Inflammatory cells in normal human fracture healing

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We studied inflammatory cells in specimens of callus taken from normally healing human fractures. Using immunohistochemistry, T-cells, B-cells, macrophages, HLA-DR expression and endothelial proliferation were assessed. Macrophages were present from an early stage but became less numerous later. T-cells were initially specifically recruited into the fracture site at the stage of granulation tissue, but subsequently excluded from areas of bone and cartilage formation. Inflammatory cells may control and coordinate fracture healing as has been proposed for soft tissue wound healing. The most likely mechanism for this is by the cytokines and growth factors which they are known to release.

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Inflammatory cells (granulocytes, macrophages and lymphocytes) form part of the fracture-healing response, being present from the earliest stages of the fracture hematoma. The role of these cells in bone repair has not been widely investigated, but in soft tissue wounds it has received considerable attention (Turck et al. 1987, Knighton and Fiegel 1989, Barbul and Regan 1990, Regan and Barbul 1991). The main finding to emerge from this work is that, in addition to their immune surveillance function, T lymphocytes and macrophage/monocytes act as initiating cells in the process of wound healing, perhaps principally as a source of growth factors. They also have a coordinating role in the later stages of wound repair. In bone repair, two studies by Hulth and coworkers (Hulth et al. 1985, Henricson et al. 1991) demonstrated that, in rats, both T lymphocytes and macrophages were present from the early stages of fracture healing.

An immune response to an antigen requires that the antigen be processed by an antigen-presenting cell. This cell then simultaneously presents, on its surface, the antigen and HLA Class II; together these are recognized by a T lymphocyte, which is activated and will continue the immune process. HLA-DR is part of the HLA Class II locus. Cells can be induced to express HLA-DR by several cytokines, therefore the presence of HLA-DR is an indicator of the activation status of the cell and does not, of itself, indicate that the cell is presenting antigen. Many of the macrophages seen in healing bone by Henricson et al. (1991) were expressing the HLA-DR antigen on their surfaces. Askalonov et al. (1987) and Liberman et al. (1990) produced effects on fracture healing by modulation of the immune response in animals. However, we have been unable to find previous studies concerning the presence of inflammatory cells in normal fracture healing in man, although the presence of inflammatory cells in delayed and non-union material has recently been investigated (Santavirta et al. 1992). We have studied inflammatory cells in normal human fracture callus.

Materials and methods

Specimens were obtained at operations to fix normally healing fractures which had developed malposition 1–8 weeks post-fracture. All of the fractures have now been followed to 1 year post-operation and have united normally. Patients were adults, and, apart from their fractures, were otherwise healthy. Ethics Committee approval and informed consent were received. Specimens of periosteal fracture callus were removed when available, in general it was a longitudinal section of the callus mass, approximately 5 mm wide. The specimens were fixed in 10 percent neutral buffered formalin for 24 hours. They were then radiographed and, if necessary, decalcified in 20 percent EDTA (pH 7.2), until radiographically decalcified. Following paraffin embedding, 7 μM tis-

Table 1. Results for inflammatory cells. Counts are per high-power field; 10 HPF were counted and the mean result is given. CD68 indicates the number of mononuclear CD68 positive cells; CD68 CLAST indicates the number of CD68 positive osteoclasts

No.	Bone	Days post fx	Grade	L26	UCHL1	OPD 4	CD4 3	Mac387	CD68	CD68 clast	LN3	CR3/43
1	Tibia	7	1a	3	200	50	500	500	100	0	0	0
2	Humerus	14	1a	4	210	42	350	250	95	0	0	10
3	Radius	7	1b	1	14	8	100	80	200	0	0	55
4	Femur	9	1b	2	100	40	500	500	200	0	10	2
5	Humerus	11	1b	0	30	30	200	10	5	0	50	200
6	Fibula	12	1b	0	200	80	100	100	80	0	0	40
7	Humerus	8	1b	0	0	10	50	3	15	0	3	150
8	Radius	11	2a	1	1	1	6	2	0	0	0	0
9	Humerus	29	2b	0	50	2	10	0	40	2	0	50
10	Femur	12	2c	12	40	30	20	2	200	25	250	500
11	Radius	15	2c	1	40	25	160	17	50	0	15	150
12	Radius	?	2c	0	40	20	30	10	10	0	3	3
13	Humerus	28	3b	10	400	200	200	5	100	90	30	400
14	Femur	17	3c	0	26	30	30	30	150	350	50	150
15	Metacarpal	17	3c	2	20	20	40	3	100	200	2	3
16	Humerus	21	3c	2	45	40	10	2	30	150	0	0

