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Analyses on the mechanisms that underlie the chondroprotective properties of calcitonin

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ABSTRACT

Introduction: Calcitonin (CT) has recently been shown to display chondroprotective effects. Here, we investigate the putative mechanisms by which CT delivers these actions.
Methods: Immortalized C-28/I2 cells or primary adult human articular chondrocytes (AHAC) were cultured in high-density micromasses to investigate: (i) CT anabolic effects using qPCR and immuhistochemistry analysis; (ii) CT anti-apoptotic effects using quantitation of Bax/Bcl gene products ratio, TUNEL assay and caspase-3 expression; (iii) CT effects on CREB, COL2A1 and NFAT transcription factors.
Results: CT (10^{-10}–10^{-8} nM) induced significant up-regulation of cartilage phenotypic markers (*SOX9*, *COL2A1* and *ACAN*), with down-regulation of catabolic (*MMP1* and *MMP13* and *ADAMTS5*) gene products both in resting and inflammatory conditions. This was mirrored by an augmented production of type II collagen and accumulation of glycosaminoglycan- and proteoglycan-rich extracellular matrix *in vitro*. Mechanistic analyses revealed only partial involvement of cyclic AMP formation in these effects of CT. Congruently, using reporter assays for specific transcription factors, there was no indication for CREB activation, whereas the COL2A1 promoter was genuinely and directly activated by cell exposure to CT. Phenotypically, these mechanisms supported the ability of CT, whilst inactive on its own, to counteract the pro-apoptotic effects of IL-1β, demonstrated by TUNEL-positive staining of chondrocytes and ratio of *BAX/BCL* genes products.
Conclusion: These data may provide a novel lead for the development of CT-based chondroprotective strategies that rely on the engagement of mechanisms that lead to augmented chondrocyte anabolism and inhibited chondrocyte apoptosis.

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1. Introduction

One of the major burdens for society is represented by joint diseases, which in large proportion, are due to osteoarthritis (OA). OA is characterized by the breakdown of the articular cartilage and abnormal bone/joint remodeling, which impairs mobility and causes debilitating pain. Although many factors are known to predispose to OA, including acute trauma and aging [1], the pathogenic mechanisms remain poorly characterized. This paucity of knowledge results in the absence of strategies to develop effective treatments for this disease other than the symptomatic relief with non-steroidal anti-inflammatory drugs, until the need for joint replacement as a last resource. Identification and development of new therapeutic approaches is then imperative and, within this vein, a viable approach could be exploited by drug repositioning.

Abbreviations: AB, Alcian Blue; ADAMT, A Disintegrin And Metalloproteinase with Trombospondin motif; AHAC, adult human articular chondrocyte; BAX, Bcl-2-associated X protein; BCL-2, B-cell leukemia-lymphoma-2 protein; BMP2, Bone Morphogenic Protein type 2; CT, Calcitonin; CTR, calcitonin receptor; CREB, cAMP response element-binding protein; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; FSK, forskolin; GAG, glycosaminoglycan; GPCR, G-protein coupled receptor; MMP, matrix metalloproteinase; NFAT, nuclear factor of the activated T-cells; OA, Osteoarthritis; PG, prostaglandin; PKA, protein kinase A; PLC, Phospholipase C; PRED, prednisolone; RA, Rheumatoid Arthritis; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling..

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Chondrocytes represent the sole cellular component of cartilage and are endowed with non-redundant roles in regulating the homeostasis of the extracellular matrix (ECM) conferring tensile strength and compressive resistance to articular surfaces during motion [2,3]. In physiological conditions, chondrocytes maintain a tight balance between synthesis and remodeling of the ECM, modulating the activity of degrading enzymes such as aggrecaneses (e.g. A Disintegrin And Metalloproteinase with Trombospondin motifs [ADAMT(s)] [4] and matrix metalloproteinases [MMPs]) [5] and the synthesis of the main constituents of the extracellular matrix like proteoglycans and collagens [6]. In OA, the equilibrium between anabolic and catabolic events is lost in favor of extensive matrix degradation by unchecked proteolysis [7]. Pro-inflammatory molecules, such as IL-1, contribute to these phenomena and promote chondrocyte apoptosis and eventually leading to cartilage breakdown in OA [8,9]. However, the issue of the pivotal role that apoptosis may play in imbalanced remodeling of the cartilage is not fully characterized, notwithstanding the opportunity that anti-apoptotic mediators may then represent as therapeutic leads.

Calcitonin (CT) is a 32-amino acid peptide secreted by the parafollicular cells of the mammalian thyroid, modulating the metabolism of Ca^{2+} in the body with a potent anti-resorptive effect in the bone [10,11]. For this powerful property, CT has been successfully used in the treatment of osteoporosis for over 40 years [12] and is also used as a safe and well-tolerated treatment for Paget's disease in patients, who cannot receive biphosphonates [13]

Evidence exists that articular chondrocytes express the calcitonin receptor (CTR) [14] and indeed CT can promote anabolic events on articular cartilage, both in *in vitro* and *in vivo* settings [15–17] although the exact molecular mechanisms involved have not been clarified so far.

