Role of multinuclear cells in granulation tissue in osteomyelitis

Immunohistochemistry in 66 patients

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ABSTRACT – We investigated the origin of multinuclear cells (MNCs) in the granulation tissue in osteomyelitis by immunohistochemical techniques in 66 patients. 12 samples were analyzed for the presence of CD68, cathepsin K, CD11b and tartrate-resistant acid phosphatase (TRAP) activity.

Many MNCs were present in the granulation tissue adjacent to a sequestrum. MNCs in contact with the sequestrum were also noted, however, no osteoblasts were found. Immunohistochemically, CD68, cathepsin K and TRAP were strongly expressed in most of the MNCs, while CD11b positive cells were not found. MNCs remote from and in contact with the sequestrum showed the same immunohistochemical features which are characteristic of osteoclasts.

Further, MNCs in contact with the sequestrum had originally developed in the granulation tissue and directly infiltrated towards the sequestrum without cell-to-cell interaction with osteoblasts.

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Osteoclasts are formed in bone marrow-derived stromal cells and need cell-to-cell interaction with osteoblasts on the bone surface to differentiate into osteoclasts (Suda et al. 1992). Recent experimental studies have shown that some of the multinuclear cells (MNCs) in inflammatory granulations have immunohistochemical and cytochemical characteristics similar to osteoclasts and can resorb bone in a dentine resorption assay (Fujikawa et al. 1996a, b, c, Darling et al. 1997, Takayanagi et al. 1997, Gravallese et al. 1998). Yasuda et al. (1998) and Lacey et al. (1998) independently identified the membrane-bound protein, termed osteoclast differentiation factor (ODF) or osteoprotegerin ligand (OPGL), which mediates an essential signal for osteoclast progenitors to differentiate into osteoclasts and is a critical factor for osteoclastogenesis. It has been shown that soluble forms of ODF/OPGL induce osteoclast formation from spleen cells (Takayanagi et al. 1997).

Using histochemical stains, we studied whether multinuclear giant cells adjacent to the sequestrum in osteomyelitis, like osteoclasts, can resorb bone.

Patients and methods

Specimens of granulation with sequestrum were obtained at the time of operation on 66 patients with severe osteomyelitis. All patients were admitted to Oita Medical University Hospital from 1983 to 1996. The average age of patients was 58 (51–67) years. All specimens were examined after hematoxylin-eosin staining, 12 of the 66 cases were also examined immunohistochemically (Table 1). Paraffin sections of 10% formalin-fixed specimens were cut at 4 mm on a Leitz base-slide microtome, placed on APES-coated slides and then dried overnight at 37 °C. Before staining, sections were dewaxed in xylene and rehydrated in graded alcohol.

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Table 1. Clinical data of patients with osteomyelitis

Sex	Age, years	Localization	Duration of disease	Operation	Bacterial strain
Female	44	R. femur	6 months	c., g.	unknown
Female	16	L. humerus	4 years	c.	unknown
Male	41	R. femur	4 years	c., g.	MRSA
Male	68	R. tibia	48 years	c., g.	SA
Male	65	L. tibia	40 years	c., g.	Pseudomonas aeruginosa
Male	19	R. tibia	4 months	c., g.	unknown
Male	8	R. tibia	4 months	c., g.	SA
Female	41	R. metatarsals	20 years	c., p.	Morganera morganii
Female	48	R. femur	4 months	c., p.	Fusobacterium species
Female	43	R. femur	5 months	c., p.	SA
Male	21	L. tibia	4 years	a.	MRSA

c. curettage, g. bone graft, p. perfusion, a. amputation, SA Staphylococcus aureus, MRSA Methicillin-resistant Staphylococcus aureus

Table 2. Monoclonal antibodies used in the immnohistochemical studies

Antibodies (manufacturer)	Molecular specificity	Target cells	Dilution
PG-M1 (DAKO)	CD68	Monocytes/tissue macrophages	100
ZY-9C5 (ZYMED)	TRAP	Osteoclasts, no macrophages	100
182-12G5 (Fuji chemical)	Cathepsin K	Osteoclasts	50
71597112 (Chemicon)	CD11b	Macrophages, no osteoclasts	75

Immunohistochemical staining

The sections were stained using the avidin-biotin complex (ABC) technique with monoclonal antibodies (mAb) (Table 2). Antigen retrieval was carried out using a microwave oven for 10 min (microwaved twice, 5 min each) before exposure to the primary antibody. Paraffin sections were examined for the presence of CD68, marker associated with macrophages; cathepsin K, marker associated with osteoclasts; CD11b, marker associated with macrophages, but not mature macrophages, and the presence of TRAP activity. After washing in Tris-buffered saline, pH 7.4, (TBS) for 20 min and incubation with 1:50 normal horse serum in TBS, the mAb was applied, diluted in 0.1% bovine serum albumin in TBS. Excess antibody was removed by washing in TBS. The sections were then incubated with a biotinylated horse anti-mouse secondary antibody diluted 1:100 in TBS for 45 min. Excess secondary antibody was removed by washing in TBS before incubation for 30 min. with streptavidin diluted 1:100. Excess conjugate was then removed by further washing with TBS before visualization of antibody-binding sites with the streptavidin substrate kit. Finally, sections were counterstained for 30 s in Carazzins' hematoxylin, "blued" in saturated aqueous lithium carbonate, dehydrated in graded alcohols, cleared in xylene and mounted in DPX (BDH).

