J Orthop Sci (2003) 8:554–559
DOI 10.1007/s00776-003-0663-5

Journal of
Orthopaedic Science

Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line

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Abstract Because of the difficulty of developing pairs of osteosarcoma cell lines and cytotoxic T lymphocytes (CTLs), no osteosarcoma tumor antigens that are useful for antiosteosarcoma immunotherapy have yet been identified. In parallel with continuous attempts to develop such pairs from osteosarcoma, we employed serological identification using a recombinant expression cloning (SEREX) method to identify B cell-defined antigens. Consequently, a human osteosarcoma cell line, OS2000, was established from a primary osteosarcoma of a patient cured of hereditary retinoblastoma. Repetitious in vitro stimulations by OS2000 cells to the autologous peripheral T cells induced cytotoxic activity in the autologous osteosarcoma cells but not in the nontumor cells. The cytotoxicity was inhibited by anti-HLA class I monoclonal antibody. SEREX analysis revealed that autologous humoral immunity reacted to two proteins expressed in OS2000. One was the self HLA-Cw*0102 molecule, and the other was wild-type smooth muscle myosin light chain (SMMLC). However, no antigenicity of these proteins was seen versus the sera of the other patients. In conclusion, our results demonstrated the presence of host cellular and humoral immune responses to autologous osteosarcoma cells. This offered the opportunity to identify osteosarcoma antigens recognized by autologous immunity.

Key words Osteosarcoma · Cytotoxic T lymphocytes · CTL · Tumor antigen · SEREX

Introduction

Aggressive treatment for osteosarcoma, such as radical resection and cytotoxic drugs, has improved clinical outcomes. However, the prognosis for patients with osteosarcoma and other cancers, particularly those who do not respond to chemotherapy, remains poor. Recently, as a new treatment modality for malignant tumors, many investigators have focused on specific immunotherapy that utilizes cellular or humoral immune responses.[5] Tumor antigen recognized by the cytotoxic T lymphocyte (CTL) is the principal prerequisite for the development of antigen-specific cancer immunotherapy. Such antigenic peptides with the ability to induce CTLs have been identified in melanomas, and their therapeutic effectiveness has been demonstrated in some immunotherapeutic clinical trials.[10,14] However, no such antigenic peptides have been identified in bone or soft tissue sarcomas so far.

Most of the tumor antigens were identified in the context of major histocompatibility complex (MHC) class I molecules using autologous pairs, CTLs and autologous tumor cell lines; and a certain number of antigenic peptides reacting to CD8+ CTLs have been determined. In terms of identifying antigens with this approach, especially from cases of osteosarcoma, there is a serious obstacle: Establishing a cell line and inducing autologous CTLs are extremely difficult tasks, which is why no antigens have been identified from osteosarcoma.

Meanwhile, some tumor antigens have been identified utilizing the antibody response to tumor antigens. That is the SEREX method, serological identification by recombinant expression cloning. With this method, all proteins produced in a tumor cell are recombinant using the cDNA expression library and screening with cancer patients' sera. Without cell line–CTL pairs, SEREX facilitates easy identification of B cell-defined tumor antigens. Importantly, some antigens identified by SEREX are simultaneously targets of CTLs,[15] suggesting that SEREX is a useful tool for defining antigens, particularly those from sarcomas in which the establishment of cell lines and CTLs is extremely difficult.

In an attempt to overcome this difficulty of establishing cell line–CTL pairs of human osteosarcoma, we tried to induce cytotoxic T lymphocytes and simultane-

Offprint requests to: Y. Nabeta
Received: December 16, 2002/Accepted: March 11, 2003

ously performed SEREX analysis. We introduced a new human osteosarcoma cell line, OS2000, and examined the immunogenicity of OS2000 against both cellular and humoral autologous immunity.

