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Synthesis of classical pathway complement components by chondrocytes

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SUMMARY

Using immunohistochemical studies, C1q, C1s, C4 and C2 were detected in chondrocytes in normal human articular cartilage and macroscopically normal articular cartilage from the inferior surfaces of hip joints of patients with osteoarthritis. Using reverse-transcribed polymerase chain reaction (RT-PCR), mRNA for C1q, C1s, C4 and C2 was also detected in RNA extracted from articular cartilage. C1r, C3, C1-inhibitor, C4-binding protein and factor I were not detected by either technique. Articular chondrocytes cultured *in vitro* synthesized C1r, C1s, C4, C2, C3 and C1-inhibitor but not C1q, C4-binding protein or factor I, as assessed by enzyme-linked immunosorbent assay (ELISA) and Northern blot analysis. Thus cultured articular chondrocytes have a complement profile that is similar to that of cultured human fibroblasts rather than that of articular chondrocytes *in vivo*. Complement synthesis in cultured chondrocytes was modulated by the cytokines interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), showing that cytokines can probably regulate complement synthesis in intact cartilage. The possible roles of local synthesis of complement components by chondrocytes in matrix turnover and the regulation chondrocyte function are discussed.

INTRODUCTION

The complement system comprises a group of proteins that promote the inflammatory response and destroy micro-organisms. The classical pathway comprises C1, C4 and C2. The C1 macromolecular complex (C1q : C1r2 : C1s2) is a product of five genes; three encode the A, B and C chains of C1q,[1] while C1r and C1s are each encoded by a single gene.[2] The classical pathway is usually activated when C1 binds to antibody complexes containing IgM or IgG antibody.[3] Activated C1 then actives C4 and C2 by a limited proteolysis. Activated C4 (C4b) binds covalently to suitable target surfaces. C2, the rate-limiting component of the classical pathway, binds to C4 prior to activation by C1. Once activated by limited proteolysis the classical pathway C3 convertase (C4b2a) is formed. The convertase cleaves C3 into C3a (anaphylotoxin) and C3b. The C3b binds covalently to acceptor groups, and that which binds to the C4b component of C4b2a converts its specificity from C3 convertase to the C5 convertase, C4b2a3b.[3] Fluid-phase regulatory proteins that modulate classical pathway activation include C1-inhibitor (C1-inh), which prevents spontaneous activation of C1 and inhibits activated C1[4] and C4-binding protein (C4-bp), which acts as a cofactor for factor I (I) in the degradation of C4b.[3]

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During classical pathway activation a number of important pro-inflammatory products are generated, including anaphylatoxins C4a, C3a and C5a which are cleaved from the N-termini of C4, C3 and C5, respectively. These peptides activate different cell types including neutrophils, macrophages and mast cells, and C5a is a powerful chemotaxin.[4] C3b and iC3b ligate the complement receptor CR1 and CR3, respectively, and ligation of CR3 results in phagocytosis.[5] Although the C5b-9 membrane attack complex (MAC) is able to lyse cells, at sublytic concentrations it will activate a variety of nucleated cells including neutrophils, macrophages and synoviocytes. This results in secretion of cytokines, arachadonic acid metabolites and reactive oxygen species.[6]

Although the liver is the major site of synthesis for most circulating complement components, C1q, properdin and factor D do not appear to be synthesized by hepatocytes *in vivo*.[7] C1q is synthesized by follicular dendritic cells, interdigitating cells and cells of the monocyte–macrophage lineage;[8] factor D is synthesized mainly by adipocytes and mononuclear phagocytes,[9,10] while properdin is synthesized by mononuclear phagocytes and T lymphocytes.[10,11] Apart from these, many of the other components can also be synthesized in extra hepatic tissues. It appears that C3 and factor B are made by most cell types, while mononuclear phagocytes, endothelial cells and fibroblasts in culture are able to synthesize a range of complement components.[7] There is good evidence that both normal and inflamed synovial membrane can synthesize all the components of the classical pathway.[12,13] It is thought that such local synthesis of complement components may be

important in host defence in the tissues and may also contribute to the inflammatory process. In this paper we report that chondrocytes in articular cartilage synthesize the classical pathway complement components C1q, C1s, C4 and C2, but do not synthesize to C1r, C3, C1-inh, C4-bp or factor I. We also show that the chondrocytes in culture *in vitro* lose the ability to synthesize the C1q but begin to synthesize C3 and C1-inh in addition to C1r, C1s, C4 and C2, a phenotype which is the same as fibroblasts.

MATERIALS AND METHODS

Immunohistochemistry

Macroscopically normal, full-thickness articular cartilage was obtained from the inferior surfaces of hip joints of four patients undergoing joint replacement surgery for osteoarthritis, and from two normal knee joints following amputation. Rheumatoid synovial tissue was obtained from remedial synovectomies and used as positive controls to check complement component staining with each of the primary antibodies. Cartilage specimens were fixed in Carnoy's solution (4 hr), washed in 90% alcohol and routinely processed to paraffin wax. Tissue sections (4 mm) were cut, dewaxed, rehydrated and examined for the presence of complement components using the following antibodies: goat anti-human C1r and C1s (Atlantic Antibodies, High Wycombe, UK); rabbit anti-human C1q, C3, C4 and C1-inh (Dako Ltd, High Wycombe, UK); and sheep anti-human C2, factor I and C4-bp (The Binding Site, Birmingham, UK). Tissue sections of rheumatoid synovium were prepared and treated in the same way.

