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Persistent expression of mitogenic/transforming factors at the site of failed orthopaedic implants: the impact on immune reactivity

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The response to wear particles from orthopaedic implants can lead to inflammation, osteolytic lesions, and aseptic loosening. To gain an insight into the development of this pathogenetic process, immunohistochemical techniques were used to identify the expression and tissue distribution of the potent cell mitogen epidermal growth factor (EGF), and the epidermal growth factor receptor (EGF-R) at the site of bone erosion in 30 patients with clinically failed orthopaedic implants. The results showed a large proportion of the macrophage subsets (Mϕ) which expressed EGF and EGF-R, also contained wear particles, indicating their expression is a consequence of Mϕ phagocytosis of implant material. The surface membrane expression of EGF-R on fusing Mϕ suggests its presence is fundamental to the formation of bone-resorbing multi-nucleated giant cells, and the development of osteolysis. Additionally, there is increasing evidence of the long-term systemic spread of wear particles and their accumulation at distal sites including lymph nodes, liver, and spleen. Elevated expression of mitogenic factors in response to wear particles may result in deviation from normal cell growth and regulation, resulting in changes to immune cell function. Such potential transformations at distal sites are clinically significant, as alterations to the patient's immune system may result in acute divergence from normal immune cell responses.

1. Introduction

Macrophage (Mϕ) phagocytosis of indigestible particles released from orthopaedic implants, and their prolonged exposure to such particles is associated with both local and systemic immunopathological changes, leading to the development of osteolytic lesions and the failure of total joint arthroplasties [1–5]. Previous studies by our group and others have demonstrated elevated expression levels of various cytokines in response to metal, polyethylene and acrylic particles [6, 7]. Regulated expression of cytokines is required for normal cell growth and differentiation; however, abnormal expression can lead to transformation of cell function and the development of a plethora of pathological processes. This study examines the expression of a cytokine growth factor, epidermal growth factor (EGF) and its receptor (EGF-R) at the bone–implant interface of aseptically loosened total joint replacements.

Growth factors comprise a diverse group of polypeptides which primarily control or modify cell division. EGF shares similar structure and functions to that of other growth factors, most notably transforming growth factor -α (TGF-α). EGF and TGF-α are processed from larger precursor transmembrane proteins which bind with a high affinity to EGF-R [8–10]. Once bound to the receptor, the intrinsic tyrosine kinase located in the cytoplasmic domain autophosphorylates the receptor and triggers a cascade of intracellular biochemical events [11]. Subsequent to binding, both the receptor and ligand are internalized [12]. Consequently, this would lead to a reduction in receptor presence on the cell membrane; however, persistent expression of the cell membrane receptor may be maintained due to the capacity of EGF to induce its own receptor synthesis [13, 14].

Cellular responses to EGF or TGF-α binding to EGF-R include mitosis [11], cell migration [15], wound healing [16], and angiogenesis [17]. Both growth factors are also potent stimulators of bone resorption [18], which may be important in the development of aseptic loosening. Previous studies have demonstrated high expression levels of such growth factors in association with the development of tumors [19, 20], and their promotion of bone resorption may explain the etiology of hypercalcemia which

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typifies some neoplasms [21, 22]. These growth factors can induce bone resorption partly due to their ability to increase the proliferation and fusion of osteoclast precursors, leading to an increase in the number of osteoclasts [23, 24]. Additionally, TGF-α and EGF have an inhibitory action on collagen synthesis and alkaline phosphatase activity in osteoblast-like cells [25, 26]. Therefore, they potentially have a dual role in aseptic loosening, by both augmenting bone resorption and reducing bone formation, resulting in an acute disruption to the normal homeostatic turnover of bone.

2. Materials and methods

2.1. Patients

A total of 36 specimens of acetabular, femoral, or tibial bone implant interface were taken from 30 patients during revision surgery of aseptically loosened hip ($n = 20$) and knee ($n = 10$) arthroplasties (Table I). There were 12 males with an average age of 61 y, and 18 females with an average age of 66 y. The underlying joint diseases that led to joint replacement were osteoarthritis ($n = 22$), rheumatoid arthritis ($n = 4$), and others diseases ($n = 4$). All specimens were obtained fresh at the time of total hip or knee revision operations.

2.2. Tissue processing

Tissue obtained from revision operations was immediately cut into 1–3 cm long pieces. These were then frozen in liquid nitrogen-cooled isopentane and stored at $-70\,^{\circ}C$ until used for cryosectioning and immunostaining.

