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The expression of CD44 in archival paraffin embedded interface tissues of failed orthopaedic implants

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CD44 hyaluronan receptor is present on large number of different cell type. It acts as one of the adhesion proteins, binding to hyaluronan and is known to play a part in cell migration from vessels in inflammation. The aim of this study was to examine the presence and distribution of CD44 in interface membrane in aseptic loosening. Immunohistochemistry (IHC) using human anti-mouse CD44 antibody studied 20 aseptically loosened interface samples. Extracted protein from all cases was examined by Western blot and RT–PCR. CD44 was detected in 85% of interfaces by IHC and the presence of protein confirmed by blotting and RT–PCR, which showed the mRNA level for CD44. CD44 was expressed by macrophage, multinucleated giant cells, mast cells and lymphocytes. Further studies are needed to characterise the role of this molecule in the inflammatory response to wear debris in aseptic loosening.

1. Introduction

Aseptic loosening, with or without florid osteolysis on radiological examination, is the most common cause of failure of cemented and uncemented endoprostheses in total joint replacement. Loosening occurs as a result of differences in materials properties between the implant and bone, micro-movement and the presence of wear debris with an accompanying inflammatory infiltrate. The reaction to wear debris is the single most important factor, particles being derived from the load-bearing articulating surfaces of the artificial joint [1]. A fibrous layer is present between implant and bone with a cellular infiltrate comprising macrophages and the closely related foreign body giant cells CFBGCs (multinucleate giant cells (MNGCs), as well as lymphocytes and mast cells [2]. These are the typical component cells of a chronic inflammatory infiltrate and produce chemical mediators (cytokines and enzymes) which perpetuate the inflammation and give rise to extracellular matrix destruction, including that of bone [1, 2]. Recent studies implicate the lymphocytes, shown to be T cell in type, in an immunologically mediated process [3–5]. These cells are all originally derived from bone marrow and migrate from the blood into the local site of inflammation. The migration is mediated by cell to cell and cell to matrix contacts through molecules expressed on the surface of different cells. Molecules known as the selectins are responsible for initial contact between circulating and endothelial cells after which the cellular adhesion molecules play an important role in the migration. There is a further molecule, CD44, known as the hyaluronan receptor, which is involved in inflammatory reactions but it has previously not been studied in details in the context of the reaction to wear particles and osteolysis.

The aim of this study is to characterise the location and extent of CD44 expression on cells in the interface membrane of cases where aseptic loosening of the implant was the cause of revision surgery.

2. Materials and methods

2.1. Subjects

Paraffin-embedded sections were prepared from archival interface tissues from 20 cases of aseptically loosened total joint replacements the Royal Free Hospital from 1989 to 2002 requiring revision surgery. There were 11 males and nine females with ages ranging from 50 to 78 years (mean age of 63.9 years). Clinical details are shown in Table I. There were 11 hips and nine knees and all but one TJR were metal and polyethylene implants. Inclusion criteria were based on the clinical details at the time of revision, namely, that original surgery was for degenerative joint disease, there was a five-year minimum duration of implantation, and radiographic indications of inflammation-related erosion were present at the site of the implant.

Two control samples of uninflamed synovium were provided by synovial samples from a fractured neck of femur and an osteoarthritic hip, respectively, while two

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TABLE I Clinical details of cases studied

Case no.	Lab ID no.	Age at revision	Sex	Duration of implant (yrs)	No. of revisions	Affected area	Types of implant
1	172/1989	53	M	6	1	Hip	CoCr
2	26/1990	60	F	6	1	Knee	CoCr
3	138/1992	60	F	10	2	Hip	SS
4	41/1993	78	F	5.5	1	Knee	CoCr
5	198/1995	50	M	5	3	Hip	Ti
6	26/1996	73	M	7	1	Hip	Ceramic HA
7	79/1996	61	F	12	1	Hip	CoCr
8	68/1997	74	F	8	1	Hip	CoCr
9	157/1997	78	F	9	1	Hip	CoCr
10	347/1997	59	M	7	1	Knee	CoCr
11	210/1998	66	M	5	1	Hip	TI
12	466/1999	50	M	5	1	Knee	CoCr
13	25/2000	62	F	6.5	1	Knee	CoCr
14	36/2000	64	M	6	1	Hip	Ti
15	37/2000	75	F	5	2	Hip	Ti
16	177/2000	63	M	5	1	Knee	CoCr
17	45/2001	59	M	7	1	Knee	CoCr
18	165/2001	71	M	6	1	Knee	CoCr
19	172/2001	70	F	6.5	1	Hip	Ti
20	25/2002	52	M	5	1	Knee	CoCr

samples of rheumatoid (RA) synovium were used as further comparators in which there was chronic inflammation.