Table 2. Histological grading

Grade	Appearance
1a	Blood clot. Areas of mesenchymal/granulation tissue invasion at periphery of section.
1b	Most of section is mesenchymal/granulation tissue. May be areas of residual clot. No bone or cartilage formation seen.
2a	Areas of cartilage formation, but no obvious woven bone seen. No remodeling or new lamellar bone.
2b	Areas of woven bone, but no obvious cartilage seen. No remodeling or new lamellar bone.
2c	Areas of cartilage and woven bone. No remodeling or new lamellar bone.
3a	Areas of woven and lamellar bone. No cartilage seen. Active bone remodeling.
3b	Areas of woven and lamellar bone and cartilage. Active bone remodeling.

Table 3. Primary antibodies used for immunohistochemistry

Primary antibody	Source	Target cell
CD43	DAKO	Granulocytes, T lymphocytes
UCHL1	DAKO	Granulocytes, T lymphocytes
OPD4 (CD4)	DAKO	T-helper cells
L26	DAKO	B lymphocytes
Mac387	DAKO	Macrophages, granulocytes
CD68	DAKO	Macrophages, granulocytes
CR3/43	DAKO	HLA-DR
LN3	Clonab	HLA-DR (subset)
CD34	DAKO	Endothelium (?pericytes)

sue sections were cut for immunohistochemistry studies.

16 specimens were obtained (Table 1). They were classified histologically from hematoxylin and eosin, and toluidine blue-stained sections (Table 2). Many of the biopsies were heterogeneous for different stages of fracture-healing, e.g., containing proliferating fibrous tissue, new bone and cartilage formation in the same section (Tables 1 and 2). Cells were characterized by the antigens they expressed, as well as their presumed phenotype, because the antigens are not completely specific to single-cell lines, and may themselves be functionally important. These antigens were defined by their reaction with the specific antibodies (Table 3).

Prior to immunohistochemistry, sections were dewaxed and then endogenous peroxidase blocked with 0.3 percent hydrogen peroxide in ethanol. If a trypsinization step was necessary for a particular antibody, sections were then treated with 0.01 percent chymotrypsin (Sigma) in 0.01M Tris buffer containing 0.1 percent $CaCl_2$ at 37 °C for 20 min. Sections were then washed in TBS, blocked with 10 percent normal rabbit serum (15 min) and incubated for 1 hour with the primary antibodies given in Table 3. Following extensive washing, secondary antibody (swine anti-rabbit IgG, conjugated with biotin) was applied for 30 min. The sections were washed again and Avidin/Biotin/Peroxidase complex (ABC kit, Dako) was applied for 30 min. After washing, the sections were immersed in 0.2 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Sigma) containing 0.05 percent (v/v) H_2O_2 for 15 min. This revealed the antibodies as a brown coloration produced by the

action of the peroxidase in the ABC complex. Slides were counterstained with hematoxylin. For each antibody, the number of positive cells per 10 high-power fields was counted, using an Olympus BH-2 microscope.

Results

Counts per 10 high-power fields are shown in Table 1, but the cell counts only give a limited representation of the appearances of the sections, due to the histological heterogeneity of the specimens.

Stage 1a. In the fracture hematoma, mixed inflammatory cells positive for CD43, UCHL1, CD4, CD68 or Mac 387 were scattered throughout. Granulocytes were distinguishable from mononuclear cells because of their polylobated nuclei. Mononuclear cells were present immediately in front of and within very early granulation tissue seen at the edge of the hematoma; there were more cells in this area than in the main body of the hematoma. Immunocytochemistry showed that the mononuclear cells in the very early granulation tissue were a mixture of lymphocytes positive for CD43, UCHL1 and/or CD4, and macrophages positive for Mac 387 and/or CD68. There were very few B lymphocytes at this or at any subsequent stage, although occasional positive cells were seen which acted as an internal control ensuring that the staining procedure was technically adequate.

Stage 1b. At the stage of established granulation tissue, there were fewer inflammatory cells than in the hematoma. Morphologically, there were many more mononuclear cells than granulocytes. Immunocytochemistry showed that the numbers of macrophages (Figure 1) and T-cells (Figure 2) were approximately equal. There were many cells expressing the HLA-DR antigen at this stage, particularly adjacent to fragments of dead bone and in a characteristic perivascular location (Figure 3). At all histological stages, the numbers of cells expressing HLA-DR varied widely between sections at apparently similar stages of healing; no clear reason could be seen for this. Many of these cells were dendritic or fusiform. As expected, there were many capillaries; their endothelium stained with CD34 but was negative for HLA- DR.