Immunohistochemical techniques

The technique used was that described by Jeziorska *et al.*,[14] the main details of which are given below.

For all antibody treatments, tissue sections were first pretreated with normal serum from the same species as the secondary antibody, diluted 1 : 10 with Tris-buffered saline (TBS). Primary polyclonal antibodies, suitably diluted, were applied to tissue sections for 2 hr at 20°. After three 10-min washes in TBS, biotinylated secondary antibodies were applied for 45 min, followed by further washing in TBS. ABC complex (Dako) conjugated with alkaline phosphatase (AP) was then applied for 45 min, washed in TBS, and developed using New Fuchsin to provide a permanent red stain. No counterstaining was used. Rabbit, goat and sheep antibodies were followed with biotinylated IgG: swine anti-rabbit IgG (diluted 1 : 300) and rabbit anti-goat IgG (diluted 1 : 400; Dako), and donkey anti-sheep IgG (diluted 1 : 500; The Binding Site).

TBS or non-immune IgG (rabbit IgG from Dako and goat and sheep IgG both from Sigma Chemical Co., Poole, UK) in concentrations of IgG similar to those of the relevant primary antibodies were substituted for the primary antibodies on control tissue sections, and consistently produced negative observations.

The sections were dehydrated, mounted in XAM Mountant (BDH, Nottingham, UK), examined and photographed using a Zeiss Photomicroscope III and TMAX 100 pro film (Kodak Ltd, Hemel Hempstead, UK).

Complement cDNA

Plasmids containing cDNAs for C1q A-chain, C1q B-chain, C1r, C1s, C4, C2, C3, C1-inh, C4-bp and factor I were used. Details of the plasmids are given in Table 1.

Detection of complement component mRNA in articular cartilage

RNA extraction. Macroscopically normal, full-thickness cartilage was obtained from the inferior surfaces of femoral heads of six patients (two male, four female) undergoing joint replacement surgery, and the femoral head of one normal patient (female) with fractured neck of femur. The joint surfaces were washed in ice-cold TBS before cartilage was removed using a scalpel, and snap-frozen in liquid nitrogen. Frozen cartilage fragments were ground into powder under liquid nitrogen using a mortar and pestle. Total RNA was extracted from the frozen powder by the method of Chirgwin *et al.*,[23] and separated by a 4·7 M caesium chloride (Fisons, Loughborough, UK) gradient at 60 000 *g* in a Beckman ultracentrifuge using the SW 41 rotor. The gradient was removed and the pellet dissolved in 200 μl diethylpyrocarbonate (Sigma)-treated water. The RNA was precipitated with ethanol overnight at −20° and pelleted by centrifugation (MSE Microcentauer Microfuge, Sanyo Gallenkamp PLC, Leicester, UK) at 20 000 *g* for 30 min. The pellet was washed with 70% (v/v) ethanol, dried on ice for 30 min, before being dissolved in diethylpyrocarbonate (DEPC)-treated water, quantified by measuring the absorbance at 260 nm, and stored at −70°.

Table 1. Details of plasmids containing cDNAs for complement components studied

Plasmid	Reference	Fragment	Insert length length (kb)	PCR product (bp)
C1qA/pBluescript KS$^+$	1	*Xba*I/*Eco*RI	1·2	421
C1qB/pAT153	1	*Bam*HI/*Hin*dIII	1·2	402
C1r/pUC9	15	*Sal*I/*Hin*dIII	2·2	524
C1s/pUC9	16	*Sal*I/*Hin*dIII	2·2	551
C4B/pBluescript KS$^+$	17	*Eco*RI/*Xba*I	5·0	500
C2/pGEM	18	*Bam*HI/*Hin*dIII	0·4	400
C3/pBluescript KS$^+$	19	*Eco*RI/only	1·8	501
C1-inh/pBluescript KS$^+$	20	*Eco*RI/*Hin*dIII	1·6	415
C4-bp/pBluescript SK$^+$	21	*Eco*RI/*Xba*I	2·0	413
Factor I/pAT153	22	*Bam*HI/*Cla*I	1·6	425

Table 2. Oligonucleotide primers used for RT-PCR*

		Annealing temperature used†
C1q A-chain	Forward: 5′ CAG GAA ACA TCA AGG ACC 3′	45°
	Reverse: 5′ TCA GGC AGA TGG GAA GAT 3′	
C1q B-chain	Forward: 5′ AAA ATC GCC TTC TCT GCC 3′	45°
	Reverse: 5′ TCA GGC CTC CAT ATC TGG 3′	
C1r	Forward: 5′ GAA GAG CTC ATG AAG CTA GG 3′	43°
	Reverse: 5′ TCA GTC CTC CTC CTC CAT CTC 3′	
C1s	Forward: 5′ CGA ACC AAT TTT GAT AAT GAC 3′	43°
	Reverse: 5′ TTA GTC CTC ACG GGG GGT GC 3′	
C4	Forward: 5′ GCT GAT CTG GGC ATC CAG CTT 3′	52°
	Reverse: 5′ TGT CAG TGC TCG GGC CGA TCT 3′	
C2	Forward: 5′ AGC CAA TCT GGC TCT GCG GAG 3′	48°
	Reverse: 5′ AGC CCT TTT GCG GGA GTT TTT 3′	
C3	Forward: 5′ AGA ACC CCA TGA GGT TCT CGT 3′	52°
	Reverse: 5′ GTA GTT CCA CCC TCA CCT TGA 3′	
C1-inh	Forward: 5′ TCC AAA TGC TAC CAG CTC 3′	45°
	Reverse: 5′ TCT TCA TTG CTG AGG AGG 3′	
C4-bp	Forward: 5′ GAT GGC GAA TGG GTG TAT 3′	46°
	Reverse: 5′ TTT TCA CAG GTA GGA GGG 3′	
Factor I	Forward: 5′ GCA AGG TCA CTT ATA CAT CTC 3′	53°
	Reverse: 5′ GAA ACC CAA GGT CAA GGC AGG 3′	
Actin	Forward: 5′GGAGCAATGATCTTGATCTT3′	
	Reverse: 5′CCTTCCTGGGCATGGAGTCCT3′	