2.3. Immunohistochemistry

Cryostat sections were fixed in an acetone : methanol solution (50 : 50 vol/vol) for 15 min at $-20\,^{\circ}C$. Immunostaining was then carried out using the biotin/streptavidin alkaline phosphatase technique. Sections were incubated with the primary antibodies (Table II) for 18 h at $4\,^{\circ}C$. This was followed by a 1 h incubation at room temperature with biotinylated horse antimouse IgG antibody (Vector laboratories) at a 1/100 dilution. After incubation with the secondary antibody, the alkaline phosphatase streptavidin conjugate used at a 1/100 dilution was added for a further hour of incubation at room temperature. Each incubation was followed by three washes in phosphate buffer saline (PBS) for 5 min each. The color reaction was developed using 5 mg Napthol AS-BI phosphate (Sigma-Aldrich Company, UK) dissolved in 200 µl dimethylformamide. This was then mixed with 10 ml 0.1 M tris-HCl buffer pH 8.2, and

TABLE I Clinical data of the patients and details of interface tissue analyzed

Case no.	Sex/Age (y)	Joint involved/ Revision no.[a]	Duration of implant (y)	Underlying joint disease[c]	Interface tissue analyzed[d]
1	M/64	Hip/1	10	OA	A/F
2	M/38	Hip/1	13	Osteonecrosis in femoral head	A/F
3	F/67	Hip/1	8	RA	A
4	F/47	Hip/2	7	RA	A
5[b]	M/69	Hip/1	32	OA	A/F
6	M/77	Hip/1	7	OA	A
7	M/77	Knee/1	10	Osteonecrosis due to cancer	F/T
8	F/58	Hip/1	14	OA	A
9	F/47	Hip/1	11	OA	A
10	M/56	Hip/2	5	OA	F
11	F/70	Knee/1	11	OA	T
12	M/53	Knee/2	2	OA	F
13	F/80	Knee/2	6	RA	T
14	M/51	Hip/1	6	OA	A
15	F/43	Hip/2	12	OA	A
16	M/76	Knee/1	6	OA	F
17	F/51	Hip/1	10	TB of L. hip	A/F
18	F/87	Hip/1	4	OA	A
19	F/70	Hip/2	8	OA	A
20	F/49	Hip/4	2	OA	F
21	F/71	Knee/1	6	OA	T
22	F/73	Hip/1	7	OA	A
23	M/60	Hip/2	6	OA	A
24	F/64	Hip/2	2	OA	F
25	F/82	Knee/2	15	OA	T
26	F/83	Hip/2	4	OA	A
27	M/49	Hip/3	4	Ankylosing Spondylitis	F
28	M/67	Knee/1	9	RA	F/T
29	F/77	Knee/1	6	OA	F
30	F/61	Knee/1	4	OA	F

[a] Revision number indicates number of revision inclusive of current revision.
[b] Replacement of Austin Morris hemiarthroploasty.
[c] OA, osteoarthrits; RA, rheumatoid arthritis; TB, tuberculosis.
[d] A, acetabular; F, fermoral; T, Tibial.

TABLE II Antibodies used in this study

Antibody	Specificity	Type/Class	Source	Working dilution
EGF	Human EGF	Mouse Monoclonal, IgG1	Oncogene Research Products	1/10
EGF-R	External domain of human EGF-R	Mouse Monoclonal, IgG2	Oncogene Research Products	1/50
TGF-α	Human TGF-α	Mouse Monoclonal IgG2	Oncogene Research Products	1/20
CD68	Macrophage associated CD68 antigen	Mouse Monoclonal, IgG2	Dako	1/200
CD13	Myeloid associated CD13 antigen	Mouse Monoclonal, IgG1	Dako	1/100
CD3	T-cell associated CD3 antigen	Mouse Monoclonal, IgGI	Dako	1/50

10 mg Fast Red TR salt (Sigma). Levamisole was added to the final developing solution as an inhibitor of endogeneous alkaline phosphatase at a concentration of 10^{-3} M. The substrate was filtered and allowed to develop on the sections for 20 min. Finally, the sections were washed and then counterstained with Mayer's haematoxylin followed by mounting in Aquamount (BDH Merck, UK). Three sets of controls were used to confirm the specificity of the immunostaining: (1) replacing the primary antibody with PBS or non-immune immunoglobulin from the same species as the first antibody; (2) staining two sections from each case with the substrates solution with or without Levamisole to exclude the detection of endogeneous alkaline phosphatase; (3) positive controls including sections of skin biopsy and rheumatoid synovium.

