Complement C1s activation in degenerating articular cartilage of rheumatoid arthritis patients: immunohistochemical studies with an active form specific antibody

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Abstract

Objective—The first complement component C1s was reported to have novel functions to degrade matrix components, besides its activities in the classic complement pathway. This study explores participation of C1s in articular cartilage degradation in rheumatoid arthritis (RA).

Methods—Normal articular cartilage (n=6) and cartilage obtained from joints with RA (n=15) and osteoarthritis (OA, n=10) were immunostained using anti-C1s monoclonal antibodies PG11, which recognises both active and inactive C1s, and M241, which is specifically reactive to activated C1s. The effects of inflammatory cytokines on C1s production by human articular chondrocytes were also examined by sandwich ELISA.

Results—In normal articular cartilage, C1s was negative in staining with both PG11 and M241. In contrast, degenerating cartilage of RA was stained with PG11 (14 of 15 cases), and in most of the cases (13 of 15 cases) C1s was activated as revealed by M241 staining. In OA, C1s staining was restricted in severely degrading part of cartilage (5 of 10 cases), and even in that part C1s was not activated. In addition, C1s production by chondrocytes in vitro was increased by an inflammatory cytokine, tumour necrosis factor a.

Conclusion—These results suggest that C1s activated in degenerative cartilage matrix of RA but not in that of OA. C1s is thought to participate in the pathogenesis of RA through its collagenolytic activity in addition to the role in the classic cascade.

Autoimmunity is thought to be one of the dominant factors in the destruction of joint cartilage in rheumatoid arthritis (RA). Previous studies have demonstrated the presence of autoantibodies reacting with cartilage components in the serum samples of RA patients. These cartilage specific antigens include components of cartilage matrix such as type II collagen, as well as of cellular elements. Although such antibodies have regulatory roles in T cell activation, the consequences of a specific antibody interaction have also been pointed out. There is little doubt that complement activation occurs adjacent to the cartilage surfaces of RA patients as evidenced by the abundant co-deposits of immunoglobulin and activated complement components on the surface. Furthermore, it has been reported that chondrocytes themselves synthesise the components. Cytotoxic effects of the complement system is thought to play an important pathological part in the processes leading to irreversible cartilage damage in RA.

The first complement component C1s is activated by C1r, which is autoactivated upon binding of C1q to an immune complex. Activated C1s cleaves C2 and C4 to form C3 convertase and starts the classic pathway. Recently, C1s was shown to have novel functions to degrade types I, II, and IV collagen, gelatin, and decorin and to activate the zymogen of matrix metalloproteinase-9. Moreover, we have found that C1s is expressed in hamster epiphyseal chondrocytes and intensely immunostained in the hypertrophic chondrocytes, but not in normal articular chondrocytes. We also found that C1s was activated on the edge of degrading cartilage fragments at the ossification centre, where the cartilage matrix is replaced by bone marrow. These findings suggest possible participation of C1s in cartilage remodelling.

Similar to physiological chondrolysis during development, pathological destruction of articular cartilage in RA and osteoarthritis (OA) is known to be caused, at least partially, by chondrocyte derived proteolytic enzymes such as matrix metalloproteinases. In inflammatory arthritis, C1s may also participate in cartilage degradation through its proteolytic activities and cytotoxicities by activating the classic complement cascade. To ascertain C1s participation in the pathogenesis of RA, we examined localisation and activation of C1s in articular cartilage with RA and OA by immunostaining using anti-C1s monoclonal antibodies.

Methods

Patients and tissue acquisition
Articular cartilage from 31 patients were studied after obtaining informed consent. Table 1 shows the clinical details of the patients. Macroscopically normal, full thickness articular cartilage was obtained from four patients with femoral neck fracture undergoing joint replacement surgery, and two patients with chondrosarcoma undergoing amputation before radiation therapy or chemotherapy.
cartilage of 10 patients with OA and 15 patients with diagnosis of RA, according to the American College of Rheumatology criteria, were obtained at time of knee or hip joint replacement surgery. All RA patients were treated with disease modifying anti-rheumatic drugs (DMARDs) and low dose prednisone (less than 10 mg daily). Cartilage tissues were acquired from not only degenerating sites but also macroscopically normal sites of OA and RA joints. All tissues were fixed with 4% paraformaldehyde in phosphate buffered saline immediately after operation.