* PCR conditions: 95°, 3 min; 30 cycles of 95°, 1 min, X°, 1 m, 72°, 7 min; 72°, 5 min.
† Annealing temperature for actin was the same as for reactions for individual components.

Reverse transcription (RT) and polymerase chain (PCR) amplification

Complementary DNA (cDNA) was produced from cartilage mRNA by RT using 1–2 μl total RNA and the Superscript™ Preamplification System for First Strand cDNA Synthesis Kit (Gibco BRL, Grand Island, NY), according to the manufacturer's instructions.

The PCR amplification was used to detect the presence of cDNA for classical pathway complement proteins. Each reaction contained final concentrations of 100 μM dNTPs (Boehringer-Mannheim, Lewes, UK), 30 μM $MgCl_2$, 5 U Taq polymerase (Gibco), 2–4 μl of RT reaction product and 1 μM of the appropriate forward and reverse primers (Table 2). For each set of reactions, positive control reactions for complement cDNA were used. These contained full or partial length cloned cDNA (Table 2) at final concentrations of 5–10 ng/reaction. Actin primers were added to each reaction to ensure that in the case of negative results, the PCR had worked. Negative control reactions contained all components except the RT template.

A Hybaid Omnigene PCR Instrument (Hybaid Ltd, Teddington, UK) was used. The PCR conditions for each reaction are given in Table 1.

Aliquots (10–12 μl) of each PCR reaction was electrophoresized on a 2% (w/v) agarose gel (ICN Pharmaceuticals Ltd, Thame, UK) containing 100 ng/ml ethidium bromide (BDH).

Southern blotting

Following inspection under ultraviolet (UV) light, gels were pretreated for 30 min with denaturing buffer (1·5 M NaCl, 0·5 M NaOH), and then for 15 min with neutralization buffer (3 M NaCl, 0·3 M Tris–HCl, pH 8·0). The DNA was then blotted onto Hybond N filter (Amersham Int. plc, Amersham, UK) and the blots fixed using UV light for 2 min (260 nm). The filters were wrapped in clingfilm and stored at room temperature. DNA probes were prepared from full-length cDNA released from plasmids by restriction endonuclease digestion or by PCR amplification of cDNA. cDNA and PCR products were separated by electrophoresis in 2% agarose gels. The DNA bands were excised and purified using Sephaglas Band Prep Kit (Pharmacia Biotechnology, St Albans, UK), according to the manufacturer's instructions. Approximately 100 ng of DNA was labelled with ^{32}[P]dCTP (Amersham) using a Random Primed Labelling Kit (Boehringer, Mannheim, Germany). The reaction was stopped by adding 80 μl column loading buffer (0·2 M EDTA/Bromophenol blue) and the labelled probe separated from free ^{32}[P]dCTP by centrifugation at 900 *g* for 5 min through a 1-ml Biogel P4 column (Biorad Laboratories Ltd, Hemel Hempstead, UK) in 0·1 × SSC/0·1% (w/v) sodium dodecyl sulphate (SDS). Filters were prehybridized at 65° in hybridization solution [7% (w/v) SDS, 1% (w/v) bovine serum albumin (BSA), 1 mM EDTA, 250 mM Na_2HPO_4] for at least 1 hr.

Labelled probes were added to the hybridization buffer to give a concentration of 8×10^6 c.p.m./ml and the filters were hybridized at 65° overnight. Washing of the filters was performed at 65° in wash solution containing 20 mM Na_2HPO_4, 1% (w/v) SDS and 1 mM EDTA (Sigma). The first two washing steps were 20 min duration, while the third lasted 10 min. The filters were then mounted on Whatman filter paper, wrapped in clingfilm and exposed to X-ray film (Blue Sensitive Film, Genetic Research Instrumentation Ltd, Dunmow, UK).

