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The role of C1q in (auto) immunity

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Chapter 7

Human chondrocytes produce and secrete C1q

Manuscript in preparation

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Abstract

Chondrocytes, the cellular constituents of cartilage, play an important role in the cartilage by producing extracellular matrix molecules. During inflammation both chondrocytes and cartilage can be damaged. Previous studies have shown that chondrocytes express mRNA encoding for C1q. This is surprising as it is currently unclear what the role of such a large molecule is in the cartilage. In this study we demonstrate that C1q is produced and secreted by chondrocytes.

Using western blot and ELISA we analysed the presence of C1q in cultured chondrocytes. By stimulation with pro-inflammatory cytokines we examined the changes in mRNA expression of matrix molecules and of C1q.

Ex-vivo isolated human chondrocytes express mRNA for the three C1q chains. Cultured primary chondrocytes stain positive for C1q and secrete C1q into the culture supernatant. Upon stimulation with pro-inflammatory cytokines, the C1q mRNA expression is upregulated. Staining human cartilage sections for C1q revealed that in-situ chondrocytes are positive for C1q.

In this study we demonstrate that C1q can be produced and secreted by chondrocytes under basal conditions, which can be enhanced by pro-inflammatory stimuli.

Introduction

Chondrocytes are the only cells present in the healthy cartilage matrix. They are responsible for the synthesis and turnover of the extracellular cellular matrix (ECM). Chondrocytes are round cells originating from mesenchymal stem cells that represent 5-10% of the cartilage volume located in matrix cavities called lacunae. The two major ECM macromolecules produced by chondrocytes are collagen type 2 and aggrecan [1].

The complement system is an important part of the innate immune defence, which is able to kill pathogens and remove dying cells and immune complexes. The complement system can be activated via three pathways; the classical pathway, the lectin pathway and the alternative pathway. C1q is the recognition molecule of the classical pathway and together with the proteases C1r and C1s it forms the C1 complex. C1q can activate the classical pathway by binding different ligands such as IgG and IgM antibodies, but also DNA, C-reactive protein (CRP) and lipopolysaccharides [2-4]. C1q producing cells are mainly originating from the haematopoietic stem cells, such as macrophages, immature dendritic cells

and mast cells [4-7]. However, other reports suggest that trophoblasts, decidual endothelial cells, microglia and osteoclasts can also produce C1q [8-10].
Previously studies describe that matrix molecules like aggrecan, fibromodulin and osteoadherin are able to bind C1q and are able to activate the classical pathway of the complement system [11-13]. The cartilage oligomeric matrix protein (COMP) is a strong ligand for C1q, but it is hypothesised that COMP interferes with the binding site of C1r and C1s resulting in inhibition of the classical pathway [14]. This interference is also applies to the cartilage fragments decorin and biglycan [15]. Based on these interesting interactions between C1q and several matrix molecules the question arises if C1q is actually present inside the cartilage.
Proteomic data from synovial fluid from osteoarthritic patients shows the presence of complement components. Wang et al described that complement is important in the pathogenesis of osteoarthritis [16], as the presence of the MAC could be detected on cartilage. Although the authors speculate that complement activation takes place outside the cartilage and then attacks damaged chondrocytes, it cannot be excluded that chondrocytes initiate complement activation leading to MAC deposition takes place in the cartilage [16]. Similarly, In the early 90's it was already described that cartilage is positive for the mRNA for C1q and that deposits of the C1q molecule were present in the cartilage, but the source and function of the production of C1q was not clear [17].
Therefore, we have investigated whether primary human chondrocytes are able to produce and secrete C1q and if the production can be modulated under inflammatory conditions such as present in OA.

Materials and methods

Patients

Cartilage was obtained as left-over tissue from total knee-replacement surgery performed on OA patients in the Alrijne hospital, Leiden, The Netherlands. The local medical ethical committee approved the study.