3. Results

Assessment of the tissue sections of the bone–implant interface identified differences in the relative thickness of these membranes and the extent of the inflammatory cellular infiltrate. All specimens exhibited a common histological feature of the predominance of Mϕ and a variable number of multinucleated giant cells (MNGC). Small and large aggregates of T lymphocytes were observed in association with Mϕ and MNGC containing biomaterial particles. Some of the specimens showed a papillary layer at surfaces on the implant side of the interface. These thin layers were comprised of cells which morphologically resemble type A and B synoviocytes, and were arranged as a synovial lining layer 2–5 cells thick (Fig. 1a). Such regions appear to capture a significant amount of the implant particulate wear debris of metal or polyethylene as demonstrated in Fig. 1b. Histological analysis also suggests that these regions may represent particles containing Mϕ migrating into spaces between the implant and bone.

Cells within the interface membrane exhibited strong immunoreactivity with the monoclonal antibody CD68, while MNGC containing a variable number of nuclei were consistently labeled with CD13. A low level of the Mϕ inflammatory infiltrate, and histological characteristics of a normal synovium, were found in 12 cases. A further 18 cases were heavily infiltrated with Mϕ and MNGC and showed a significant increase in their thickness. Immunostaining for EGF-R was predominantly seen on the majority of Mϕ in the deeper layers of the interface, and localized in both the cytoplasm and on the plasma membrane (Fig. 2, and Table III). Intense staining was seen on Mϕ containing large numbers of metal or polyethylene wear debris, and also on a large number of perivascular aggregates of Mϕ. MNGC in the interface consistently showed no staining for the receptor, although Mϕ surrounding MNGC possibly undergoing fusion showed strong membrane expression (Fig. 3).

EGF staining was mostly confined to cells with the morphological resemblance to type A and B synoviocytes in or near the lining layer of the interface and on cells containing polyethylene wear debris (Fig. 1a and Fig. 4). The staining of EGF on Mϕ in the deeper layers of the interface was minimal relative to that of EGF-R. TGF-α showed proportionally more widespread positive labeling on a greater number of cells than both EGF and EGF-R in the lining layers and the deeper layers of the interface. Prominent staining for TGF-α was also seen on the

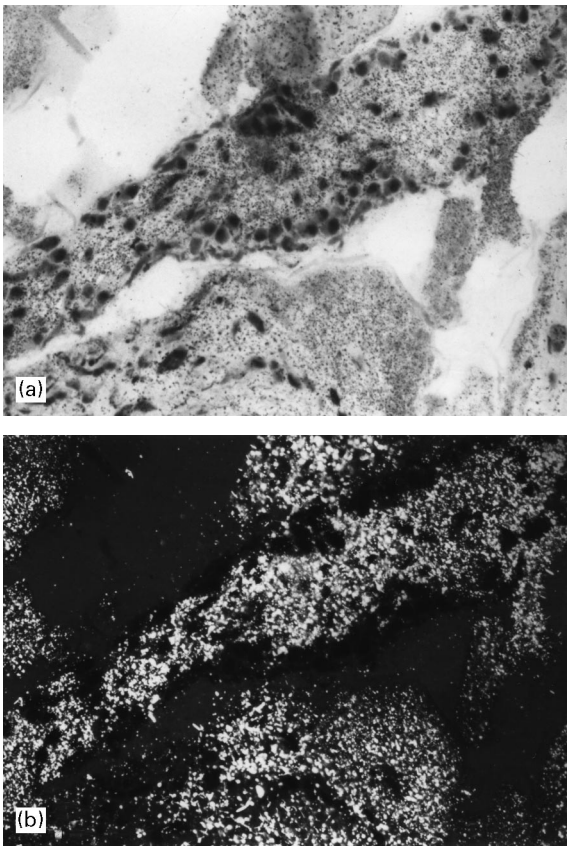

Figure 1 (a) Immunolocalization of EGF by polyethylene debris containing cells at the lining layer. Note their resemblance to type A and B synoviocytes, ×350. (b) The corresponding section but viewed under polarized light highlighting the presence of polyethylene wear debris, ×350.

