Infectious versus non-infectious loosening of implants: activation of T lymphocytes differentiates between the two entities

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Abstract

Purpose Loosening of implants occurs mainly for two reasons: bacterial infection of the implant or “aseptic loosening” presumably due to wear particles derived from the implant. To gain further insight into the pathomechanism, we analysed activation of the T cell response in these patients.

Methods Activation of peripheral T lymphocytes was determined by cytofluorometry as down-regulation of CD28 and up-regulation of CD11b. In addition, tissue samples obtained during surgery were analysed by quantitative RT-PCR for gene expression of CD3, CD14 and cathepsin K, as markers for T cells, monocytes/macrophages or osteoclasts, respectively.

Results Activated T lymphocytes were detected in patients with infection but not in patients with aseptic loosening. Gene expression of CD3 was significantly enhanced in tissues of patients with infection compared to those with aseptic loosening. Expression of CD14 and of cathepsin K did not differ between the two groups.

Conclusion Implant-associated infection and aseptic loosening are associated with a local inflammatory response, which eventually results in osteoclastogenesis and bone resorption. Systemic T cell activation, in contrast, occurs only in patients with implant-associated infection, and hence analysis of T cell

activation markers could serve as a diagnostic tool to differentiate between the two entities.

Keywords Aseptic loosening · Implant-associated osteomyelitis · T cell response · Cathepsin K · CD14

Introduction

Loosening of implants occurs mainly for two reasons: infection around the implant that leads to persistent and progressive inflammation and eventually to bone erosion, and the so-called aseptic loosening, where activation of phagocytic cells by implant-derived wear particles is presumed, but also other mechanisms including low-grade infection are discussed [1–8]. In both conditions, activation of phagocytic cells and production of pro-inflammatory cytokines is described [9, 10]. Eventually, the pro-inflammatory environment promotes osteoclast generation and osteolysis [6, 11].

The participation of cells of the innate immune response in infection and in aseptic loosening is well established, whereas the role of T lymphocytes in implant-loosening is less well defined. Activation of T lymphocytes is invariably associated with virus infection, there is, however, also evidence for an activation of T cells in the course of bacterial infection, notably, in implant-associated infection [12–14]. Activated T cells might provide a link between infection and bone degradation, because the receptor activator of NFκB ligand (RANKL) which is released from activated T cells, has been identified as one of the major protagonists of bone resorption in periodontal disease, rheumatoid arthritis or in tumour- or tumour-metastasis associated bone degradation [15–18].

In aseptic loosening, participation of T cells is still under investigation. T lymphocytes are rather rare among the infiltrated cells, or do not bear the well-established activation markers, which, however, does not rule out a participation of

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these cytokines in aseptic loosening [19, 20]. To gain more information on a possible participation of T cells in implant loosening, we phenotypically analysed peripheral blood T cells in patients. As parameter for T cell activation, expression of surface CD28 and CD11b was studied. CD28, a co-stimulatory receptor of T cells, is down-regulated when T cells are activated (reviewed in [21, 22]); concomitantly, a subpopulation of T cells acquires CD11b, and hence CD11b is taken as a marker for a recent activation [23–25], particularly within the CD8 compartment, and to a lesser extent in the CD4 compartment [26]. CD11b expression is transient, but CD8+CD28- cells may persist, particularly in patients with chronic inflammatory disease or in the elderly (reviewed in [27]).

We now found an increased percentage of CD28 negative T cells and an up-regulation of CD11b, indicative of T cell activation, in patients with bacterial infection and implant loosening, but not in patients with aseptic loosening. In line with this interpretation a higher gene expression of CD3 was seen in tissues of patients with infection.

Material and methods

Patients

Ten patients undergoing revision surgery due to aseptic loosening and 25 patients with infected implants were included in the study. Diagnosis of loosening was based on patient's complaints, clinical examination and by conventional X-ray and/or CT-scan. The patients were considered "aseptic" when no bacteria were detectable by standard diagnostic techniques and when CRP concentrations in blood and the white blood cell count were within the normal range. Because failure to detect bacteria and the lack of systemic indicators of inflammation do not necessarily rule out infection, further criteria for aseptic loosening were the time interval between arthroplasty and beginning of symptoms (median value 8.25 months for infectious cases versus 90 months for aseptic cases) and evidence on the implant of excessive wear (i.e. pronounced wear of polyethylene inlay).

Diagnosis of infected implants was based on clinical evaluation (reddening, swelling, hyperthermia, pain, pus seen intraoperatively, and existence of a sinus tract) and laboratory results (elevated CRP levels and white blood cell count). For comparison, healthy individuals ($n=10$) were included. The study was approved by the local ethic committee, and informed consent was obtained from the patients and the healthy individuals as well.

Collection of tissue and blood samples

During surgery, tissue samples from various sites around the implant were taken in a standardized fashion and conserved

for histological analysis, microbiological examination or placed into RNA later (RNA later, Ambion Cat. # 7021) for quantitative PCR analysis. Before surgery, 5 ml blood was drawn into heparinized tubes (Sarstedt, Nümbrecht Germany), and cytofluorometry was performed within five hours.