Serial sections 5 μm thick were taken from each case, placed on Apes coated slides and one section stained using haematoxylin and eosin stain (H&E). These slides were then examined for the histological appearances and the presence of wear debris. The other sections were used for immunohistochemistry (IHC), Western blotting and RT–PCR studies.

2.2. Immunohistochemistry

Sections were immunostained with human monoclonal anti-CD68 (Clone KP-1) and anti-CD44 antibodies (Dako) using the biotin streptavidin alkaline phosphatase technique. The deparaffinised sections for CD68 were digested with 0.1% trypsin for 15 min and sections for CD44 were subjected to citrate buffer treatment for 2 min, followed by rinsing in Tris-buffered saline (TBS) (0.1 M Tris, 0.5 M sodium chloride, pH 7.3) and blocking with 2% bovine serum albumin (BSA) (Sigma) for 1 h. After blocking, primary antibodies were applied at the appropriate optimised concentrations (determined by serial dilutions) namely 1 : 100 for CD68 or 1 : 40 for CD44, then incubated overnight at 4 °C. Subsequently, sections were washed in TBS and secondary biotin-labelled rabbit anti-mouse IgG antibody was added. After three washes in TBS the presence of the biotin label on the secondary antibodies was detected with DAKO streptavidin alkaline phosphatase and visualised with the naphthol AS-MX phosphate/fast red TR salt capture method. Stained sections were counterstained with Harris's haematoxylin and mounted using DPX.

2.3. Western blotting

A modified SDS-PAGE and Western blotting study was performed, using deparaffinised sections. Tissues were incubated in citrate buffer pH 6.0 and lysed in 500 μL SDS (20%), 200 μL Tris pH 6.8 (800 mM), 20 μL (PMSF 100 mM) (Sigma). Cellular debris was removed by centrifugation and protein concentration determined by the BSA protein assay.

2.4. Enzymatic treatment

Cases were treated with 5% B-mercapthanol and 1% bromophenol blue at 70 °C for 10 min. Following this 20 μg of extract was subjected to SDS-PAGE on a 10% agarose gel.

After separation on SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane. The membrane was incubated in casein solution (blocking solution) (Vector) for 30 min at room temperature, followed by incubation with anti-CD44 antibody diluted 1 : 1000. Tris buffer was used for washing, followed by the addition of biotinylated antibody for 30 min, then streptavidin/alkaline phosphatase. The antigen on the membrane was then detected using BCIP/NBT chromogenic substrate (Vector).

2.5. Reverse transcriptase–polymerase chain reaction

Total cellular RNA was extracted from paraffin sections with Proteinase K (Life Technologies) using standard procedures. Two micrograms of total RNA was reverse transcribed into cDNA. After incubation at 65 °C for 10 min and 37 °C for 60 min, the resulting cDNA was amplified using specific primers for CD44 with beta-actin primers used as a control.

The sense and anti-sense primers had the following sequences: CD44 (forward primer: 5′-AGACATCTACCCCAGCAAC-3′, reverse primer: 5′-CGTTGAGTCCACTTG-G TTTC-3′); beta-actin, (forward primer 5′-CCT AAG GCC AAC CGT GAA AAG-3′ and reverse primer 5′-TCT TCA TGG TGC TAG GAG CCA-3′).

Polymerase chain reaction amplification was per-

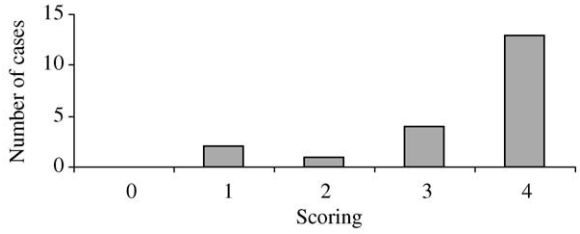

Figure 1 Intensity of CD44.

formed using 2 µg of cDNA with a Perkin-Elmer 480 thermal cycler. The resulting mixture was subjected to the following amplification profile: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min for duration of 32 cycles. The PCR products along with hyperladder 1, molecular weight marker (Bioline) was then separated on a 0.4% agarose gel, stained with ethidium bromide, and visualised using UV transillumination.

