JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 762–767

Assessment of the role of cytokines in bone resorption in patients with total joint replacement

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Periprosthetic osteolysis is known to be a consequence of a local chronic inflammatory reaction in the synovial tissue and the bone-implant interface membrane, and is mediated by macrophages (Mϕ) and foreign body multinucleated giant cells (MNGC) in these tissues. Activated Mϕ produce major classes of cytokines which have been documented in the regulation of bone cell formation, function and activity. In rheumatoid arthritis, inflammatory mediators released by Mϕ participate significantly in articular tissue destruction. In this study we have analysed the production and tissue distribution of 4 cytokines in the interface membranes obtained from patients with osteolysis associated aseptic loosening and in rheumatoid synovium to determine their role in the functional transformation of effector cells in these two conditions. The production of IL-1, GM-CSF, IL-6 and TNF-α was assessed using immunohistochemistry on cryostat sections of the interface and the synovial tissues. IL-1 and GM-CSF were detected in significantly high numbers of the inflammatory Mϕ in both RA and aseptic loosening. A specific pattern of expression was noted in the interface. IL-1 production was sporadic throughout the sections, while GM-CSF was immunolocalized in a distinct subset of phagocytic macrophages on the implant side. IL-6 showed moderate expression in both conditions and was more widely produced at sites near the bone side in the interface. TNF-α expression was absent or reduced in the interface but was more abundant in RA synovial Mϕ. The differential expression of cytokines indicates that bone lysis in these two pathological conditions is mediated by different mechanisms and regulated by different cytokines.

1. Introduction

Cytokines include a large group of biologically active glycoproteins that mediate a number of cellular interactions and immune inflammatory responses. Data has also accumulated describing the potential of different cytokines in the regulation of bone cell formation, function and activity, particularly the bone resorbing capacity of osteoclasts (OC), which is essential in bone remodelling [1]. The majority of these findings are based on *in vitro* and *in vivo* studies of the bone marrow microenvironment, the main source of bone cells [2]. It is now well established that bone-resorbing OC are derived from haemopoietic mononuclear cells [3], while osteoblasts (OB), the bone-forming cells, originate from bone marrow stromal cells [4]. It is therefore apparent that the differentiation of haemopoietic and bone cells is regulated by the same network of cytokines and growth factors produced within the bone marrow. Although a large number of these factors is produced by macrophages (Mϕ), there is evidence that stromal cells, OB, T cells, fibroblasts and endothelial cells also synthesize high levels of these factors [1].

Interleukin-1 (IL-1) and tumour necrosis factor (TNF-α) are the first known potent stimulators of bone resorption *in vitro* [5, 6] and *in vivo* [7]. They are often termed as OC activating factors. IL-1 has also been shown to have an inhibitory effect on OB function and bone collagen synthesis [8]. Reports also suggest that IL-1 is partly responsible for postmenopausal osteoporosis [9], and increased hypercalcemia in relation to various malignancies [10].

In addition to its production by the Mϕ/monocyte lineage, IL-6 has also been demonstrated in stromal cells, OB cell lines and non transformed human bone cells [11]. It is the major cytokine responsible for the development of focal osteolytic lesions in multiple myeloma [12]. IL-6 acts as an autocrine/paracrine factor in Paget's disease of bone [13]. Elevated levels of IL-6 produced by OC-like giant cells have been

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detected in the blood and marrow plasma from patients with this disease. Similar findings have been reported in patients with osteoclastoma [14].

Granulocyte/monocyte colony stimulating factor (GM-CSF) is a haemopoietic growth factor with a stimulatory effect on the growth and differentiation of OC precursors [15], therefore a potential regulator of bone resorption.

