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An intra-articular salmon calcitonin-based nanocomplex reduces experimental inflammatory arthritis

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ABSTRACT

Prolonged inappropriate inflammatory responses contribute to the pathogenesis of rheumatoid arthritis (RA) and to aspects of osteoarthritis (OA). The orphan nuclear receptor, NR4A2, is a key regulator and potential biomarker for inflammation and represents a potentially valuable therapeutic target. Both salmon calcitonin (sCT) and hyaluronic acid (HA) attenuated activated mRNA expression of NR4A1, NR4A2, NR4A3, and matrix metalloproteinases (MMPs) 1, 3 and 13 in three human cell lines: SW1353 chondrocytes, U937 and THP-1 monocytes. Ad-mixtures of sCT and HA further down-regulated expression of NR4A2 compared to either agent alone at specific concentrations, hence the rationale for their formulation in nanocomplexes (NPs) using chitosan. The sCT released from NP stimulated cAMP production in human T47D breast cancer cells expressing sCT receptors. When NP were injected by the intra-articular (I.A.) route to the mouse knee during on-going inflammatory arthritis of the K/BxN serum transfer model, joint inflammation was reduced together with NR4A2 expression, and local bone architecture was preserved. These data highlight remarkable anti-inflammatory effects of sCT and HA at the level of reducing NR4A2 mRNA expression *in vitro*. Combining them in NP elicits anti-arthritic effects *in vivo* following I.A. delivery.

1. Introduction

Prolonged inappropriate local and systemic inflammatory responses contribute to the pathogenesis of rheumatoid arthritis (RA), which affects 0.5–1.0% of adults in the industrialized world [1]. Therapeutic strategies involve the use of disease-modifying anti-RA drugs (DMARDs) including oral methotrexate and leflunomide, along with injected TNF-α inhibitors [2]. Short courses of oral glucocorticoids facilitate subsequent lower dose levels of long-acting DMARDS. In addition, steroids are also injected locally *via* the intra-articular (I.A.) route to specific RA joints displaying swelling and aggressive flare-up, where they can cause pain relief for up to 8 weeks [3]. In contrast to RA, osteoarthritis (OA) is a chronic degenerative joint condition and is more prevalent than RA, affecting 60–70% of people older than 65 years, and 13% of the world's population [4]. Knee OA displays degeneration, reflected by loss of articular cartilage components due to an imbalance between extracellular matrix destruction and repair [5,6], inappropriate osteophyte formation and synovial inflammation [7]. Key aspects of OA cartilage pathogenesis include degradation driven by cytokine cascades associated with production of inflammatory mediators. The activated synovium releases proteinases and cytokines that accelerate cartilage destruction. Chondrocytes from OA patients produce increased levels of inflammatory cytokines including IL-1β and TNF α, which in turn decrease collagen synthesis and increase production of degradation proteases, including matrix metalloproteinases (MMPs) [8]. In destructive hip OA, patients displayed significantly elevated MMP-3 and -9 concentrations in synovial cells, synovial fluid, plasma, and sera [9,10]. Unlike RA, synovial inflammation in OA is mostly confined to local areas adjacent to pathologically damaged cartilage and bone. Although Type-1 cytokine inflammatory pathways mediated by TNF-α, IL-1 and IL-6 are major targets for RA and form the basis of effective biologic therapies including etanarcept, rituximab, and abatacept, inflammatory targets for OA joints have yet to be confirmed [11,12].

Current treatment options for OA are far more limited than for RA and there are no approved disease-modifying drugs. Local I.A. injections of corticosteroids [13] and the viscosupplement, hyaluronic acid (HA) [14], are widely used for symptomatic treatment. Efficacy of I.A. steroids on pain relief is moderate and typically last only for 3 weeks [15], while systemic side-effects due to resorption include temporary suppression of the hypothalamic-pituitary adrenal axis,

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reduction in serum cortisol and transient elevation of serum glucose [16]. Effects of I.A.-delivered HA for knee OA are similar to those of I.A.-delivered steroids with respect to pain scores, but it is thought to cause less side effects and benefits may last several weeks longer [17]. There is recent evidence that HA restores matrix metalloproteinase (MMP)-13 in osteoarthritic subchondral bone [18], so additional anti-inflammatory effects as well as lubrication may contribute to its benefits in OA.

Oral options for symptomatic treatment of OA include paracetamol, NSAIDs, and opioids, however there is a risk of serious side effects with long term use. OA therefore requires new drug formulations that address long term pain relief, the arresting of structural joint degeneration and sustained reduction of local inflammation. Of the potential candidates to alleviate inflammatory arthritis, the analgesic peptide, sCT, is already approved as a second line nasal and injectable anti-resorptive treatment for osteoporosis (OP) [19]. Interest in sCT as a potential treatment for OA has increased due to its beneficial metabolic actions on cartilage and bone turnover [20]. sCT also inhibits MMP expression and blocks collagen degradation in articular chondrocytes exposed to TNFα/oncostatin M *in vitro* [21]. Intriguingly, while an oral formulation of sCT recently completed Phase III clinical trials for OP [22], direct evidence of anti-inflammatory effects of sCT is scant.

