Expression profile of T cell associated molecules in the interfacial tissue of aseptically loosened prosthetic joints

BETH HERCUS*, SUHUR SAEED, PETER A. REVELL
Department of Histopathology and IRC in Biomedical Materials, Royal Free Campus, Royal Free and University College, School of Medicine, Rowland Hill Street, London NW3 2PF, UK
E-mail: bethhercus@hotmail.com

The involvement of T cells in the progression of inflammation in response to wear debris at the interface of aseptically loosened joints is currently undefined. This cell type has repeatedly been demonstrated to be a common component of the cellular membrane, the interface, which forms between the bone and implant of total joint replacements (TJRs) [1, 2]. Three further insights into the role of this cell type in the interface were investigated here. Immunostaining demonstrated CD4 expression in 80% of the 15 cases tested while CD8 expression was present in 60% of the cases. Polymerase chain reaction (PCR) detected IFN-γ mRNA expression in 75% of eight cases tested; in contrast IL-10 mRNA was only demonstrated in 50% of these same cases. Proteins extracted from another eight cases of revision tissue were analyzed using Western blotting for IL-17, fractalkine (Fkn) and CD40. IL-17 and Fkn were a consistent feature of all cases tested (8/8), while CD40 was undetectable in one case (7/8). These results show that T cells present in the interface are more commonly of the helper T cell phenotype, although cytotoxic T cells are also present. Helper T cells (Th) are responsible for the polarization of the immune response through their production of key mediators. The PCR results obtained in this study suggest that a Th1 response characterized by the production of IFN-γ predominates over the Th2, IL-10 mediated response. Furthermore the demonstration of the expression of IL-17, Fkn and CD40, all of which are Th1 associated molecules, supports this conclusion.
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Introduction

The role of the T cell in aseptic loosening is as yet poorly defined. Several studies have confirmed that the T cell is a common feature of the inflammatory membrane which forms between the implant and the bone [1, 2]. While T cell presence at the interface is now generally accepted, their state of activation in the interface membrane is uncertain. Conflicting results exist, since one group has shown T cell expression of characteristic lymphokines [2], another has demonstrated that T cells in the interfacial tissue fail to express these proteins [3]. T cells have previously been shown to play an important role in the inflammatory joint disease rheumatoid arthritis (RA) [4] and their involvement in allergic contact dermatitis (ACD) to nickel is also well documented [5, 6]. This study aims to expand the current knowledge on the role of the T cell in aseptic loosening, by defining the profile of T cell associated mediators and cell surface molecules (Table I) expressed at the interface. These molecules are both those expressed by T cells and those expressed by other cell types which, influence T cell behavior.

T lymphocytes are detected by their expression of the specific cellular marker, CD3, but they can be further classified due to their differential expression of CD4 and CD8. T cells of the helper phenotype express CD4 while those of a cytotoxic phenotype are characterized by their expression of CD8. The balance between helper T (Th) cells and cytotoxic T (Tc) cells is of interest, as Th cells possess the ability to control the immune response while their cytotoxic counterparts can cause tissue damage and to a certain extent influence the local response.

Th cells can be subdivided further into Th1 and Th2 cells dependent on their lymphokine profiles. The two predominant mediators that distinguish each division and also inhibit the other are IFN-γ (Th1) and IL-10 (Th2). Immune responses associated with Th1 cell control are cell mediated while Th2 associated responses are classically humoral. The polarization of Th cell responses is commonly studied in human diseases.

* Author to whom all correspondence should be addressed.

TABLE I Molecules associated with T cell responses, their activity and cellular expression

Molecule	Cellular expression	Activity
CD4	T helper cells	Control of the immune response
CD8	Cytotoxic T cells	Cytotoxicity
IFN-γ	Th1 cell	Macrophage activation, inhibition of Th2 development
IL-10	Th2 cells	Th2 cell growth and activation factor, inhibition of Th1 development
IL-17	Activated T cells	Pro-inflammatory
Fractalkine	Activated endothelium	T cell chemoattraction and adhesion/migration
CD40	Activated endothelium	T cell adhesion and stimulation

Diseases showing a predominant Th1 response include rheumatoid arthritis and the granulomatous response to persistent antigenic stimulation, while humoral Th2 responses are associated with allergic conditions such as asthma. The inflammation observed at the interface membrane of loosened joints is highly cellular. Cells commonly identified include macrophages, foreign body giant cells and fibroblasts, B lymphocytes, however, are a rare feature. Furthermore the interface has many features in common with the delayed type hypersensitivity granulomatous response including fibrosis, lymphocytic involvement and giant cell formation.