**SERUM C REACTIVE PEPTIDE MEASUREMENT**

Serum C reactive peptide (CRP) of each patient was measured by using N Latex CRP Kit (Hoechst Japan, Tokyo, Japan).

**ANTIBODIES**

Characterisation of anti-human C1s monoclonal antibodies M81, M365, and M241 have been reported. M81 and M365 recognise around the C4 binding site of human C1s, and react with both active and inactive C1s. M241, a neutralising antibody, binds specifically to the active centre of activated C1s. These three antibodies are kind gifts from Dr M Matsumoto. Anti-hamster C1s monoclonal antibodies PG11 and NG7 have also been characterised previously. PG11 recognises the same epitope as M81, and cross reacts with human C1s. NG7 shows low cross reactivity with human C1s, and was used for control staining.

**IMMUNOHISTOCHEMICAL STUDIES**

Articular cartilage fixed with paraformaldehyde was decalcified with 20% EDTA in 0.15 M NaCl solution, and frozen in OCT compound (Miles Scientific, Naperville, Ill, USA). Serial sections were prepared for haematoxylin and eosin, toluidine blue (pH 7.0), and immunohistochemical staining. For immunostaining, the sections were treated with PG11 (50 µg/ml) or M241 (50 µg/ml) at 4°C overnight, followed by an incubation with biotinylated goat antimouse IgG F(ab)2 (Boehringer Mannheim Biochemicala, Mannheim, Germany) for three hours. The samples were then incubated with horseradish peroxidase (HRP) conjugated streptavidin (Boehringer Mannheim Biochemicala, Mannheim, Germany), and counter stained with haematoxylin.
CELL CULTURE AND QUANTIFICATION OF C1s IN CONDITIONED MEDIA

Normal articular cartilage was obtained from amputated lower extremities of young patients with informed consent. For isolation of chondrocytes, the finely diced tissues were digested with 0.2% collagenase (type IV, Sigma Chemical, St Louis, MO, USA) in minimal essential medium plus 10% fetal calf serum (FCS) for three hours. Isolated chondrocytes were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F-12 (DMEM+F-12), supplemented with 20% FCS. To examine effects of inflammatory cytokines on C1s production, the first passage human articular chondrocytes were incubated at a density of $3 \times 10^4$ cells/well in 96 well plates and grown in 0.2 ml of the medium described above. After reaching confluence (four days after inoculation), the culture medium was switched to 0.1 ml of serum free DMEM+F-12 supplemented with human transferrin (10 µg/ml), bovine insulin (10 µg/ml), and hydrocortisone (10⁻⁸ M). Various concentrations of recombinant human interleukin 1α (IL1α, Boehringer Mannheim Biochemica, Mannheim, Germany) or human tumour necrosis factor α (TNFα, Boehringer Mannheim Biochemica, Mannheim, Germany) were added and incubated for 48 hours. Conditioned media were collected and C1s in the media was quantified by sandwich ELISA as described previously.29

Briefly, wells of 96 well flexible assay plates (Becton Dickinson Labware, Oxnard, CA, USA) were coated with M365 (50 µg/ml), and reacted with samples for one hour at room temperature. After washing, the wells were incubated with biotinylated M81 (50 µg/ml), followed by alkaline phosphatase labelled avidin (10 µg/ml, E-Y Laboratories, Inc, San Mateo, CA, USA). The enzyme activity was assayed for 30 minutes at 37°C in 10% diethanolamine buffer (pH 9.8), 3.8 mM p-nitrophenyl phosphate (Sigma Chemical, St Louis, MO, USA) and 1 mM MgCl₂. Absorbance was measured at 405 nm with Immuno reader NJ-2000 (Nippon Inter Med, Ltd, Tokyo, Japan). C1s in the conditioned media was quantified using a standard curve drawn as previously described.29

IMMUNOBLOTTING

Serum free conditioned medium of human articular chondrocytes was concentrated 50 times by ammonium sulphate precipitation, and the amount of total protein was examined by Lowry's method. Samples (20 µg protein / lane) were analysed on SDS-PAGE (10% gel) under non-reducing condition. Immunoblotting was carried out using M81 (50 µg/ml).

