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Osteocalcin Expression in Primary Bone Tumors
— In Situ Hybridization and Immunohistochemical Study —

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Osteocalcin is one of the most abundant noncollagenous proteins found in adult bone. It is a highly conserved gamma-carboxyglutamic acid-containing protein that is believed to be produced exclusively by osteoblasts. In this study, intracellular and extracellular localization of osteocalcin in osteosarcoma was examined with anti-osteocalcin antibody and in situ hybridization using a synthetic oligonucleotide.

Immunohistochemically, osteoblastic osteosarcomas were all positive for osteocalcin. The chondroblastic osteosarcomas were positive on the neoplastic chondrocytes. The five fibroblastic osteosarcomas out of seven were positive for osteocalcin immunostaining over the neoplastic spindle cells. Five cases of osteoblastic osteosarcomas out of seven were positive for osteocalcin in situ hybridization. Two cases of chondroblastic osteosarcomas and three cases of fibroblastic osteosarcomas were positive for in situ demonstration of osteocalcin. The malignant tumor giant cells were positive for osteocalcin immunostaining in 83 %. They were also positive for in situ hybridization. The benign giant cells in five giant cell tumors and five aneurysmal bone cysts were negative for osteocalcin immunostaining. The benign giant cells in three chondroblastoma and three Paget's disease were positive for osteocalcin. In this study, the osteocalcin in situ hybridization and immunostaining has very important meaning for making differential diagnoses of, especially giant cell rich bone forming tumors.

Key Words : *Osteocalcin, Osteosarcoma, Giant cell tumor, In situ hybridization, Immunohistochemistry*

INTRODUCTION

Osteocalcin is a low molecular weight protein found abundantly in the extracellular matrices of collagenous mineralized tissues (Price, 1985 ; Lian and Gundberg, 1988 ; Hauschka et al., 1989). The presence of several residues of gamma-carboxyglutamic acid (Gla) enhances the ability of this protein to bind calcium ions (Hauschka and Gallop, 1977 ; Price et al., 1977 ; Hauschka and Carr, 1982 ; Svard et al., 1986) and promotes its adsorption onto hydroxyapatite (Hauschka et al., 1975 ; Romberg et al., 1986). Indeed, levels of osteocalcin in bone increase concurrently with advancing mineralization (Hauschka et al., 1975 ; Price and Baukol, 1980). Based on these characteristics, the potential role of osteocalcin in

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mediating certain events in the mineralization process has been extensively discussed in several recent reviews (Price, 1985; Lian and Gundberg, 1988; Hauschka et al., 1989). Despite significant advances in characterizing its physicochemical interactions with calcium ions and solid calcium-phosphate and its ability to act as a chemoattractant for peripheral blood monocytes (Malone et al., 1982; Mundy and Poser, 1983), mesenchymal cells (Lucas and Caplan, 1988), and cells with some of the characteristics of osteoclasts (Glowacki and Lian, 1987; Glowacki et al., 1991), the precise function of osteocalcin as a bone matrix component remains unclear. Immunohistochemical studies on extracellular localization of osteocalcin showed positive osteocalcin in the mineralized bone matrix and in the early foci of calcification of normal bone by light and electron microscopic examination combined with immunohistochemical staining (Bronckers et al., 1978; Groot et al., 1986; Camarda et al., 1987).

In this study, intracellular and extracellular localization of osteocalcin in osteosarcoma was examined with anti-osteocalcin antibody and in situ hybridization using synthetic oligonucleotide. The authors attempted to investigate the availability of osteocalcin immunohistochemical study and in situ hybridization for differential diagnosis of osteosarcoma.

MATERIALS AND METHODS

Thirty two osteosarcomas from 29 patients in various stages of differentiation were studied for immunohistochemistry. The tumors were subtyped according to their main component as 16 osteoblastic, 9 chondroblastic, and 7 fibroblastic. Six giant cell tumors, five aneurysmal bone cysts, three chondroblastomas, three Paget's diseases, and one osteochondroma were done for osteocalcin immunohistochemical staining for control. Twelve cases of osteosarcomas from 12 patients were studied for in situ hybridization for mRNA expression of osteocalcin. There were 7 osteoblastic type, two chondroblastic and three fibroblastic osteosarcomas. The antibody for osteocalcin was a generous gift from Larry Fisher (National Institute of Dental Research, National Institutes of Health, Bethesda, MD)and Lorraine A. Fitzpatrick, M.D. (Endocrine Research Unit, Mayo Clinic, Mayo Foundation, Rochester, MN). It was a bovine monoclonal IgG and could recognize human and bovine bone osteocalcin.

An osteocalcin antisense oligo-DNA probe was synthesized. It was a 50-mer complementary to the human mRNA sequence that begins with nucleic acid 237 (Kiefer et al., 1990). It was 5' GAA AGC CGA TGT GGT CAG CCA ACT CGT CAC AGT CCG GAT TGA GCT CAC AC 3'.