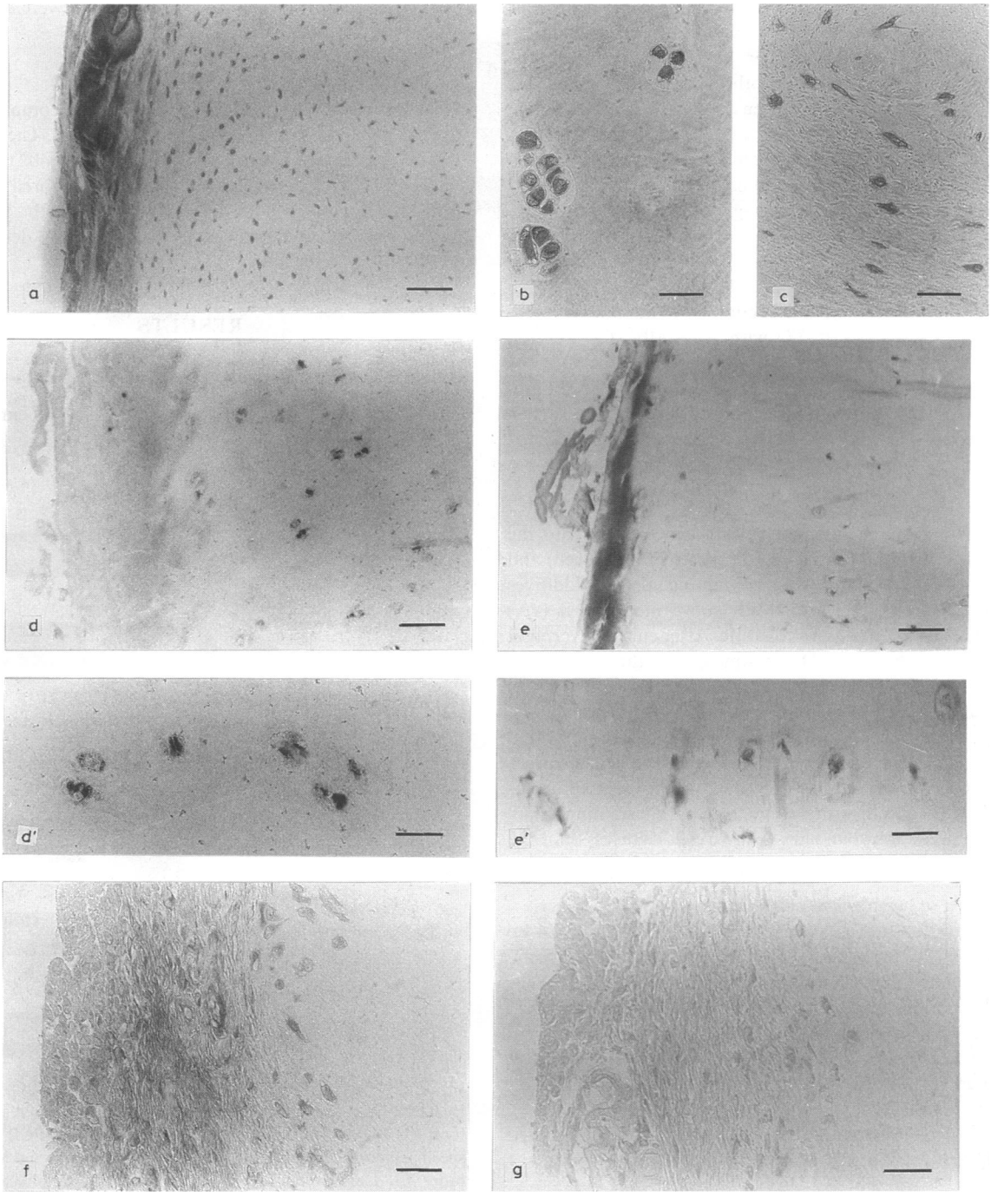

Figure 1. Immunolocalization of components C1q, C1s, C2 and C4 in human articular chondrocytes of osteoarthritic cartilage using the antibody-coupled alkaline phosphatase alkaline (ABC–AP) technique. (a) Low power micrograph showing moderate staining of C2 in chondrocytes and heavier staining of the matrix at the cartilage surface (bar = 50 μm). Examples of C2 staining in chondrocyte clusters and at sites of fibrillation are shown in the high power micrographs (b) and (c), respectively (bar = 20 μm). The apparent nuclear staining is probably due to the presence of immunoreactive material in the Golgi apparatus, which cannot be resolved under this magnification. (d) Low power micrograph showing chondrocytes staining for C1q (bar = 50 μm). Note the restricted intracellular distribution of C1q in chondrocytes shown in d' (bar = 20 μm). (e) Low power micrograph showing moderate staining of C4 in chondrocytes, and more densely stained matrix at the cartilage surface (bar = 50 μm). At high power, the C4 staining appears to be restricted to chondrocytes (e') (bar = 20 μm). (f) Distribution of C1s shown by weak staining in superficial chondrocytes and part of the matrix close to cartilage surface (bar = 20 μm). (g) Cartilage examined for C1r showing negative staining (bar = 20 μm). Such negative observations were seen for all the controls using non-immune immunoglobulins, as described in the Materials and Methods.

Chondrocyte culture

Human articular chondrocytes were obtained by proteolytic digestion of macroscopically normal articular cartilage derived from femoral heads obtained from remedial surgery, as described previously.[24] In previous studies the cells in all cultures prepared in this manner have been shown to be chondrocytic as they express both histamine H1 and H2 receptors,[25] types II and IX collagens and the large aggregating proteoglycans aggrecan, versican and link protein, but not syndecan.[26] The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing sodium pyruvate and glucose (1 g/l) (Gibco), to which L-glutamine (1% w/v), non-essential amino acids 1% (w/v), penicillin, streptomycin, fungizone and 20% (w/v) heat-activated fetal calf serum (FCS) (2 hr at 56°) (all from Gibco) had been added. Medium was changed twice weekly, and at confluence monolayers were detached by incubation at 37° with trypsin/EDTA solution (Gibco). Trypsinization was stopped using 10 ml DMEM containing 20% (v/v) FCS (DMEM–FCS). After washing in DMEM–FCS, the cells were divided into three aliquots, each being added to a fresh culture flask. Chondrocytes were studied between passages three and five.