STATISTICAL ANALYSIS

Statistical analysis was performed using Student’s t test with Welch’s correction. Differences between groups were considered significant when the p value was less than 0.05.

Results

IMMUNOHISTOCHEMICAL DETECTION OF ACTIVATED C1s IN HUMAN ARTICULAR CARTILAGE WITH RA

In normal articular cartilage, neither chondrocytes nor cartilage matrix was stained with an anti-C1s monoclonal antibody PG11 that recognises both active and inactive C1s (fig 1A). Naturally, normal cartilage was non-reactive to both M241, recognising only active C1s (data not shown), and NG7, an antihamster C1s monoclonal antibody that is poorly cross reactive to human C1s (fig 1B). In superficial areas with cartilage fibrillation, a

**Table 2** Summary of immunohistochemical examinations of C1s in human articular cartilage

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Diagnosis</th>
<th>Chondrocytes (no of positive/no of examined)</th>
<th>Degenerating matrix (no of positive/no of examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG11</td>
<td>Normal</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>M241</td>
<td>OA</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>PG11</td>
<td>RA active</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>M241</td>
<td>RA inactive</td>
<td>4/5</td>
<td>3/5</td>
</tr>
</tbody>
</table>

Active RA: patients whose serum CRP was higher than 1.0 mg/dl. *Positive cells were localised to only degenerating superficial zone.

**Figure 2** Differential reactivities of PG11 and M241 in RA articular cartilage. Articular cartilage of RA patients (A, C, E: 36 year old woman, B, D, F, G: 51 year old woman) was fixed and frozen sectioned as described in the text. The samples were immunostained with PG11 (A, B), M241 (C, D), and NG7 (E, F, for control staining) and stained with toluidine blue (G). Degenerative cartilage matrix was equally reactive to both PG11 (A, B arrowheads) and M241 (C, D arrowheads). Whereas, chondrocytes were stained with PG11 (A, B arrows) but not with M241 (C, D). Bar: 100 µm.
coincidental C1s detection and loss of proteoglycans was revealed by toluidine blue staining (fig 1F). However, C1s activation is thought to be minimal, if at all, as staining intensity by M241 (fig 1D) was equivalent to that of NG7 (fig 1E).

The surface of degenerative articular cartilage of RA patients showed histopathological features such as fibrillation, clustering of chondrocytes, fibrocartilage formation (fig 2), and faint metachromasia by toluidine blue staining (fig 2G). In these regions, cartilage matrix was equally reactive to both PG11 (fig 2A, B arrow heads) and M241 (fig 2C, D arrow heads), indicating presence of C1s activation. In regions of pannus invasion, synovial fibroblasts (fig 3C) and tartrate resistant acid phosphatase (TRAP) positive multinucleated cells (fig 3E arrows) were infiltrating into the matrix. Invading synovial cells, degrading cartilage matrix were intensely stained with PG11 (A). They also stained with M241 (D), indicating that C1s was activated there. Bar: 50 µm.

Figure 3  Immunolocalisation and activation of C1s at the site of pannus formation. Articular cartilage with pannus was fixed, and serial frozen sections were prepared as described in the text. They were stained with haematoxilin and eosin (C), toluidine blue (F) and immunostained with PG11 (A), M241 (D), and NG7 (B). Tartrate resistant acid phosphatase (TRAP) staining was also performed (E). Invasion of synovial cells and TRAP positive multinucleated cells (E arrows) was observed in the cartilage matrix. Invading synovia and degrading cartilage matrix were intensely stained with PG11 (A). They also stained with M241 (D), indicating that C1s was activated there. Bar: 50 µm.