Isolation of chondrocytes

Chondrocytes were isolated from cartilage obtained from total knee-replacement surgery. Cartilage pieces were incubated with pronase (2 mg/ml, Roche) for 1,5 hours, followed by overnight incubation with collagenase type 2 (225 U/ml, Worthington). After digestion, chondrocytes were directly lysed for western blot analysis or RNA isolation. The purity of the chondrocytes was determined using

Flow Cytometry, whereby CD14 (APC) and CD45 (FITC) were measured using a BD LSRFortessa cell analyser (BD Biosciences, San Jose, CA, USA) and analysed using FlowJo v10.1 software (Tree Star, Ashland, OR, USA).

Stimulation of chondrocytes

Primary chondrocytes were plated in a cell concentration of 5*105 cells/mL in F12 DMEM culture medium (Gibco) supplemented with 10% BSA (Bovine Serum Albumin), L-glutamine, penicillin and streptomycin. Chondrocytes were stimulated for 24 hours with IL-1ß (30 ng/ml, R&D systems), TNF-α (100 ng/ml, R&D systems), TGF-ß (10 ng/ml, peprotech) or LPS (100 ng/ml, Sigma). After stimulation RNA isolation was performed. For western blot analysis primary chondrocytes were lysed and C1qA, C1qB, C1qC was determined as previously described [18]. Culture supernatants of unstimulated chondrocytes were harvested after 72h and analysed by ELISA to determine C1q levels as described before [18].

qPCR

RNA was isolated from cultured chondrocytes using RNeasy mini kit (Qiagen) and analysed with the NanoDrop (NanoDrop Technologies). Subsequently, the RNA was treated with DNase I, Amplification Grade (Invitrogen) and cDNA was synthesized using superscript III (200U/µl, Invitrogen). The cDNA was diluted 1:25 and qPCR was performed using SensiFast Sybr no-ROX (Bioline) and primers with a start concentration of 10 pmol. Primers specific for MMP1, collagen type 1,type 2, type 10, C1r, C1s, C3, C5 and the C1q genes C1qA/B/C, as well as the reference RPL5 were used (see table 1 for primer sequences). The qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and an annealing temperature of 64°C for 5 seconds followed by an elongation step for 20 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr. The qPCR for the C1q genes was performed with annealing temperatures ranging from 58-65°C for 10 seconds.

Immunofluorescence staining of C1q in chondrocytes

Freshly isolated chondrocytes were cultured on poly-d-lysine (Sigma 50 µg/ml) chamber slides (Thermo Scientific) and fixated using 1% paraformaldehyde and acetone. Cells were subsequently incubated with rabbit anti-C1q (DAKO) or isotype control rabbit Ig (DAKO) for 1 hour at RT. After washing, slides were incubated with goat anti-rabbit ALEXA 488 (Invitrogen) and ActinRed 594 (Applied bioprobes) for

1 hour at RT. Finally, the slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei and analysed on a confocal microscope (Leica SP8 confocal).

Gene	5' Forward	3' Reverse
MMP1	CTGGCCACAACTGCCAAATG	ATTCTGTCCCTGAACAGCCC
Collagen type 1	CATGTTCAGCTTTGTGGACCTC	ATGGTACCTGAGGCCGTTCT
Collagen type 2	TCCTAAAGGAGCCCGAGGTGCC	CAGGAGCTTGGAGGCCGGGTT
Collagen type 10	CCCAGCACGCAGAATCCATCT	TTCCAGCCGGTCCAGGGATT
C1qA	CCAGGAAGAACCGTACCAGA	GACGATGGACAGGCAGATTT
C1qB	TCCAGATATGGAGGCCTGAC	TTCACTCAGCAGCATTCACC
C1qC	AAGGARGGGTACGACGGACT	GTAAGCCGGGTTCTCCCTTC
C1r	TCACAGTCCCCACGGGATAC	CCAGTGGAGAACCCAGTTGC
C1s	CTGCAGAGGGAGCGTCAA	TGGTAGGCTCAGCATAAACCC
C3	TGGCCAATGGTGTTGACAGA	GCGTAGACCTTGACTGCTCC
C5	TACCTTGCCTGTTGAAGCCC	CCAGGGAAAGAGCATACGCA
RPL5	TGGAGGTGACTGGTGATG	GCTTCCGATGTACTTCTGC

Table 1. qPCR primers

Immunohistochemical staining of C1q in cartilage

C1q was detected on paraffin embedded cartilage of OA patients (5µm). Slides were deparaffinised and stained with 10 µg/ml rabbit anti-C1q (DAKO) or matching isotype control for 1 hour at RT . Next, slides were incubated with anti-rabbit HRP for 1 hour at RT and were analysed on a Zeiss Axio ScopeA1 microscope.