Stages 2a, b, c. At the stages of bone and cartilage formation, there were fewer inflammatory cells present. T lymphocytes appeared to be completely absent from these areas, but were present in areas of fibrous tissue formation on the same sections. In some areas, macrophages were present adjacent to areas of early ossification or cartilage formation (Figure 4). Cells in these areas also expressed HLA-DR, and those sections with more macrophages present had more HLA-DR expression. Many of the cells positive for HLA-DR had a dendritic morphology and, as before, there were increased numbers present close to fragments of dead bone. Again, capillary endothelium expressed CD34 but not HLA-DR.

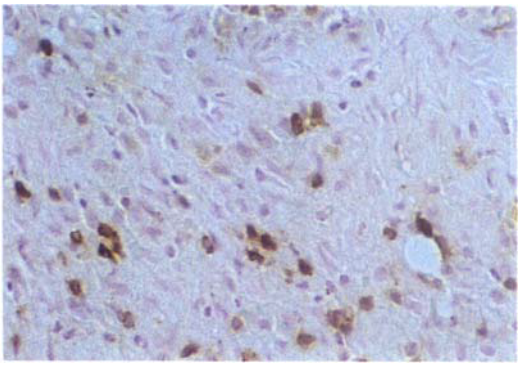

Figure 1. Case 5. Macrophages around capillaries in 11-day fracture. Mac 387, ×400.

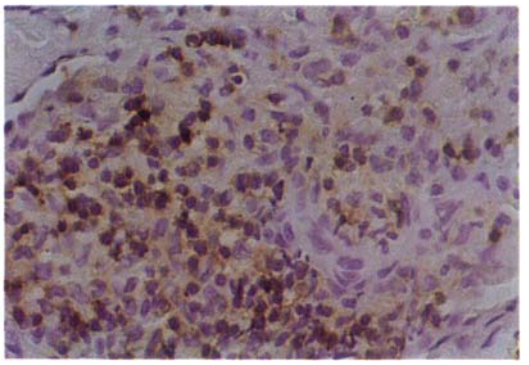

Figure 2. Case 5. Large numbers of T Lymphocytes adjacent to a vessel in 11-day fracture. UCHL1, ×400.

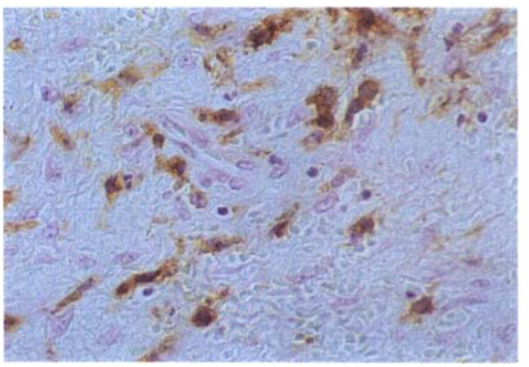

Figure 3. Case 7. Perivascular HLA-DR positive cells in 8-day fracture of the humerus. CR3/43, ×200.

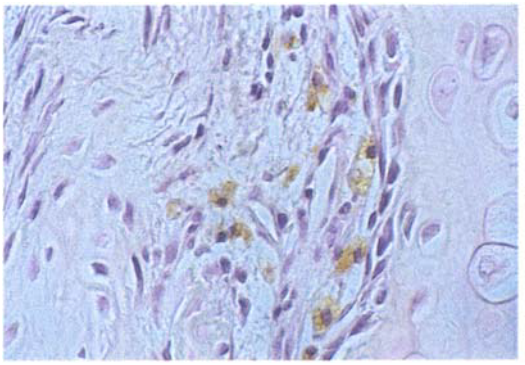

Figure 4. Case 11. CD68 positive macrophages adjacent to area of new bone formation. Note hypertrophic chondrocytes trapped within woven bone. 15-day radial fracture. CD68, ×400.

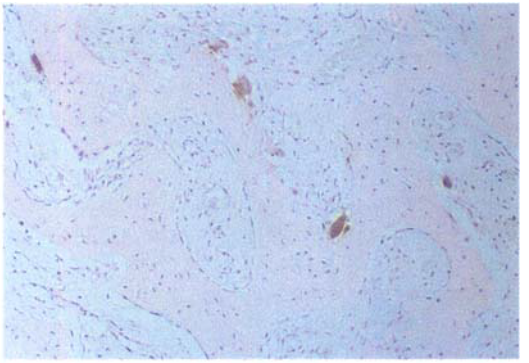

Figure 5. Case 14. Osteoclasts engaged in resorption of woven bone. 17-day femoral fracture. CD68, ×200.

