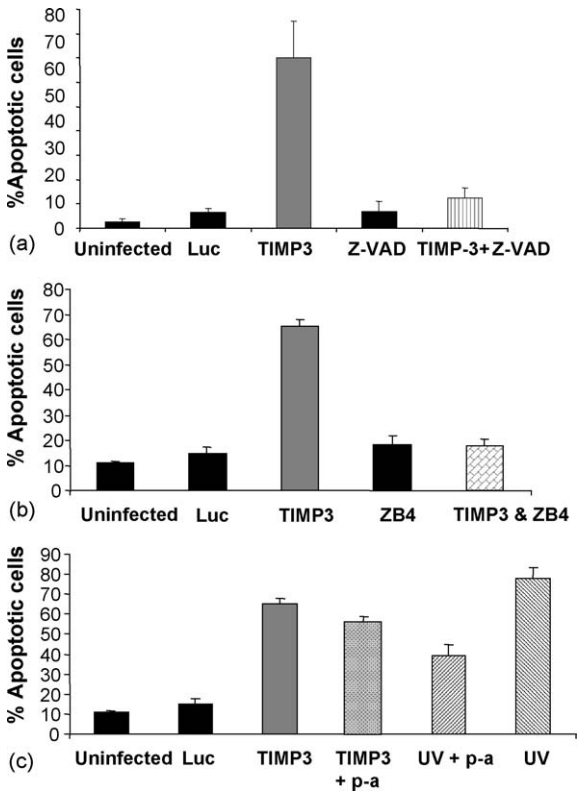

Fig. 5 The effects of blocking specific integral receptor activity in the apoptotic pathway. (a) The caspase inhibitor (Z-VAD) inhibits the effects of AdCMVTIMP-3 on A549 cells, (b) the Fas receptor inhibitor (ZB-4) inhibits the apoptotic inducing effects of TIMP-3 on A549 cells and (c) the p53 inhibitor pifithrin-α (p-a) does not significantly affect apoptosis induced by TIMP-3.

istry and found expression was increased ($67.5 \pm 5.4\%$ positive cells) versus uninfected, AdCMVLuc, AdCMVTIMP-1 and AdCMVTIMP-2 infected cells ($28.3 \pm 3.2\%$, $34.4 \pm 5.3\%$, $43.1 \pm 3.2\%$, $22.6 \pm 3.6\%$, respectively). We thus proceeded to p53 inhibition studies in an effort to determine whether changes in p53 function were involved in TIMP-3 mediated apoptosis.

3.5. Inhibitory experiments

We conducted inhibitory experiments to determine the relative roles of Fas, caspase and p53 in the apoptotic process initiated by TIMP-3. As previously discussed TIMP-3 has been shown to act via a Fas death domain dependent Type II apoptotic pathway in melanoma cells [29]. The caspases are central to the development of apoptosis initiated by both the intrinsic and extrinsic mechanisms. The demonstration of active caspases in cells transfected with TIMP-3 is consistent with involvement of the caspases in the apoptotic process initiated by TIMP-3. The addition of the pan-caspase inhibitor Z-VAD to the cells infected with TIMP-3 inhibited the development of apoptosis up to 72 h, thus confirming the pivotal role of caspases in the TIMP-3 apoptotic process (Fig. 5a).

The Fas inhibitor ZB-4 is a neutralising monoclonal antibody to Fas receptor that inhibits Fas receptor-mediated apoptosis. As expected A549 cells developed apoptosis following infection with TIMP-3 ($65.1 \pm 2.8\%$ positive for Annexin V and PI staining). There was no toxicity associated with the addition of ZB-4 to uninfected cells compared to the standard viral control ($18.07 \pm 3.7\%$ v $14.7 \pm 2.7\%$). However, the addition of ZB-4 to the TIMP-3 treated cells inhibited the development of apoptosis. Only $17.69 \pm 2.5\%$ of these cells were positive for Annexin V and PI staining. This confirms the role of the Fas death receptor in the transformation of the extrinsic apoptotic signal to the activation of DISC and subsequent caspase activation following transduction of A549 cells with adenoviral TIMP-3 (Fig. 5b).