Synthesis of complement components

Chondrocytes from a confluent culture were seeded into the wells of 24-well Linbro tissue culture plates (2×10^4/well) and cultured in DMEM (1 ml/well) containing all the additives added above except FCS, which was substituted by 2% (v/v) Ultroser-G (Gibco), a serum substitute. After culture overnight at 37° in a humidified 5% CO_2/air atmosphere, the supernatant was removed and replaced by fresh DMEM/Ultroser-G (day 0) and culture continued as before. Daily, for 7 days, the supernatant in each of three wells was removed and stored frozen at −70°. The cells from these wells were detached in a trypsin–EDTA treatment and counted using a haemocytometer. Viability was determined by trypan-blue staining. In all cases the number of trypan blue-positive cells were less than 5% of the total.

Measurement of complement components

The concentrations of C1q, C1r, C1s, C4, C2, C3 C1-inh, C4-bp and factor I in culture supernatants were measured by antibody-capture enzyme-linked immunosorbent assay (ELISA). Wells of ELISA plates (Immulon 4, Dynatech Laboratories Inc., Chantilly, VA) were coated with IgG fractions of polyclonal monospecific antisera, and bound components detected using biotinylated IgG antibodies and avidin-peroxidase (Sigma), as described previously.[27]

The functional activities of C4, C2 and C3 were measured haemoalytically using sera deficient in these components.[28]

Effect of cytokines on chondrocyte synthesis of complement components

These studies were undertaken in 75-cm^2 Nunc tissue culture flasks. Once the cells were confluent, the medium was changed and the culture continued in DMEM containing Ultroser-G (2% v/v) in the absence or presence of the recombinant cytokines interleukin-1α (IL-1α), tumour necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) (all from R&D Systems, Minneapolis, MN), at 10 ng/ml. The culture supernatants were sampled at 24 hr and removed at 48 hr, when the monolayers were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 4 M guanidinium isothiocyanate, and RNA prepared as described for cartilage. The concentrations of complement components in the culture supernatants were measured by ELISA, as described above. Specific mRNA were detected by Northern blot analysis.

Northern blot analysis

RNA was electrophoresed in a formaldehyde-containing 1·2% agarose gel and blotted onto Hybond N filters. The RNA was cross-linked to the filters by UV irradiation (402 nm) for 2–5 min, and baking at 80° for 2 hr.[29] Blots were stored in the dark at 4°. Blots were prehybridized, hybridized with ^{32}[P] cDNA probes, washed and exposed to X-ray film as described for Southern blots (see above).

RESULTS

Immunohistochemical studies of articular cartilage

Chondrocytes stained for C1q (six of six specimens were

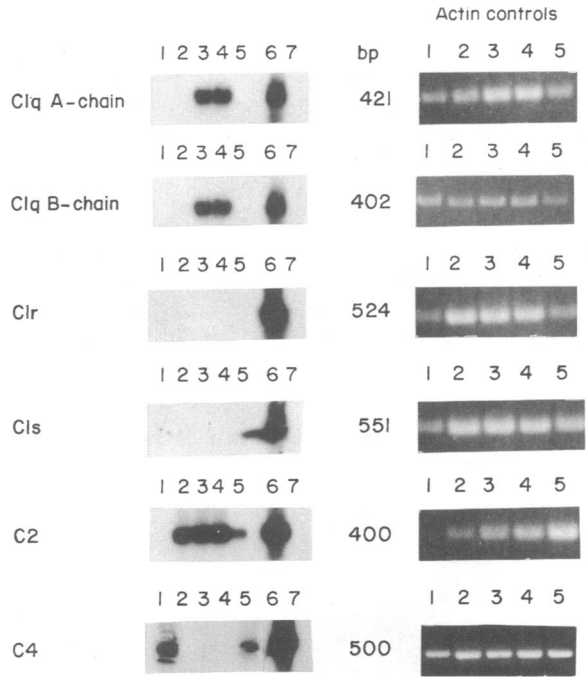

Figure 2. Detection of mRNA transcripts for human C1q (A-chain and B-chain), C1r, C1s, C2 and C4 in articular cartilage. Preparations of total RNA from five specimens were analysed by RT-PCR using gene-specific oligonucleotides, as described in the Materials and Methods. In order to demonstrate the specificity of PCR amplification, PCR products were separated in a 1% agarose gel, blotted to Hybond-N® membrane and hybridized with ^{32}P-labelled cDNA probes for each of the tested complement components. The RT-PCR products from the different cartilage specimen were loaded in lanes 1–5, the positive control of each reaction was loaded on lane 6, and the negative (H_2O) control on lane 7. Although none of the specimens in this series of experiments was positive for C1s mRNA, positive results were found with two specimens of normal articular cartilage (data not shown). As a positive control for the RT-PCR reaction, a 201 bp fragment of the β-actin mRNA sequence was amplified (see actin primers in Table 2). The ethidium bromide staining of the actin–PCR product for each RT-PCR reaction is shown on the right-hand side of this figure.

positive), C1s (five of six positive), C4 (five of six positive) and C2 (six of six positive), but not for C1r, C3, C1-inh, C4-bp or factor I (Fig. 1). C1q staining was most pronounced and mainly located in the superficial zone immediately below the articular surface (Fig. 1d). Not all chondrocytes in the superficial zone stained for C1q. C1s staining of chondrocytes was weak and located in the superficial layers (Fig. 1f). Chondrocyte staining for C4 was seen mainly in the mid-zone (Fig. 1e). Only occasionally did chondrocytes in the superficial zone stain with moderate intensity for C4, while none of those in the deep zone (adjacent to bone) was positive. Chondrocytes in the superficial zone stained heavily for C2 (Fig. 1a). Characteristically, heavily staining cells occurred in clusters and at sites of fibrillation. Chondrocyte staining for C1q, C1s, C4 and C2 was most intense in areas of cartilage fibrillation, but was also present in areas of normal cartilage. Intense staining of the matrix for C4 and C2 was seen, but only in areas of surface fibrillation. There was no surface staining for any of the other components studied. There was no matrix staining for any component around positively staining chondrocytes.