Immunohistochemical method

Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method (DAKO LSAB kit, Carpinteria, CA), as previously described. Briefly, 6mm paraffin sections were adhered to silanized slides (DAKO, Carpinteria, CA) and dried. After deparaffinization and rehydration, the tissue sections were incubated for five minutes with a 3 % hydrogen peroxide and blocking reagent. The sections were exposed to the primary antibodies (1 : 200 working dilution with DAKO antibody diluent) for 30 minutes at 37℃. After washing with TRIS-buffered saline (DAKO), biotinylated link antibody was applied for 15 minutes followed by streptavidin peroxidase for an additional 10 minutes. Color development was performed with substrate-chromogen (3-amino-9-ethylcarbazole) solution for 10 minutes.

In situ hybridization

^{35}S-ATP-labelled single-stranded antisense for cDNA was prepared with terminal deoxynucleotidyl transferase using a NEP-100 labelling kit (Du Pont, Boston, MA). ^{35}S-labelled probe was used for hybridization at a concentration of 5.6 kcpm/ml. Treatment of the slides and hybridization conditions were as previously described (Simmons et al., 1989)with our modification. After hybridization, the sections hybridized with osteocalcin were treated with 10mg/ml of RNAse in 5M NaCl, 1 M Tris, pH 8.0, 0.5 M EDTA at 37℃ for 30 minutes. Sections were washed twice in 2 X SSC and once in 1 X SSC and once in 0.5 X SSC. They were then dehydrated in graduated ethanol and dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1 : 1 with pure water. The dipped slides were placed in a well ventilated area for 4 hours to dry at room temperature, and were exposed at 4℃ in desiccated slide boxes. The exposed slides were developed in a D-19 developer for 5 minutes at room temperature, fixed in a fixative for 5 minutes and washed with water for 20 minutes. They were counterstained with hematoxylin-eosin.

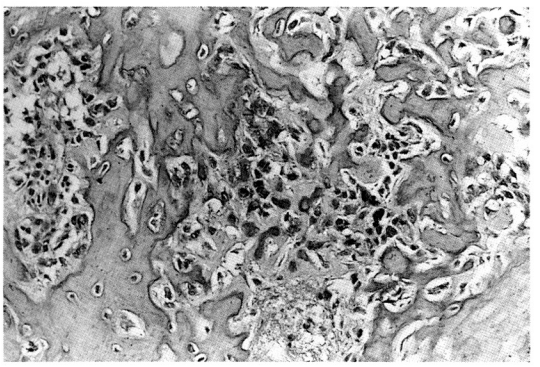

Fig. 1. Immunohistochemical expression of osteocalcin in osteoblastic osteosarcoma. The tumor cells between the neoplastic osteoid were positively stained (×200).

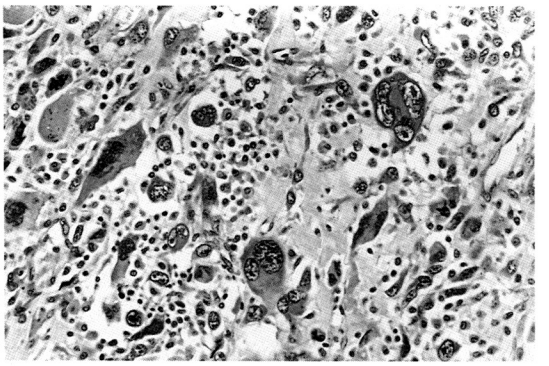

Fig. 2. The neoplastic tumor giant cells were stained intensely in their cytoplasms (×200).

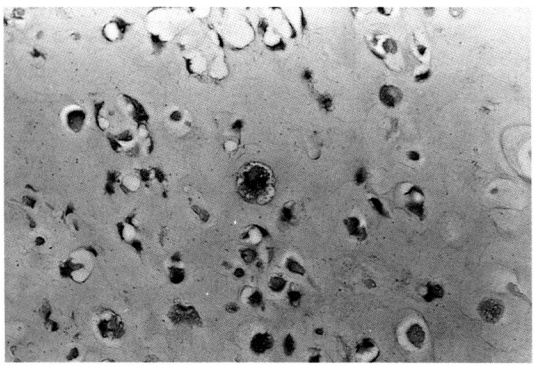

Fig. 3. The neoplastic chondrocytes in chondroblastic osteosarcoma showed positive staining in their cytoplasms (×200).

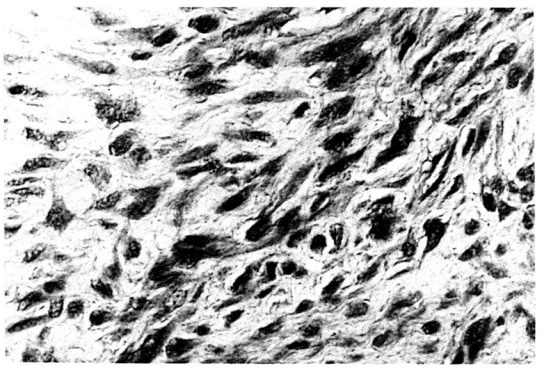

Fig. 4. The fibroblastic osteosarcoma showed intense cytoplasmic positive reaction (×200).

