EXTENDED REPORT

Expression of the peptide C4b-binding protein β in the arthritic joint

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Background: C4b-binding protein (C4BP) is a plasma oligomeric glycoprotein that participates in the regulation of complement and haemostasis. Complement-regulatory activity depends on the C4BP α -polypeptide, whereas the C4BP β -polypeptide inactivates protein S, interfering with the anti-coagulatory protein C-dependent pathway.

Objective: To investigate the expression of C4BP β in the rheumatoid joint.

Methods: Expression of C4BP was studied in synovial explants from patients with rheumatoid arthritis, osteoarthritis and healthy controls, using immunohistochemistry and in situ hybridisation. C4BP isoforms and free C4BP β were studied in synovial effusions from patients with rheumatoid arthritis, osteoarthritis and microcrystalline arthritis (MCA) by immunoblotting; total and free protein S levels were studied by enzyme immunoassay.

Results: C4BP β was overexpressed in the synovial membranes of patients with rheumatoid arthritis, in close association with the severity of synovitis and the extension of interstitial fibrin deposits. As many as 85% fluids from patients with rheumatoid arthritis contained free C4BP β , whereas this unusual polypeptide was present in 50% fluids from patients with MCA and 40% fluids from patients with osteoarthritis. Free protein S at the effusions was pathologically reduced in patients with rheumatoid arthritis and MCA, and remained normal in patients with osteoarthritis.

Conclusion: C4BP β is expressed by the inflamed synovial tissue, where it can participate in processes of tissue remodelling associated with invasive growth.

The C4b-binding protein (C4BP) regulates complement activation and haemostasis. These two systems are active participants in the inflammatory reaction, and changes in their functioning may lead to perpetuation of inflammation. C4BP is an oligomeric protein present in plasma as $\alpha_7\beta_1$, $\alpha_7\beta_0$ and $\alpha_6\beta_1$ isoforms.¹ Each C4BP α chain has a binding site for C4b² and this interaction inhibits the classic pathway of complement activation by reducing the formation and stability of C3 convertase (C4bC2b). By this mechanism, potentially harmful complement overactivation is prevented. The C4BP β chain binds the vitamin K-dependent protein S. Protein S is found in plasma coupled with C4BP or as a free protein, but only the free fraction is active.^{3–4} After activation by thrombin–thrombomodulin complexes at the cell surface, protein C binds circulating protein S, which acts as a cofactor for the inactivation of coagulation factors V and VIII.⁵ The interaction of C4BP β and protein S shows high affinity and all C4BP β^+ molecules circulate in plasma bound to protein S. Thus, the free (active) protein S plasma level corresponds to the molar excess of total protein S over C4BP β^+ molecules.⁶ As congenital defects of protein S and reduction of free protein S levels are risk factors for thrombosis,⁷ it is thought that raised levels of C4BP β^+ may also favour thromboembolic events.^{3–7–8} Despite C4BP oligomers being expressed exclusively in the liver, we have recently shown that C4BP β , without C4BP α , is expressed in the adult human ovary.⁹ The ovary C4BP β polypeptides are locally produced by fibroblast-like cells activated after contact with fibrin clots in the corpus luteum and participate in its invasion to form a corpus albicans. This finding suggests that C4BP β is not only associated with coagulation but may also have a role in processes of connective tissue remodelling by fibroblasts.

Fibrin deposition inside the synovial tissue is a prominent feature of synovitis, particularly in rheumatoid arthritis.^{10–11} Fibrin–fibroblast interaction may be a determinant for the activation of synovial cells to acquire an invasive phenotype in this disease.^{12–16} Invasion of fibrin matrices by activated fibroblasts is, therefore, a feature shared by the ovary cells associated with the resorption of the corpus luteum and the cells participating in pannus formation. We explored whether synovial fibroblasts could also express C4BP β polypeptides, looking into a potential role of this peptide in the pathophysiology of synovitis.