Cytofluorometry

The following mouse monoclonal antibodies were used: IgG (mouse)-FITC (Beckman Coulter, Krefeld, Germany), isotype control IgG1-kappa-PE, IgG PerCP, anti-CD4-PerCP, anti-CD8-PerCP, anti-CD28-FITC, and anti-CD11b-PE, all purchased from BD Biosciences, Heidelberg, Germany. Of whole heparinised blood, 100 µl were incubated with the respective antibodies (5 µl) for 30 minutes at room temperature in the dark. Then erythrocytes were lysed using FACS Lysing Solution (Becton and Dickinson, Heidelberg, Germany). Fluorescence was measured by FACScalibur using CellQuest as software (Becton and Dickinson). The gate was set for lymphocytes; 10,000 events within the gate were counted. Results are given as percent positive cells of all lymphocytes. To control for non-specific binding, isotype controls were used, and the markers were set accordingly (shown as bars in the images).

Gene expression analysis

RT-PCR of tissue samples were collected and stored in RNAlater (Ambion), disrupted with a RiboLyser device (ThermoHYBAID, Heidelberg) in lysing matrix „D“ tubes (Q-BIOgen, Heidelberg) containing 400 µl lysis buffer from the MagnaPure mRNA Isolation Kit I containing 1%DTT (v/w) (ROCHE Diagnostics, Mannheim). Lysates were centrifuged (13000 rpm for five minutes) transferred to MagnaPure sample cartridges and mRNA was isolated with the MagnaPure-LC device using the standard protocol for cells. mRNA was reversely transcribed using AMV-RT and oligo-(dT) as primer (First Strand cDNA synthesis kit, Roche) according to the manufactures protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 µl and stored at -20°C until PCR analysis. Primer sets optimized for the LightCycler® (RAS, Mannheim Germany) were purchased from SEARCH-LC GmbH (www.Search-LC.com). The PCR was performed with the LightCycler® FastStart DNA Sybr GreenI kit (RAS) according to the protocol provided in the parameter specific kits. To control for specificity of the amplification products, a melting curve analysis was performed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR-cycle number (CP) at which the detected fluorescence intensity reaches a fixed value.

Tissue and immunohistochemistry

Biopsies were formalin fixed, decalcified in ethylenediaminetetraacetic acid (EDTA), paraffin embedded and stained with hematoxylin/eosin. Cathepsin K was visualized immuno-histochemically as previously described [11]. The antibody to cathepsin K was purchased from Calbiochem (San Diego, USA).

Statistical evaluation

Comparison between patient groups was made by descriptive analysis, calculating mean values and standard deviation, as well as the median. Data are displayed as box-and-whiskers blot, showing the mean ($\square$) and the median value (horizontal bar) and the highest and lowest values, with the box containing 50 % of the values. Differences between groups (either tissue or patients) were calculated by Friedman test, followed by Mann–Whitney test using Origin 9.0 as software.

Results

Expression of CD28 and CD11b on cells of patients with implant-associated osteomyelitis or aseptic loosening

By cytofluorometry, surface expression of CD28 and CD11b was determined on CD4+ and CD8+ peripheral blood lymphocytes. As exemplified in Fig. 1 for one patient with implant-associated osteomyelitis and one patient with aseptic loosening, CD4+ and CD8+ cells were found which were either positive for CD28 (CD28+) or did not express CD28 (CD28-). Also CD4+ and CD8+ cells were found, that either expressed CD11b (CD11b+) or did not (CD11b-). CD11b was predominantly expressed by cells lacking CD28, but there were small populations of CD28+CD11b+ cells. CD8+ and CD4+ cells showed essentially similar expression patterns, the percentage of CD28- and CD11b+ was, however, considerably lower on CD4+ cells compared to CD8+.

A quantitative analysis revealed that the percentage of CD28 negative cells and of CD11b positive cells was significantly higher in patients with implant infection than in patients with aseptic loosening. For the latter, it was within the same range as for healthy individuals (Fig. 2). CD4+CD28+CD11b+ and CD8+CD28+CD11b+ cells were only found in patients with infection.

T lymphocytes and T-cell derived cytokines in tissue of patients with infection and patients with aseptic loosening

In both aseptic loosening and implant-associated infection, infiltration into affected sites by myeloid cells is seen by histology. An example of a patient with infection is shown

in Fig. 3 where a mixed inflammatory infiltrate consisting of neutrophils, macrophages and mononuclear cells including T cells is seen, as is necrotic tissue, degraded bone and osteoclasts, forming resorption lacunae.

To quantify infiltration of T cells and monocytes/macrophages, respectively, tissue was recovered during surgery from three sites at or in the vicinity of the osteolytic lesion. By quantitative PCR, gene expression of CD3 as a marker for T cells, and expression of CD14 as a marker for monocytes was determined, as was expression of cathepsin K, which is characteristic for osteoclasts. In patients with infection, the number of transcripts for CD3 was significantly higher compared to expression in patients with aseptic loosening of the implant, whereas gene expression of CD14 and of cathepsin K did not differ between the groups (data summarised in Fig. 4).

