Virchows Archiv A Pathol Anat (1992) 420:507–511

Virchows Archiv A
Pathological Anatomy and Histopathology

Usefulness of a novel monoclonal antibody against human osteocalcin in immunohistochemical diagnosis

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Received July 22, 1991 / Received after revision December 16, 1991 / Accepted January 14, 1992

Summary. A novel monoclonal antibody against human osteocalcin, recently established in our laboratory, was shown by immunoblotting and immunohistochemistry to react specifically with human osteoblasts. In the present study, the antibody was applied to the immunohistochemical diagnosis of human bone tumours, especially osteoblastic tumours. The antibody reacted with all 27 osteosarcomas. No positive reaction was found either in chondrosarcoma, giant cell tumours of bone, soft tissue tumours or epithelial tumours. A positive reaction was found preferentially in the cytoplasm of most of the osteosarcoma cells, but not in the extracellular matrix. Since the antibody reacted with formalin-fixed and paraffin-embedded tissues, it will be a useful tool for routine immunohistochemical diagnosis of osteoblastic lesions.

Key words: Human osteocalcin – Monoclonal antibody – Immunoblotting – Immunohistochemistry

Introduction

Osteocalcin, a low-molecular-weight calcium-binding protein, localizes preferentially to bone (Hauschka et al. 1975; Price et al. 1976), where it comprises 10–20% of non-collagenous protein. Although the exact physiological functions of osteocalcin are still not fully understood, the major role of osteocalcin is to bind to calcium ions and hydroxyapatites at the γ-carboxyglutamic acid (Gla) residues (Poser and Price 1979). Gla residues appear in osteocalcin by a post-translational carboxylation of glutamic acid (Glu) residues in the presence of vitamin K (Vermeer 1984). Human osteocalcin, consisting of 49 amino acids, contains three Gla residues (Poser et al. 1980), thus the protein appears to participate in the process of calcification of human bones.

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In a previous study, we isolated osteocalcin from human bones and generated a polyclonal antibody against osteocalcin (Ohta et al. 1989). The antibody was shown to react specifically with osteocalcin by immunoblotting, but a non-specific reaction was not ruled out. We have recently succeeded, for the first time, in establishing a monoclonal antibody against human osteocalcin (Ohta et al. 1991). Since the antibody was shown to localize preferentially in the cytoplasm of osteoblasts immunohistochemically, but did not react with the extracellular matrix of normal human bones, we expected it to be a helpful immunohistochemical diagnostic tool for osteoblastic tumours.

Materials and methods

The procedure for producing a monoclonal antibody against human osteocalcin has been described in a previous paper (Ohta et al. 1991). Briefly, human osteocalcin was purified from human bones according to the method of Gundberg with a slight modification (Gundberg et al. 1984; Ohta et al. 1989). Female BALB/c mice (6–8 weeks old) were immunized intraperitoneally once a week for 5 weeks with 50 μg osteocalcin. Four days after the last immunization, spleen cells from the immunized mice were fused with a mouse myeloma cell line (NS-1), at a ratio of 5:1. Approximately 2 weeks later, the hybridoma supernatants were screened for the presence of anti-osteocalcin antibody by enzyme-linked immunosorbent assay (Engvall 1980) and the antibody-producing clones were selected by a limiting dilution method. Five clones were obtained, but there was no difference in the immunohistochemical reactions (Ohta et al. 1991). Thus, the 10E8 monoclonal antibody was used in this study.

BALB/c mice were injected intraperitoneally with cloned hybridoma cells (10E8) and the ascitic fluid was used in the present immunohistochemical studies as the antibody, after passing through a sepharose 4B column coupled with goat anti-mouse immunoglobulin. The antibody was named OCL-1.

Bone and soft tissues were obtained at biopsy or surgery in Sapporo Medical College Hospital and Sapporo National Hospital. The material consisted of 27 cases of osteosarcoma, including 5 metastatic lesions to the lung (Table 1), 3 giant cell tumours of bone, 3 chondrosarcomas, 2 cases of osteomyelitis, 3 osteoid osteomas, 2 osteochondromas, 1 case of non-ossifying fibroma, and 11 soft tissue tumours. The soft tissue tumours included malignant

Table 1. Clinicopathological findings of osteosarcomas

1)	Location	
	Femur	11 cases
	Tibia	6 cases
	Humerus	4 cases
	Fibula	1 case
	Lung metastasis	5 cases
2)	Histological pattern	
	Osteoblastic type	16 cases
	Chondroblastic type	5 cases
	Teleangiectatic type	1 case
3)	Age	
9–35 years (average 16.9 years)		
4)	Sex	
	Male	14 cases
	Female	8 cases