2.6. Semi-quantitative assessment and statistical analysis

For statistical analysis, the intensity of staining was scored as the average value: no staining (0), very weak staining (1 points), weak staining (2 points), moderate staining (3 points) and strong staining (4 points) (Fig. 1). The distribution of the staining was assessed using a low-light charge-screen coupled 12-bit PC digital image camera mounted on a Leitz (Germany) light microscope. Distribution scores were recorded as the percentage area immunostained: no staining (0), staining of less than 20% of the area (1 point), staining of 20–40% of the area (2 points), staining of 40–60% of the area (3 points), staining of more than 60% of the area (4 points) (Fig. 2) The students unpaired *t* test was used to analyse the differences in both intensity and distribution between interface cases and controls.

2.7. Quantitative analysis

From RT–PCR done the band intensity of ethidium bromide fluorescence was measured using a BioRad GS-700 graphic densitometer, quantity 1 version 4.21, the intensity of the bands in each lane were quantitatively determined by pixel density (Fig. 3).

3. Results

On examination, the interfaces revealed the presence of large numbers of macrophages and variable numbers of

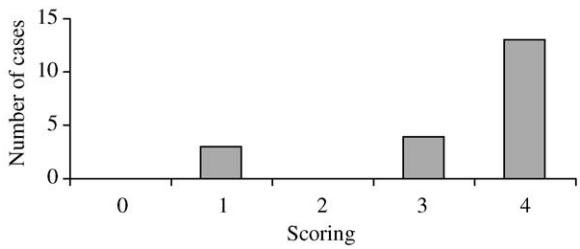

Figure 2 Distribution of CD44 staining.

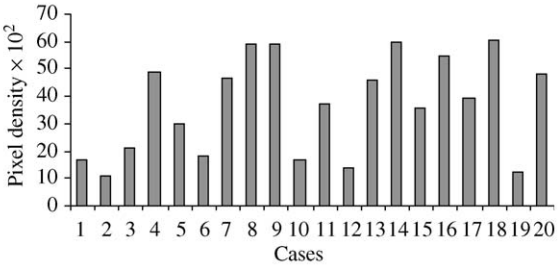

Figure 3 Quantitative analysis of RT–PCR.

MNGCs as shown using the anti-CD68 immunostain (Fig. 4(a)). The greatest concentrations of macrophages were seen at the implant side of the interface and around the blood vessels. Some of the CD68 positive cells contained intracellular metallic wear debris (Fig. 4(b)).

Moderate to strong positivity for CD44 activity was present in 85% of cases, where intense positivity was observed in the synovium-like lining and the deeper interface tissues (Fig. 4(c) and (d)). The cell types that showed CD44 activity were macrophages, perivascular mast cells and lymphocytes.

In the control non-inflamed and RA syovia, the pattern of distribution was similar to the interface membrane. However, the staining of OA sample was much weaker in intensity and less extensive in distribution compared to the RA samples and the interface tissues.

Positive Western blotting results were obtained from 14 out of the 20 interface samples. They all demonstrate a band correlating to the molecular weight marker 85 kDa (Fig. 5). Western blot indicated higher levels of CD44 in cases with a heavy wear debris distribution. In comparison the CD44 expression in the controls was present but in smaller amounts and less extensive in distribution.

RT–PCR was used to demonstrate the presence of the mRNA for CD44 (Fig. 6). This was confirmed in 12 out of 20 interfaces. There was a correlation between CD44 positive cases by Western blotting, RT–PCR analysis for the CD44 message and those that interfaces, which had moderate to strong expression of CD44 using IHC.