Detection of mRNA for complement components in articular cartilage

Insufficient RNA was obtained from these cartilage samples to perform RT-PCR reactions for all the complement components under investigation on all seven cartilage samples. Amplification products demonstrated the presence of mRNA for following components: C1q A-chain (two of five positive), C1q B-chain (two of five positive), C1s (two of seven positive), C4 (two of five positive) and C2 (four of four positive) (Fig. 2). Amplification products corresponding to C1r, C3, C1-inh, C4-bp and factor I were not detected in any of the seven specimens. In all cases the amplification product of actin mRNA was detected.

Synthesis of classical pathway component by cultured articular chondrocytes

Using ELISA, C1r, C1s, C2, C3 and C1-inh were detected in the supernatants of chondrocyte cultures, and their concentrations increased with time (Fig. 3). The patterns of increase varied for each component. C1q, C4-bp and factor I were not detected. Concentrations of C1r, C1s, C4, C2, C3 and C1-inh in the culture supernatant were reduced significantly by the addition of cycloheximide [1·0 μg/ml (w/v)] to the culture supernatants (data not shown). Chondrocyte C4, (5×10^5 effective molecules/ng), C2 ($1{\cdot}4 \times 10^5$ effective molecules/ng) and C3 ($2{\cdot}5 \times 10^3$/effective molecules/ng) were shown to have levels of haemolytic activity that were similar to those obtained for serum C4 ($8{\cdot}3 \times 10^5$), C2 ($1{\cdot}8 \times 10^5$) and C3 ($3{\cdot}7 \times 10^3$).

Northern blot analysis of mRNA from chondrocytes revealed the presence of mRNA for C1r, C1s, C2, C3 and C1-inh (Fig. 4). Messenger RNA (mRNA) for C1q A chain, C1q B chain, C4-bp and factor I were not detected by Northern blot or by RT-PCR analyses (data not shown). Although C4 mRNA was not detected in unstimulated chondrocytes, it was detected in INF-γ-treated cells (see below).

Effect of cytokines on complement synthesis by chondrocytes

IFN-γ (10 ng/ml) increased synthesis of all components except C2, as shown by increased concentrations in the cell culture supernatants and increased abundancies of specific mRNA on Northern blots (Table 3 and Fig. 4). TNF-α (10 ng/ml) increased secretion of C1r, C1s and C3 and reduced secretion of C4, C2 and C1-inh.

In general, changes in mRNA abundances were in good agreement with changes in protein secretion. However, there were some discrepancies, e.g. the effects of IL-1β on C1s

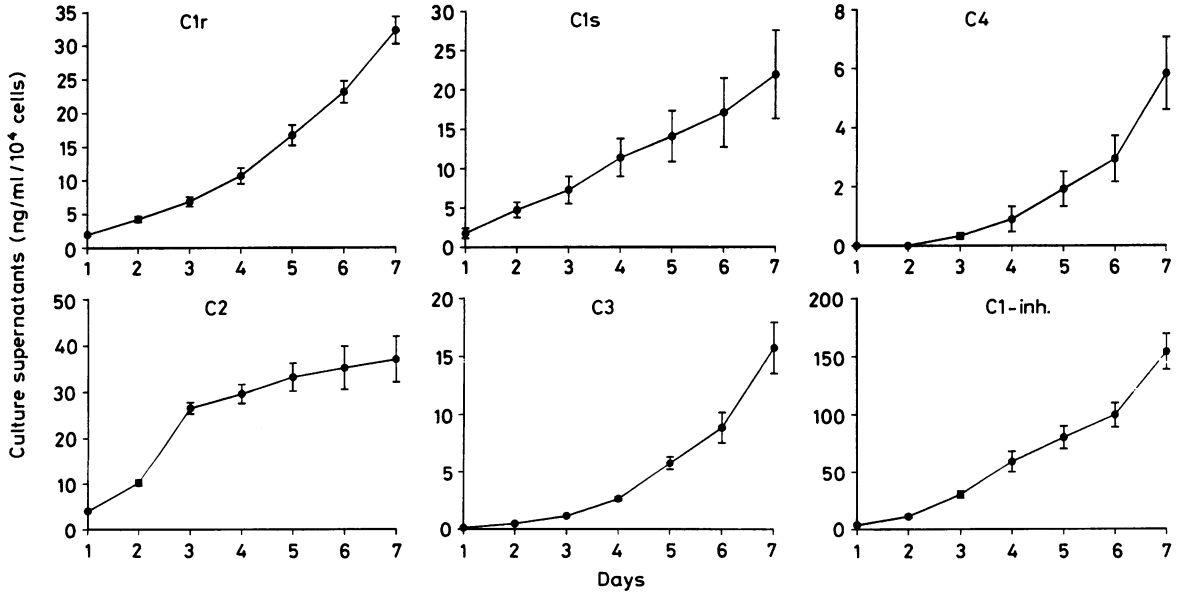

Figure 3. Cumulative synthesis of C1r, C1s, C1-inh, C2, C4 and C3 by articular chondrocytes cultured *in vitro*. Culture supernatants were expressed as ng/ml/10^4 cells. Each point represents the mean of three replicate cultures and the horizontal bars show the standard deviations. C1q, C4-bp and factor I were not detected. The same results have been seen on cultured articular chondrocytes from 12 separate donors.