Table 2. Immunoreactivity of monoclonal antibody with Human bone and soft tissues

Histology	No. of cases	No. of positive cases
Osteosarcoma	22	22
Osteosarcoma (metastasis)	5	5
Osteomyelitis	2	2
Osteoid osteoma	3	3
Giant cell tumour	3	0
Chondrosarcoma	3	0
Osteochondroma	2	0
Non-ossifying fibroma	1	0
Malignant fibrous histiocytoma	4	0
Liposarcoma	3	0
Rhabdomyosarcoma	2	0
Malignant haemangiopericytoma	1	0
Synovial sarcoma	1	0

fibrous histiocytoma, liposarcoma, rhabdomyosarcoma, malignant haemangiopericytoma, and synovial sarcoma. Twenty-two epithelial tumours obtained at autopsy or surgery were also examined.

The tissues were fixed in buffered 10% formaldehyde at room temperature for 2 days and routinely processed for embedding into paraffin.

For indirect immunoperoxidase staining, de-paraffinized sections were treated with methanol containing 0.6% hydrogen peroxide for 30 min, then incubated with 1% normal goat serum (Tago, Burlingame, Calif., USA) for 1 h at room temperature to inhibit non-specific binding. They were incubated with the monoclonal antibody for 1 h at room temperature. The other sections were incubated with normal mouse serum and served as a control. After washing with phosphate buffered saline (PBS, pH 7.4), the sections were reacted with biotinylated goat anti-mouse immunoglobulin (Tago, 1:400 dilution in PBS) and subsequently reacted with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif., USA) for 1 h at room temperature. After washing with PBS, the sections were treated with 3-3′diaminobenzidine in 5 mM TRIS hydrochloride buffer at pH 7.6, containing 0.01% hydrogen peroxide for 10 min, followed by the counterstaining with methyl green. A positive reaction was when more than 50% of cells reacted.

Results

Reactivity of the antibody with normal human bone has been described in a previous paper (Ohta et al. 1991). The antibody reacted with all 27 osteosarcomas, not only in the primary tumours, but also at the sites of metastasis to the lung. As shown in Table 2, the positive

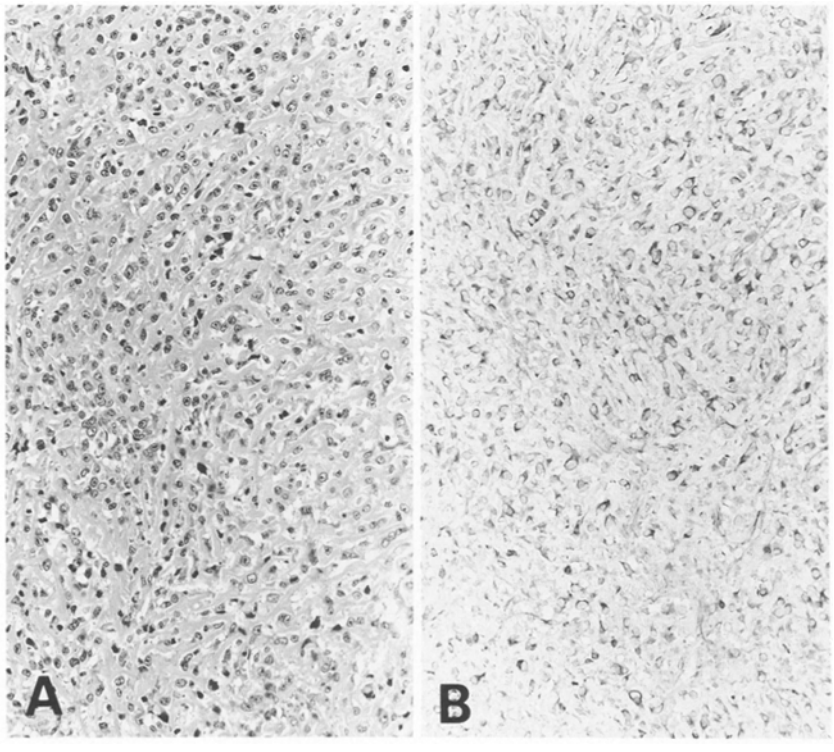

Fig. 1 A, B. Tissue sections of a human osteosarcoma (osteoblastic type). **A** Haematoxylin and eosin; **B** immunoperoxidase OCL-1, ×100