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Results

Chondrocytes are able to produce and secrete C1q

Primary human chondrocytes were isolated and the purity of the isolated chondrocyte population was analysed using flow cytometry. As shown in figure 1A, chondrocytes are negative for the hematopoietic marker CD45 and the myeloid marker CD14, indicating no contamination of cells from hematopoietic or myeloid origin (Figure 1A).

Next, we investigated whether chondrocytes can produce and secrete C1q. Using western blot analysis we detected C1q in chondrocyte lysates from 2 OA patients (Figure 1B). We found the molecular size of C1q in the chondrocytes to be similar to the C1q band observed in the lysates of PBMCs and Normal Human Serum (NHS) (positive controls). No C1q was observed in the lysate of T cells (negative control).

Using confocal microscopy we observed C1q inside the cultured chondrocytes (Figure 1C). We confirmed the data by ELISA on lysates of unstimulated chondrocytes cultured for 72 hours (Figure 1D). Moreover, secretion of C1q into the culture supernatant could be shown in one out of 2 OA patients (Figure 1D). To confirm that chondrocytes express C1q in the natural environment of cartilage, we performed an immunohistochemical staining on OA cartilage. Also staining of the human cartilage showed positivity for C1q, which is mainly located around the nucleus of the chondrocytes (Figure 2A-C).