More recently other molecules have been associated with the Th1 responses (IL-17, Fkn & CD40). IL-17 is a newly described cytokine only expressed by activated $CD4^+$ T cells with pro-inflammatory effects on a wide range of cells including endothelial cells and fibroblasts. In these cells it stimulates the production of IL-6, IL-8, G-CSF and PGE_2 [7], in addition IL-17 stimulation of osteoblasts up-regulates the potent osteoclastogenic mediator RANKL [8]. Fractalkine is a relatively newly described chemokine produced by endothelium in response to pro-inflammatory mediators such as IL-1 and TNF-α. This chemokine can be either membrane bound where it is capable of mediating the firm adhesion of T cells, monocytes and NK cells or soluble where it acts as a chemoattractant for the same cell types. It has been shown to be capable of amplifying the polarized Th1 response due to the preferential expression of its receptor CX3CR1 on Th1 cells when compared to Th2 cells [9]. Fkn expression is a common feature of rheumatoid arthritis and psoriasis, but not seen in reactive lymph node hyperplasia and Castleman's disease, showing its preferential expression in Th1 associated disease conditions.

The relationship between T cells and the endothelium in inflammation is becoming increasingly intimate. The initial interaction between these cell types represents an essential step in mediating T cell entry into a site of immune challenge. One particular cellular adhesion molecule CD40 which is up-regulated by IFN-γ mediates T cell adhesion and migration into inflammatory sites through its counter-ligand CD154. Furthermore, this ligation induces the up-regulation of other cellular adhesion molecules (CAMs) and chemokines by endothelial cells, thus promoting inflammation through the enhanced recruitment of leukocytes. It has also recently been shown that CD40 ligation on endothelial cells, as with monocytes, stimulates the production of IL-12 [10]. IL-12 production by endothelial cells in response to CD40 ligation may therefore represent a positive feedback loop for Th1 cell proliferation.

Materials and methods

Sample preparation

Fifteen cases of human interfacial tissue removed at revision surgery were chosen from cases between the years of 1996 and 2000. Age at time of revision surgery ranged from 54 to 87 years, duration of implant from 7 to 15 years and the male to female ratio was 4–6. There were 14 hips and one knee. The original disease was osteoarthritis in all cases. No microorganisms were cultured from any case.

Immunostaining

Frozen sections were mounted on polylysine coated slides and immunostained using the APAAP technique. Primary antibodies used for this technique were anti-human CD8 and CD4 (Novocastra) used at dilutions 1/100 and 1/50, respectively. Sections were counter-stained with haematoxylin prior to mounting in Aquamount (BDH).

Polymerase chain reaction

RNA extraction was achieved using Trizol reagent (GIBCO). RNA was converted to cDNA using reverse transcription (RT) PCR (Bioline). The resulting cDNA was amplified using primers for IFN-γ and IL-10 (Biostat).

Protein extraction

Three to five tissue sections were cut using a cryostat and placed in a 1 ml Eppendorf. To this was added 500 µl of lysis buffer (640 µl dH_2O + 100 µl 800 mM Tris stock solution (4.844 g/50 ml) + 250 µl SDS stock solution (1 g/50 ml) + 10 µl PMSF in 100% isopropanol). The mixture was vortexed and incubated for 1 h at room temperature. After 1 h 5 µl each of glycerol (Sigma) and bromophenol blue (Sigma) were added to each sample. The samples were inverted and heated to 95 °C for 5 min. Samples were centrifuged at 14 000 × g for 1 min, prior to loading. The supernatant was removed and placed in a clean Eppendorf for storage at −80 °C.