Materials and methods

Preparation of tumor and blood samples

Between April 1997 and March 2000, consecutive patients with a putative diagnosis of osteosarcoma gave informed consent to provide tumor specimens and blood samples according to the institutional guidelines for the use of human subjects in research. Tumor specimens and peripheral venous blood samples were obtained at biopsy. The tumor specimens were minced into small pieces (approximately 2 mm diameter) and separately cultured with Iscove's modified Dulbecco modified Eagle medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) containing 15% fetal calf serum in a 5% CO_2 incubator. When cells had grown to confluence, half of them were removed with 0.25% trypsin and passaged. Lymphocytes were isolated from blood samples using Ficoll-Conray density gradient centrifugation and stored at −80°C until use. If indicated, T cells and B cells were purified from peripheral lymphocytes, respectively, using a nylon wool column. T cells were subjected to CTL induction as described below, and B cells were infected with Epstein Barr (EB) virus as described elsewhere.[6]

Cell lines

Among the tumor cell cultures tried, one has been persistently maintained for more than 1 year and thus was regarded as a cell line, designated OS2000. EB-virus-transformed B cells were also established from the blood sample of the same patient and designated LCL-OS2000. The osteosarcoma lines HOS and NY were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). A muscular fibroblast line was developed from the primary culture of a normal quadriceps muscle that had been obtained from a biopsy specimen of malignant fibrous histiocytoma from a 44-year-old man. An osteosarcoma cell line, KIKU, was established in our laboratory. A leukemia cell line, K562, was purchased from American Type Culture Collection (Rockville, MD, USA). These cell lines were maintained in RPMI1640 containing 10% fetal bovine serum and antibiotics.

In vivo tumorigenicity and histological analysis

OS2000 cells (1×10^7) were injected subcutaneously into the back of a 6-week-old severe combined immunodeficiency (SCID) mouse, C.B-17/IcrCrj-scid (Charles River Japan, Yokohama, Japan) that had been irradiated (30 Gy) beforehand. On day 12 the mouse was killed and the tumor specimen removed. The specimen was fixed in 10% phosphate-buffered formalin for 24 h, embedded in paraffin, and sections prepared. The sections were stained with hematoxylin and eosin (H&E) and examined microscopically.

Detection of osteocalcin and type I collagen mRNAs

The expression of osteocalcin and type I collagen mRNAs in the OS2000 cells was examined using the reverse transcriptase-polymerase chain reaction (RT-PCR). In experiments to determine the expression of osteocalcin, OS2000 cells were cultured with and without 1,25$(OH)_2$ vitamin D_3 [1,25$(OH)_2D_3$] (10^{-8} M) for 7 days prior to extraction of total RNA. Total RNA was isolated from 10^7 cells of the OS2000 line with ISOGEN (Nippon Gene, Toyama, Japan). The first strand cDNAs were synthesized with 1 mg of total RNA using the SUPERSCRIPT preamplification system (GIBCO BRL). Target cDNAs were amplified by PCR with KOD Dash polymerase (Toyobo, Tokyo, Japan) and each gene-specific primer set. Primers used for amplification of type I collagen were as follows: forward, 5′-GGTGGTGGTTATGACTTTGGTT-3′; reverse, 3′-TGGACTTTTGTAGGGTCGGTTC-5′.[18] Primers for osteocalcin were as follows: forward, 5′-CATGAGAGCCCTCCACA-3′; reverse, 3′-AGAG CGACACCCTAGAC-5′.[13] PCR amplification was performed in a GeneAmp thermal cycler (Perkin-Elmer). The thermal cycle was initiated with 3 min of denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 2 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Reaction products were analyzed by electrophoresis in 1.2% agarose gels with ethidium bromide staining.

CTL induction and cytotoxicity assay

In vitro CTL induction was performed as described elsewhere.[16] Peripheral lymphocytes of the OS2000 patient were thawed from the frozen stocks, and 5×10^6 T cells were cultured with 5×10^5 cells of the 30-Gy-irradiated OS2000 in AIM-V medium (Life Technologies, Gaithersburg, MD, USA) with recombinant human interleukin-2 (rIL-2) 10 U/ml (gift from Takeda Pharmaceuticals, Osaka, Japan) in 24-well flat-bottom culture dishes. Three days later the final concentration of IL-2 in the cultivation was raised to 200 U/ml. At intervals of more than 10 days, stimulation of the T cells by OS2000 was repeated after reducing the IL-2 concentration to 10 U/ml on the previous day. The same

stimulations were repeated at least five times, each time after an interval of at least 10 days.