METHODS

Patients and material

Synovial samples were obtained during knee joint replacement from patients fulfilling the American College of Rheumatology criteria for rheumatoid arthritis¹⁷ (n = 7) and patients with knee osteoarthritis (n = 7). Synovial samples from young adults who underwent knee arthroscopy for reconstruction of meniscal tears were used as controls (n = 3). Synovial fluid was aspirated from the swollen joints of patients with rheumatoid arthritis (n = 13) and the joints of those without rheumatoid arthritis (n = 9). A plasma sample was obtained during joint aspiration from seven patients with rheumatoid arthritis. Informed consent and approbation of the local ethics committee were obtained. A paraffin wax-embedded human liver section, kindly provided by the Department of Pathology of the Fundación Jiménez

Abbreviations: BSA, bovine serum albumin; C4BP, C4b-binding protein; MCA, microcrystalline arthritis

Díaz Hospital, was used as a source of C4BP for positive control in the in situ hybridisation techniques.

Synovial membranes were washed in phosphate-buffered saline (PBS), fixed in formalin, dehydrated and embedded in paraffin wax. Slices of 7 μ m were placed in poly-L-lysine-treated crystals and were used for haematoxylin-eosin staining, immunohistochemistry and in situ hybridisation. Synovial fluids were frozen at -80°C . After thawing they were centrifuged, treated with Triton X and used in protein studies.

Reagents

Primary antibodies were a polyclonal mouse antibody against the recombinant fusion protein gold sodium thiomalate:C4BP β and a rabbit anti-human C4BP α polyclonal immunoglobulin G (IgG) developed in our laboratory,¹⁸ a polyclonal anti-fibrinogen IgG tailed with fluorescein (The Binding Site, Birmingham, UK), a polyclonal rabbit anti-human CD3, a mouse anti-human CD20 and a mouse anti-human CD68 (Dako, Glostrup, Denmark). Horseradish peroxidase-labelled immunoglobulins (Diacclone, St Louis, Montana, USA) and an anti-digoxigenin alkaline phosphatase-labelled Fab fragment (Roche, Mannheim, Germany)

were used as secondary antibodies. Bovine serum albumin (BSA), trypsin, proteinase K, levanisole and triton X were purchased from Sigma (Besançon, France). The C4BP β probes used for the in situ hybridisation studies were obtained from the complete coding region of the C4BP β cDNA subcloned into the pBS plasmid—after *Hind* III restriction and transcription with T3 polymerase (anti-sense fragment of 866 bp) or after *Bam*HI restriction and T7 transcription (sense C4BP β product of 506 bp)^{9,19}—and digoxigenin-labelled (Roche). Diaminobenzidine was purchased from Dako, and nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate from Roche.

Histochemistry

Synovial membranes were incubated in 1% hydrogen peroxide-methanol and digested with 0.1% trypsin, pH 7.8, at 37°C . Incubation with primary antibodies was carried out overnight, at 4°C , after blocking with BSA. Preimmune sera were used as negative control in each reaction. Secondary antibodies were applied after washing for 30 min at room temperature. Diaminobenzidine was used for colour developing and tissues were counterstained with haematoxylin, before dehydration and mounting in DPX medium.

Figure 1 Representative synovial membranes of patients with rheumatoid arthritis (RA), $\times 100$ (A), osteoarthritis (OA), $\times 100$ (B) and controls, $\times 200$ (C) stained with haematoxylin and eosin (HE). (D) Synovitis scores of the different groups (mean (standard error of mean)), $*p < 0.02$. (E) Fibrin scoring at the synovial tissues (0–4-point scale), $**p < 0.005$. (F) A representative RA synovial tissue after incubation with the specific fluorescein isothiocyanate-tailed anti-fibrinogen antibody, original magnification $\times 100$ (left) and the same tissue stained with HE (right). (G) Correlation between synovitis and fibrin scores. $\rho = 0.804$; $p < 0.01$.