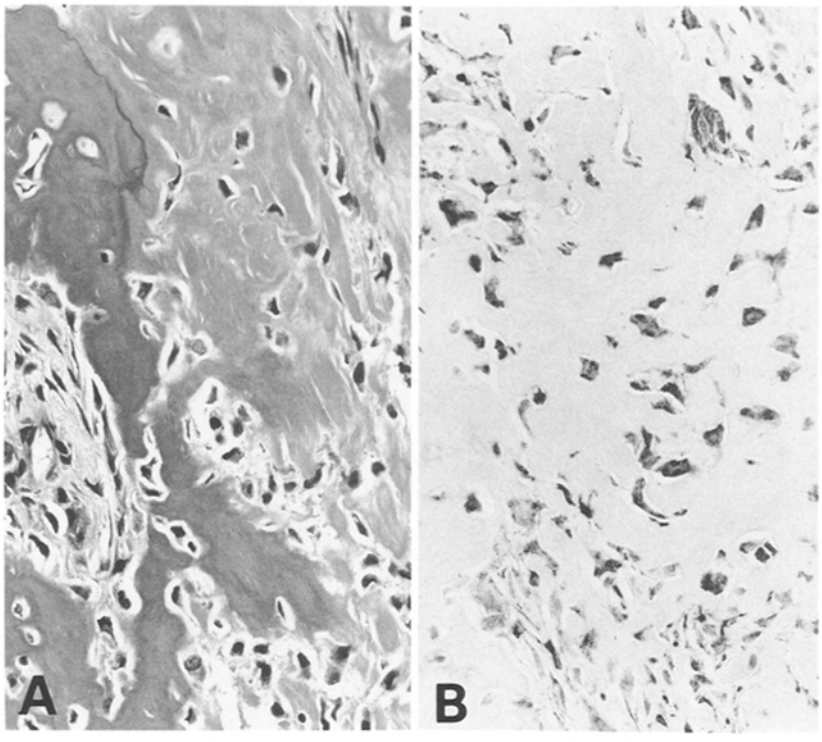

Fig. 2A, B. Tissue sections of a human osteosarcoma (osteoblastic type). **A** Haematoxylin and eosin; **B** immunoperoxidase OCL-1, ×400

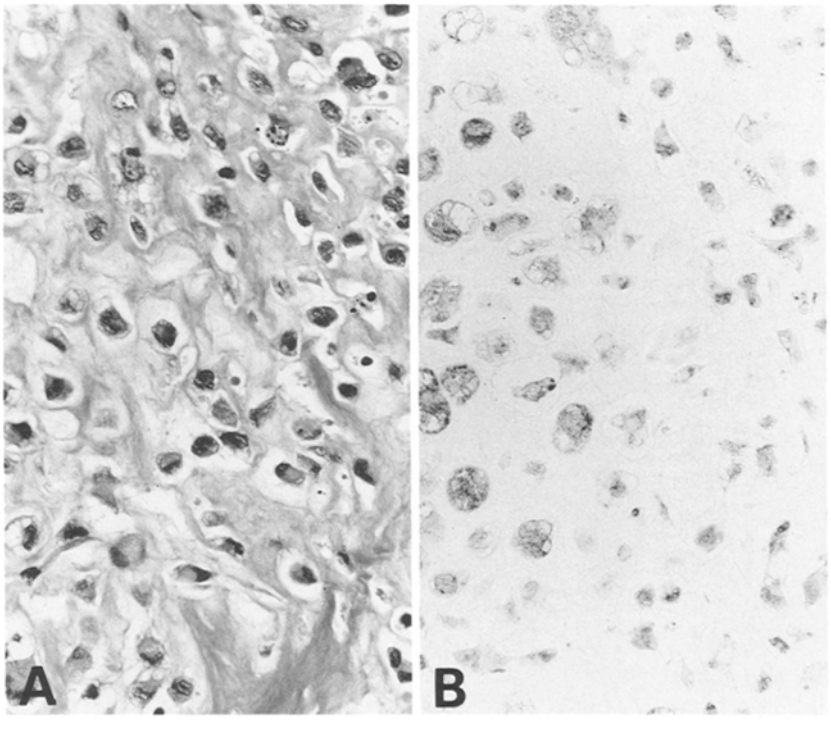

Fig. 3A, B. Tissue sections of a human osteosarcoma (chondroblastic type). **A** Haematoxylin and eosin; **B** immunoperoxidase OCL-1, ×400

reactions were found in more than 50% of tumour cells in all 27 cases, and the intensity of the reaction observed in each osteosarcoma cell was essentially uniform among individual cases. Osteocalcin was localized in the cytoplasm of sarcoma cells (Figs. 1–3), but not in the osteoid trabecula.