Discussion

Activation of T lymphocytes is typically antigen-driven and results in expansion of antigen-specific cell clones. Accordingly, only low numbers of T cells are activated in dependence—among others—on the number of distinct antigenic epitopes that are recognised by the individual. Activation and expansion of T cell clones are transient and associated with up-regulation of cytokines and receptors, the latter rather useful for phenotypic analyses. As described in the introduction, we chose down-modulation of CD28 and up-regulation of CD11b as markers for activation, because according to data in the literature and also to our previous findings these receptors are easy to trace markers for T cell activation in patients with viral or bacterial infection [12, 14, 22, 23].

In patients with implant-associated osteomyelitis higher percentages of CD28- and CD11b+ T cells, and hence activated T cells, were detected compared to patients with aseptic loosening. In the latter, the percentage of T cells with activation-associated receptor pattern was within the range seen for healthy donors where activated CD4 cells were scarce or not detectable. Because CD8+CD28- were also present in patients with aseptic loosening—representing most likely senescent T cells which persist to some extent also in healthy individuals [27–29]—particularly activation of CD4+ T cells is a suitable parameter to distinguish between loosening due to infection and aseptic loosening, at least in the patients with either metal-on-polyethylene or ceramic-on-polyethylene implants. The situation might be different in patients with metal-on-metal implants, where there is evidence for systemic T cell activation, which is most likely due to metal ions, and not to metal wear particles [30].

Analysis of the biopsies confirmed this notion; thus, using CD3 as marker for infiltrated T cells, significantly lower CD3 gene expression was noted in tissue of patients with aseptic

Fig. 1 Characterisation of T lymphocytes by cytofluorometry. In whole blood, T cells of the helper type (CD4; left panel) or cytotoxic T cells (CD8; right panel) were tested for expression of CD28 or CD11b, respectively. In the upper panel, data of a patient with implant-associated infection is shown; in the lower panel data of a patient with aseptic loosening. The data are presented as dot blots, and for CD4 and CD8 cells expressing CD28+ (upper right quadrant) and cells lacking CD28

(upper left quadrant) were seen, as were cells expressing CD11b (upper left) or those which did not (upper right). The percentage of cells lacking CD28 is given, as well as the percentage of CD11b+ cells in the respective quadrant. To determine the coincidence of CD28 and CD11b expression is shown on the respective histograms (The gates were set according to the isotype controls, as were the markers (M1, M2) in the histograms)

Fig. 2 Expression of CD28 and CD11b on CD4+ and CD8+ T lymphocytes of patients and healthy donors. CD28 and CD11b were determined as shown in Fig. 1 and the percentage of CD4+ cells lacking CD28 or expressing CD11b is shown, as well as of CD8+CD28- and CD8+CD11b+. Data are summarised for patients with implant-associated infections ($n=25$), patients with aseptic loosening ($n=10$), and healthy donors ($n=10$). The data are shown as box-and-whiskers blot with the box containing 50 % of the values. The differences between the groups were calculated by Mann–Whitney test. The p values refer to the comparison between septic and aseptic patients. There was no difference between aseptic patients and healthy donors

loosening compared to patients with infections. The latter is in line with previous data, showing infiltration into the infected site of T cells, particularly of CD8+ and the fact that only activated T cells can infiltrate tissue [12]. Gene expression of CD14, a marker for monocytes, did not differ between aseptic and septic samples, and also gene expression of cathepsin K, which is indicative of osteoclast generation, was within the range, in line with the observation that monocytes are activated in aseptic loosening, and that osteolysis occurs [8].

In conclusion, in both implant-associated infection and in aseptic loosening, the local innate immune response is activated, as is osteoclastogenesis. Systemic T cell activation, in contrast, occurs only in patients with implant-associated

Fig. 3 Analysis of a patients' biopsy with infection. **a** Eroded bone is seen (thick arrow), and next to it osteoclasts identified by an antibody to cathepsin K and multiple nuclei (arrows). **b** Following haematoxylin/eosin staining again eroded bone is seen as is a mixed cellular infiltrate, consisting of neutrophils (thin arrows) and mononuclear cells (arrow heads)

infection. Because determination of activation-associated receptors by cytofluorometry is a well-established and easy method, analysis of peripheral blood T cells could serve as a diagnostic tool to differentiate between the two entities.

Fig. 4 Gene expression of CD3, CD14 and cathepsin K in tissue. By qRT-PCR gene expression was quantified in tissue derived from patients with aseptic loosening (open boxes) or patients with infection (striped boxes). From each patient three samples were taken; data are again summarised as box-and-whiskers blot. The difference between the groups was calculated using Mann–Whitney test (note the log scale)

Conflict of interest The authors declare that they have no conflict of interest.

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