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Calcium pump expression in human bone and soft tissue tumours

Masahiko Kuroda[1], Hajime Horiuchi[1], Teruaki Oka[1], Tsuyoshi Ishida[1], Akira Ono[2], Masao Kawakita[2], Rikuo Machinami[1]

[1] Department of Pathology, Faculty of Medicine, University of Tokyo, Hongo 7–3–1, Bunkyo-ku, Tokyo 113, Japan
[2] Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3–18–22, Bunkyo-ku, Tokyo 113, Japan

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Abstract. Ninety-one cases of human bone and soft tissue tumours were studied for calcium pump expression by strepto-avidin-biotin immunohistochemical staining with a monoclonal antibody against sarcoplasmic reticulum calcium-ATPase (mAb6F5). Two out of 5 cases of embryonal rhabdomyosarcoma, 1 out of 5 cases of biphasic synovial sarcoma, 4 of 4 cases of chordoma and all of 3 chondrosarcoma cases were positive for mAb6F5. Although this novel monoclonal antibody can be used as a marker of myogenic tumours, the present positive result for endoplasmic reticulum calcium-ATPase (calcium pump) in other tumours including chordoma, chondrosarcoma and synovial sarcoma indicates a wider immunoreactivity. The findings further suggest that intracellular calcium may play an important role in cell proliferation and/or differentiation.

Key words: Endoplasmic reticulum – Sarcoplasmic reticulum – Calcium ATPase – Bone and soft tissue tumours – Immunohistochemistry

Introduction

The calcium-ATPase of mammalian skeletal muscle sarcoplasmic reticulum (SR) is a representative ion-transport enzyme, first isolated in 1970 (MacLennan 1970), and extensively investigated at the molecular level (MacLennan and Reithmeier 1982; MacLennan et al. 1985). Recently, it has been pointed out by using a cDNA probe for calcium-ATPase of cardiac muscle (MacLennan and Lytton 1988; Wuytack et al. 1989) that endoplasmic reticulum (ER) calcium-ATPase shares a high structural homology with SR calcium-ATPase. Furthermore, it was shown that the calcium-ATPase of ER regulates calcium charges during the cell cycle and contribute to various regulatory mechanisms of cellular functions (Gronda et al. 1992; Lodish et al. 1992). A recent study identified the existence of three distinct sarco(endo)plasmic reticulum calcium-ATPase (SERCA) genes. One of the genes encodes fast-twitch muscle (SERCA1) and the other slow-twitch/cardiac muscle (SERCA2), while the third gene (SERCA3) is expressed in both muscle and non-muscle tissues (Burk et al. 1989; Zarain-Herzberg et al. 1990). The SERCA 1 gene encodes two alternatively spliced transcripts which are developmentally regulated; the adult (SERCA1a) and neonatal (SERCA1b) skeletal forms. The SERCA2 gene also encodes two alternatively spliced calcium-ATPase isoforms; one is present in the SR of cardiac and slow-twitch muscles (SERCA2a) and the other is present in the ER of smooth muscle and non-muscle tissues (SERCA2b). The SERCA3 gene encodes calcium-ATPase isoforms of the ER of non-muscle tissues (Burk et al. 1989; Zarain-Herzberg et al. 1990).

We raised a monoclonal antibody 6F5(mAb6F5) reacting with the A1 fragment of SR calcium-ATPase of human skeletal muscle, and found it also recognizes ER calcium-ATPase in some epithelial tissues including the pituitary gland, parathyroid and islets of Langerhans (Kuroda et al. 1992). The present paper documents findings on the expression of this enzyme in bone and soft tissue tumours as assessed by immunohistochemical analysis and focuses attention on the usefulness of mAb6F5 for histopathological diagnosis of tumours in comparison with other cellular markers.

Materials and methods

Ninety-one cases of human bone and soft tissue tumours examined in the Department of Pathology, Tokyo University Hospital from 1965 to 1992 were analysed after review of the established diagnoses (Table 1). All were fixed in 10% formalin and embedded in paraffin. Sections were cut, 3 μm thick dewaxed and submitted to immunohistochemical study.