The immunoreactivity of the antibodies to other bone tumours not of an osteoblastic nature was also assessed (Figs. 4, 5). Two cases of osteomyelitis and 3 cases of osteoid osteoma showed a positive reaction only in osteoblast-like cells. Three cases of giant cell tumour of bone showed no immunoreactivity to the antibodies. Three cases of chondrosarcoma were also non-reactive. Eleven soft tissue tumours, including 4 malignant fibrous histiocytomas, 3 liposarcomas, 2 rhabdomyosarcomas, 1 case of malignant haemangiopericytoma and

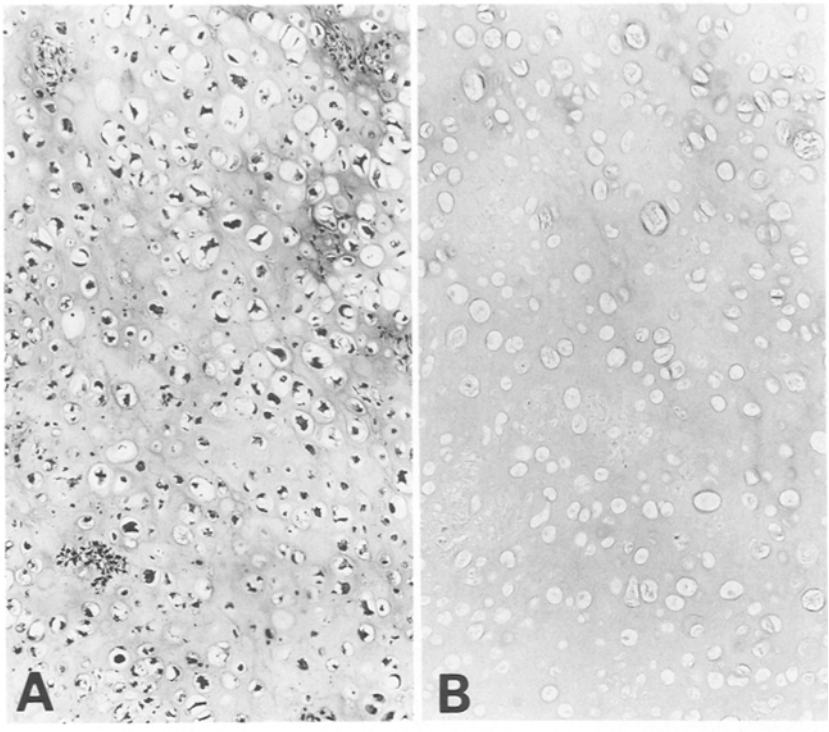

Fig. 4A, B. Tissue sections of a human chondrosarcoma. **A** Haematoxylin and eosin; **B** immunoperoxidase OCL-1, ×100