As these cytokines regulate bone cell activity, an imbalance in their production may result in accelerated OC production or activation which subsequently leads to local or generalized bone defect/loss, as in the case of rheumatic diseases such as rheumatoid arthritis (RA) or osteoarthritis (OA) [16] and in osteoporosis [17]. Periprosthetic osteolysis is well documented in association with loosened and well fixed orthopaedic implants. This process is mediated by a local tissue reaction in the bone-implant interface fibrous membrane, and is caused by the response to wear particles released from different implant components [18]. We have previously reported that the persistent presence of different forms of Mϕ and the frequent appearance of foreign-body multinucleated giant cells (MNGC) in these tissues, and in close contact with the damaged bone may contribute to this process [19]. Mϕ are known to produce major classes of cytokines, growth factors and enzymes with potential in stimulating bone resorption. In this study we have analysed the production and tissue distribution of four cytokines in the bone-implant interface obtained from patients with aseptic loosening and in RA synovia. The objective of the study was to determine their role in the functional transformation and differentiation of effector cells in these two conditions.

2. Materials and methods

2.1. Patients

Twenty two patients who had osteolysis associated loosening were studied. Brief clinical details are summarized in Table I. The underlying disease that led to joint replacement was rheumatoid arthritis (RA) in eight and osteoarthritis (OA) in 14. All patients had degenerated joint condition on revision and there was radiographic indication of osteolysis at various locations around the implant components. The duration of the implants ranged from two to 17 years.

2.2. Tissue processing

Specimens of the bone-implant interface on the femoral, acetabular, or tibial side obtained during revision surgery were immediately cut into small pieces and frozen in isopentane-cooled liquid nitrogen. Representative blocks of these tissues were fixed in buffered formal saline and processed to paraffin wax, to be used for the histopathological assessment. In addition to these cases, 5 synovial membranes collected from RA patients undergoing total knee replacement were also used.

TABLE I Details of the patients whose interface tissues were obtained during revision

Number of patients studied		22
Sex	M	9
	F	13
Age range (years)	34–85	
Original joint disease	OA	14
	RA	8
Joint in which revision surgery was performed	Hip	17
	Knee	5

2.3. Immunohistochemistry

The cytokines IL-1, GM-CSF, TNF-α and IL-6 were immunolocalized on cryostat sections cut from at least two frozen tissue blocks of all the cases analysed. The sections were dried for 2 hours at room temperature (*rt*), fixed in acetone/methanol (1:1) at − 20 ° C for 15 minutes, and stained with the biotin/streptavidin alkaline phosphatase technique. The sections were incubated overnight at 4 ° C with the specific antibody to each cytokine (listed in Table II). This was followed by 1 h incubation at *rt* with one of the following biotinylated secondary antibodies: goat anti rabbit, horse anti mouse, or horse anti goat IgG depending on the species in which the first antibody was raised. Finally streptavidin alkaline phosphatase was added for another hour incubation. All conjugated antibodies were purchased from Vector Laboratories. The substrate reaction was developed with Naphthol AS-BI phosphate and Fast Red TR salt (Sigma) in 0.1 M Tris-HCl buffer (pH 8.2), and used for 25 mins. Sections were washed after each step with 0.05 M Tris-HCl buffered saline (TBS), pH 7.6. Positive controls used and confirmation of the specificity of immunostaining as has been described previously [20, 21]. Negative controls to check for non-specific binding included replacing the primary antibody with TBS or non immune immunoglobulin from the same species as the first antibody. One section from each case was also incubated with the substrate only to exclude the detection of endogenous alkaline phosphatase in endothelial cells or bone cells.