In situ hybridisation of the C4BP β gene product

Hybridisation was carried out as previously reported.²⁰ Briefly, tissues were fixed in glutaraldehyde-paraformaldehyde, incubated in 50 mM levamisole, permeabilised with 0.1% hydrochloric acid pH 1.5, and with 10 μ g/ml proteinase K for 20 min and fixed again in 4% paraformaldehyde. Pre-hybridisation (65°C, 2 h) was followed by incubation with the anti-sense probe (40 ng/ml) overnight at 50°C. For control, incubation with the sense C4BP β probe (40 ng/ml) was carried out in parallel. After washing, the anti-digoxigenin antibody labelled with alkaline phosphatase was added to the tissues (1:2000) and colour was developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Immunoblotting in synovial fluids

To detect C4BP isoforms, electrophoresis was carried out in 5% polyacrylamide gels in the presence of sodium dodecyl sulphate using the buffer system described by Laemmli. Proteins were transferred to nitrocellulose membranes using a Trans-blot electrophoretic transfer cell from Bio-Rad (Richmond, California, USA). After transfer, the membranes were blocked with BSA-Tween 20 and incubated with the polyclonal anti C4BP α antibody, which has previously been shown to recognise C4BP $\alpha\beta$ complexes but not unbound C4BP β polypeptides.¹⁸ Detection of C4BP β was carried out using 12% polyacrylamide gels and incubating the transferred membranes with the anti-C4BP β antibody. All the experiments were carried out under non-reducing conditions, to

prevent separation of the polypeptides from the C4BP isoforms. After incubation with secondary horseradish peroxidase-labelled antibodies, the enhanced chemiluminescent method (Amersham, Little Chalfont, UK) was used for development.

Determination of protein S levels in the synovial fluids and plasma

Determination of total and free protein S antigen was carried out by a quantitative enzyme immunoassay (Grifols, Barcelona, Spain). To measure free protein S levels, the test was conducted on polyethylene glycol-treated samples, to precipitate protein S–C4BP complexes. Plates were developed with a chromogenic substrate and read at 450 nm. Results were calculated with a standard curve developed with dilutions from a reference normal serum and expressed as a percentage. Detection range for both tests was 5–150%. The ratio of free to total protein S with regard to reference samples was calculated for each sample (and was considered normal at above 70%).

Data analysis

Morphological features of the synovial tissues were evaluated by a blinded expert (OS-P) using Krenn's 0–9 points synovitis score.²¹ Deposition of fibrin and immunostaining of C4BP β were separately scored on semiquantitative scales. These data and protein S ratios were analysed with non-parametric Mann–Whitney U test and Spearman's test. Data were expressed as mean (SEM) and level of significance was set

Figure 2 Localisation of complement C4b-binding protein (C4BP) chains by immune-peroxidase techniques at the synovial tissues (positive staining shown in brown (A–D)) C4BP β , (E–G) C4BP α . (A, B, E, F) A representative case of rheumatoid arthritis (RA). (C, G) A case of osteoarthritis (OA). (D) Controls. (H) Negative control of the C4BP β staining shown in (B). Original magnification $\times 100$ (A, E); $\times 200$ (B, C, D, F, G, H). (I) Scoring of C4BP β immune-reactivity. (J) Correlation between C4BP β and fibrin immune-reactivities. $\rho = 0.80$; $p < 0.01$. (K) Correlation between C4BP β and synovitis scores. $\rho = 0.89$; $p < 0.01$. HE, haematoxylin and eosin.

at $p < 0.05$. The analysis was carried out with SPSS V.8.0 software.