Electrophoresis and blotting

Samples were separated using a 10% SDS-PAGE gel run for 30 min at 120 mA and 200 V. After separation the proteins were transferred to the nitrocellulose membrane at 250 mA and 100 V for 1 h. The membrane was blocked using a 1% Marvel solution in TBS-Tween (TBS-T). Proteins were detected using a panel of specific

antibodies for human IL-17 (R&D), fractalkine (Santa Cruz), CD40 (DAKO) and IFN-γ (R&D) used at a dilution of 1/1000 in TBS-T. These were then washed three times for 5 min each in TBS-T. 1 ml per membrane of primary antibody solution was added and incubated at room temperature for 1 h. Membranes were washed as before. The membranes were then incubated for 1 h at room temperature with a secondary biotinylated antibody (Vector) diluted to 1/1000 in TBS-T. Membranes were washed as before. Finally the membranes were incubated for 20 min at room temperature with standard ABC vectastain reagent (Vector). Equal volumes of detection reagent 1 and detection reagent 2 of the ECL Western blotting detection kit (Amersham) were mixed. 1 ml of the mixture was added to the membrane, and exposed for 5 min. Membranes were visualized using an Alpha Imager.

Results

Immunostaining

CD3 expression by cells in the interface confirmed that T cells are a common feature of the periprosthetic inflammation. To further classify these T cells it was necessary to determine whether they expressed CD4 and were therefore Th cells or if they were CD8 positive Tc cells. Immunohistochemistry for these molecules demonstrated that out of the 15 samples stained CD4 expression was detected in 80% of the cases while CD8 expression was only detected in 60%. As with CD3 expression the distribution of both of these cell types was diffuse and present throughout the sections. Neither cell type was found in all cases tested, and in some samples both cell types co-existed but in all cases tested one or other of these two cell types was found.

Polymerase chain reaction

CD4 positive Th cells are responsible for the polarization of the immune response to either a cell-mediated or humoral response. This polarization is mediated through Th cell production of key mediators. Two cytokines in particular are characteristically used to determine which Th cell response predominates. IFN-γ is characteristically associated with Th1 responses, a potent activator of macrophages and inhibitor of the Th2 response. In contrast IL-10 is a potent inhibitor of both macrophage activation and the Th1 response. PCR was used to assess the mRNA expression of these two cytokines in the nucleic acids extracted from revision tissue. The eight cases tested using PCR were selected from the 10 cases immunostained for CD4 and CD8, Table II summarizes these results. IFN-γ was expressed in 75% (6/8) of the cases tested while IL-10 was expressed in 50% (4/8) of the cases. PCR showed there to be three cases where only IFN-γ was detected, one case where only IL-10 was detected, three cases where both were detected and one case where neither were detected. IFN-γ production was also overall a more common feature of cases tested.

TABLE II Summary of immunostaining and PCR results for eight cases tested

Case No.	CD4	CD8	IFN-γ	IL-10
79/96	+	+	+	+
284/97	−	+	+	−
343/97	+	+	+	+
19/98	+	−	−	+
442/99	+	−	−	−
467/99	+	−	+	+
468/99	+	−	+	−
160/00	+	+	+	−

Western blotting

It is important to understand both the conditions under which the T cell is acting and the profile of mediators, which those T cells are producing in the interface. T cells, as with all cell types, are altered in response to the mediators and CAMs with which they come into contact. Protein mixtures extracted from eight cases of interface tissue were shown to be positive for both Fkn (Fig. 1) and IL-17. Seven of these cases were also positive for the endothelial molecule CD40 (Fig. 2), which mediates T cell adhesion and activation.

Discussion

T cells, and in particular the Th cell sub division of this cell type, are responsible for determining the progression of an immune response. This involvement allows the immune system to mount the most appropriate and therefore most effective response to any given antigen. Th cells are characterized by their expression of the CD (cluster of differentiation) 4, while their cytotoxic

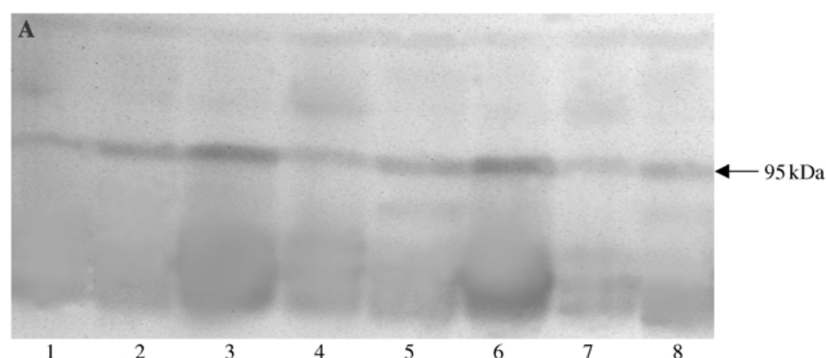

Figure 1 Western blot for fractalkline showing positive bands at 95 kDa for all eight samples examined.