4. Discussion

The demonstration of CD44 on the macrophages and MNGCS of the interface membrane in aseptic loosening provides further information regarding the cellular mechanisms involved in the inflammatory process. Failure to show CD44 in three out of 20 samples does not necessarily mean that this was absent, since there are limitations to the use of archival material, including the duration of formaldehyde fixation, the actual composition of this fixative, and duration and conditions of storage. This said there was reproducible labelling of cells in the interface of the vast majority of cases. The intensity of staining and numbers of cells labelled has also been evaluated in this study. This again may vary with fixation, embedding and storage conditions. It is, however, interesting to observe that the findings using IHC and those obtained with Western blotting and RT–PCR are parallel. The same cases showed abundant CD44 expression in each of these methods. The number

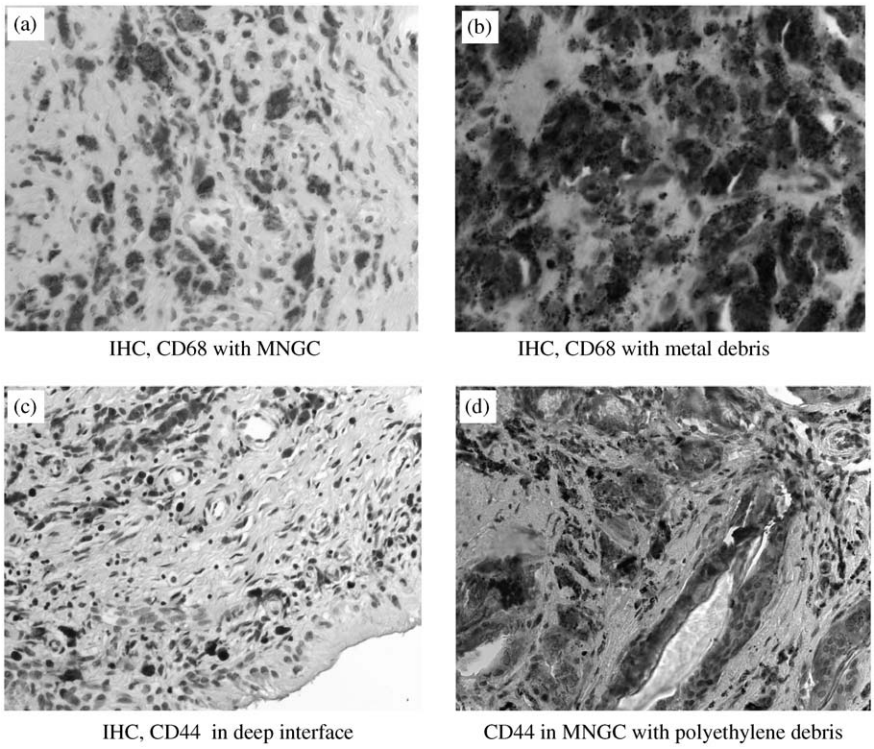

Figure 4 (a) IHC, CD68 with MNGC. (b) IHC, CD68 with metal debris. (c) IHC, CD44 in deep interface. (d) CD44 in MNGC with polyethylene debris.

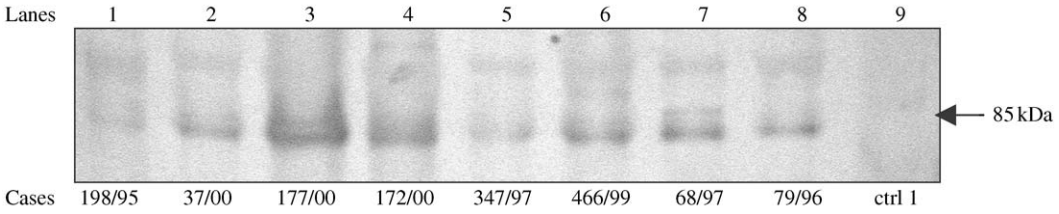

Figure 5 Western blot of different cases and the expression of CD44 along with control.

of CD44 expressing cells was also related to the amount of particulate debris present, as assessed semi-quantitatively/qualitatively. The type of debris present varied both metal and polyethylene being abundant in different cases. It is recognised that the particles seen by light microscopy represent only a small percentage of the total debris load, since over 95% of wear debris in tissues is below 1 μm in size [9–11]. Nevertheless, the number of particles seen by light microscopy may be a reflection of the total number of particles, albeit they are invisible without ultra-structural methods [9]. Thus, with this caveat, the present work has shown that the upregulation of CD44 is related to debris content using IHC and Western blotting. One other general observation is that CD44 expression was much stronger in interface tissues than in control osteoarthritic synovium, an observation in confirmation of the only other study of this adhesion molecule in interface tissues [12].

