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The potential role of free chitosan in bone trauma and bone cancer management

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ABSTRACT

Bone defects caused by fractures or cancer-mediated destruction are debilitating. Chitosan is commonly used in scaffold matrices for bone healing, but rarely as a free drug. We demonstrate that free chitosan promotes osteoblast proliferation and osteogenesis in mesenchymal stem cells, increases osteopontin and collagen I expression, and reduces osteoclastogenesis. Chitosan inhibits invasion of endothelial cells, downregulating uPA/R, MT1-MMP, cdc42 and Rac1. Better healing of bone fractures with greater trabecular bone formation was observed in mice treated with chitosan. Chitosan induces apoptosis in osteotropic prostate and breast cancer cells *via* caspase-2 and -3 activation, and reduces their establishment in bone. Chitosan is pro-apoptotic in osteosarcoma cells, but not their normal counterpart, osteoblasts, or chondrosarcoma cells. Systemic delivery of chitosan does not perturb angiogenesis, bone volume or instinctive behaviour in pregnant mice, but decreases foetal length and changes pancreatic secretory acini. With certain controls in place, chitosan could be useful for bone trauma management.

1. Introduction

Chitosan, an abundant biopolymer derived from naturally-available chitin, is a polyelectrolyte with a variety of applications including, but not limited to, antimicrobial activity [1], biomedical devices [2], and implants for controlled drug release [3]. In bone tissue engineering, chitosan has quite frequently been used in conjunction with other bone-forming matrices [4] such as hydroxyapatite, and/or collagen, and artificial scaffolds consisting of poly(lactide-co-glycolide) (PLGA) or poly(L-lactic acid) (PLLA). However, to date, chitosan's bone-associated activities *per se* have not been properly tested, and the current study sheds light on some of these.

Chitosan has been used in scaffold form to deliver growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and tumour growth factor β (TGF-β) to mesenchymal stem cells (MSCs) for more efficient repair of fractures [5]. Low molecular weight chitosan (LMWC) has previously been shown to inhibit osteoclastogenesis at a concentration of 0.044% (w/v) in culture [6], but there is a paucity of data looking at the role free chitosan plays in all four types of cells investigated herein – osteoblasts, osteoclasts, MSCs and endothelial cells. All these cell types play important roles in bone development and homeostasis [7,8]. For bone tissue engineering, MSCs are widely used due to their high proliferation rate and osteogenic differentiation potential [9].

Chitosan has also been found to possess anticancer activity *per se*. One study showed that chitosan inhibited the growth of bladder tumour cells [10]. Although anticancer activity was observed in a dose-dependent manner, it was not significant until high doses above 0.001%, showing almost 50–60% inhibition. Treatment of human breast cancer cells with increasing concentrations of chitosan led to a concentration-dependent decrease in cell migration, inhibition of cell invasion, and reduced amounts of secreted matrix metalloproteinase-9 (MMP-9) [11]. The activity of metastasis-

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promoting MMP-9 could also be inhibited by chitosan in murine lung carcinoma cells [12]. *In vivo*, chitosan inhibited the growth of liver cancer cells in severe combined immunodeficient (SCID) mice. In a lung carcinoma-bearing mouse model, chitosan inhibited tumour growth and the number of lung colonies as well as metastasis, and it prolonged survival. Chitosan treatment inhibited the invasiveness of cytokine-treated human colorectal cancer cells in a dose-dependent manner and inhibited cytokine-induced MMP-2 activity [13].

In this study, we describe seminal results from a variety of *in vitro* and *in vivo* studies with free chitosan. We tested whether chitosan can differentiate MSCs to osteoblasts *in vivo*. We also tested whether chitosan had any effects on survival of primary osteoblasts and osteoclasts *in vitro*. Human endothelial cells were also exposed to chitosan in viability and 3-dimensional invasion assays. Effects of chitosan on bone fracture were also investigated. We evaluated the effects of chitosan in osteolytic primary human bone and cartilaginous tumour cell lines (osteosarcoma and chondrosarcoma respectively). We examined pro-apoptotic effects of chitosan on osteotropic prostate and breast cancer cells *in vitro* and tumour establishment in bone *in vivo*. Finally, the safety of sustained systemic delivery of chitosan was tested *in utero*. This series of studies provide evidence that chitosan can be used for bone health, specifically for bone healing and against bone tumours.