Microscopic analysis

All areas of each tissue block section, comprising at least 5 separate samples, were examined at a magnification of 200×. Immunohistochemical features were scored on a 5-point scale by two independent observers who were unaware of the clinical data. For example, the score of CD68 expression in the macrophages and giant cells was determined as follows: 0 = no positive cells, 1 = 1–15% stained, 2 = 16–50% stained, 3 = 51–85% stained, 4 = 86–100% stained.

Results

With HE staining, we saw many sequestra in the granulation tissue of all 66 cases, but no osteoblasts attached to them. Osteoclast-like MNCs

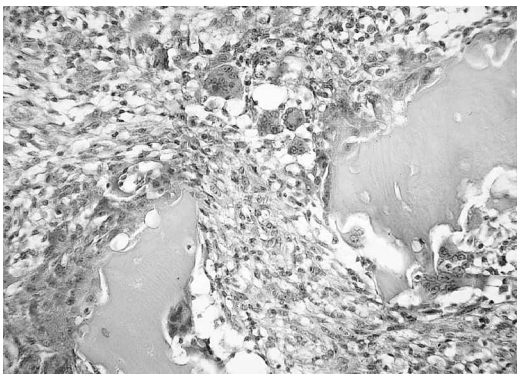

Figure 1. HE staining (×100) showed many sequestra in the granulation tissue in all 66 cases, but no osteoblasts were attached to them. Osteoclast-like MNCs tended to surround the sequestrum. Howship lacunae with many MNCs were present on the sequestrum, a result of the dissolution of bone mineral. MNCs were common far from the sequestrum. In the granulation tissues, fibroblasts and infiltrates of mononuclear cells, such as histiocytes, lymphocytes and plasma cells, were also seen, but rarely granulocytes.

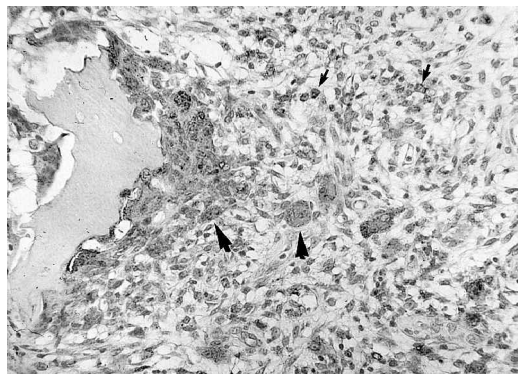

Figure 2. MNCs adjacent to the sequestrum in the granulation tissue of osteomyelitis are stained with anti-CD68 monoclonal antibody (×200) (large arrow). 10% of mononuclear cells were also positive to CD68. The positive cells were mononuclear histiocytic stromal cells (small arrow).

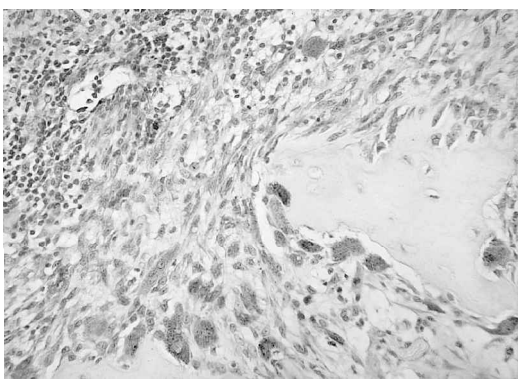

Figure 3. MNCs adjacent to the sequestrum in the granulation tissue of osteomyelitis are stained with anti-TRAP monoclonal antibody (× 200). No activity of TRAP was seen in mononuclear cells.

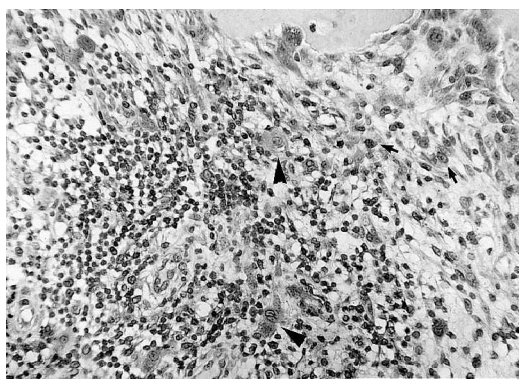

Figure 4. MNCs adjacent to the sequestrum in the granulation tissue of osteomyelitis stain with anti-cathepsin K monoclonal antibody (×200). Cathepsin K is expressed in most MNCs (large arrow) and in 50%–70% of mononuclear cells in the granulation tissue. The positive cells may contain osteoclast progenitor cells in addition to mononuclear histiocytic stromal cells (small arrow).

tended to surround the sequestrum. Howship lacunae with numerous MNCs were seen on the sequestrum, a result of the dissolution of bone mineral. MNCs were frequently found far away from the sequestrum (Figure 1). In the granulation tissue, fibroblasts and infiltrated mononuclear cells, such as histiocytes, lymphocytes and plasma cells, were present, but hardly any granulocytes.