RESULTS

Histopathology and synovial fibrin deposition

Synovial specimens from patients with rheumatoid arthritis were characterised for prominent subintimal inflammatory infiltrates, granulation tissue and fibrotic hypertrophic changes, whereas intimal hyperplasia was not different from that of membrane samples from patients with osteoarthritis (fig 1A,B). One of the three membrane samples from healthy controls showed some inflammatory changes and moderate lining hyperplasia, which was attributable to trauma (fig 1C). Synovitis scores were 5.4 (0.3) in the rheumatoid arthritis group, 3.3 (0.6) in the osteoarthritis group, ($p < 0.025$) and 1.0 (1.0) in the control group (fig 1D). Interstitial fibrin immune reactivity scored on a five-point scale was markedly different between groups: 2.7 (0.2) in rheumatoid arthritis, 0.8 (0.3) in osteoarthritis and 0 in healthy synovial tissues ($p < 0.005$ between rheumatoid arthritis and osteoarthritis and $p < 0.01$ between rheumatoid arthritis and controls; fig 1E). In fact, rheumatoid tissues showed fibrin-rich areas alternating with others of low-fibrin signal at the interstitium (fig 1F,G), whereas synovial tissues from patients with osteoarthritis did not show this alternating pattern and only occasional superficial fibrin deposits. The extension of interstitial fibrin deposits was not only a distinguishing feature between osteoarthritis and rheumatoid arthritis but

also correlated with the severity of synovitis ($\rho = 0.804$, $p < 0.01$; fig 1H).

Inflamed synovial membranes express C4BP polypeptides

Membranes from patients with rheumatoid arthritis showed a remarkable expression of C4BP β by immunohistochemistry. The polypeptide was located at the cytoplasm of the lining and in most subintimal fibroblast-like cells throughout the tissues (fig 2A,B; negative control in H). Specimens from patients with osteoarthritis had a much lower signal for C4BP β at the lining than rheumatoid arthritis tissues and large areas of synovium did not show C4BP β immune reactivity. The difference between osteoarthritis and rheumatoid arthritis was more evident at the subintimal area, where tissues from patients with osteoarthritis showed scant positive cells (fig 2C). In turn, one of the three specimens from healthy donors showed some C4BP β immune-reactivity, although to a much lesser extent than the synovial specimens from patients with rheumatoid arthritis or osteoarthritis (fig 2D). By contrast, there was no intracellular binding of anti-C4BP α at the synovial tissues, where it was only found in patches at interstitial areas, particularly in rheumatoid tissues (fig 2E–G). The semiquantitative scoring of C4BP β was 3.6 (0.3) for rheumatoid tissues, 1.4 (0.4) for the group with osteoarthritis ($p < 0.005$ v rheumatoid arthritis) and 0.7 (0.7) for controls ($p < 0.005$ v rheumatoid arthritis; fig 2I). The respective semiquantitative scores of fibrin deposition and C4BP β immune reactivity increased in parallel ($\rho: 0.80$;

Figure 3 Characterisation of complement C4b-binding protein (C4BP) distribution in synovial tissues of patients with rheumatoid arthritis by serial immune-peroxidase techniques (positive shown in brown–dark brown). (A–D) Serial cuts of the same specimen, $\times 200$, stained with haematoxylin and eosin (HE), anti-C4BP α , anti-fibrinogen fluorescein isothiocyanate and C4BP β , respectively. (E) Anti-C4BP β in comparison with (F) anti-CD68 binding in serial cuts, $\times 100$; (G, H) detail of the binding pattern of C4BP β and CD68, respectively, $\times 400$; (I–K) a large mononuclear aggregate showing the different patterns of immune reactivity between C4BP β (I), CD20 (J) and CD3 (K), $\times 200$. An $\times 400$ magnification of the bipolar cells expressing C4BP β is also shown in the inset (I). Insets in (J, K) are negative controls of each procedure.

Figure 4 Complement C4b-binding protein β (C4BP β) mRNA expression by in situ hybridisation in the synovial membranes (positive signal shown in dark blue). (A) Rheumatoid arthritis, $\times 200$. (B) Osteoarthritis, $\times 200$. (C) Control, $\times 200$. (D) Detail of C4BP β mRNA expression by interstitial fibroblasts in an RA specimen, $\times 400$ and (E) its negative control incubated with the sense probe.