3. Results

3.1. Histological features of the bone-implant interface

Sections of the bone-implant interface were characterized by the presence of different morphological forms of Mϕ and MNGC, which form the major part of the cellular infiltrate. These were identified with the monoclonal antibody (MAb) CD68 and CD11b (Fig. 1). Perivascular aggregates of T cells were also present. The number of T cells varied, and showed slight increase in cases with metal debris. The extent and the histological pattern of the cellular infiltrate correlated strongly with the amount of the wear particulate debris seen in the sections. The appearance of few layers of Mϕ and small MNGC (2–5 nuclei) in the lining layer towards the implant side represents the early onset of the cellular response to the released particles

TABLE II Antibodies used in this study

Antibody	Host species	Dilution	Specificity	Source
IL-1α	Rabbit	1/100	IL-1α and IL-1α precursor	Genzyme
IL-1β	Rabbit	1/100	IL-1β and IL-1β precursor	Genzyme
GM-CSF	Mouse	1/25	Natural and recombinant human GM-CSF	Genzyme
GM-CSF	Goat	1/80	Natural and recombinant human GM-CSF	R & D Systems
TNF-α	Mouse	1/40	Human TNF-α	Celltech
TNF-α	Mouse	1/25	Natural and recombinant human TNF-α	R & D Systems
IL-6	Rabbit	1/80	Natural and recombinant human IL-6	Genzyme
IL-6	Mouse	1/40	Natural and recombinant human IL-6	Genzyme
EBM11	Mouse	1/100	CD68 macrophage associated antigen	Dako
CD11b	Mouse	1/100	C3bi complement receptor	Dako
CD13	Mouse	1/100	Aminopeptidase-N	Dako

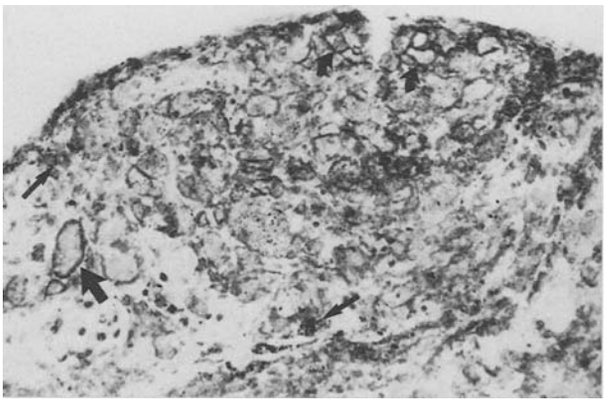

Figure 1 Section of the bone-implant interface stained for CD68, Mϕ associated antigen. Large number of Mϕ and small MNGC are seen in the lining layer (small arrow heads), while large MNGC are in the deeper layers (large arrow heads). Metal wear debris is seen throughout the section (arrows).

of metal, polyethylene or bone cement. The deeper layers of the interface in these cases consisted mainly of a fibrous stroma with some blood vessels.

Cases in which elevated concentrations of particulate material were detected in the sections demonstrated the presence of a prominent Mϕ infiltrate in the deeper layers, and the frequent appearance of large MNGC (6–40 nuclei) particularly towards the bone side of the interface membrane. MNGC accounted for more than 50% of the cellular infiltrate in some cases, and were characterized by their strong immunoreactivity with the MAb CD13, regardless of their size and location (Fig. 2). Fifteen of the cases analysed revealed the presence of intramembranous osteoid and mineralized bone at different sites within the interface membrane. Bone pieces were often lined with a thin layer of fibrocartilage tissue and showed features of trabecular bone. This observation highlights the capacity of the interface membrane to display both osteogenic and inflammatory characteristics in comparison to the inflammatory erosive lesion of the rheumatoid synovium. Another important feature that distinguished the interface from RA synovia was the complete absence of B cells and lymphoid follicles, and the infrequent presence of polymorphonuclear cells.