Stages 3a and b. At the stages of remodeling of woven bone, T-cells were present in small numbers in the spaces between trabeculae of bone and, again, in variable numbers in areas of fibrous tissue. CD68 positive mononuclear cells were present in small numbers, but many CD68 (and to a lesser extent Mac387) positive osteoclasts were present (Figure 5). There were strikingly few chondroclasts present considering the extensive areas of cartilage in many of the sections, but these cells, when seen, were also CD68 positive. None of the chondroclasts or osteoclasts expressed HLA-DR.

Within the T-cell population, the helper/suppressor ratio was calculated from the numbers of T-helper cells (shown by OPD4) and total T-cells (shown by CD43). In the hematoma and granulation tissue a helper/suppressor ratio of 3:2 was found, which is approximately that of peripheral blood in the healthy adult, but in the other stages it was found to be quite variable.

Discussion

Macrophages, and the growth factors they produce, play a vital role in coordinating soft tissue wound-healing (Nathan 1987, Knighton and Fiegel 1989). This cell line is likely to serve a similar function in fracture healing. Lymphocytes are also important and it has been proposed that, in soft tissue healing, T-helper cells promote healing initially while T-suppressor cells help to reduce fibrosis in the later stages of healing (Regan and Barbul 1991). These effects may be indirect via lymphocyte modulation of macrophages. The effects of T-lymphocyte depletion are less marked than that of macrophage depletion, but the healing of soft tissue wounds (Regan and Barbul 1991) and fractures (Askalonov et al. 1987, Henricson et al. 1991) is slower and weaker than normal.

What can be deduced from the present study of human material about the role of lymphocytes and macrophages in fracture healing? The most striking finding was that T lymphocytes were selectively recruited into fracture hematoma and granulation tissue, but subsequently excluded from areas of bone and cartilage formation. We know that T-cells were selectively recruited because they outnumbered B-cells at all times: if they were present purely due to hemorrhage rather than active migration, the 2 different types of lymphocyte would be expected to be present in approximately equal numbers.

Selective recruitment of T-cells into soft tissue wounds was reported by Fishel et al. (1987). Hulth et al. (1985) found that T-cells were only seen at the early stages of fracture healing. Subsequently, IL-2 receptor positive (i.e., activated) T-cells were only present in very small numbers in areas of actively forming bone induced by demineralized bone matrix but not in fracture healing (Henricson et al. 1991). However, they did not directly address the spatial relationship of T-cells to bone formation. In our sections, T-cells were absent from areas of bone formation, but were present in fibrous tissue in normally healing fractures. The finding that the predominant inflammatory cell in delayed union material is the CD4 T lymphocyte (Santavirta et al. 1992) is of interest, and raises the question of whether T lymphocytes are important in the pathogenesis of failure of bone healing. The finding that these cells are absent from areas of woven bone formation but present in both granulation tissue and delayed union material suggests that areas of active bone formation are characterized by a paucity of T-cells.

Macrophages were present in large numbers in early wound repair and in granulation tissue, but in much smaller numbers in areas of bone formation. They were largely not related to areas of debris, and did not seem to be merely acting as scavengers, as has previously been proposed in fracture healing. It seems likely that they are acting as coordinating cells in a similar fashion to soft tissue healing. The expression of HLA-DR by cells of dendritic morphology probably demonstrates activation of a subset of macrophages to perform an antigen presentation function. This has previously been demonstrated in a rat fracture model (Henricson et al. 1991). This function of macrophages is vital to a normal immune response to foreign antigens, and in this situation it may reflect a relatively non-specific immune surveillance function related to tissue injury and repair.

The principal way in which lymphocytes and macrophages influence fracture healing is likely to be via the growth factors and cytokines which they produce. Lymphocytes produce interleukin 1 and 2, which may both influence mesenchymal cells directly and control other inflammatory cells (Male et al. 1991). Macrophages produce a wide variety of growth factors, including TGFb and PDGF. These have both been shown to be involved in fracture healing in the rat (Nemeth et al. 1988) and human (Andrew et al. 1993).

The role of inflammatory cells in fracture healing has hitherto been relatively neglected. Our findings indicate that early fracture healing has some inflammatory cell events in common with soft tissue healing, but that the two processes may diverge at the stage of both bone and cartilage formation. The dysfunction of lymphocytes and macrophages caused by HIV infection may prove to be an important cause of compromised fracture union in the future.

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