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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