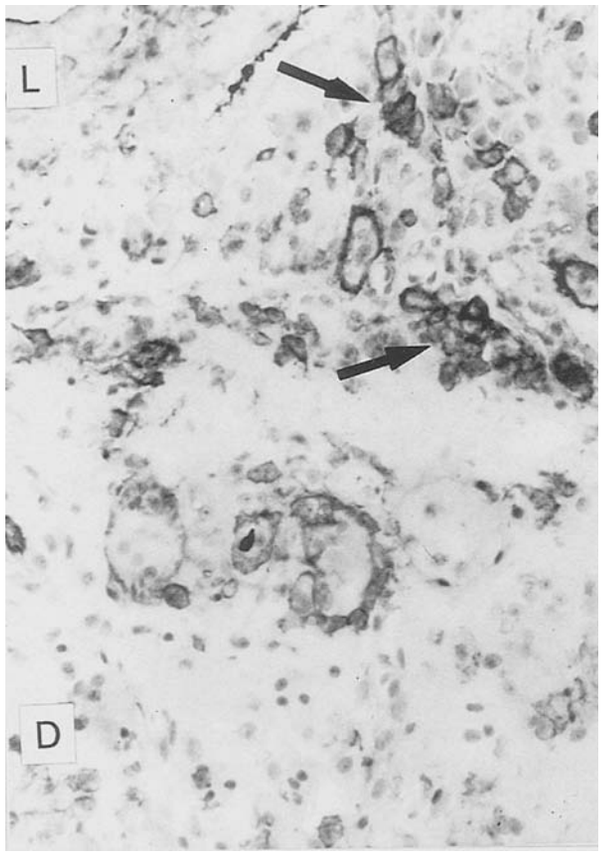

Figure 2 CD13 monoclonal antibody labels a proportion of the phagocytic Mϕ on the implant side (arrows). It also marks small and large MNGC. L, indicates the lining and D, the deeper layers of the interface.

3.2. Immunolocalization of cytokines in tissue sections

IL-1 and GM-CSF were detected in a significantly high number of the inflammatory Mϕ in both the interface membranes and the RA synovia. IL-1β form of this cytokine and not IL-1α was immunolocalized in 15 specimens of the interface membranes. IL-1β was mostly produced by Mϕ in the deeper layers, although in two cases, histiocytic Mϕ in the lining layers were also positive. A variable number of MNGC showed membrane and cytoplasmic staining for IL-1β. This was more evident in cases with metal wear debris, and

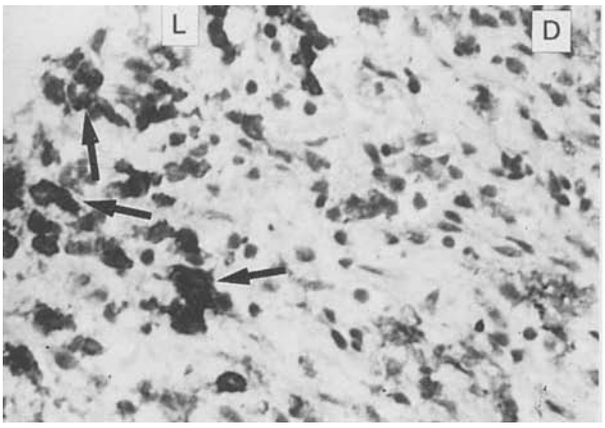

Figure 3 Strong expression of GM-CSF by phagocytic Mϕ on the implant side (arrows). No significant positive staining in the deeper layers, D.

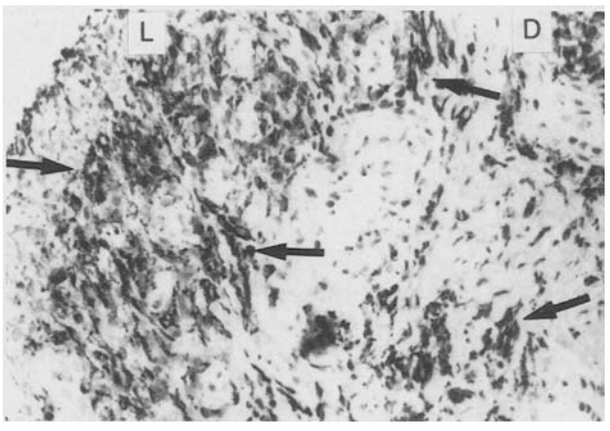

Figure 4 GM-CSF expression in the RA synovium. Large proportion of the Mϕ infiltrate throughout the section produce GM-CSF (arrows).

particularly in cases that showed high proportion of IL-1β producing Mϕ (up to 80% of the total cellular infiltrate). In RA synovia both IL-1α and IL-1β were identified on separate Mϕ populations. No significant difference in the number of cells producing each form of this cytokine was noted in the positive cases (3 of 4 synovia analysed). Induction of IL-1β in endothelial cells of blood vessels, particularly in the lining layer, was noted in both conditions.