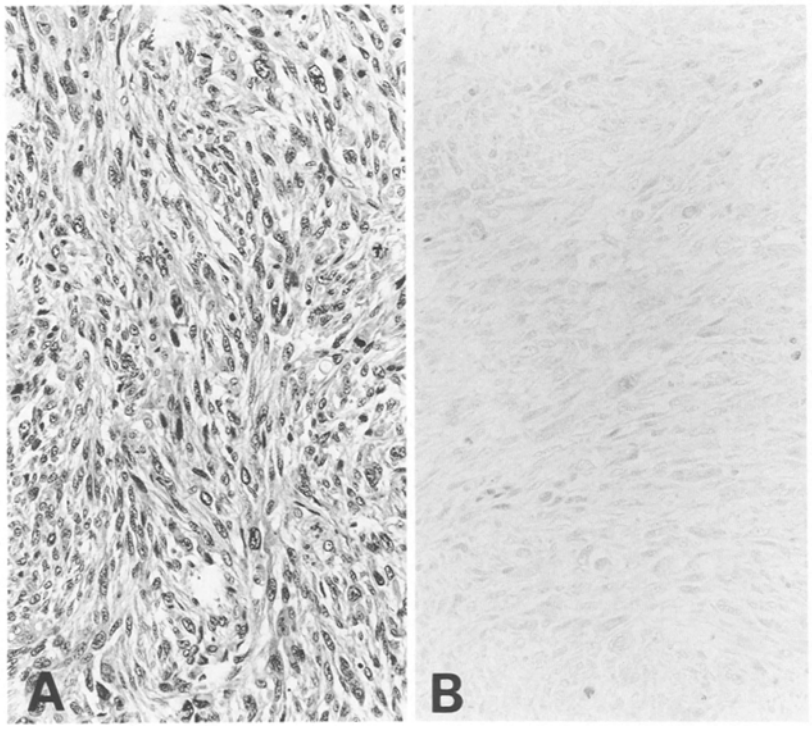

Fig. 5A, B. Tissue sections of a human malignant fibrous histiocytoma. **A** Haematoxylin and eosin; **B** immunoperoxidase OCL-1, ×100

1 case of synovial sarcoma, did not react with the antibody (Table 2).

Twenty-two epithelial tumours from various organs, including adenocarcinomas, squamous cell carcinomas, hepatocellular carcinomas, and transitional cell carcinoma, did not show positive reaction to the antibody (Table 3).

Discussion

This monoclonal antibody against human osteocalcin was shown to react preferentially with human osteocalcin by immunoblotting. The antibody reacted most intensely with osteoblasts in normal human bone but nor with osteoid trabecula. Although the reason for this is

Table 3. Immunoreactivity of monoclonal antibody with Human epithelial tumours

Organ	Histology	No. of cases	No. of positive cases
Lung	Adenocarcinoma	2	0
	Squamous cell carcinoma	2	0
Oesophagus	Squamous cell carcinoma	2	0
Stomach	Adenocarcinoma	5	0
Colon	Adenocarcinoma	3	0
Pancreas	Adenocarcinoma	2	0
Liver	Hepatocellular carcinoma	3	0
Urinary bladder	Transitional cell carcinoma	1	0
Uterus	Squamous cell carcinoma	2	0

not fully understood, we surmise that the epitope was masked because osteocalcin is bound to calcium ions and hydroxyapatites. Therefore, we extended the immunohistochemical studies to human neoplasms to assess whether the antibody can be utilized as an immunohistochemical diagnostic marker for tissues of osteoblastic origin.

Histochemistry for alkaline phosphatase has long been utilized in the diagnosis of osteosarcoma, but osteocalcin is more specific to bone. Although osteonectin and osteopontin are also typical proteins of bone, osteonectin may be localized in platelets (Stenner et al. 1986) and osteopontin in kidney and nervous tissues (Mark et al. 1988). Thus, osteocalcin is a more specific marker than osteonectin and osteopontin.

The results of the present study indicate that our antibody reacted strongly with osteosarcoma cells and was not influenced by the degree of osteoid formation. The antibody reacted with all osteosarcomas examined, but did not react with either giant cell tumour of bone or chondrosarcoma. The antibody reacted with 5 cases of chondroblastic osteosarcoma, but not with 3 cases of chondrosarcoma, probably because sarcoma cells in chondroblastic osteosarcoma have the function of bone formation, unlike the chondrosarcoma cells.

Positive reactions were limited to the cytoplasm of most, if not all, of the sarcoma cells in all cases of osteosarcomas. They were not found in the osteoid trabecula. These results are consistent with the recent observations presented by Vermeulen et al. (1989) that osteocalcin is localized in osteoblastic cells in normal and neoplastic bones, but not in osteoid trabecula of human osteosarcomas.

Our monoclonal antibody reacted not only in osteosarcoma, but also in benign tissues (normal osteoblast, osteomyelitis and osteoid osteoma). Thus the antibody cannot be used for the differential diagnosis of benign versus malignant tumours. However, our antibody reacted well with the formaldehyde-fixed and paraffin-embedded tissue sections and could be utilized widely for the routine immunohistochemical diagnosis of human tumours of osteoblastic origin.

The antibody is also expected to be useful for the serum diagnosis and clinical follow-up study of patients with osteosarcomas. It was shown that osteosarcoma cells in vitro secrete osteocalcin into the culture medium (Nishimoto and Price 1980; Price and Baukol 1980), and our preliminary studies have shown that the secreted osteocalcin is detected by immunoprecipitation with the antibody in the culture media of osteosarcoma cell lines. Further studies in this line are now in progress.

Acknowledgement. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

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