The cytotoxic activity of the stimulated T cells was measured with the conventional ^{51}Cr-releasing assay.[16] Cell lines used as targets were OS2000, autologous fibroblasts, and K562. The autologous fibroblasts were obtained by chance while culturing the biopsy specimens of the OS2000 patient. The target cells were labeled with 100 mCi of ^{51}Cr for 1 h at 37°C. The stimulated T cells were mixed with the labeled target cells in the wells at a concentration of 5×10^3 cells/well. After a 6-h incubation at 37°C, the ^{51}Cr level in the supernatant of the culture was measured by quantification in an automated gamma counter. The percentage of specific cytotoxicity was calculated as the percentage of specific ^{51}Cr release: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). In blocking experiments, T cells were incubated with anti-HLA class I antibody W6/32 (American Type Culture Collection) for 30 min at 37°C before the cytotoxicity assay.[9]

SEREX analysis

Construction of cDNA library in bacteriophage lambda vector

Total RNA was isolated from 10^7 cells of the OS2000 line with ISOGEN (Nippon Gene), and poly(A) RNA was selected from the total RNA with PolyATtract (Promega, Madison, WI, USA). From 5 mg of the poly(A) RNA, cDNA was synthesized using a cDNA synthesis kit (Stratagene, La Jolla, CA, USA), then ligated into ZAP Express (Stratagene) lambda phage vector. The titer and mean insert size of the constructed cDNA library were 3×10^6 pfu/ml and 1.5 kbp (200–3000 bp), respectively.

Serological screening of expression library with autologous serum

The constructed library was transfected into strain XL-1 Blue MRF in agar, plated, and cultured at 42°C for 5 h. The filters, impregnated with isopropylthio-β-D-galactoside (IPTG), were laid on top of the developing plaques on the agar surface. The filters were peeled off after 4 h of incubation and rinsed in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). Subsequently, they were agitated in PBS containing 0.05% Tween 20 and 5% nonfat dried milk (blocking buffer) for 1 h. The filters were then treated by the autologous serum, which was pretreated with *Escherichia coli* for absorption of anti-*E. coli* natural antibodies and diluted in the blocking buffer at 500-fold dilution. After rinsing three times in PBS-T, 2000-fold diluted horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin G (IgG) antibody was treated. Antibodies binding to proteins on the filter surface were detected by 3,3′-diaminobenzidine (DAB) and peroxidase.

Cloning and sequencing

The plaque corresponding to the positive spot in the previous immunoscreening was cultured and stocked in SM buffer. Positive clones were excised into the pBK-CMV phagemid vector from the ZAP Express vector using the in vivo excision protocol supplied by Stratagene.

Cloned DNAs in the pBK-CMV vector were sequenced with vector-specific primers, the ABI PRISM Dye Terminator Sequencing Ready Reaction Kit (Perkin-Elmer), and an ABI automated DNA sequencer (Perkin-Elmer).

Detection of antigens in patients and normal donors

The response of serum from other patients and normal donors was examined using the same method as described in the section on serological screening with autologous serum. Altogether, 14 patients with osteosarcoma, 8 with other cancers, and 25 normal donors gave informed consent to use their sera.

Results

Characterization of osteosarcoma cell line OS2000

Between April 1997 and March 2000 tumor specimens obtained from 32 patients with a putative diagnosis of osteosarcoma were subjected to cell culture. One of them, which has been maintained for longer than 1 year with more than 50 passages, was designated OS2000. This cell line was obtained from a primary tumor in the left femur of a 16-year-old Japanese female patient who had been cured of bilateral retinoblastoma at infancy. The histological diagnosis of the tumor was fibroblastic osteosarcoma. The patient had multiple metastatic lesions in both lungs and in the retroperitoneal space at the time of biopsy. The HLA phenotype of the patient's peripheral blood lymphocytes was HLA-A24, HLA-B-55, HLA-B-61, HLA-Cw-1, HLA-DR-2, and HLA-DR-4.

We characterized the OS2000 cells, focusing on their neoplastic and osteoblastic features. As shown in Fig. 1, subcutaneous inoculation of these OS2000 cells into an irradiated SCID mouse produced a tumor consisting of large cells with nucleic dysplasia and increased chromatin condensation, although osteoid was not produced. With the RT-PCR analysis, the OS2000 cells expressed type-I collagen mRNA equivalent to other osteosarcoma cell lines (Fig. 2). In contrast, although osteocalcin mRNA was not expressed constitutively in the OS2000 cells, it was induced by treatment with $1,25(OH)_2D_3$.