Positive staining for GM-CSF was observed in 18 cases. The expression was significantly higher in the Mϕ of the lining layer at the implant side (Fig. 3). This layer often showed cellular hyperplasia (10–25 cells in thickness) and a variable number of fused Mϕ (small MNGC 2–5 nuclei). These cells showed significant upregulation in GM-CSF production in terms of the number of positive cells and intensity of staining. Perivascular Mϕ expressing GM-CSF in the deeper layers were also present in some cases. In RA, GM-CSF producing cells outnumbered those seen in the interface, and showed more sporadic distribution throughout the synovium (Fig. 4).

In contrast to IL-1β and GM-CSF, TNF-α was expressed at a very low level in the interface. This was confirmed by using two monoclonal antibodies to TNF-α in this study. Production of this cytokine was

Figure 5 Production of IL-6 by dendritic Mϕ in the deeper layers of the interface.

noted in only a few cells in two cases. Endothelial cells of blood vessels particularly in the lining layer were more frequently positive. The RA synovia showed upregulated expression of TNF-α. Positive cells had a similar distribution to that of IL-1α and IL-1β.

Both antibodies used for the immunolocalization of IL-6 gave a strong membrane and cytoplasmic staining in all the positive cases. In the group of revision tissue analysed ($n = 22$), IL-6 was detected in five cases only. The number of IL-6 producing cells varied, but generally did not exceed 50% of the total cellular infiltrate (Fig. 5). Mϕ in the lining layer of the interface, MNGC throughout the sections, and endothelial cells were consistently negative. Interestingly a proportion of the bone-lining cells (possibly osteoblasts) in cases with intramembranous formation of bone, showed strong immunoreactivity for IL-6. The number of IL-6 producing Mϕ in the RA synovia was noticeably elevated in four of five cases analysed. Positive cells had no distinct distribution. High concentration of membrane and cytoplasmic IL-6 was detected on Mϕ in the intimal/lining layer as well as the deeper layers.

4. Discussion

This study describes the level of production and tissue distribution of four potential cytokines which were localized in sections of the bone-implant interface in patients with osteolysis associated aseptic loosening. The cytokines analyzed in this study have been implicated as important factors in the regulation of bone cell formation and function [1]. In RA, the synovial tissue represent a model of inflammatory erosive lesion that participates in articular tissue destruction in this pathological condition [22]. Mϕ which constitute a major part of the cellular infiltrate in these tissues, have been shown to elaborate a high level of pro-inflammatory cytokines. IL-1, TNF-α, GM-CSF and IL-6 have all been demonstrated in abundance within the synovial tissue and synovial fluid in patients with RA [23]. For that reason RA synovia were used in this study as positive controls for our analysis of the bone-implant interface.

It is well established that Mϕ are the main component and mediator of the inflammatory process in both

conditions. Furthermore the lining layer of the interface that develops towards the implant side is indistinguishable from the RA synovial intima. Similarities include the cellular content, morphological features and most phenotypic markers. However our histological assessment and cytokine analysis highlights the presence of significant differences in the deeper layers. In RA, B cells, polymorphonuclear cells and lymphoid follicles are common features of the inflammatory infiltrate. In comparison, the bone-implant interface rarely show any of these cellular components. In addition Mϕ in the sub-lining and deeper layers of the interface exhibit marked heterogencity. Histiocytic, dendritic and stellate Mϕ are frequently seen [24]. More important, MNGC arc present at a significantly higher proportion compared to the RA synovia. These observations suggest gradual and persistent cellular transformation of phagocytic Mϕ into MNGC which may have a pivotal role in bone resorption. Indeed our group has recently demonstrated that a large proportion of MNGC in the interface membrane express OC markers (the vitronectin receptor) [25], therefore may exhibit functional similarity to bone-resorbing OC. Accordingly the bone-implant interface appears to have histological features that are distinct from the erosive lesions in RA synovia. In this context the differential expression of cytokines reported here correlates strongly with the nature of the cellular response.

