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Chitosan microparticles encapsulating PEDF plasmid demonstrate efficacy in an orthotopic metastatic model of osteosarcoma

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Abstract

The major stumbling block for most therapies against deep-seated disease, including tumours, is inefficient drug delivery. Such a concern is particularly important for osteosarcoma, the predominant form of bone cancer, and the largest cancer of its type in the paediatric age group. Pigment epithelium-derived factor (PEDF) is the most potent anti-angiogenic factor found endogenously in the body, with an increasing number of reports pointing to its direct antitumour activity. In this report, when a plasmid expressing PEDF (pPEDF) was encapsulated within two types of chitosan microparticles, anti-invasion and increased adhesion of the osteosarcoma cell line SaOS-2 was noted. Microparticles were formulated using two methods of complex coacervation and were ~400–600 nm in diameter. The plasmids were strongly attached to the particles which were polymorphic in shape as determined by electron microscopy. Preliminary experiments with the green fluorescent protein (GFP) reporter plasmid revealed that cells were efficiently transfected with the particles, with particles outlasting transfection with lipofectamine cationic liposomes at 5 days. *In vivo*, the better pPEDF microparticle resulted in a decrease in primary tumour growth, reduced bone lysis and reduced establishment of lung metastases in a clinically relevant orthotopic model of osteosarcoma. Thus, this new mode of localised gene delivery may hold promise for molecular therapy of osteosarcoma.

Keywords: Chitosan; Microparticle; PEDF; Cancer; Gene delivery

1. Introduction

Tumorigenesis entails the sequential acquisition of numerous genetic defects that renders neoplastic cells capable of growing, invading and eventually metastasising [1]. Various specific events such as unregulated angiogenesis, aberrant cell-signalling pathways and disrupted cell–matrix interactions are rife in tumours. These events in turn form the foci for targeted molecular therapy. Therapy for non-superficial tumours is tricky due to the need for limiting effects of the transgene on neoplastic tissue and not affecting normal healthy tissue.

One tumour type that underscores the need for targeted treatment is osteosarcoma. Osteosarcoma is the second highest cause of cancer-related death in young people, essentially in the prime years of life. Although the current treatment of this aggressive tumour, which comprises of resection with pre- and post-operative multi-agent chemotherapy, has resulted in substantial improvements in survival, 1 in 3 patients still develop pulmonary metastases, and this remains the major cause of death from this condition [2,3]. Therefore, it is the systemic spread of osteosarcoma which fails to be adequately eradicated with current regimes. Furthermore, dose intensification of chemotherapeutic agents, to the point of requiring hematopoietic stem cell reconstitution, has failed to consistently offer any added survival benefit to these patients [4]. One alternative source of potential osteosarcoma therapeutics is endogenous biologicals, which should preclude the problems faced with current cytotoxic agents such as drug resistance and toxicity. The progression of osteosarcoma adjacent to the growth plate cartilage in bone has revealed that this relatively avascular structure

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acts as a natural barrier to the progression of this tumour, which is attributable to the expression of anti-angiogenic factors such as pigment epithelium-derived factor (PEDF) in the growth plate cartilage [5].

PEDF is a widely expressed 50-kDa secreted glycoprotein that has been identified as one of the most potent endogenous inhibitors of angiogenesis, inducing endothelial cell apoptosis through the Fas/FasL death pathway, as well as decreasing the expression of important pro-angiogenic factors such as VEGF [unpublished data, 6–8]. PEDF also plays a role in promoting cell differentiation and influencing cell proliferation by regulating the cell cycle [9] and inducing apoptosis [10]. Decreased levels of intratumoral PEDF have been correlated with higher microvessel density and a more metastatic phenotype and a more poorer outcome in numerous malignancies, highlighting the key role PEDF plays in neoplasia [11].

Previously, we have demonstrated the therapeutic potential of PEDF against osteosarcoma using two clinically relevant orthotopic models of osteosarcoma and a panel of cell-based assays relevant to osteosarcoma proliferation, apoptosis, differentiation, angiogenesis and metastasis [12]. The feasibility of PEDF as a biological drug candidate was also discussed with promising preliminary results for stability and toxicity, as well as bioactivity of short cost-effective peptides derived from the parent molecule (manuscript under consideration). However, the proof-of-principle studies used PEDF-over-expressing cells to demonstrate PEDF activity *in vivo*. We have extended the findings to include injection of cells with microparticles in an attempt to more closely emulate drug delivery that can be adapted in the clinic. Thus, what is needed is a realistic approach for delivery of genes to osteosarcoma primary tumours, one that can be translated into clinical usage.