K562 erythroleukemia cells, used as a negative control, did not show such characteristics. These findings support the neoplastic and osteoblastic nature of OS2000 cells.

Induction of autologous CTLs

We attempted to induce autologous CTLs that recognize OS2000 tumor cells from peripheral T cells. After stimulation by co-culturing with OS2000 six times, we established a CTL line, designated TcOS2000, that can lyse OS2000 cells. TcOS2000 was then tested in a ^{51}Cr-releasing assay using autologous OS2000, fibroblast, or K562. As shown in Fig. 3A, it exerted a cytotoxic influence on OS2000 in a dose-dependent manner. The cytotoxicity of TcOS2000 was not detected in the autologous fibroblast or K562 targets. Anti-HLA class I W6/32 antibody apparently blocked approximately 60% of the cytotoxicity of TcOS2000 against autologous OS2000 (Fig. 3B), indicating that these autologous CTLs could specifically recognize the patient's osteosarcoma cells.

Antigens defined by SEREX analysis

To identify cDNA clones reacting with autologous serum, a total of 5×10^5 independent plaques were screened. Subsequently, two positive clones were obtained and sequenced. One clone was the cDNA encoding HLA-Cw*0102, and the other was smooth muscle myosin light chain (SMMLC). The sequencing analysis of cDNA nucleotides indicated that both cDNAs were wild type without any mutations in their coding frame of proteins (data not shown).

Because HLA-Cw*0102 was likely to be an HLA genotype of the patient examined, we focused the

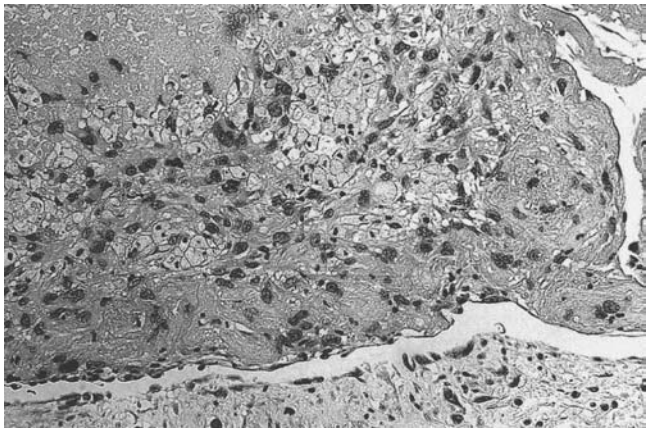

Fig. 1. Large cells that have nucleic dysplasia are proliferating in fibrous necrotic tissue in a specimen inoculated into a SCID mouse. H&E

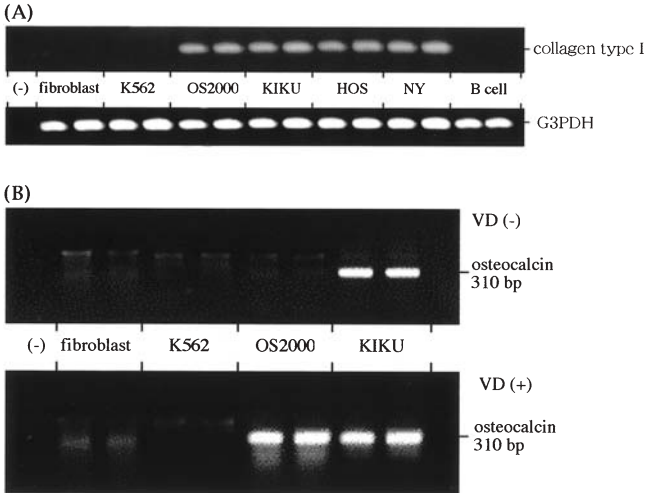

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis. **A** Expression of type I collagen in osteosarcomas (KIKU, HOS, NY, OS2000), fibroblasts, and Epstein-Barr (EB) virus-transformed B cells. **B** Expression of osteocalcin before and after culture in the presence of vitamin D_3 (*VD*)