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Figure 4  Effects of IL1 and TNFα on C1s production by human articular chondrocytes. (A) Serum free culture medium of human articular chondrocytes was analysed on SDS-PAGE (10% gel) under non-reducing condition and immunoblotted with M81. Lane 1; culture medium, lane 2; molecular marker, arrow; C1s. (B) Chondrocytes were seeded at a density of 3 × 10⁴ cells/well in 96 well plates and grown in DMEM+F12 supplemented with 20% FCS. After they reached confluency (four days), the culture medium was switched to serum free medium, DMEM+F-12 supplemented with human transferrin (10 µg/ml), bovine insulin (10 µg/ml), and hydrocortisone (10⁻⁸ M), containing various concentration of IL1α or TNFα, (control: no addition). After a 48 hour incubation period, C1s in the medium was quantified by sandwich ELISA and the amounts were normalised to cell number. Values are the average (SD) of four determinations (bars). * p<0.05; ** p<0.01 v control.
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well as that of C1r and C1 inhibitor by normal dose dependent manner. IL1 of 10), whose serum CRP was over 1.0 mg/dl.

Table 2 summarises all C1s immunohistochemical examinations. C1s was detectable in none of the normal articular cartilage examined (0 of 6). In contrast, in half of the OA cases (5 of 10) and in almost all RA cases (14 of 15), cartilage was positive in C1s staining. In particular, intense C1s staining in both chondrocytes and cartilage matrix was observed in all cases diagnosed as active RA (10 of 10), whose serum CRP was over 1.0 mg/dl.

EFFECTS OF CYTOKINES ON C1s SYNTHESIS BY ARTICULAR CHONDROCYTES IN VITRO

The first passage chondrocytes grew into multilayer cell sheets after they reached confluency and produced proteoglycan rich matrix around them. Secretion of type II collagen into the medium was ascertained by immunoblotting with M81 (fig 4A). As cytokines are important mediators for inflammation, we examined if IL1α and TNFα had any effect on C1s production by articular chondrocytes by sandwich ELISA using anti-type II collagen monoclonal antibodies. The articular chondrocytes were shown to secrete C1s into the culture media by immunoblotting with M81 (fig 4A). As cytokines are important mediators for inflammation, we examined if IL1α and TNFα had any effect on C1s production by articular chondrocytes by sandwich ELISA using anti-type II collagen monoclonal antibodies. The articular chondrocytes were shown to secrete C1s into the culture media by immunoblotting with M81 (fig 4A).

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In RA, it is likely that C1s derived C1s, secreted into the lacunae, participates in this reaction. In addition, C3b on the cartilage is reversible damage to chondrocytes. It is possible that chondrocyte derived C1s, secreted into the lacunae, participates in this reaction. In addition, C3b on the cartilage is thought to stimulate the phagocytosis by macrophages, resulting in further cartilage degradation. Another possible contribution of C1s activation in RA pathogenesis is its potency of direct matrix degradation, as it degrades matrix components such as type I, II, and IV collagens and decorin, and activates the zymogen of matrix metalloproteinase-9. Other types of proteolytic enzymes, such as matrix metalloproteinases, were shown to be produced by chondrocytes and play an important part in pathological destruction of articular cartilage in RA. In RA, it is likely that de novo synthesised C1s promotes degradation of pericellular matrix.

In addition to type II collagen of other animals, we demonstrated that human C1s also degrades human type II collagen (data not shown). Type II collagen fragments cleaved by C1s at different sites from that of typical collagenase could be antigens for the autoimmune disease. There is considerable evidence showing the presence of antibodies against type II collagen fragments in serum samples of RA.
patients.4–14 Terato et al demonstrated that a major immunogenetic and arthritogenic epitope on type II collagen resides in a cyanogen bromide-generated fragment (CB11), using a collagen induced arthritis model.30–34 CB11 was shown to contain epitopes of type II collagen, which are recognised by T cells that regulate collagen induced arthritis.35–41 The analysis of CB11 generated type II collagen fragments is a vital factor in proving CB11 participation in the pathogenesis of RA. Ochi et al have reported that the amounts of another C1 component C1q in serum samples have a close correlation to the activity and prognosis of RA.42–46 As C1q activation was evident in articular cartilage with RA but not with OA, it may be possible to discriminate RA from OA by detecting activated C1q and/or CB11 generated type II collagen fragments in cartilage.

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