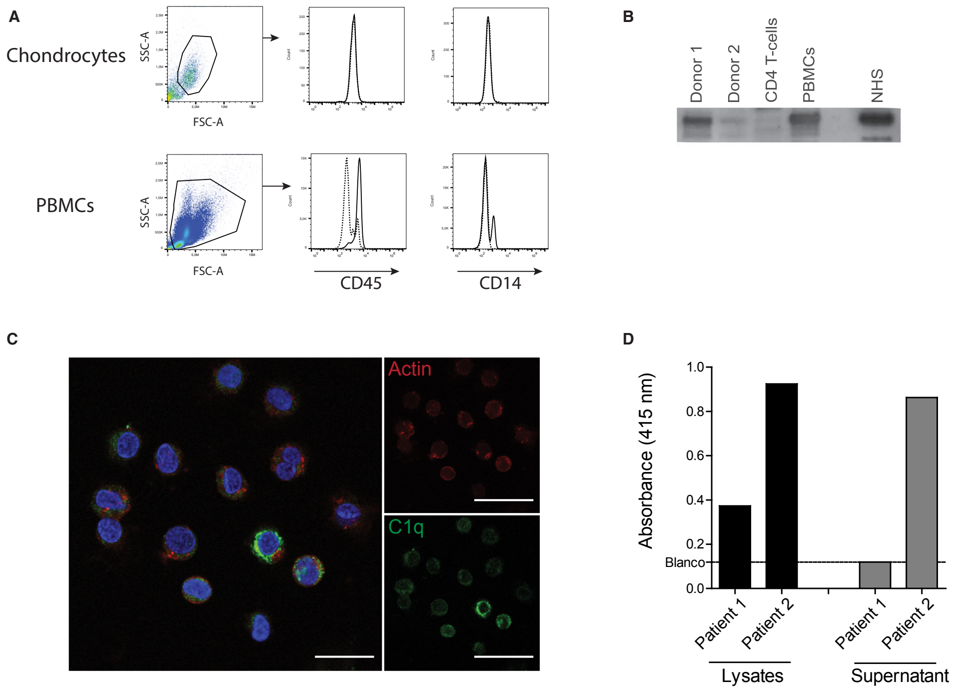

Figure 1. Chondrocytes are positive for C1q.
A. Flow cytometry of chondrocytes isolated from human cartilage compared to PBMCs, **B.** Western blot analysis of lysates of cultured chondrocytes (72 hours, N=2), CD4 T-cells and PBMCs. As positive control NHS is used, **C.** Cultured chondrocytes on chamber slides. C1q is visualized with ALEXA488. Actin is used as counterstaining at 594 nm. The nucleus is stained with DAPI, 630x magnification, the scale bars indicates 6.3 µm, **D.** C1q ELISA on lysates and supernatants of cultured chondrocytes (72h). Read out is the absorbance on 415 nm.

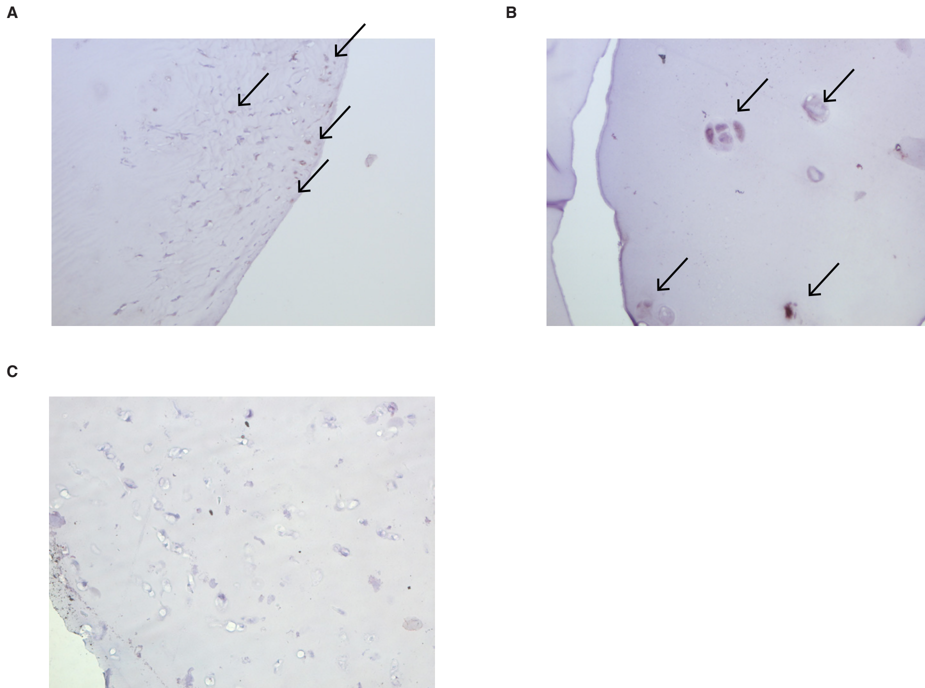

Figure 2. The expression of C1q in human cartilage. A. Expression of C1q (HRP) with a co-staining of haematoxylin with a magnification of 400x, **B.** With a 1000x magnification. **C.** Isotype control on 400x magnification.

mRNA expression of C1q is increased in a pro-inflammatory environment

Next, we wished to determine the mRNA expression pattern of C1qA, C1qB and C1qC by qPCR. We investigated the basal expression pattern as well as the changes that occur after stimulation with cytokines that are relevant for OA [19]. Stimulation with IL-1ß and TNFα led to an increased expression of C1qA, C1qB and C1qC (Figure 3A-C). Furthermore, we investigated whether the expression of other complement components could be modulated by these inflammatory cytokines. Expression levels of C1qA, C1qB and C1qC were upregulated by IL-1ß and TNFα stimulation, while no or limited enhancement was observed for stimulation with TGF-ß and LPS (Figure 3). The expression pattern of the serine protease C1r and C1s and of complement component C3 was largely comparable to the C1q genes, with the exception that LPS did induce a modest increase in their expression after 24 hours of stimulation (Figure 4A, C), while C5 was unaffected by any of the studied cytokines. LPS had little or no effect on the complement genes we studied (Figure 4D).