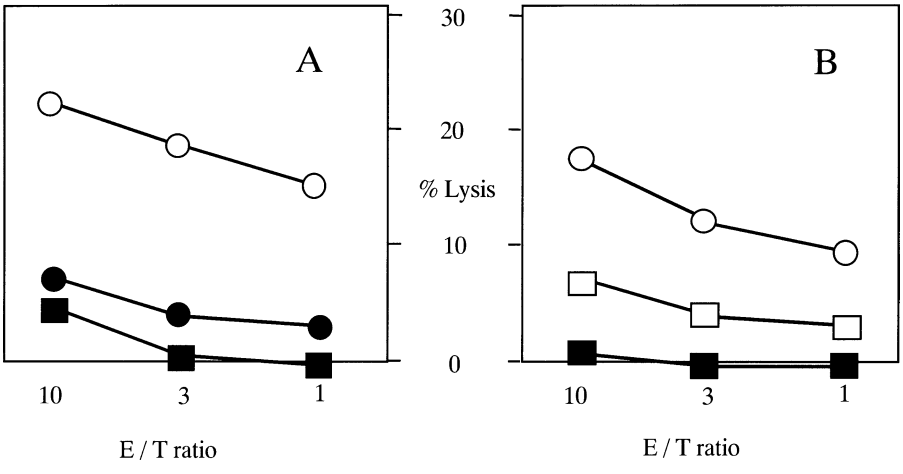

Fig. 3. A Cytotoxicity assay. Cytotoxicity of autologous cytotoxic T lymphocytes (CTLs), TcOS2000, against OS2000 cells was examined by a ^{51}Cr release assay at various E/T ratios. Targets used were OS2000 (*open circles*), autologous fibroblasts (*closed circles*), and K562 cells (*filled squares*). **B** Blocking assay. Cytotoxicity of TcOS2000 against OS2000 was examined in the presence (*open squares*) or absence (*open circles*) of anti-HLA class I monoclonal antibody W6/32. K562 cells (*filled squares*) were used as a target for the negative control

Table 1. Response of sera from patients and normal donors against cloned proteins

Sera used	Serum response to smooth muscle myosin light chain (positive/total no. of cases)
OS2000 patient	+
Osteosarcoma patients	– (0/13)
Cancer patients	– (0/8)
Normal donors	– (0/25)

The response of sera of osteosarcoma patients other than the OS2000-derived patient, patients suffering from other cancers (four gastric, three colon, one pancreas), and normal donors (healthy students of our school) was examined by the same method as SEREX screening

analysis on the SMMLC. The plaques expressing SMMLC were reacted with sera from 13 patients with osteosarcoma, 8 patients with other malignant tumors, and 25 healthy individuals. As shown in Table 1, none of these 46 individual sera responded to the plaques expressing SMMLC molecules.

Discussion

A human osteosarcoma cell line that has neoplastic and osteoblastic characteristics was established from the primary osteosarcoma of a patient who had been cured of retinoblastoma during infancy. To analyze the antigenicity of OS2000 to cell-mediated and humoral autologous immunity, we tried to induce cytotoxic T lymphocytes and to perform SEREX analysis. In consequence, HLA class I-restricted CTLs were induced from the autologous peripheral blood, and HLA-Cw*0102 and SMMLC reacted with autologous sera. To our knowledge, no such cell lines recognized by both cellular and humoral autologous immunity have so far been established from bone and soft tissue sarcomas.

Although osteosarcoma is the most frequent primary bone malignancy, there is a serious delay in the identification of tumor antigens. This is due mainly to the exceptional difficulty of establishing cell line–CTL pairs, associated with the relatively poor adaptability of osteosarcoma to in vitro culture. Despite culturing more than 30 individual osteosarcoma specimens, only OS2000 cells were continuously growing in vitro. It should be noted that OS2000 was established not from a conventional osteosarcoma but from an incidental osteosarcoma in a patient with hereditary retinoblastoma; furthermore, OS2000 had a defective of retinoblastoma (*RB*) gene, as shown by RT-PCR analysis (data not shown). Thus, the unique genetic aspects of OS2000 and the parental patient might have contributed to the successful development of an autologous osteosarcoma cell–CTL pair. In this regard, only two other cell lines have been reported to have been established from a second primary osteosarcoma in hereditary retinoblastoma patients.[7] In contrast, osteosarcoma cell lines have been sporadically developed from conventional osteosarcomas, 60% of which have loss of heterozygosity of the *RB* gene[2]; and some of the established osteosarcoma cell lines (e.g., HOS, U2OS, MG63) express a normal *RB* gene.[12] Therefore, deficiency or mutations in the *RB* gene appears not to be a critical requirement for osteosarcoma to acquire in vitro adaptability.