A novel mouse monoclonal antibody against SR calcium-ATPase (mAb6F5) was raised by the authors (Kuroda et al. 1992). The monoclonal antibody 6F5 was diluted 1:100 with phosphate buffered saline(PBS) containing 3% bovine serum albumin (BSA).

Correspondence to: M. Kuroda

Table 1. Calcium pump in expression bone and soft tissue tumours

Lesion	mAb 6F5 (Positive/total)
I. Benign lesions	
Leiomyoma	0/3
Epithelioid leiomyoma	0/1
Angioleiomyoma	0/1
Dermatofibroma	0/4
Haemangioendothelioma	0/3
Schwannoma	0/5
Neurofibroma	0/4
Pigmented neurofibroma	0/4
Fibrous dysplasia	0/5
Chondroblastoma	0/2
Osteoblastoma	0/1
Giant cell tumour	0/3
Brown tumour	0/1
II. Borderline/malignant	
Osteosarcoma	0/5
Chondrosarcoma	3/3
Leiomyosarcoma	0/4
Dermatofibrosarcoma protuberans	0/3
Malignant fibrous histiocytoma	0/5
Malignant haemangioendothelioma	0/2
Angiosarcoma	0/1
Malignant Schwannoma	0/1
Synovial sarcoma	1/5
Rhabdomyosarcoma	2/5
Clear cell sarcoma	0/3
Epithelioid sarcoma	0/3
Paraganglioma	0/6
Chordoma	4/4
Primitive neuroectodermal tumour	0/1
Alveolar soft part sarcoma	0/3
Total	10/91

Table 2. Primary antibodies: their sources and working dilutions

Antibody	Source	Dilution
6F5	(Kuroda et al. 1992)	1:100
KL-1	Immunoteck	1:100
AE1/AE3	ICN ImmunoBiologicals	1:100
EMA	Dako Corp	1:100
CEA	Dako Corp	1:50
Vimentin	Dako Corp	1:100
Desmin	Dako Corp	1:100

Other antibodies used and their working dilutions are listed in Table 2. Control incubations were performed with pre-immune mouse sera. Autoclave pre-treatment (Shin et al. 1991) of 3 μm thick sections was performed before immunolabelling with mAb6F5. All sections were treated with methanol containing 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity prior to staining. PBS containing 10% normal rabbit serum was added for 30 min to block non-specific binding. After its removal, all the sections were incubated with the mAb at the appropriate concentration achieved by dilution of stock mAb with PBS containing 3% BSA. A commercial kit for the strepto-avidin-biotin-peroxidase complex (SAB) method (Histofine, Nichirei, Tokyo, Japan) (Zuo-Rong et al. 1988) was applied for the immunoperoxidase staining. Sections were scored for staining reaction on a scale from − to 4+; −: no immunostaining, 1+: 1% to 25% positive cells, 2+: 26% to 50% positive cells, 3+: 51% to 75% positive cells, 4+: >75% positive cells.

SR membranes were purified from human skeletal muscle and rabbit fast-twitch muscle according to the method of Kawakita (Kawakita et al. 1980). Microsomes from pig liver was prepared as described by Prpic et al. (1984).

The purified SR fraction of human skeletal muscle containing calcium-ATPase molecules at high concentration and the microsomal fraction of pig liver were stored at a protein concentration of 15 mg/ml at −80° C. For SDS-PAGE, some microsomal preparations were mixed with 2× sample buffer and analysed according to the method of Laemmli (1970) and electrophoretically transferred to nitrocellulose filter papers. Filter papers were incubated in TRIS-HCl buffer (pH 7.5) containing 5% dried milk, 150 mM sodium chloride and 0.025% Tween 20 (TBS-T) at 4° C overnight to block non-specific binding of mAb to the papers. Then, the filter papers were incubated for 1 h at room temperature with mAb6F5 diluted 1:100 in TBS-T and washed three times with the same solution for 10 min. Control incubations were performed with pre-immune mouse sera. Finally the filter papers were incubated for 1 h with affinity purified goat anti-mouse whole Ig conjugated with peroxidase, followed by several washes in TBS-Tween, and developed with chloronaphthol.