2. Materials and methods

2.1. Cell culture

Human osteosarcoma cell line SaOS-2, human breast cancer cell line MDA-MB231, human prostate cancer cell line PC3, and human colorectal cancer cell line HT29 were obtained from the American Tissue Culture Collection, ATCC (Manassas, VA, USA). Human chondrosarcoma cell line JJ012 was provided by Dr. J. A. Block, (Rush University Medical Centre, Chicago, USA). Human microvascular endothelial cell line HMEC-1 was obtained from the Centre for Disease Control, CDC (Atlanta, GA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute medium (RPMI) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotics and antimycotics at 37 °C within a humidified 5% CO_2 chamber. Medium and supplements were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Chitosan nanoparticle (CNP) formulation

Ultra-pure chitosan (110–150 kDa molecular weight range, high purity; Sigma–Aldrich, St Louis, MO, USA) produced in accordance with cGMP was used. The crystalline structure is in the α form, and the linkage between glucosamine units is β (1,4). The bacterial endotoxin level of this product is ≤10 EU/g, and it is ≥93% (w/w) chitosan. The chitosan powder was first dissolved in 1% acetic acid (v/v) prior to further dilution in water prior to use. CNPs were formulated using a complex coacervation method with modifications [14,15]. Chitosan (0.4%) in 25 mM sodium acetate (SA) buffer was added to an equal volume of 5 μg Dz13Scr in 50 mM sodium sulphate (SS) buffer and vortexed at high speed for 30 s at room temperature (20 °C). The formulation was then allowed to sit for 5 min to complete the formulation process. Particles were subsequently spun at 4000*g* for 4 min before the supernatant fraction was removed, and the resultant pellet was resuspended gently in sterile water and stored at 4 °C prior to use.

2.3. Dynamic light scattering (DLS) analysis of nanoparticles

DLS analysis was performed at an angle of 90° and a temperature of 25 °C using a Malvern 4700 apparatus with a 10 mW AR+ ion laser at 488 nm. The nanoparticle preparations were diluted to ensure that multiple scattering and particle–particle interactions were negligible in this system. In addition, particle concentration was adjusted so that the scattering from the particles dominated that of the polymer by several orders of magnitude. The time autocorrelation functions were analysed by an inverse Laplace transform algorithm, CONTIN, and the hydrodynamic radii interpreted using the Stokes–Einstein equation and appropriate viscosity and temperature as previously [15].

2.4. Electron microscopy of nanoparticles

Electron microscopy was performed on a Siemens 102 transmission instrument at 60 kV (Australia). Samples were spotted onto formvar grids and dried at room temperature. The particles were then negatively-stained with uranyl acetate and observed.

2.5. Harvesting and treatment of osteoblasts

Approval for calvariae harvesting was attained from the St. Vincent's Health Animal Ethics Committee. For osteoblast cultures, calvariae of neonatal mice were digested with 0.1% collagenase and 0.2% dispase five times, and cells isolated by the last three digestions were combined and cultured in α-MEM containing 10% FBS, 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate at a density of 1×10^5 cells/well in a 12-multiwell plate [16]. Cells were incubated with 0.005% chitosan or CNPs (equivalent to 0.005% chitosan – based on mass of chitosan used) for a period of 48 h prior to viability assay using a resazurin-based assay (CTBlue, Promega, Melbourne, Australia).