The present study begun by investigating the cellular and biochemical properties of CT on articular chondrocytes using a medium-throughput chondrocyte culture system we recently described [18]. New insights into potential mechanisms were gained by identifying significant modulation of chondrocyte apoptosis and gene promoter activation as new actions underlining the potent chondroprotection afforded by this hormone.

2. Material and methods

Unless otherwise stated, all materials were from Sigma-Aldrich (Poole, UK).

2.1. Chondrocyte isolation and culture

The immortalized chondrocyte cell-line C-28/I2 was kindly provided by Dr. Mary Goldring [19]. Cells were cultured in monolayer (2×10^4 cells/cm^2), under normoxic conditions, before being released in trypsin–EDTA and re-cultured in micromasses with density of 0.5×10^6cells/micromass for 2 days in DMEM/Ham's F12 medium, supplemented with 1% ITS (Insulin Transferrin Sodium Selenide) [18]. Micromasses were incubated for 48 h with salmon CT (generous gift of Dr. N. Mehta, Unigene Corporation, NJ) in the presence of different stimuli as described in individual sets of experiments.

Primary adult human articular chondrocyte (AHAC) were isolated from cartilage obtained from OA cartilage obtained from knee joint replacement after obtaining informed consent (samples provided by Mr P. Achan, Barts Health NHS Trust, London, UK; procedures approved by the East London and The City Research Ethics Committee 3). Cartilage tissue was dissected from preserved areas (Mankin score ≤4) of the femoral condyles and the patellar groove and articular chondrocytes were then isolated as described before by Nalesso et al. [20]. All experiments were performed using freshly isolated or confluent P_0 cells.

In all cases, micromasses were activated with interleukin (IL)-1β (20 ng/ml) and treated with different concentrations of CT (10^{-10}–10^{-8} M). In some cases the glucocorticoid prednisolone (PRED) was also tested at the selected concentration 10^{-8} M [18], whilst the melanocortin alpha-melanocyte stimulating hormone was used at 10^{-6} M [21]. Forskolin (FSK) was tested at 10 μM. In most cases analyses of cell phenotype and markers were done at the 48 h time-point.

2.2. Alcian blue (AB) staining *in vitro*

For semi-quantitation of cartilage-specific highly-sulfated glycosaminoglycans (GAGs) generated under different culture conditions, a protocol optimized by Greco et al. [18] was used. Briefly, micromasses were fixed with 4% glutaraldehyde solution and incubated with AB dye (1% Alcian Blue 8GS in 0.1 N HCl; pH < 1; Carl Roth, Karlsruhe, Germany) at room temperature. After extensive washing, extraction was done overnight in guanidine–HCl (6 M). OD of the extracted dye was measured at 630 nm (Spectronic 2000 spectrophotometer; Bausch & Lomb, Rochester, NY), and concentration values were interpolated with an AB standard curve, and normalized to DNA content (μg/ng). To this end, DNA content was measured by fluorescence using SYBR Green I dye (Invitrogen, Paisley, UK) against a DNA standard curve (double-stranded(ds)DNA for Standard Curve–Lambda DNA, Invitrogen, Paisley, UK) with measuring at 485/535 nm by spectrophotometry (TECAN-M200; Tecan, Männedorf, Switzerland).

2.3. Real-time PCR

2.3.1. Relative quantification

Total RNA was extracted using a commercially available kit (Qiagen RNeasy Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer's instructions. Complementary DNA (cDNA) was generated by reverse transcription (RT) of total RNA employing Superscript III reverse transcriptase system (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol and using oligo$(dT)_{15}$ as a primer. cDNA was diluted to a final concentration 10 ng/μL and 20 ng of cDNA was used as a template to determine relative amounts of mRNA by real-time PCR (ABI Prism 7900 Real-time PCR system, Applied Biosystems Inc, CA, USA) using specific primers (Quantitect Primer Assay, Qiagen) and 2X Power SYBR Green Mastermix (Applied Biosystems Inc., Carlsbad, CA). The predesigned primers (Qiagen) were used to probe for the expression levels of *SOX9*, *COL2A1*, *ACAN*, *MMP1*, *MMP13*, *CALCR* and *ADAMTS5*. *GAPDH* was amplified as internal control. Cycle threshold values (Ct) were detected and measured by sequence detector software. Relative mRNA contents were inferred from the equation $2^{-\Delta\Delta Ct}$, providing the relative amount of target gene normalized to endogenous control (*GAPDH*) and to the untreated control samples with the expression set as 1 [22].

2.3.2. Absolute quantification

Absolute quantification was performed, as described [20], for determination of Bcl-2-associated X protein (*BAX*) and the anti-apoptotic B-cell leukemia-lymphoma-2 (*BCL-2*) ratio using specific primers (Table 1) following differential stimulation of AHAC cells

Table 1
Primers used for detection of the apoptotic modulator gene products.

Gene	Primer	Primer sequence
Hs_BCL2	Sense	AGTGGGATGCGGGAGATGTG
	Antisense	CCACCGAACTCAAAGAAGGC
Hs_BAX	Sense	GCGTCCACCAAGAAGCTGAG
	Antisense	TGAAGATGGGGAGAGGGCAC

Bcl-2-associated X protein (BAX); B-cell leukemia-lymphoma-2 (Bcl2)

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