Results

Western blotting revealed that mAb6F5 reacted not only with the SR calcium-ATPase from human skeletal muscle but also with peptides from the microsomal fraction of pig liver expressing SERCA2b mRNA. The reaction was revealed by a main band at 110 kDa (Fig. 1).

The results of immuno histochemistory for 91 cases of bone and soft tissue tumours are summarized in Table 1. Two of 5 cases of rhabdomyosarcoma, 1 of 5 cases of

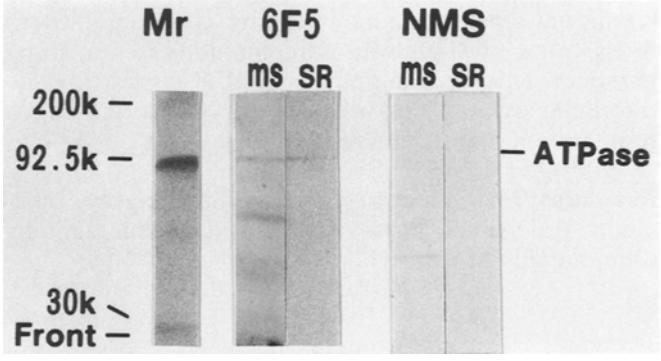

Fig. 1. Binding of mAb6F5 against calcium-ATPase to human skeletal muscle SR and pig liver. Microsomes(*ms*) from pig liver and SR membranes of human skeletal muscle(*SR*) were separated on 10% SDS-PAGE, and transferred to a nitrocellulose membrane sheet. 6F5 reacted with the calcium-ATPase molecule both in microsome fractions from pig liver and in SR showing a main band at 110 kDa. No reaction products were observed in the negative control study using normal mouse serum (*NMS*). Aliquots (2 μg) of microsomal and SR membranes were applied to each lane

Table 3. Immunohistochemical data for rhabdomyosarcomas

Case	Age/Sex	Site	Type	6F5	Desmin
1	7/M	Orbit	Em	4+	4+
2	14/F	Forearm	Em	4+	4+
3	18/M	Thigh	Em	−	4+
4	25/M	Thigh	Em	−	−
5	30/M	Buttock	Al	−	−

Em, Embryonal type; Al, alveolar type; −, no immunostaining; 4+: >75% positive cells

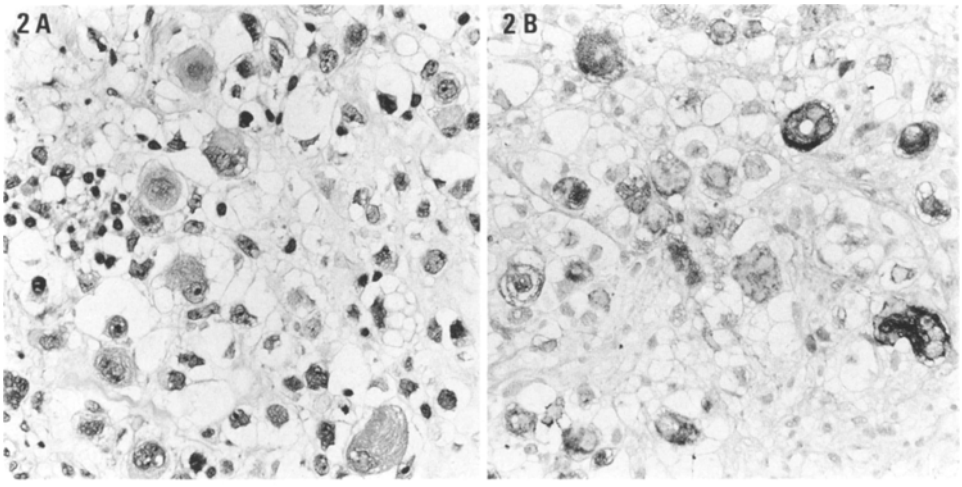

Fig. 2. A. Embryonal rhabdomyosarcoma (case 1). Undifferentiated round cells and scattered rhabdomyoblasts are seen (H & E, ×343). **B.** Immunostaining of the same tumour reveals mAb6F5 immuno-reactivity in the cytoplasm of rhabdomyoblasts (×343)