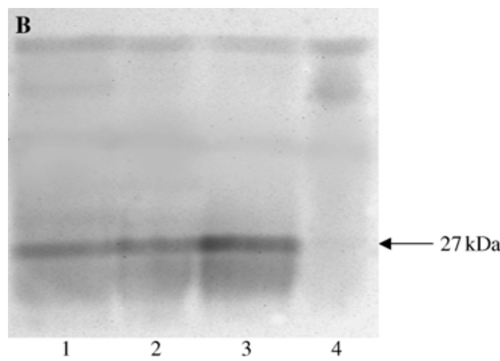

Figure 2 Western blot for CD40 showing positive bands at 27 kDa in three of the four cases examined in one experiment.

counterparts are characterized by their expression of CD8. In this study, immunohistochemistry for these two molecules showed that Th cells were a more common feature of the interface membrane, although Tc cells were still detected in a number of cases. These findings are in keeping with previous studies showing a predominance of Th cells [2]. The two most common situations seen with the PCR results is either a Th1 dominant response, or a co-dominance of both responses. Testing of more cases is required to resolve this situation. Although CD4 positive Th cells are more commonly recognized as cytokine producers, CD8 positive Tc cells are also capable of producing cytokines and in particular IFN-γ. In cases where CD4 positive T cells were absent, IFN-γ production was still detected showing that Tc cells are also involved in the control of inflammation in aseptic loosening. Having determined that T cells in the interface are more commonly of a Th cell phenotype PCR was conducted to determine the polarization of these cells.

In addition to IFN-γ several other mediators and adhesion molecules have been classified as Th1 associated molecules. These include IL-2, Il-12, IL-15, IL-17, Fkn, and CD40. Previous studies by this group have shown IL-15 [11], and IL-2 [2] to be expressed in the interface. In this study, proteins extracted from revision tissue were analyzed for the presence of IL-17, Fkn and CD40. All three of these molecules were a common feature of the protein mixtures extracted from the interface tissue. In combination the results suggest that the Th cell response at the interface is predominantly Th1.

The detection of IL-17 in all of the cases tested, furthermore, shows that T cells in the interface are capable of potentiating the inflammation present and more importantly, through this cytokine, they are capable of inducing RANKL expression by osteoblasts [8]. As a potent stimulator of osteoclastogenesis, RANKL up-regulation by IL-17 may represent an important mechanism by which bone resorption is increased in aseptic loosening. Fractalkine is chemoattractive not only for Th1 cells but also to monocytes and natural killer (NK) cells [9], so expression of this chemokine by the endothelium of the interface promotes leukocyte migration and therefore inflammation. CD40 is also expressed in the interface and the cells likely to be expressing it include macrophages, dendritic cells and the endothelium. This molecule mediates the adhesion of these cells to T cells via CD154 (CD40L). This receptor ligand coupling has been implicated in the amplification of the Th1 polarization circuit as CD40 ligation induces the host cell to produce IL-12 [10]. The results in this study and those of other groups investigating the role of the T cell in the interface are increasingly showing that, with respect to the helper T cell response, a Th1 polarization is occurring [1, 2]. Other conditions where a Th1 polarization occurs such as rheumatoid arthritis and the DTH granulomatous response also occur as a result of persistent antigenic stimulation. In the case of aseptic loosening, particulate wear debris represents that persistent antigen, and as the removal of this material is not possible, other solutions to the problem must be found. It is possible that drugs targeting key points of the immune response may be effective, therefore a further understanding of the response at the interface is required. This study has provided a greater understanding of the behavior of T cells in this response and will provide the basis for future research.

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