There is growing evidence that dysregulation of intracellular nuclear receptors plays a pivotal role in chronic inflammation [23]. Expression of the nuclear orphan receptor, NURR1 (NR4A2), correlated with disease activity in synovial tissue from human RA joints, an effect that was reduced by low dose MTX [24]. Furthermore, NR4A-activated genes are rapidly activated by inflammatory mediators in chondrocyte and endothelial cells [24,25] and NR4A receptors have been identified as intermediate effector molecules for cytokine signalling in macrophages [26]. Macrophage exposure to lipopolysaccharide (LPS), cytokines, or oxidized lipids also triggers induction of the NR4A1, 2, and 3 receptors [27]. In addition, functional studies demonstrate both pro- and anti-inflammatory roles for NR4A1, suggesting that expression levels of these receptors in distinct cell types may permit differential effects on gene expression. There is considerable interest therefore in examining NR4A receptor as a potential molecular target in both RA and OA [28]. While increased expression of MMPs has long been associated with inflammation and joint destruction [29], there are no specific inhibitors for either condition, so it may be opportune to target other implicated proteins including NR4A.

Joint-specific features of both OA and RA make a long-acting I.A. injection an attractive approach as the drug can be retained locally to generate increased efficacy while minimizing systemic side effects at the lower dose levels required [8,30]. An impediment to translation is that unformulated molecules are rapidly cleared from the synovial space and have short half-lives [31], highlighting the need for nanomedicine formulations to provide sustained release. Notable preclinical examples include I.A. injections of betamethasone in poly(lactic-co-glycolic acid) (PLG) nanospheres in the antigen-induced rabbit, where joint swelling and cell infiltration were reduced [32], while methotrexate entrapped in PLG microspheres was also retained longer in rabbit joints compared to solution [33]. There is a considerable debate over the design of the optimal particle composition and diameter for targeting to the articular cartilage, and this is in part due to the wide range of species and disease induction models used.

Our aim therefore was to examine whether sCT and/or HA could reduce expression of induced NR4A1-3 and MMP mRNA levels in human chondrocytes *in vitro*. We then ad-mixed the two agents to see if expression of NR4A2 mRNA in human chondrocytes could be further attenuated. Subsequently, sCT and HA were combined using chitosan into an electrostatic-based nanocomplex (NP), which preserved bone, reduced inflammation and inflammatory gene expression when injected by the I.A. route to mice induced to display acute joint arthritic inflammation by administration of K/BxN serum [34,35].

2. Materials and methods

2.1. Materials

sCT was purchased from PolyPeptide Laboratories (Denmark). The Parameter™ cAMP (EIA) kit was obtained from R&D systems, UK. Tissue culture reagents were obtained from BioSciences, Ireland. Hyaluronic acid (HA) sodium salt from *Streptococcus equi sp*. was purchased from Sigma, Ireland. The ultrapure chitosan chloride, Protasan UP CL113, was obtained from Nova Matrix, Norway. SW1353, T47D, THP-1 and U937 cells were obtained from ATCC (USA). All other reagents, chemicals and solvents were of analytical grade.

2.2. Tissue culture and reagents

Human chondrosarcoma cells (SW1353) and monocytes (U937 and THP-1) were maintained in RPMI 1640 medium containing 10% foetal calf serum, HEPES, and penicillin/streptomycin (Invitrogen, UK) at 37 °C and 5% CO_2 in air. Cells were passaged with 0.25% Trypsin/EDTA (Invitrogen, UK) and seeded on 6 well plates.

2.3. Preparation of sCT–HA-chitosan nanocomplexes (NPs)

Nanoparticle dispersions were prepared according to a formulation process previously described [36]. HA (MW 257 kDa) was obtained by ultrasonicating a solution of native HA in deionized water. Briefly, a predefined aliquot of chitosan chloride (Protosan UP CL113 (CS), MW 110 ± 6.78 kDa) solution made up in deionized water was added to a known volume of HA and sCT with stirring. A dispersion of NP was obtained instantly after adding CL113 solution to the HA/sCT liquid phase. The combined total polymer concentration was 0.1% w/v and the final sCT concentration in the NP dispersion was 100 μg/ml. Stirring was carried out for 10 min and no cross-linking agents were used.

2.4. NP size, zeta potential analysis and morphology

The intensity-averaged mean particle size (mean particle size) and the polydispersity indices (PDI) of the NP were determined by dynamic light scattering (DLS) with the use of 173° backscatter detection. Electrophoretic mobility values were measured by laser Doppler velocimetry (LDV) and were converted to zeta potentials [36]. Both DLS and LDV measurements were carried out using a Zetasizer Nano series Nano-ZS ZEN3600 fitted with a 633 nm laser (Malvern, UK). Samples in deionized water were placed directly into the folded capillary cells without dilution. Each analysis was carried out at 25 °C with an equilibration time of 5 min. The readings were carried out at least three times for each batch and the average zeta potential values of at least three batches were calculated and corrected for viscosity [36]. Transmission electron microscopy (TEM) (Jeol 2100, Japan) was used in order to examine NP morphology. The samples were immobilised on copper grids and stained with either 1% (w/v) ammonium molybdate solution for 60 s or 1% (w/v) uranyl acetate solution for 30 s, followed by overnight drying and TEM viewing.

2.5. Association efficiency of sCT

The association efficiency of sCT in NP was calculated by the difference between the total amount of sCT used to prepare the complexes and the remaining amount of free sCT in the aqueous medium. The amount of free sCT was determined in the supernatant by RP-HPLC (described below) following separation of NP from aqueous medium by a combined ultrafiltration–centrifugation technique (Amicon Ultra-15, MW cut off, 50 kDa; Millipore, USA). Briefly, 5 ml sample was placed in the sample reservoir of a centrifugal filter device and centrifuged (5810R centrifuge, Eppendorf, UK) for 60 min at 3000 rpm. The filtrate was collected, its volume measured and the amount of sCT quantified