RESULTS

Immunohistochemical study of osteocalcin

We evaluated the expression of osteocalcin in paraffin embedded sections using immunohistochemistry. Twenty-nine cases out of 32 osteosarcomas with various histologic subtypes reacted positively with the antibody against human and bovine osteocalcin. The intensity of the reaction was variable, however, there were no differences between the tumor subtypes. The 16 classical osteoblastic osteosarcomas composed of tumor cells and neoplastic osteoid revealed one positive to three positive staining intensity in the cytoplasm of neoplastic osteoblasts (Fig. 1). In areas, scattered tumor giant cells were positive for osteocalcin immunostaining (Fig. 2). Eight chondroblastic osteosarcomas out of nine with abundant cartilage formation showed positive marking in the cytoplasm of neoplastic chondrocyte with variable staining intensities (Fig. 3). However, one chondroblastic osteosarcoma was negative. Five fibroblastic osteosarcomas out of seven revealed various staining intensities in the tumor cells (Fig. 4) and some of the tumor giant cells. Two fibroblastic osteosarcomas were negative for osteocalcin. The tumor giant cells were observed in twelve cases of osteosarcoma regardless of their subtypes. Among them 10 cases showed positive immunostaining for osteocalcin and two were negative.

Six cases of giant cell tumors were done for osteocalcin immunostaining. The giant cells were negative for osteocalcin (Fig. 5) in four cases and the remaining two cases showed focal grade 1 positive for osteocalcin. In these occasions, the giant cells

age- and gender-related changes in the distribution of 4 different staining patterns of osteocalcin. In newly apposed bone, the matrix did not show any osteocalcin staining, suggesting that osteocalcin deposition may be a relatively late event in the process of new bone formation (Groot et al., 1986). Although osteocalcin has been reported to be a bone-specific protein, the localization of osteocalcin in bone tumor has not been well demonstrated (Vermeulen et al., 1989). As for differential diagnosis of bone and/or soft tissue sarcoma, the specific antigens, such as S-100 protein for neurogenic tumor and cartilagenous tumor (Weiss and Dorfman, 1986) and alpha-1-antitrypsin and alpha-1-antichymotrypsin for malignant fibrous histiocytoma (du Boulay, 1982) have been used in immunohistochemical studies. These proteins and exzymes, however, are by no means tumor specific.

The purpose of this study is to investigate any specific markers of osteoid forming tumor. The osteoblastic osteosarcomas were all positive for osteocalcin immunostaining. The chondroblastic osteosarcomas showed positive staining on the neoplastic chondrocytes. We also performed osteocalcin immunostaining on the cartilaginous cap as a control. These benign chondrocytes were negative for osteocalcin. Therefore, osteocalcin immunostaining might be helpful in making differential diagnoses of chondroblastic osteosarcoma. We had three cases of osteosarcomas with dissociation between immunohistochemistry and in situ hybridization. In two cases of osteoblastic type, immunohistochemically osteocalcin was strongly positive, however, in situ hybridization was negative. One fibroblastic osteosarcoma showed a positive signal for in situ hybridization, but was negative for immunohistochemical osteocalcin staining. This might be due to the posttranslational and/or posttranscriptional modification of osteocalcin. It was reported that true osteoclast was not stained (Bronckers et al., 1978 ; Ohta et al., 1989), however the multinucleated cells observed in 12 cases of osteosarcoma were stained positively with osteocalcin antibody in 10 cases (83 %). The multinucleated giant cells in osteosarcoma also showed positive signal for in situ hybridization of osteocalcin oligonucleotide probe. The authors performed osteocalcin immunostaining on the benign giant cell tumors. Most of the benign giant cells were negative for osteocalcin. The giant cells shown in the aneurysmal bone cysts were negative for osteocalcin immunostaining. The giant cells in the chondroblastoma showed focal grade one positive staining. The osteoclastic giant cells in three cases of Paget's disease showed focal grade one positive staining. In 1992 Iwasaki et al.(1992) demonstrated that giant cells on the benign giant cell tumors were negative for osteocalcin immunostaining. However based on our results, immunohistochemical determination of osteocalcin can not be used as a marker of malignant giant cells versus benign osseous tumorous lesions with giant cells.

Park et al.(1995) performed osteonectin immunostaining and in situ hybridization on the various types of osteosarcoma. According to their study, osteonectin immunostaining was positive on the both neoplastic osteoblasts as well as chondrocytes. They did osteonectin staining on the chondrosarcomas also. The neoplastic tumor cells of the chondrosarcomas were also positive for osteonectin immunostaining. However, in situ demonstration of osteonectin on neoplastic chondrosarcomas was negative. The osteonectin were negative for tumor giant cells on both immunostaining and in situ hybridization. In summary, this osteocalcin immunostaining and in situ hybridization have very important meaning in making diagnoses of, especially giant cell rich bone tumors and also are helpful in making differential diagnoses of the osteoid forming tumors.

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