The p53 inhibitor, pifithrin-α is a reversible inhibitor of p53 mediated apoptosis. It improves cell survival after genotoxic stresses. The effect of the UV light on the A549 cells was as anticipated. It caused almost 80% of the cells to become apoptotic. Following the addition of the p53 inhibitor, the number of cells induced by UV light to undergo apoptosis was reduced by half. TIMP-3 initiated apoptosis in $65.1 \pm 2.8\%$ of cells and the addition of the p53 inhibitor did not significantly affect the number of cells undergoing apoptosis (Fig. 5c). The controls of uninfected and AdCMVLuc showed results consistent with previous findings. This suggests that the p53 pathway is not directly involved in the initiation of apoptosis in A549 cells.

3.6. Bystander effect of TIMP-3

For the best therapeutic efficacy, the anti-tumour effect of any gene therapy application should ideally extend beyond the transduced cells (''bystander effect''). Our vector validation studies had shown that TIMP-3 was detectable in the cell-matrix rather than freely in conditioned media. We used two methods to determine the bystander effect of TIMP-3. Firstly, we infected cells then 24 h later (before signs of toxicity) mixed these at various ratios with uninfected cells. Secondly, we infected cells, then waited 72 h (at which time toxicity was apparent) then took the conditioned medium from these and applied it to uninfected cells. We used standard cell counting techniques to determine cell numbers on completion of the protocol. The mixed cultures containing the TIMP-3 infected cells were significantly affected by the presence of TIMP-3 transduced cells. In fact almost all the cells were dead in all ratios examined (Fig. 6a). The addition of media containing 15% and 35% of the TIMP-3 conditioned media resulted in the death of all the uninfected cells. The media from the parallel viral cultures of AdCMVLuc, AdCMVTIMP1 and AdCMVTIMP2 did not have any significant effect on the numbers of viable cells present (Fig. 6b). The slightly greater killing effect seen with the conditioned media experiment possibly reflects the greater lead time for TIMP-3 generation in that experiment

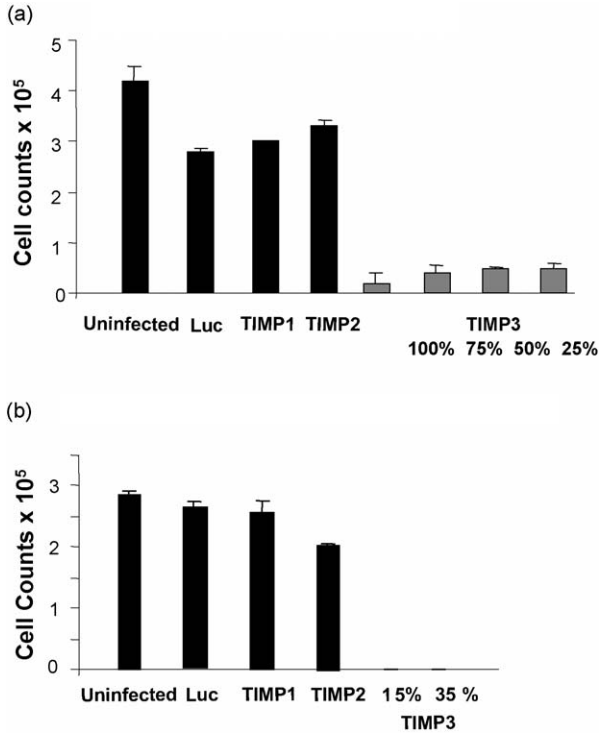

Fig. 6 (a) Bystander effects of cells infected with AdCMVTIMP-3 and mixed with uninfected cells at various % of infected cells as indicated. Luc, TIMP-1 and -2 columns are 100% infected with the indicated virus. Cell counts performed at 96 h post-infection. (b) Conditioned media (CM) from cells transduced with TIMP-3 for 72 h was then transferred to naïve A549 cells and cell counts performed 96 h later. Media from control cells was used at 35%.