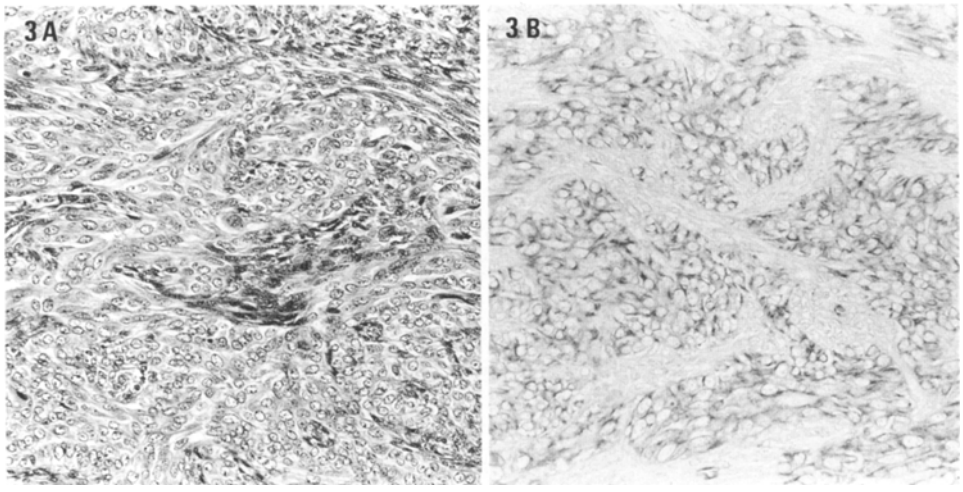

Fig. 3. A. Biphasic synovial sarcoma (case 3), showing transition between pale-staining epithelial and dark-staining spindle cell elements (H & E, ×274). **B.** The same tumour exhibiting cytoplasmic immunostaining for mAb6F5 in most of the epithelial cells (×343)

Table 4. Immunohistochemical data for synovial sarcomas

Case	Age/Sex	Site	Type	6F5		KL-1		AE1/AE3		EMA		CEA		Vim	
				EP	SP	EP	SP	EP	SP	EP	SP	EP	SP	EP	SP
1	20/M	Thigh	B	−	−	2+	+	2+	+	4+	+	−	−	+	4+
2	23/F	Lower leg	B	−	−	3+	+	2+	+	4+	+	−	−	+	4+
3	52/M	Thigh	B*	2+	−	4+	+	4+	+	4+	+	+	−	+	4+
4	59/F	Lower leg	ME	−	−	3+	+	2+	+	4+	+	−	−	+	4+
5	33F	Hip	MF		−		−		−		−		−		4+

EMA, Epithelial membrane antigen; CEA, carcinoembryonic antigen; Vim, vimentin; Ep, epithelial component; Sp, spindle cell component; B, biphasic type; ME, monophasic epithelial type; MF, monophasic fibrous type; *, with squamous differentation; −, no immunostaining; +, 1% to 25% positive cells; 2+, 26% to 50% positive cells; 3+, 51% to 75% positive cells; 4+, >75% positive cells

synovial sarcoma, all 4 of the cases of chordoma and all 3 cases of chondrosarcoma showed positive staining with mAb6F5. Of the rhabdomyosarcomas, only 2 out of 4 cases of embryonal type were positive for mAb6F5, positive reactivity being present in the cytoplasm of undifferentiated round cells and rhabdomyoblasts and being particularity strong in the latter (Fig. 2). Of 3 cases of rhabdomyosarcoma which were positive for desmin 2 were also positive for mAb6F5 (Table 3). In the synovial sarcomas, mAb6F5 reacted with biphasic type epithelial components. In the gland-like structures, positive staining was found mainly along the apices of the cells and also in the lumina. As a consequence of this staining pattern, lumina that are difficult to identify by routine light microscopy could be visualized by this monoclonal antibody (Fig. 3). The epithelial components of such cases were also positive for various epithelial markers including AE1/AE3, KL-1, EMA and CEA (Table 4). In one case foci of squamous differentiation which did not react with mAb6F5 were found to be positive for AE1/AE3, KL-1 and EMA. As for chordomas and chondrosarcomas, all the lesions investigated demonstrated