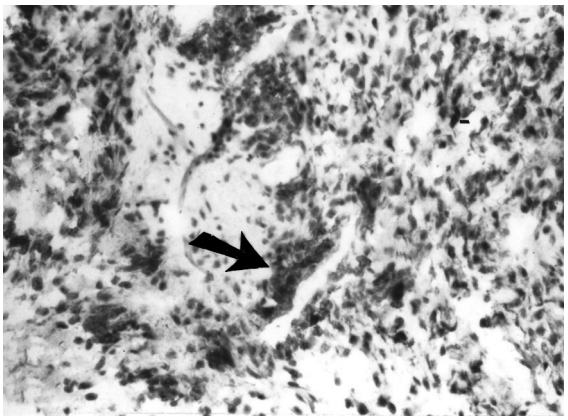

Figure 2 Immunostaining for EGF-R on Mϕ which is particularly prominent near vascular endothelium (see arrow), ×200

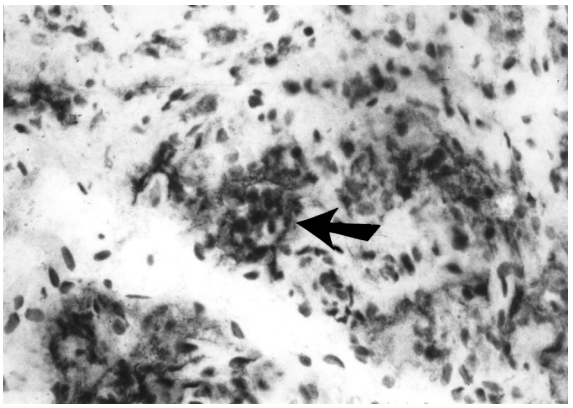

Figure 3 EGF-R positive staining on Mϕ fusing to form MNGC (see arrow), ×270.

TABLE III Summary of the level of expression and tissue distribution of EGF and EGF-R

Cells at bone–implant interface	Cytokine expression level	
	EGF	EGF-R
Mϕ, implant lining layer	++	++
Mϕ, deeper layers	+	++++
MNGC (3–6 nuclei)	+	−
MNGC (7 or more nuclei)	+	−
Endothelial cell	+	−
Fibroblasts	−	+
T-lymphocytes	−	−
Bone lining cells	+	++

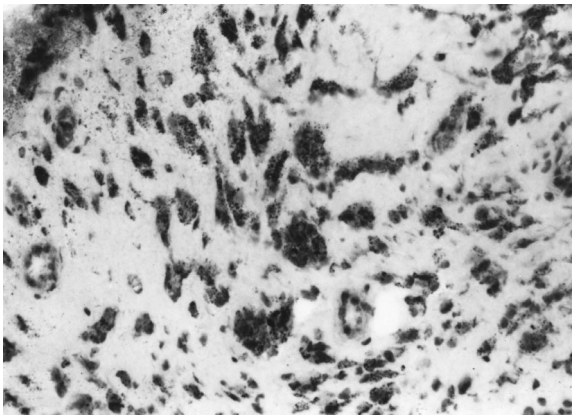

Figure 4 An example of expression of EGF by Mϕ near the lining layer. These cells also contained a large amount of wear debris, ×320.

Figure 5 Positive staining for TGF-α by Mϕ lining the bone surface. Note the presence of two distinct resorption pits with TGF-α positive cells lining their surface (see arrow), ×330.

membrane of all MNGC, whereas EGF was weakly expressed in the cytoplasm of these cells and in relatively few cases.

Of the cases examined, 12 contained areas of fibrocartilage, osteoid, and/or perimplant bone with morphologically varied cells lining their surface. Many of the cells showed staining for both TGF-α and EGF-R (Fig. 5). These cells were subsequently identified as Mϕ using serial sections as they demonstrated immunoreactivity to CD68.

4. Discussion

The results from immunostaining show, for the first time, elevated levels of EGF-R expression by activated phagocytic Mϕ containing large amounts of implant wear debris. Induction of receptor expression in Mϕ indicates a role for the receptor in altering immune cell function and the development of this inflammatory response. Additionally, immunolocalization of the receptor in both the cytoplasm and the membrane demonstrated a rapid turnover of the receptor in both its synthesis and internalization. The number of cells expressing EGF in the interface was found to be considerably less than TGF-α, examined by this group in previous studies [27]. Therefore, in the development of this pathological state, TGF-α is the dominating growth factor binding to and activating EGF-R.