The distribution of CD44 in the inflamed interface is of interest as it relates well to other observations. First, there is localisation to the synovium-like layer found adjacent to the implant. This layer has a structural organisation like that of the synovium, comprising a deep layer of fibroblast-like cells each surrounded individually by type IV collagen, laminin and other matrix proteins including hyaluronan [13–15]. Since hyaluronan is the chief counter-ligand for CD44, this distribution may be expected. However, other CD44 ligands include fibronectin [16, 17], which may be through a heparin-binding fragment of fibronectin [17], as well as chondroitin sulphate [18]. Both of these are also localised to the synovium-like layer at the implant–tissue interface [14, 15]. It has been suggested that the hyaluronan synthase/hyaluronan/hyaluronan receptor (CD44) signalling system is actually responsible for the induction of the synovium-like lining at the implant interface [12].

It is not known whether the hyaluronan in the lining cell layer of the implant interface is in the fragmented (low molecular weight) form or is high in molecular

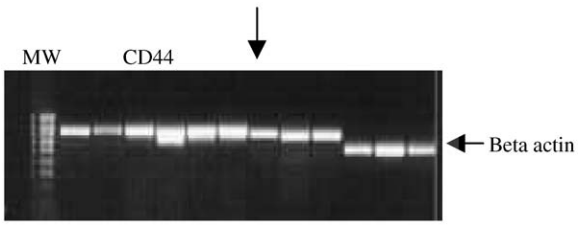

Figure 6 CD44 mRNA expression in interface tissues. RT–PCR products were visualised by ethidium bromide staining. The first nine (9) bands represent CD44 mRNA and the later three (3), bands represent beta actin control.

weight. Low molecular weight hyaluronan is known to be present in sites of inflammation and induces cytokine and chemokine production by macrophages [19]. There thus may be an activation of macrophages by a CD44–hyaluronan mediated mechanism, particularly in the diffuse, underlying macrophage and MNGC infiltrate containing wear debris. It seems likely that CD44, a transmembrane glycoprotein takes part in signal-transduction processes, leading to Fas expression and transcription [20]. CD44 is also implicated in signal transcription processes associated with the actin cytoskeleton [21]. Actin expression has recently been demonstrated as occurring in macrophages activated by ingestion of metal wear particles [22] and it is possible that CD44 plays a part in this process.

There are other cell adhesion molecules involved in macrophage migration and CD44 has been shown to be linked to these. The interaction of CD44 with fragmented hyaluronan induces VCAM-1 and Fas on the synovial cells of the inflamed rheumatoid synovium [23]. VCAM-1 has been demonstrated in the synovium-like layer of the implant–tissue interface [24].

Not only is CD44 implicated in cell to matrix interactions, but also with cell to cell attachments, both in transmigration through the vessel wall and in the formation of MNGCs. Thus, the important molecules in rolling and attachment of cells to the endothelium are the selectins. P-selectin has been demonstrated on the endothelial cells of the newly formed vessels in the interface in aseptic loosening [25] while E-selectin is expressed at a similar site in cases where an immune reaction may be occurring with T cell migration [26]. It is possible that CD44 plays some role in common with these selectins in the attachment process. While not directly related, L-selectin is known to have chrondroitin sulphate proteoglycan as a common ligand with CD44 [18]. That ICAM-1 is present in the interface membrane has also been demonstrated [26]. The expression of this molecule is important not only in cell migration but also in homotypic adhesion, mediated by ICAM-1/CR3 linkage to give aggregation and fusion of cells leading to MNGC formation [26]. TNFα mediates the fusion of macrophages from macrophages and T helper 1 cells, interferon γ from T helper 2 cells and IL4 from T helper 2 cells and mast cells, all of which have been demonstrated in the interface membrane, as well as the particular cytokines characteristic of the T helper subtypes [28–30]. It seems entirely likely that CD44 is among the factors which not only induces inflammatory cytokine production but is also in turn induced itself by the presence of these cytokines. Proinflammatory cytokines (IL1, TNF and GM-CSF) are known to induce peripheral blood monocytes to bind hyaluronan with CD44 [6].

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