The upregulated expression of IL-1 in both conditions is consistent with the role of this cytokine in the process of Mϕ activation and the maintenance of the inflammatory response [26]. IL-1 is also a potential inductive factor in the activation of vascular endothelial cells. This function is essential for the recruitment of mononuclear cells to sites of inflammation [27]. Furthermore IL-1 stimulates both OC formation and the bone-resorbing capacity of these cells [1]. This cytokine has also been shown to regulate the fusion of immature Mϕ into MNGC [28].

GM-CSF showed specific localization pattern and more frequent expression in Mϕ in the lining layer of the interface. It is of interest in this context to note that previous studies have demonstrated that adherence to fibronectin induce the expression of GM-CSF [29]. Phagocytosis and the presence of inflammatory stimuli have also been shown to upregulate GM-CSF production [30]. Fibronectin is known to be present at a considerable level at the lining layer of the interface. It has also been considered as one of the major plasma proteins that enhances the early stages of attachment of phagocytes to the implant surface. The demonstrated expression of GM-CSF in our study therefore may reflect the onset of the cellular inflammatory response which follows the release and phagocytosis of implant particulate debris. This specific localization pattern is also of a particular interest in light of the well defined role of GM-CSF as stimulatory factor for the proliferation of Mϕ [31]. Furthermore GM-CSF plays a major role in increasing the proliferation of OC and MNGC precursors, and enhances the initial stages of fusion of these cells [15]. These findings support our observation of the appearance of small giant cells towards the implant side. In addition GM-CSF production reported here was more evident and correlated strongly with the increased thickness of the lining layer and the lining cell hyperplasia seen in cases with excessive metal debris.

This study revealed a significant difference in the level of production of IL-6 between the RA synovia and the interface. Elevated concentrations of IL-6 in the synovial fluid of patients with RA has been shown to promote chronic synovitis and to correlate with the histological changes in the inflammed synovium [32]. In RA, IL-6 may induce B cell activation and the local production of IgG, an essential component in the formation of immune complexes [33]. IL-6 is also known as a powerful bone-resorbing factor and hypercalcemic agent *in vivo* [34]. Other studies suggest that IL-6 mediates bone resorption by stimulating IL-1 and TNF-α production [10]. The role of IL-6 in the interface is not yet clarified. However its production by Mϕ in the deeper layers, and towards the bone side may regulate the differentiation of OC precursors and the formation of functional OC in the nearby bone. *In vitro* culture studies has demonstrated that recombinant IL-6 induce the formation of MNGC and the expression of vitronectin receptor, a marker of functional OC [35]. Our observation of the expression of IL-6 by the osteoblasts lining the newly formed bone is of interest. Production of this cytokine may have a regulatory role in maintaining the inflammatory response within the interface, and inhibitory effect on the process of bone remodelling.

In conclusion this study demonstrated that the bone-implant interface exhibits histological features that is distinct from the erosive lesions in RA. The differential expression of cytokines indicates that bone lysis in these two pathological conditions is mediated by different mechanisms and regulated by different cytokines.

Acknowledgement

This work was supported by the Interdisciplinary Research Centre in Biomedical Materials, London, UK. We thank the orthopaedic surgeons at the Royal Free Hospital and the Royal London Hospital for their collaboration in providing the tissue samples used in the study.

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Received 29 June
and accepted 4 July 1995