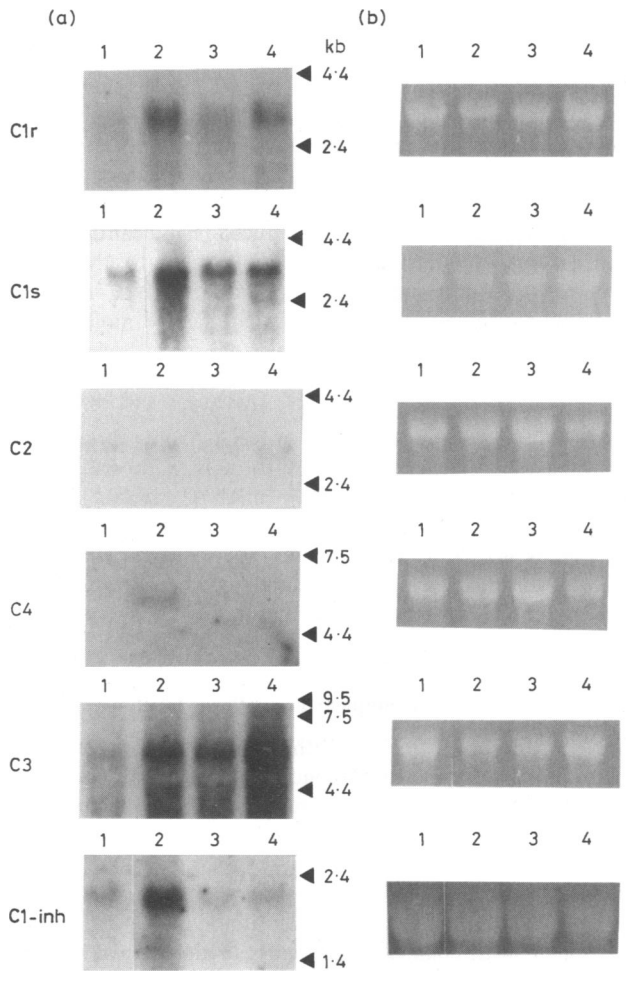

Figure 4. Northern blot analysis of total RNA preparations from cultured human chondrocytes cultured for 48 hr in the presence and absence of cytokines. Approximately 15 μg of total RNA was loaded on each lane. RNA of cells cultured in the absence of cytokines was loaded in lane 1; RNA of cells cultured in the presence of IFN-γ (10 ng/ml) was loaded in lane 2; RNA of cells cultured in the presence of TNF-α was loaded in lane 3; RNA of cells cultured in the presence of IL-1 was loaded in lane 4. Hybridization signals with cDNA probes specific for C1r, C1s, C2, C4, C3 and C1-inh demonstrated the presence and the abundance of specific mRNA transcripts in these cells. The autoradiographs of these Northern blots are shown in (a), whereas the loading of total RNA of each Northern blot is stated by the ethidium bromide staining of the ribosomal 28 s rRNA shown in (b). The positions of the size markers (◁) are shown to the right of the gels in (a).

expression and TNF-α on expression of C2 and C1-inh. These inconsistencies were not unexpected as cytokines act on transcriptional and a number of post-transcriptional events.

DISCUSSION

Chondrocytes in articular cartilage exist within the cartilage matrix without any direct cell–cell contact. Each cell is responsible for the synthesis, maintenance and turnover of the extracellular matrix in its vicinity. These functions are achieved by the synthesis of a number of matrix components, including collagens and proteoglycans, and enzymes capable of degrading cartilage such as collagenase. Chondrocytes also synthesize a tissue inhibitor of metalloproteinase that regulates cartilage degradation. In this paper, we have shown that some chondrocytes in articular cartilage express the classical pathway complement components C1q, C1s, C4 and C2, as judged by the immunohistochemical detection of each component. As chondrocytes are the only cell type present in cartilage, and as molecules larger than 65 000 MW cannot diffuse through cartilage matrix,[30] the presence of complement components in or on cells in the sections studied must be due to synthesis of these components by these immunohistochemically positive chondrocytes. The detection of mRNA of these components by RT-PCR supports this conclusion. The failure to detect specific mRNA for all components in all samples may well be due to sampling error as the distribution of immunohistochemically-positive cells was not uniform in any specimen. Furthermore, we do not know whether a single articular chondrocyte *in vivo* synthesizes all the complement components simultaneously.

Although some of the samples used in immunohistochemical and RT-PCR studies were from joints affected with osteoarthritis, all the specimens taken were from the inferior surface of the femoral heads and were full-thickness cartilage. Despite this, there was histological evidence of cartilage fibrillation in some areas but most of the sections appeared normal. C1q, C1s, C4 and C2 were detected in histologically normal cartilage from joints of patients with osteoarthritis and in cartilages from two normal knee joints. As the immunohistochemical data were supported by the results of RT-PCR, we believe that it is reasonable to assume that these components are synthesized in normal articular cartilage. This conclusion is supported by the immunohistochemical detection of C1s in normal hamster articular cartilage.[31] The failure to detect C1r, C3, C1-inh, C4-bp or factor I by either technique suggests that these components are not made by articular chondrocytes *in vivo*. Previous studies on complement synthesis by hepatocytes, monocytes, fibroblasts, intestinal epithelial cells and umbilical vein endothelial cells, have shown that whenever C1s is synthesized, C1r is also present.[32] Often the levels of C1r and