Several cases examined in this study contained MNGC at various locations in the bone–implant interface. MNGC are derived from macrophage fusion, and have been well documented in connection to osteolytic lesions around aseptically loosened orthopaedic implants [28]. They are also associated with many other pathological processes including giant cell tumors of bone [29] and tendon sheath, [30], and also in arthritic synovium [31]. MNGC at the bone–implant interface have previously been identified as producing high levels of tartrate-resistant

acid phosphatase (TRAP), and expressing antigenic markers such as the vitronectin receptor which have previously been defined as osteoclastic markers [32]. Further similarities to osteoclasts include MNGC ability to resorb bone [33], and their presence at sites of osteolytic lesions indicates a pivotal role in the development of aseptic loosening.

In agreement with a previous study carried out by our group, a large number of MNGC at the bone–implant interface showed intense positive staining on the membrane for TGF-α [27]. As this study showed no staining for EGF-R on MNGC, it can be presumed that TGF-α is not bound to the receptor but is being produced by the cell in the form of a precursor. The presence of the precursor on the membrane is due to inefficient proteolytic cleavage into the fully mature growth factor [34]. EGF-R was, however, identified on Mϕ surrounding MNGC. The TGF-α precursor can bind to EGF-R on adjacent cells [34, 35] providing a form of cell to cell adhesion between Mϕ and MNGC. Such interactions may therefore be pivotal for the fusion of Mϕ and the genesis of MNGC. The binding of the precursor to the receptor also activates EGF-R, potentially producing juxtacrine stimulation and a mitogenic stimulus [36], which may lead to a prolonged biological response relative to that stimulated by the secreted growth factor [35].

Serial sections of the bone–implant interface containing a piece of peri-implant bone revealed that a large number of EGF-R positive cells lining the bone surface were CD68 positive Mϕ. Growth factor receptors are known to regulate integrin molecule expression and therefore affect cell adhesion. The binding of EGF or TGF-α to EGF-R has been shown to increase cell adhesion to fibronectin and collagen, as a result of modulating integrin-mediated adhesion [37–39]. Activation of the EGF-R through autocrine/paracrine stimulation is, therefore, important for enhancing the attachment of Mϕ to bone. The potential role for such cells in the development of osteolytic lesions is highlighted by their ability to resorb bone in response to implant wear particles [40]. Additionally, the increased Mϕ presence on the bone surface could interfere with osteoblastic attachment, preventing new bone formation.

The increased expression of TGF-α and EGF-R by activated Mϕ lining the bone surface may also be preventing osteoblastic maturation. As the expression of EGF-R has been identified on osteoblastic precursors [41, 42], elevated levels of growth factors binding to these cells may interfere with their development into the fully mature osteoblast. This, in turn, would result in the reduction of bone-forming osteoblasts and alteration to the normal balance between bone formation and bone resorption.

The expression of potent mitogenic factors and their receptor in response to wear particles has widespread as well as local implications. Increasingly of concern is the mounting evidence for the systemic distribution and accumulation of a variety of wear particles originating from orthopaedic implants to distal organs, including lymph nodes, spleen, and liver via the lymphoreticular system [43, 44]. Upregulation of both TGF-α and EGF-R expression have been described at the sites of asbestos fiber deposition in the lungs and following exposure of mesothelial cell lines to such fibers [45, 46]. The release of such a potent cell mitogen and the expression of its receptor were identified as having a role in cellular transformation and the development of interstitial pulmonary fibrosis with possible induction of neoplastic transformation [46]. Evidence that elevated growth factor expression alone can contribute to or cause a predisposition to cellular transformation has been identified using the creation of transgenic mice. Gene transfer using molecular constructs to overexpress TGF-α caused extreme alterations in the development of adult organs, and was found to be oncogenic in mammary epithelium [47, 48].

In addition to the toxic effects of the metallic elements [49], increased risks of lymphomas and leukemia [50, 51] following insertion of orthopaedic implants may be due to the presence of wear particles at these sites. Systemic activation of immune cells and the subsequent elevated expression of factors in response to wear particles may therefore have clinically detrimental effects on immune cell function and cause alterations to normal immune cell phenotype.

5. Conclusion

This study demonstrates, for the first time, the consistent and elevated expression EGF-R by Mϕ in response to wear particles from orthopaedic implants. In addition to a number of other factors, the abnormal expression of TGF-α and its receptor may alter immune function at the bone–implant interface, contributing to this pathological state and aseptic loosening of the implant. Owing to the widespread dissemination of wear particles, the subsequent release of growth factors at other sites may cause alterations to immune cells resulting in long-term clinical problems.

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