$p < 0.01$; fig 2J). Similarly, a positive correlation was observed between C4BP β immune reactivity and synovitis score (ρ : 0.89; $p < 0.01$; fig 2K).

Distribution of C4BP in the synovial membranes of patients with rheumatoid arthritis

Serial tissue sections were used to compare the binding of the anti-C4BP α and C4BP β antibodies with different markers to characterise the distribution of C4BP polypeptides at the inflamed synovial tissues. As mentioned, C4BP α was not found inside cells, but interstitial areas showed immune reactivity to anti-C4BP α at the subintimal region of many tissues from patients with rheumatoid arthritis. As shown in fig 3A–D, the extracellular reactivity for both C4BP chains had a similar distribution and was restricted to fibrin-rich areas.

Intracellular binding of anti-C4BP β in the tissues from patients with rheumatoid arthritis was not homogeneous. The maximal reactivity to the polypeptide was found in fibrin-rich areas than in non-fibrinous zones (where lymphatic aggregates were more frequent). Most of the C4BP β -expressing cells had the typical bipolar shape of interstitial fibroblasts. On the other hand, macrophages, detected by CD68, and CD20 or CD3-positive lymphocytes, did not seem to show any anti-C4BP β binding in these serial studies (fig 3E–K).

Local production of C4BP β was further confirmed with in situ hybridisation of the specific anti-sense RNA probe (fig 4). Both intimal and subintimal fibroblast-like cells of rheumatoid tissues showed a strong hybridisation signal. Also to a lesser extent, tissues from patients with osteoarthritis showing severe inflammatory changes had a moderate binding of the probe, mostly at the synovial surface. Some

of the specimens were incubated with the sense probe as control.

Presence of C4BP β polypeptides in the synovial effusions

Investigation of C4BP was carried out in synovial effusions from 13 patients with rheumatoid arthritis and 9 without rheumatoid arthritis. Of these, five had osteoarthritis and four had MCA. By western blotting, all samples tested showed the presence of three isoforms that, according to their size and electrophoretic pattern, were identified as $\alpha_7\beta_1$, $\alpha_7\beta_0$ and $\alpha_6\beta_1$ complexes.¹⁸ We did not find differences between aetiological groups with regard to predominance of C4BP β^+ isoforms (not shown). On the other hand, free C4BP β polypeptides appeared in the synovial effusions from 11 of 13 patients with rheumatoid arthritis; in lesser amounts in synovial effusions from 2 of 5 patients with osteoarthritis

Figure 5 Immunoblot of complement C4b-binding protein β (C4BP β) in the synovial fluid of patients with different joint pathologies. Purified C4BP β (5 ng) was used as control (right-end lane). A major band was found after incubation with the specific antibody at 45 kDa. OA, osteoarthritis; MCA, microcrystalline arthritis; RA, rheumatoid arthritis.

Figure 6 Ratios of free protein S to total protein S in synovial fluids from the different groups. The dotted line represents the threshold below which ratios are considered to be pathologically reduced. Individual bars show the mean ratios for each group of patients. As shown in the graph, both rheumatoid arthritis (RA) and microcrystalline arthritis (MCA) groups showed values below the normal range, with regard to the osteoarthritis (OA) group.

and in fluids from 2 of 4 patients with MCA. Figure 5 shows one of two C4BP β immunoblots.

Total and free protein S were measured in the synovial fluids by enzyme immunoassay. The ratio of free to total protein S was calculated and found to be below normal in 85% synovial fluids from the group with rheumatoid arthritis and in all the synovial fluids from the group with MCA, whereas 1 of 5 synovial fluids from patients with osteoarthritis showed this change. On the whole, rheumatoid arthritis (53 (7)%) and MCA (40 (7)%) ratios were markedly lower than ratios of osteoarthritis (92 (11)%, $p < 0.01$ *v* rheumatoid arthritis and $p < 0.05$ *v* MCA, respectively; fig 6). As control, we determined plasma protein S levels in seven patients with rheumatoid arthritis and found that they were normal in all cases (data not shown).