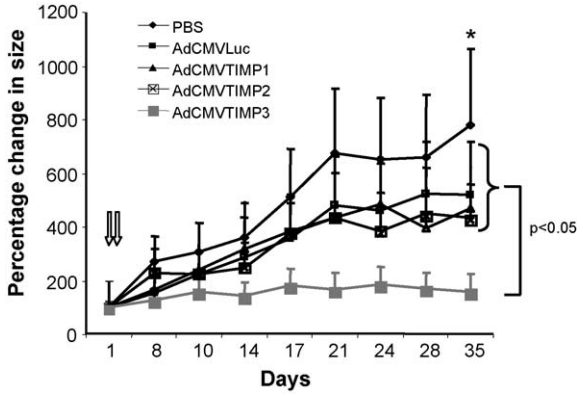

Fig. 7 Inhibition of A549 subcutaneous tumour growth following intra-tumoural injection with AdCMVTIMP-3 compared to treatment with AdCMVTIMP-1 and -2. Tumour volumes were expressed by the mean volume (mm^3) ± S.D. from eight mice of each group.

(total 72 h before medium transfer, versus 24 h infection before mixing). It is possible that factors released by TIMP-3-mediated cell death contributed to the net death effect, rather than TIMP-3 alone, but the key finding was that it is not necessary to achieve 100% transduction of target cells to achieve efficient cell death.

3.7. AdCMVTIMP3 effect on subcutaneous tumours in nude mice

The effects of adenovirally delivered TIMP-3 were compared to effects of TIMP-1, -2 and control virus AdCMVLuc. Subcutaneous A549 tumours were injected with PBS, AdCMVLuc and AdCMVTIMP1, 2 and 3. Those tumours injected with AdCMVTIMP3 showed significantly impaired growth when compared to PBS, AdCMVLuc, AdCMVTIMP1 or AdCMVTIMP2 injected tumours (Fig. 7). On completion of the tumour protocol at day 35 the animals were euthanised and the tumours and livers extracted for examination. TUNEL analysis of the tumour sections was used to identify apoptotic cells within the sections. Tumours injected with AdCMVTIMP3 had significantly more apoptotic cells than tumours injected with PBS, AdCMVLuc and either TIMP-1 or -2 (Fig. 8-1(a—e)). H&E staining and TUNEL assay was used to assess inflammation or apoptosis in the livers of animals given tail vein injections of adenoviral vectors. There were no significant differences between the livers of those animals injected with AdCMVTIMP3 and control animals. Serological examination of hepatic enzymes showed that there was no significant evidence of hepatic derangement in any of the groups.

3.8. Immunohistochemistry of the vasculature within the tumours

We examined both Factor VIIIR-Ag and CD31 on the tumours from each of the treatment groups. We determined that there was significant expression of both Factor VIIIR-Ag and CD31 on the PBS and AdCMVLuc injected tumours consistent with normal development of blood vessels with in these tumours. The tumours injected with the TIMP adenoviral