Table 3. Complement levels in culture supernatants and specific mRNA levels determined by densitometry from human articular chondrocytes (HAC) cells cultured with cytokine for 48 hr, expressed as a proportion of the unstimulated level

	Cytokine					
	IFN-γ		TNF-α		IL-1β	
Complement component	Protein[1]	RNA	Protein	RNA	Protein	RNA
C1r	1·7	2·8	1·4	1·3	1·1	2·2
C1s	2·0	1·8	1·5	0·8	1·5	0·5
C4	5·2	ND†	0·4	ND	0·6	ND
C2	0·9	2·3	0·7	1·9	1·1	1·2
C3	2·3	2·0	1·8	2·1	2·3	1·7
C1-inh	5·6	4·7	0·6	0·1	0·8	0·4

*Unstimulated levels of complement components were: C1r, 75; C1s, 64; C4, 3·2; C2, 4·1; C3, 84; C1-inh, 57 ng/ml. Cells were used for RNA extraction so cell counts were not available.

† C4 mRNA was only detected in IFN-γ-treated cells.

C1s are similar; however, in umbilical vein endothelial cells very small amounts of C1r were synthesized in comparison with C1s, and levels in culture supernatants were often at the lower limit of detection. Perhaps in chondrocytes C1r synthesis occurs, but at a level below the limits of detection.[32] If this is the case, articular chondrocytes would synthesize intact C1 (C1q, C1r, C1s), C4 and C2 *in vivo* but not C3 or any of the classical pathway regulatory proteins.

The role of complement components synthesized by articular chondrocytes is unknown. These components do not enter the cartilage matrix, as shown by the lack of staining around the lacunae. This is not unexpected, because, as mentioned above, only molecules smaller than 65 000 MW can diffuse through the matrix[30] and all the complement components studied are larger than this. Thus, if the complement components synthesized by chondrocytes have any biological role, it must be on the matrix at the chondrocyte–matrix interface and/or on the chondrocytes themselves. Furthermore, if activation of complement occurs all the components required for activation must be synthesized by a single chondrocyte.

There is evidence that C1s will degrade collagen type I and type II,[33] the latter being the most abundant type found in articular cartilage. In addition, it has been shown that C1q and the C1 macromolecule can bind to decorin and, in a fluid phase system, decorin inhibited C1 functional activity.[34] However, the binding of C1 to decorin in cartilage matrix, where more than one subunit of a sight C1q molecule could be engaged to aggregated decorin, may lead to C1 activation. Other complement enzymes, e.g. C4b2a, may also cleave cartilage matrix components.

The possibility that locally synthesized complement acts on chondrocytes themselves would require the presence of complement receptors. We have been unable to detect CR1, CR2, CR3 or CR4 on chondrocytes in articular cartilage or in chondrocyte cultures *in vitro*. However, in both these situations, chondrocytes express membrane cofactor protein (MCP) (CD46), decay accelerating factor (DAF) (CD55) and CD59 (K. Whaley, unpublished data). It is possible that C4b or C4b2a could act as ligands for MCP and/or DAF to modulate chondrocyte function. Ligation of DAF on T cells leads to proliferation involving $p56^{ick}$ or $p59^{fyn}$.[35]

In contrast to the absence of staining for complement components around lacunae, the superficial matrix in damaged areas stained for C4 and C2. It is unlikely that these components have diffused from the synovial fluid into the cartilage, as there was no staining for any of the other components, in particular C3 which could bind covalently to matrix components. It is therefore more likely that this represents chondrocyte C4 and C2, which diffuse more readily through the damaged cartilage and bind to matrix components.

The studies on cultured articular chondrocytes showed that, unlike chondrocytes in cartilage, C1q was not synthesized whereas C3 and C1-inh were. The profile of components synthesized by cultured chondrocytes (C1r, C1s, C4, C2, C3 and C1s-inh) is the same as that produced by fibroblasts,[13] suggesting that chondrocytes might differentiate towards a fibroblast phenotype on prolonged culture *in vitro*.[36–38] The ELISA data for all these components (except C2) have been confirmed by Western blot analysis (K. Whaley, unpublished data). An experiment using primary cultures of chondrocytes showed that, within 1 week, synthesis of C1q had ceased and synthesis of C3 and C1-inh had started (K. Whaley, unpublished data). Prior to this report, the only cells known to synthesize C1q were dendritic cells, mononuclear phagocytes and glial cells.[8,39] It is possible that C1q synthesis occurs in many different cells *in vivo* and the inability to detect C1q synthesis in *in vitro* culture systems may be due to suppression of synthesis in this environment. The response of cultured chondrocytes to cytokines suggests that in the cytokine-rich environment of the inflamed joint, synthesis of rates of complement components may change and synthesis of other components, such as C3, may occur.

In summary, these data provide strong evidence for synthesis of some classical pathway complement components by articular chondrocytes *in vivo*. Future studies must be directed to determine if single chondrocytes are able to synthesize each of these components simultaneously. The finding of C1q, C1s and C2 in the superficial zone suggests that this might be the case, but C4 was mainly detected in chondrocytes in the mid-zone. However, we looked at a small number of specimens and detailed studies of larger numbers of specimens from normal joints and from those with different types of articular disease must be studied by immunohistochemistry and *in situ* hybridization to resolve this issue. Investigations should also be directed towards understanding the physiological and pathological roles of articular chondrocyte complement.

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