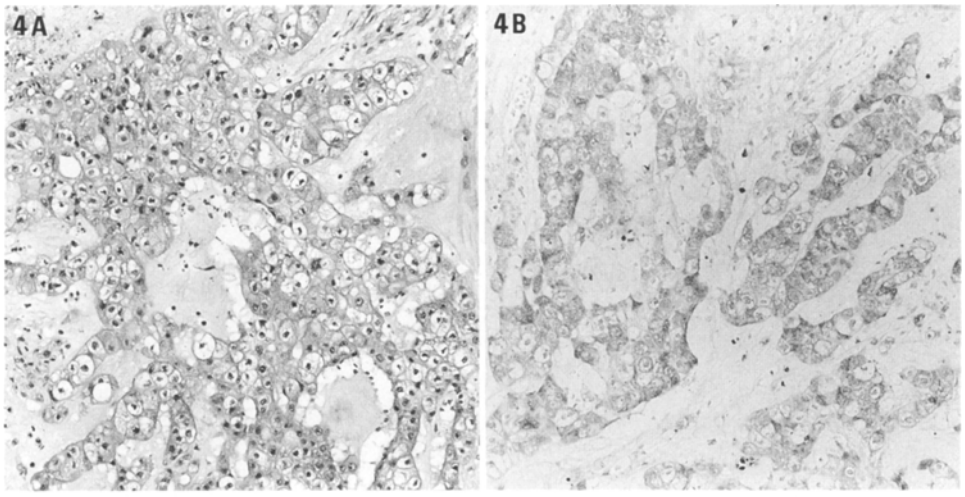

Fig. 4. A. Histology of chordoma (case 3) illustrating the cord-like arrangement of tumour cells and extracellular mucinous materials (H & E, ×137). **B.** Chordoma cells are strongly positive for mAb6F5. The reactivity is present diffusely in the cytoplasm (×137)

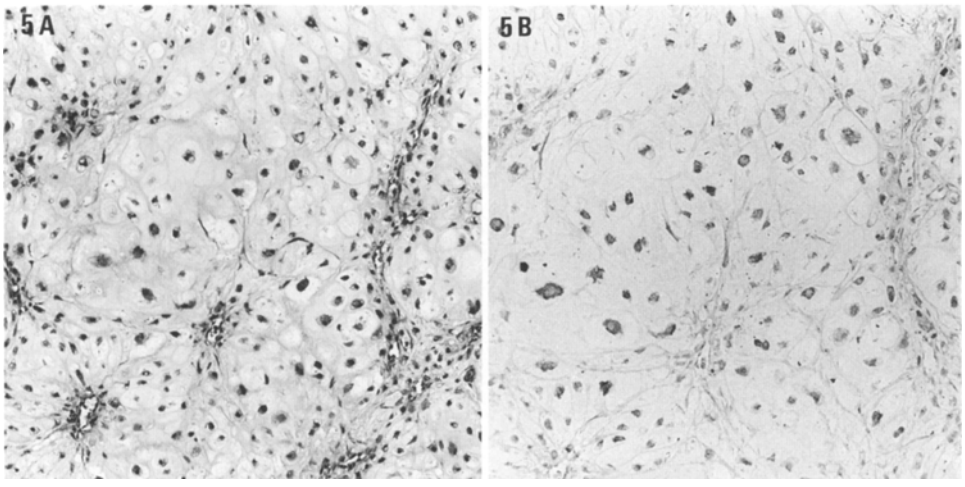

Fig. 5. A. Chondrosarcoma (case 2). Lobular architecture, increased cellularity, chondrocytes with enlarged nuclei and some binucleate cells are seen. (H & E, ×171). **B.** Same tumour showing immunostaining for mAb6F5. The immuno-reactivity is present in the perinuclear cytoplasm. (×137)

Table 5. Immunohistochemical data of chordoma

Case	Age/Sex	Site	6F5	KL-1	EMA	Vim	CEA
1	40/M	Sacrum	4+	4+	3+	3+	–
2	50/F	Sphenoid sinus	4+	4+	4+	3+	–
3	44/F	Cervical vertebrae	4+	4+	4+	3+	–
4	27/F	Ethmoid sinus	4+	4+	4+	3+	–

EMA, Epithelial membrane antigen; Vim, vimentin; CEA, carcinoembryonic antigen; –, no immunostaining; 3+, 51% to 75% positive cells; 4+, >75% positive cells

binding of mAb6F5. The immuno-reactivity in both tumour types was present diffusely in the cytoplasm and most of the tumour cells were positive (>80%) (Figs. 4, 5). The cases of chordoma were all positive for KL-1 and EMA but showed no immunoreactivity for CEA (Table 5). The cytoplasm of physaliferous cells was also positive for mAb6F5. Control immunostaining gave negative results in all cases.