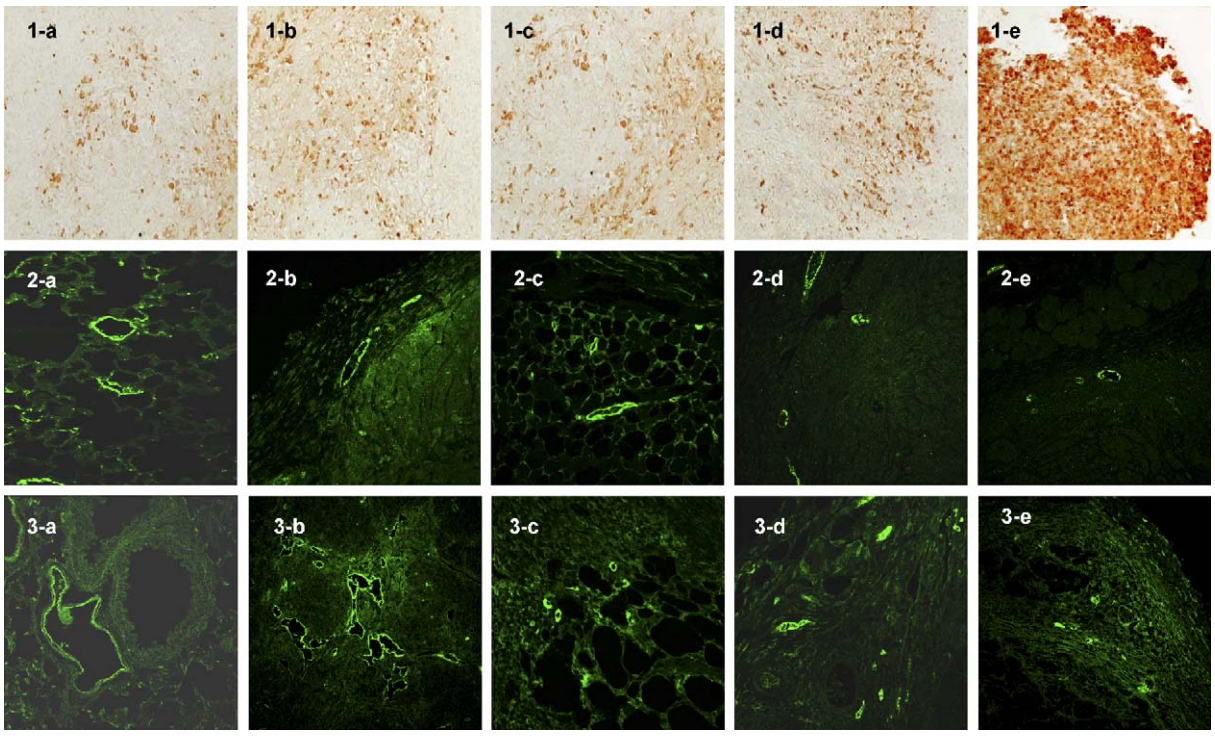

Fig. 8 Effect of intratumoural injection of AdCMVTIMP3 on apoptosis, and angiogenesis within subcutaneous A549 tumours. 1(a—e) TUNEL assay demostrating significant apoptosis within TIMP-3 treated tumours: (a) PBS, (b) AdCMVLuc, (c) AdCMVTIMP-1, (d) AdCMVTIMP-2 and (e) AdCMVTIMP3. 2(a—e) Factor VIIIR-Ag expression within tumours following treatment with adenoviral vectors: (a) normal lung control, (b) PBS, (c) AdCMVTIMP-1, (d) AdCMVTIMP-2 and (e) AdCMVTIMP-3. 3(a—e) CD-31 expression within tumours following treatment adenoviral vectors: (a) normal lung control, (b) PBS, (c) AdCMVTIMP-1, (d) AdCMVTIMP-2 and (e) AdCMVTIMP-3.

vectors had significantly reduced expression of both Factor VIIIR-Ag and CD31 (Fig. 8-2, 8-3(a—e)). However, the TIMP-3 injected tumours demonstrated less cohesive expression of CD31 indicating that there is failure to maintain vessel integrity within these tumours. The illustrated sections are taken away from areas of necrosis. The specificity of immunostaining was demonstrated by the absence of signal in sections processed in the absence of the primary antibody and lack of staining seen in uninfected cells or cells infected with an irrelevant virus.

4. Discussion

The development of cancer therapeutics based on modulation of MMP activity is a rational paradigm but as yet it has failed to achieve significant meaningful clinical advances. Gene-based approaches for MMP inhibition using TIMPs may have several advantages over conventional pharmaceutical approaches. The potential for high-level locoregional expression of the therapeutic agent and the anti-tumour properties of the TIMPs beyond MMP inhibition offer the potential of multifaceted therapeutic agents. A number of disturbances in the TIMP/MMP relative ratios have been described in lung cancer [30]. High levels of MMP expression are found in non-small cell carcinoma [31]. The number of MMPs expressed tends to increase with increased tumour stage and the levels of individual MMPs tends to be higher with advanced tumour stage [30]. Decreased TIMP-3 expression at the invasive front of human colon carcinomas suggests that a regional loss of TIMP-3 may facilitate tumour invasion and metastasis [14]. The delivery of TIMP-3 to cancer cells would allow restoration of the MMP/TIMP balance and inhibit the effects of excessive MMP expression. TIMP-3 delivery would offer other potential benefits independent of MMP inhibition such as the initiation of apoptosis and cell death in lung cancer cells, and the inhibition of angiogenesis.