As a positive control for TGF-ß stimulation we determined the mRNA expression of Collagen type 1, 2 and 10. As expected TGF-ß increases the expression of Collagen

type 1 (figure 5A) [20], while it had little effect on type 2 and type 10 (Figure 5B,C). A small decrease of the expression of Collagen type 1, type 2 and 10 was seen after stimulation with IL-1ß., while TNF or LPS stimulation had little or no effect on the collagen gene expression. MMP1 expression was determined as a positive control for the pro-inflammatory stimulations and shows an increase after stimulation of IL-1ß, TNFα and LPS (figure 5D). The pattern of up/down regulation of the C1q genes is largely similar to C3, C5 and MMP but different from Collagen type 1,2, and 10.

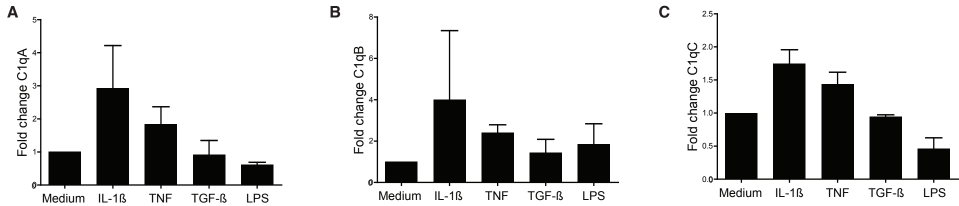

Figure 3. mRNA fold change of C1q genes after 24 hours stimulation (N=3). **A.** Fold change expression after correction for RPL5 of C1qA, **B.** C1qB, **C**. C1qC. Data represents mean and SEM of 3 different OA donors measured in duplicate.

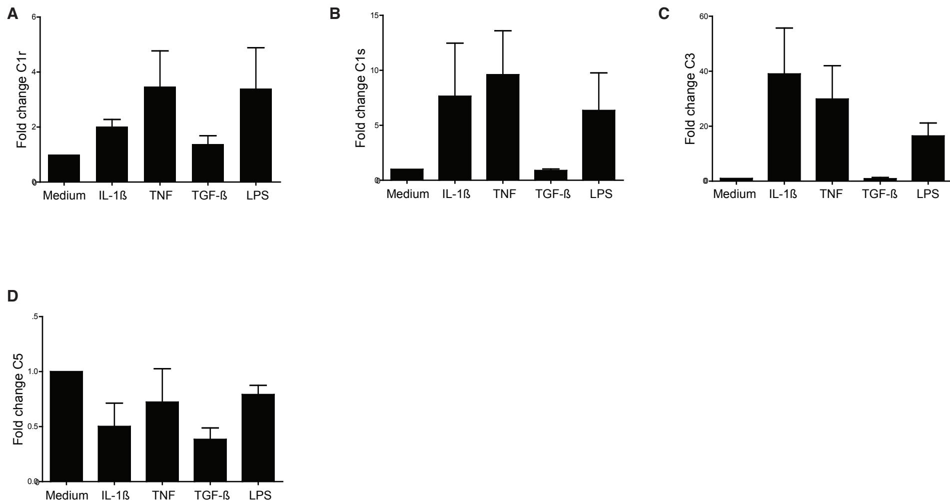

Figure 4. mRNA fold change of complement components after 24 hours stimulation (N=3). A. Fold change expression after correction with RPL5 of C1r, **B.** C1s, **C**. C3. **D.** C5. Data represents mean and SEM of 3 different OA donors measured in duplicate.