An ideal delivery vehicle has to have the following features: be biocompatible, biodegradable, non-immunogenic, non-toxic, able to carry a variety of types of molecular agents without changing its own or their chemical constitution, able to release the ferried agent in a sustained (controlled) manner, be relatively easy and inexpensive to formulate, not require biohazardous chemicals or unsafe formulation procedures for manufacture, and be formulated from abundant natural raw materials. One such material is chitosan, a natural polysaccharide found in crustacean shells, which has been used for plasmid delivery *in vivo* via the oral route for vaccination against peanut allergy in mice [13]. Plasmid expression has been found to be higher *in vivo* than that achieved with naked plasmid or transfected with lipofectamine cationic liposomes when particles were injected intramuscularly [14]. Most importantly, expression in the muscle lasts up to 12 weeks post-transfection [15]. Apart from gene delivery, chitosan has also been used widely for sustained drug delivery mainly due to its biocompatible, low immunogenic, and biodegradable nature [13].

Here, we demonstrate the feasibility of using chitosan microparticles, prepared using two different methods of complex coercavation, for delivery of plasmids into a osteosarcoma cell line. One microparticle, performing better than the other in cell culture, was then chosen for *in vivo* analysis with PEDF. It was shown to be efficacious in an orthotopic model of osteosarcoma [16], reducing both primary and secondary tumour growth in mice.

2. Materials and methods

2.1. PEDF plasmid

Human PEDF cDNA was generated from muscle mRNA and cloned into a pCR-II vector (Invitrogen) for transformation. A BamH1-Xba1 insert was then cloned into pcDNA3.1-his-myc(-)A vector (Invitrogen) for transfection. The plasmids expressing green fluorescent protein (pGFP), pPEDF and the pcDNA3.1 empty vectors were amplified using TOP10 bacterial cells (Invitrogen, Australia). Plasmids were extracted and purified using the Midi-prep kit (Invitrogen, Australia).

2.2. Formulation of particles

Low viscous chitosan ([2-amino-2-deoxy-(1 > 4)-β-D-glucopyranan]) was from Sigma-Aldrich (Australia). Particles were formulated using either of the two methods. In the first, a 0.25% chitosan solution in 25 mM sodium acetate solution (pH 6.0) was prepared. The plasmid was diluted into an equal volume of 50 mM sodium sulphate solution (pH 8.0) to a final concentration of 100 μg/mL. The plasmids were mixed at 500 rpm and into this mix was added an equal volume of the 0.25% chitosan solution [15]. Samples were stirred for 60 min and stored at 4 °C post-formulation. In the second method, a 0.02% chitosan solution in 25 mM sodium acetate solution was prepared. The plasmid was diluted into an equal volume of 50 mM sodium sulphate solution to a final concentration of 100 μg/mL. The plasmids were vortexed on maximum setting, into this mix was added an equal volume of the 0.02% chitosan solution [13], and contents were vortexed for a further 30 s. Samples were stored at 4 °C post-formulation, without further processing.

2.3. Loading capacity

A plasmid dilution series of 100% down to 3% was run alongside the microparticle preparation on a Tris-acetate-EDTA/0.5% agarose gel at 90 V for 90 min. The gel was visualised and imaged using a BioRad Gel-Doc system with Quantity One software (Australia).

2.4. Dynamic light scattering (DLS)

DLS measurements were performed using a Malvern 4700 apparatus (Australia) with a 10 mW AR+ ion laser at 488 nm. Unless stated otherwise, analysis was performed at an angle of 90° and a temperature of 25 °C. The dilute particle concentration in the samples ensures that multiple scattering and particle–particle interactions are negligible in this system. The particle concentration was adjusted so that the scattering from the particles dominated that from the polymer by several orders of magnitude. The time autocorrelation functions were analysed by an inverse Laplace transform algorithm, CONTIN to obtain a distribution of relaxation times and diffusion coefficient. The hydrodynamic radii were interpreted using the Stokes–Einstein equation and appropriate viscosity and temperature.