DISCUSSION

This work describes the expression of C4BP at the synovial tissue. Both C4BP α and C4BP β were found together at the fibrin-rich interstitial areas of the inflamed synovium. However, the interstitial immune reactivity to C4BP polypeptides probably indicated a plasma origin of these molecules, as C4BP α was not found in the cytoplasm of synovial cells. In fact, our data suggest that the inflamed synovial tissue produced C4BP β without C4BP α . The production of C4BP β by activated synovial cells is not totally surprising. Although it was initially thought that the expression of both C4BP genes was restricted to the liver, our group has recently shown production of C4BP β in the ovary. In the same manner as in the synovial tissue, ovarian fibroblast-like cells expressed C4BP β in the absence of C4BP α .⁹ This dissociation indicates that both peptides are independent in role, activity and regulation. The affinity between C4BP α and C4BP β may be critical for the presence of the whole molecule at inflammatory sites, where both chains could collaborate in the resolution of the process. In this sense, C4BP α helps to prevent excessive complement-mediated damage, whereas inactivation of the protein C anti-coagulatory pathway by C4BP β coupling of protein S possibly contributes to restore tissue integrity by the formation of haemostatic plugs. But

our data suggest that C4BP β can have an additional and perhaps more prominent role in reparative processes carried out by the resident fibroblasts.

Although C4BP β was not detected at the healthy synovial tissue, its expression seemed inducible in the case of acute or chronic injuries, and prominently up regulated in rheumatoid arthritis. Thus, in one of our controls, diagnosed with a trauma-associated internal derangement, the intimal layer showed some C4BP β immune reactivity and the peptide was also commonly expressed at the surface of synovial tissues of patients with osteoarthritis. On the other hand, a widespread expression of C4BP β by intimal and subintimal fibroblasts was found in tissues with severe synovitis, mostly rheumatoid cases. In the ovary, C4BP β expression was not constitutive either, but restricted to cells associated with a process of invasion and absorption of the degenerating corpus luteum.⁹ In some way, fibroblasts in both processes—synovitis and corpus albicans formation—share a common phenotype in that they become invasive and migrate in a fibrin-rich environment. The difference between both situations is that absorption of the corpus luteum by omen cells is a physiological process, key for tissue homeostasis, whereas in synovitis deposition of fibrin absorption may act as a stimulus for perpetuating fibroblast invasion and as a physical scaffold for synovial overgrowth.¹²

C4BP β -positive cells were more abundant in the vicinity of fibrin exudates and we also found a correlation between both immune reactivity scores. This was expected, as both molecules clearly seemed to be markers of inflammation severity. On the other hand, C4BP β may act as a pro-fibrinogenetic molecule inside the joint,⁵ as the protein C anticoagulatory pathway prevents fibrin formation and also participates in its physiological removal.^{8, 22} In a closed environment such as the synovial cavity, this type of action would help to sustain inflammation.^{10–16, 23–27} But to intercept the protein C-dependent system, both local production and secretion of C4BP β to the inflamed milieu would be required.⁵

Our work did not directly show secretion of C4BP β by synovial fibroblasts, although our data suggested the possibility. One finding was that of free C4BP β polypeptides at many synovial effusions, which was notorious in patients with rheumatoid arthritis, because it is extremely unusual to find the molecule not bound in α - β complexes in peripheral blood. Additionally, C4BP β is the principal regulator of free protein S levels due to the high affinity between both molecules. In this sense, a reduced ratio of free to total protein S, as we found in most synovial fluids from the groups with rheumatoid arthritis and MCA groups, reflects an excess of C4BP β locally.⁶

In summary, our observations suggest that besides its role in the regulation of haemostasis, C4BP β is associated with processes of fibroblast migration and invasion of fibrin-rich substrates such as synovitis. However, neither the changes found at the synovial effusions nor the up regulation of C4BP β at the synovial tissue were specific to rheumatoid arthritis, but were probably related to the magnitude of the inflammatory reaction.

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