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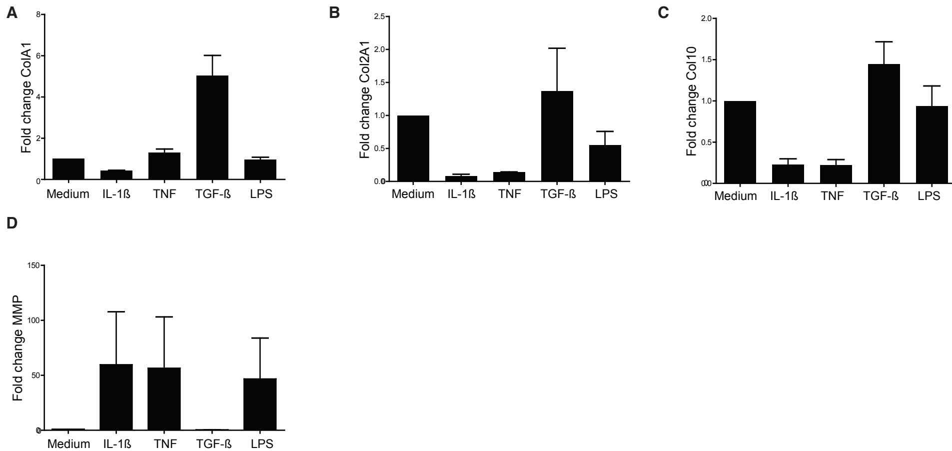

Figure 5. mRNA fold change of chondrocyte components after 24 hours stimulation (N=3). A. Fold change expression after correction with RPL5 of Collagen type 1, **B.** Collagen type 2, **C.** Collagen type 10. **D.** MMP1. Data represents mean and SEM of 3 different OA donors measured in duplicate.

Discussion

The main producers of C1q are originating from haematopoietic stem cells. Chondrocytes are originating from the mesenchymal stem cell and are important in the production of extracellular matrix molecules to build up the cartilage. Wang et al have described that the dysregulation of complement in the synovial joints in has a central role in the pathogenesis of osteoarthritis [16]. In the early 90's it was demonstrated that the cartilage was positive for several complement components like C1q on RNA level, but the presence of C1q could not be shown in vitro. Also the function or relation with the cartilage was not described [17]. Here, we demonstrate that chondrocytes are able to produce and secrete C1q and that the production of C1q is increased by cytokines present in osteoarthritic joints and believed to play a role in disease pathogenesis.

To evaluate which factors could modulate the production of C1q by chondrocytes we stimulated the chondrocytes with cytokines that are known to be present in the joint during inflammation [19]. Stimulation with IL-1ß and TNF-α showed an increase in mRNA expression of C1q compared to unstimulated chondrocytes. Furthermore, the expression patterns of the C1q genes were different compared to the expression of the collagen genes, which are of importance in the maintenance of the cartilage, as seen after TGF-ß stimulation where the gene expression of collagen types 1,2 and 10 were upregulated and the expression of the C1q genes were downregulated. C1q released by chondrocytes could deposit on cartilage or synovium which is damaged by inflammation however, C1q is a large (460kD)

molecule and the size limit of proteins that can move freely in cartilage is estimated to be around 65 kD [17]. Therefore it is likely that C1q produced by chondrocytes must serve a local rather than a systemic function. Conceivably, C1q would function in an autocrine setting in stimulating the chondrocyte or alternatively C1q would be involved in local complement activation, involving C1r and C1s directly outside the chondrocyte. We speculate that C1q and complement activation may be involved in maintenance of a lacuna for the chondrocyte.

Previous studies have demonstrated that the serine protease C1s can degrade collagen type 1 and type 2 when it is activated [21] and that it can play a role in the degenerative cartilage matrix in RA [22]. In our results we also see an increase of RNA expression of C1s and for C1r after stimulation. This could indicate that in pro-inflammatory conditions complement activation will occur in the cartilage or at the surface of the cartilage. Together with the production of C1q and C3 it is highly conceivable that the production of different complement components by chondrocytes plays a role in the degradation of the cartilage.

In our study we only used cartilage and chondrocytes derived from patients who underwent a total knee replacement due to osteoarthritis, indicating that the cartilage is already in inflammatory conditions. In further investigations the role of complement in cartilage it would be interesting to compare these results with healthy donors.

In conclusion, our results indicate that chondrocytes are able to produce and secrete C1q, suggesting a previously unexpected role of this molecule in the cartilage.

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