In the immunohistochemical study, most MNCs expressed CD68 (Figure 2), TRAP (Figure 3) and cathepsin K (Figure 4), but not expressed CD11b (data not shown). Only about 10% of mononuclear cells were positive to CD68 but not TRAP. The positive cells were mononuclear histiocytic stromal cells. Cathepsin K was expressed in most multinuclear cells and in about 50%–70% of mononuclear cells (Table 3).

Table 3. Expression of CD68, TRAP, cathepsin K and CD11b in 12 cases of osteomyelitis

	MNCs					Histiocytic stromal cells					Osteoclast progenitor cells				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Antibody															
CD68					12					12	9	2	1		
TRAP				2	10	9	2	1			11	1			
cathepsin K				1	11	8	2	1	1					1	11
CD11b	11	1							4	8	12				

0 no positive cells, 1 1–15% stained, 2 16–50% stained, 3 51–85% stained, 4 86–100% stained.

Discussion

Our previous studies have suggested that MNCs resorb bone, and that MNCs in the synovial granulation tissue of patients with rheumatoid arthritis show TRAP activity and can form resorption pits on dentine slices in cell cultures (Fujikawa et al. 1996a, b, c).

Osteoclasts are acid phosphatase-positive cells and TRAP has been widely used as a specific histochemical marker of osteoclasts (Wilinson et al. 1993, Mulherin et al. 1996). Immunohistochemical studies indicate that TRAP activity occurs only in osteoclasts, but not in macrophages present in bone (Hammarstrom et al. 1971, Minkin 1982). The most distinguishing features of osteoclasts are the presence of calcitonin and vitronectin receptors and the ability to form resorption pits on dentine slices as a functional hallmark of mammalian osteoclasts (Ashton et al. 1993, Fujikawa and Athanasou 1998). Human macrophages, but not mature osteoclasts, expressed CD14, CD11a, CD11b, CD11c, CD16, and CD18 (Athanasou et al. 1990).

In this study, we examined specimens from patients with osteomyelitis. It is noteworthy that many MNCs seemed to cluster in the granulation tissue far away from and in contact with the sequestrum. These MNCs were shown immunohistochemically to originate from macrophages, as they were CD68 positive, and stained positive for TRAP activity and cathepsin K protein (Evans et al. 1997, Hummel et al. 1998). Thus MNCs in the granulation tissue far away from and in contact with the sequestrum resembled osteoclasts immunohistochemically. In the granulation tissue,only about 10% of mononuclear cells were positive to CD68 but not to TRAP. These cells were mononuclear histiocytic stromal cells. Cathepsin K was expressed in about 50%–70% of mononuclear cells. The positive cells should contain osteoclast progenitor cells in addition to mononuclear histiocytic stromal cells. As there are no living cells, like osteocytes, osteoclasts, or even osteoblasts in the sequestrum, it is possible that osteoclast-like MNCs in contact with the sequestrum originally form in the granulation tissue and infiltrate towards the sequestrum.

Osteoclasts are currently believed to be the only bone-resorbing cells responsible for bone remodeling and are formed from osteoclast progenitor cells. Hematopoietic cells in bone marrow proliferate and differentiate into mononuclear preosteoclasts and fuse with each other to form multinucleated osteoclasts involving cell-to-cell interaction with osteoblasts (Suda et al. 1992). It is thought that ODF is expressed on osteoblast/stromal cells in response to bone-resorbing factors, and that it induces osteoclastogenesis by signaling to osteoclast progenitors (Lancey et al. 1998, Yasuda et al. 1998). However, in the osteomyelitic sequestra there are no living cells including osteoblasts.

Matsuzaki and his associates confirmed the existence and function of a soluble osteoclast differentiation factor produced by osteoblasts (Matsuzaki et al. 1998). Takayanagi et al. (1997) showed that rheumatoid synovial cells contain both osteoclast progenitors and stromal cells which help them to differentiate. As in rheumatoid arthritis, there are presumably preosteoblasts in the granulation tissue in osteomyelitis, which are derived from stromal cells. These cells are recruited and differentiate into osteoblasts. It is well

known that osteoblasts are needed to recruit and activate osteoclasts. In osteomyelitis, some of the viable stromal cells should be able to activate osteoclasts. We strongly support the view that MNCs in the granulation tissue can also differentiate into osteoclasts without cell-to-cell interaction with osteoblasts on the bone surface.

In conclusion, immunohistochemically, MNCs far away from and in contact with the sequestrum of the granulation tissue were shown to have the same immunohistochemical characteristics as osteoclasts. Our results confirmed that MNCs in the granulation tissue of osteomyelitis have the same immunophenotype as osteoclasts which are resorbing necrotic bone. There is some evidence that MNCs in contact with the sequestrum develop in the granulation tissue and then directly infiltrate towards the sequestrum without cell-to-cell interaction with osteoblasts on the bone surface, as there are no living cells on the sequestrum.

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