The evaluation of TIMPs with respect to their role in cancer has lead to potentially conflicting results [16]. TIMP-1 has been associated with increased survival of lymphoma cell lines. It also has been shown to confer resistance to Fas ligand dependent and independent apoptosis [32]. TIMP-2 has been shown to promote apoptosis in an in vivo colorectal cancer model [33], but, protects B16 melanoma cells from apoptosis [34]. TIMP-4 can also instigate apoptosis in transformed cardiac fibroblasts but inhibits apoptosis in human breast cancer cells in vitro and mammary tumours in vivo [35]. Baker et al. [5] have previously demonstrated that adenoviral gene delivery of TIMP-3 to melanoma cells and vascular smooth muscle cells in vivo inhibits invasion and induces apoptosis. The same group also demonstrated that gene delivery of TIMP-3 inhibited the invasion of HeLa (moderately invasive cervical carcinoma cells) and HT1080 (fibrosarcoma cells) through artificial basement membranes [18]. This phenomenon was also demonstrated by TIMPs 1 and 2 whereas the pro-apoptotic effect of TIMP-3 was unique and was therefore the focus of the current study. Direct

comparisons between the TIMPs have not been reported. Thus, the question remained as to which TIMP approach may ultimately prove to be the optimal anti-tumour agent. We undertook the current study to ascertain whether, in the context of lung cancer, TIMP-3 had distinct advantages over TIMPs-1 and -2, whether this correlated to in vivo utility. Questions of toxicity in normal organs, due to the use of adenoviral vectors to deliver TIMP-3 have not been addressed previously. We attempted to examine this.

TIMPs are known to have divergent effects on programmed cell death [5], and the current series of investigations demonstrates for the first time the ability of TIMP-3 delivered via an adenoviral vector to induce apoptosis and cell death in vitro in lung cancer cells. We have shown that overexpression of AdCMVTIMP3 effectively induces apoptosis and causes cell death within 96 h of its delivery to A549 lung cancer cell lines. This effect is not seen with either TIMP-1 or -2. However, we found that the effect of TIMP-3-induced cell killing in vitro was rather limited, given that most of the cell lines we tested were resistant to this effect. We attempted to determine the basis for the variable susceptibility by looking at levels of expression of various components of the apoptosis pathway, as well as differences in the basic susceptibility of the lines to infection with Ad, but no consistent differences emerged. While we assessed death receptor levels and p53 levels, a more detailed analysis of functional p53 status could provide more insight. Of note, A549 cells are known to have functional p53 [28]. Nevertheless, we did not find any impact on TIMP-3 mediated apoptosis by p53 inhibition. Variability in the susceptibility of tumour lines to TIMP-3 has been noted by others [17]. Thus, for clinical utility, some method for identifying those tumours likely to be susceptible would be preferable for optimal application of this treatment strategy.

Mitochondria and cell surface receptors mediate the two main pathways of apoptosis [36]. The death receptors transduce the pro-apoptotic signal from the extracellular space to the intracellular environment. Fas and TNF-R1 members of the TNF superfamily are among the best characterised of the death receptors [37,38]. Fas ligand is also a member of the TNF family and is the principle ligand for Fas [39]. The binding of Fas ligand to the receptor allows the formation of death inducing signalling complex (DISC) by inducing clustering of Fas associated death domain (FADD), caspase-8 and caspase-10. This results in proteolytic cleavage of these caspases, release of the active proteases and subsequent activation of the downstream effector caspases [37]. TNF-R2 does not contain a death domain motif but is thought to be able to signal apoptosis directly or through ligand passing to TNF-R1 [40,41]. Exposure of metastatic melanoma cell lines to TIMP-3 results in stabilisation of three distinct death receptors, TNF-R1, Fas and TRAIL-R1 on cell surface and makes these cells more susceptible to apoptosis induced by their respective ligands [14,42]. We have shown that treatment of A549 lung cancer cells, which have abundant expression of the Fas death receptor, with TIMP-3 up-regulates the presence of Fas ligand, TNF-R1 and TNF-R2 on the cell surface consistent with the significant apoptosis induced by the delivery of TIMP-3 to these cells. Similar treatments with AdCMVTIMP1 and AdCMVTIMP2 did not induce these changes.