2.6. Harvesting and treatment of osteoclasts

Approval for bone marrow harvesting was attained from the St. Vincent's Health Animal Ethics Committee. For the osteoclast differentiation assay, cells from mouse bone marrow were cultured in α-MEM containing 10% FBS with 50 ng/ml macrophage colony-stimulating factor (M-CSF) for 24 h [16]. Adherent cells were harvested and cultured with 10 ng/ml M-CSF and 200 ng/ml receptor activator of nuclear factor-kappaB ligand (sRANKL) ± chitosan at 0.005% or ± (equivalent to 0.005% chitosan – based on mass of chitosan used) CNPs. After 3–5 days of culture, cells were fixed and stained for tartrate resistant acid phosphatase (TRAP). TRAP+ cells containing more than three nuclei were considered to be multinucleated osteoclasts.

2.7. Endothelial cell proliferation and invasion assay

HMEC-1 cells were seeded at a density of 2000 cells/well in a 96 well plate overnight, prior to treatment ±0.005% chitosan. Cells were then assayed with a resazurin-based method (CTBlue, Promega, Melbourne, Australia) at $570_{Ex}/610_{Em}$. For the invasion assay, chamber inserts with 8 μm filter membranes (BD Biosciences, USA) were coated with a 1:3 dilution of matrigel (BD Biosciences, USA) and the wells filled with complete growth medium. HMEC-1 cells were seeded in serum-free medium at a density of 5×10^4 cells per insert, and incubated for 48 h at 37 °C in a 5% CO_2 incubator in one of these conditions: no treatment, 0.005% free chitosan, 100 nM PEDF (Bioproducts MD, Bethesda, MD, USA) or 1 mM suramin (Sigma–Aldrich). Cells which invaded through the matrigel-coated pores to the underside of the membranes in response to stimulus by seeded (1×10^4) PC3 cells were stained with Quick-Dip stain (Froline, Sydney, Australia) and invaded cells were quantitated.

2.8. Mesenchymal stem cell isolation and culture

The bone marrow stromal cell (BMSC) isolation protocol was prior approved by the Victoria University Animal Experimentation Ethics Committee. MSCs were isolated from the marrow resident in the long bones of 6 week-old Balb/c mice. Cells were cultured at 37 °C under 5% CO_2 humidified atmosphere and expanded in DMEM, with low glucose, supplemented with 10% FBS, and 1% penicillin/streptomycin. The medium was changed every day for 1 week, then every 2–3 days until adherent cells reached approximately 80% confluence. Cells were trypsinised and grown until passage 4 for derivation of a pure stem cell population. For obtaining a high yield of MSCs, cells where cultured in expansion medium (R&D Systems, Melbourne, Australia) for 3 passages and all experiments were performed within 10 passages of harvesting [17]. Stemness of cells was confirmed for expression of CD73 and CD105 and lack of expression of CD34 and CD19 [18,19].

2.9. Assay of prodifferentiation potential of chitosan on MSCs – Alizarin red assay

To examine prodifferentiation potential, 0.005% free chitosan was added to MSCs in monolayer culture. The cell seeding density for monolayer culture was 500, 1000 and 1500 for 3 different time-points (days 7, 14 and 21) respectively. Different numbers of cells were added in order to avoid wells becoming 100% confluent prior to the end of the study at days 14 and 21). The cells were treated the next day ±0.005% chitosan. Osteogenic medium (R&D Systems, Minneapolis, MN, USA) was used as positive control. The monolayer culture was evaluated for formation of calcium deposits by staining with 4% Alizarin red solution (ARS) after fixation with 4% paraformaldehyde, then heated to 85 °C followed by centrifugation at 18000*g*. Supernatants (pH 4.5–5) were removed to a 96-well plate and read at OD_{405}. A standard curve was prepared using ARS [20].

2.10. von Kossa staining

MSCs in monolayer culture treated for 14 d ± 0.005% free chitosan were subjected to von Kossa staining. Post-fixing the cells with 4% paraformaldehyde for 30 min, mineralised nodules were stained with 5% silver nitrate and the plate placed under light for 30 min. Wells were rinsed once with water, treated with 5% sodium thiosulphate solution for 5 min, then washed with water once more, prior to images being acquired.

2.11. Treatment of cancer cell panel with chitosan

Human tumour cells – SaOS-2, JJ012, MDA-MB231 and PC3 – were seeded in 96 well plates at a density of 2000 cells per well in triplicate in 100 μl complete