Discussion

The present results clearly demonstrated a variety of bone and soft tissue tumours to be immunoreactive with mAb6F5 against calcium-ATPase. Calcium is believed to play an important role in cytoplasmic signal transduction not only in muscle cells but also in non-muscle cells. Although it has been postulated that the endoplasmic reticulum of non-muscle cells constitutes a cytoplasmic calcium pool equivalent to the SR of muscle cells, little is known about the distribution of human SR type calcium-ATPase in tumour tissues. We earlier demonstrated immunohistochemically that mAb6F5 reacts not only with SR calcium-ATPase but also with calcium-ATPase of the pituitary gland, parathyroid and islet of Langerhans as documented in a previous paper (Kuroda et al. 1992). Furthermore, western blot analysis in the present study revealed mAb6F5 to react with a 110 kDa protein of microsomal membranes from pig liver which express the SERCA2b gene. This result indicates that mAb6F5 indeed reacts with calcium-ATPase of ER.

There are a number of reports concering expression of SR calcium-ATPase in rhabdomyosarcomas (Kahn et al. 1983; Krenacs et al. 1990). However, nothing has been described of the expression of ER calcium-ATPase in bone and soft tissue tumours. This was the rationale for the present study of expression of SR and ER calcium-ATPase using a novel monoclonal antibody (mAb6F5).

All 5 cases of rhabdomyosarcoma including 2 calcium-ATPase positive cases, bound antibody against

desmin. The fact that the 2 cases which reacted with both mAb6F5 and anti-desmin antibody belonged to the embryonal type suggest that our monoclonal antibody might be useful to some extent for differential diagnosis. The applicability of antibody against calcium-ATPase of SR as a marker for rhabdomyosarcoma has already been stressed (Kahn et al. 1983; Krenacs et al. 1990).

Synovial sarcomas are malignant soft tissue neoplasms which primarily occur in close association with the tendon sheath, bursae, or joint capsule. It is generally accepted today that they are derived from primitive mesenchymal precursor cells (Katenkamp and Raikhlin 1985) with a capability of differentiation toward both epithelial and fibroblast-like cells. Hence it has been proposed by Miettinen and Virtanen (1984) and Ghadially (1987) that they be termed soft tissue carcinosarcomas. Five primary synovial sarcomas including 3 biphasic, 1 spindle cell monophasic and 1 epithelial monophasic cases were examined in this study and mAb6F5 binding was limited to the epithelial component of one of the biphasic lesions. The epithelial components in this case were also positive for KL-1, AE1/AE3, EMA and CEA.

The fact that antigens recognized by mAb6F5 were expressed in all 4 cases of chordomas investigated, along with KL-1, EMA and vimentin is of interest. Chordomas are rare neoplasms believed to arise from notochordal remnants and are thus itself thought to have the same dual epithelial and mesenchymal nature as notochord (Heaton and Turner 1985; Salisbury and Isaacson 1985; Hruban et al. 1990).

The positive reaction for mAb6F5 found in the cytoplasm of chondrosarcoma cells was also consistent with the documented expression of calcium-ATPase in chondrocytes in the fetus (Kuroda et al. 1992).

There was no relationship between proliferation markers (PCNA-PC10) and mAb6F5 positive cells (data not shown).

In conclusion, the present investigation confirmed and extended the finding that mAb6F5 reacts with rhabdomyosarcomas and tumours with epithelioid features other than epithelioid sarcomas. The results of western blot analysis, the observed positive labelling of synovial sarcomas, chordomas and chondrosarcomas indicates an abundance of ER calcium-ATPase in these tumours, analogous to the expression of SR calcium-ATPase in rhabdomyosarcomas.

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