With respect to the immunological aspects of osteosarcoma, Ichino and Ishikawa[3] induced CTLs from six patients that lysed autologous fresh osteosarcoma cells, although they failed to obtain stable cell line–CTL pairs. This supports the presence of substantial immunogenicity in osteosarcomas. In the present case, we assume that T cells were primed for osteosarcoma antigens during the development of multiple blood-borne metastases, as seen in our recent HLA/peptide tetramer analysis[17] in which increased in vivo frequency of CTL precursors to SYT-SSX-derived peptides was significantly associated with the present or past history of distant metastasis in synovial sarcoma patients.

Induction of CTLs ensured the existence of antigenic peptides in the context of the HLA class I molecules on OS2000 cells. Beyond this point, there are several steps of antigen analysis before identification of a T cell-defined antigenic peptide, including: (1) determination of the antigen-presenting molecules from four candidates (HLA-A*2402, B5502, B*4002, Cw*0102); (2) single cell cloning of the CTLs; (3) construction of an OS2000 cDNA library; (4) cloning of cDNAs encoding the HLA molecules; and (5) expression cloning of the gene encoding the antigenic peptide using cytokine-releasing assays. In our preliminary study, HLA-A*2402 and B*5502 were defined as antigen-presenting molecules. Furthermore, we succeeded in single-cell cloning of the CTLs (data not shown). At any rate, identification of candidate antigens is of great value for antiosteosarcoma immunotherapy.

In addition to identifying antigenic peptides recognized by CD8+ CTLs, it is equally important to define tumor antigens recognized by CD4+ T cells, which play a central role in the regulation of antitumor immune responses.[11] Because isotype switching from IgM to IgG implies the presence of specific help from CD4+ T cells, antigens defined by SEREX likely contain epitopes for CD4+ T cells.[4]

SEREX analysis using a cDNA library of OS2000 and autologous serum defined HLA-Cw*0102 and SMMLC. It is evident that peptides derived from HLA-Cw*0102 molecule have a property that allows binding to HLA-

DR4 molecules.[1] Because the OS2000 patient was positive for HLA-DR4, certain epitopes of the HLA-Cw*0102 molecule in antigen presenting cells that had processed tumor debris during progression, invasion, and widespread necrosis of the osteosarcoma might have been presented to CD4+ T cells in the context of HLA-DR4. Then CD4+ T cells might stimulate B cells to produce autoantibodies through production of Th2 cytokines.

With reference to SMMLC, autoantibody to cardiac myosin light chain I was detected in the sera of patients with dilated cardiomyopathy.[8] Mutated myosin class I was also reported to serve as a tumor antigen recognized by CTLs that regress autologous melanoma.[19] Given these insights, we further investigated the antigenicity of SMMLC, although this molecule is known to be widely and constitutively expressed in human tumors and normal tissues. Sequencing of the full-length cDNA encoding SMMLC in the library of OS2000 revealed no mutations; and none of the sera obtained from other osteosarcoma patients, cancer patients, or normal donors reacted substantially with SMMLC. The present patient had no history of blood transfusion, pregnancy, or autoimmune diseases such as cardiomyopathy. Therefore, the reasons for the limited antigenicity of SMMLC remained a mystery.

Conclusions

We introduced a human osteosarcoma autologous system that included an osteosarcoma cell line, a CTL line that can lyse the self osteosarcoma cells in an HLA class I–restricted fashion, and autoantibodies reacting to two osteosarcoma-derived proteins. Establishment of this system not only offers the opportunity to identify osteosarcoma antigens recognized by autologous immunity, it can further the progress of immunotherapeutic approaches to bone and soft tissue sarcomas other than osteosarcoma, for which current treatment protocols have limited efficacy.

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