A number of pharmaceutical inhibitors of MMPs (MMPIs) have been developed. Preclinical studies in experimental animal model systems demonstrated significant benefits from the delivery of MMPIs in the form of reduced number of metastases and prolonged survival [43,44], although these results have not yet translated to consistent benefits in human clinical trials. Importantly, we have shown that the promising anti-tumour effects of TIMP-3 gene delivery in vitro can be translated into a therapeutic effect in vivo, for susceptible cells. Delivery of intra-tumoural AdCMVTIMP-3 inhibits the growth of pre-established subcutaneous A549 tumours in Balb/c nude mice and this reduction in growth is related to an increase in apoptosis within these tumours. There was no increase in toxicity as determined by both histological examination of livers and examination of hepatic enzymes. Concurrent analysis of Balb/c nude mice treated with AdCMVTIMP-1 and -2 did not show evidence of a reduction in growth.

The anti-angiogenic effects of the TIMPs were initially postulated to be due to their MMP inhibitory abilities. However, it has been shown that each of the TIMPs 1—3 have MMP independent mechanisms for inhibiting angiogenesis. TIMP-1 has an influence on tumour progression through MMP inhibition, specifically MMP-9, and also by impairing angiogenesis thus restricting tumour growth and progression. It has also been shown to inhibit microvascular cell migration thus having a negative impact on tumour angiogenesis [45—48]. TIMP-2 has been shown to specifically inhibit angiogenesis via induction of MKP-1 leading to p38 MAPK pathway inactivation [49]. TIMP-2 also inhibits the mitogenic response of human microvascular endothelial cells to growth factors [50]. There has also been significant investigation into the mechanisms of TIMP-3 induced inhibition of angiogenesis. There is an effect exerted via MMP inhibition which could potentially lead to a positive or negative effect on angiogenesis. However, there is a net negative effect seen in the presence of TIMP-3 overexpression in both in vitro and in vivo experiments. The in vitro effects of TIMP-3 on endothelial cell proliferation, migration and EC tube formation have been documented by Anand-Apte et al. during their investigation into the role of TIMP-3 mutations in the development of Sorsby's fundal dystrophy [51]. It was demonstrated that TIMP-3 inhibited the chemotaxis of vascular endothelial cells toward vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and inhibited the invasion of collagen gels, capillary morphogenesis in vitro and also inhibited bFGF induced angiogenesis. Spurbeck et al. [52] demonstrated that retroviral gene delivery of TIMP-3 to murine neuroblastoma and melanoma tumour cells in vivo affected functional capillary morphogenesis. In this model the endothelial cells did not form functional tubules. There was decreased expression of vascular endothelial (VE)—cadherin by endothelial cells in the presence of TIMP-3. Further, the work of Qi et al. [9] demonstrated that the ability of TIMP-3 to inhibit VEGF mediated angiogenesis was mediated via the inhibition of the binding of VEGF to VEGF receptor-2 and the subsequent inhibition of the downstream signalling and angiogenesis. Further investigations in animal studies have shown there is a decrease in vascular endothelial-cadherin expression on endothelial cells in the presence of increased levels of TIMP-3. This supports the concept that TIMP-3 overex-

pression leads to failure of vascular integrity in tumours [52].

At present, TIMP-3 is emerging as the most attractive anti-tumour agent. It has shown promise in animal models of malignant glioma and human leukaemia xenografts in mice [17,53]. It inhibits MMPs effectively, induces apoptosis in tumour cells and inhibits angiogenesis [5,18,54]. It is bound to the extracellular matrix and its ability to induce an effect beyond the transduced cell is directly related to its localisation here [55]. Clearly however, the therapeutic utility of TIMP-3 will not be universal, but for those tumours susceptible to the apoptosis-inducing effects, therapeutic gains can be achieved over alternate TIMPs. Further vector development to enhance efficacy might increase the therapeutic value of this promising agent but better understanding of the molecular basis of TIMP-3 susceptibility would help to better target this therapeutic approach.

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