Detection of Human Osteosarcoma-associated Antigen(s) by Monoclonal Antibodies

Susumu Hosoi, Takashi Nakamura, Shoichiro Higashi, Takao Yamamuro, Sakuji Toyama, Keisuke Shinomiya, and Haruki Mikawa

INTRODUCTION

There has been much evidence suggesting that human osteosarcomas have unique tumor antigens. It has been reported that patients with osteosarcoma have antibodies to autologous tumor cells, or antibodies common to sarcomas (2, 5, 14, 16–18, 20), and that they express specific cellular immunity to osteosarcomas (4, 6, 24). Xenoantisera have also been used to detect osteosarcoma-specific antigens (12). Since xenantisera contain many species-specific antibodies, the monospecificity of an antibody preparation should be rigorously established after extensive absorption with normal tissue. The method of Köhler and Milstein (10) of producing monoclonal antibodies to single antigenic determinants circumvents these problems. Recently, several monoclonal antibodies to tumor-associated antigens have been reported (3, 7, 9, 11, 19, 21–23). These investigators used established tumor cell lines as antigens to obtain antibody-secreting hybridomas. The use of established cell lines has many advantages in the construction of sensitive and reproducible assay systems. However, the possibility that the expression of tumor-associated antigens may vary with tissue culture conditions cannot be excluded. The use of tumor cells from surgically resected material should rule out the possibility of tissue culture artifact.

This paper reports the production of monoclonal antibodies that bind to osteosarcoma cells but not to autologous skin fibroblasts with the use of freshly resected osteosarcoma cells to immunize mice.

MATERIALS AND METHODS

Cell Lines. The BALB/c myeloma cell line P3-NS1/1-Ag4-1 used for fusion was maintained in RPMI 1640 containing 15% fetal bovine serum. TE 85 human osteosarcoma cells established by McAllister et al. (13) were kindly provided by Dr. W. A. Nelson-Rees. MG 63 osteosarcoma cells (1) were obtained through the generosity of Dr. Kawade (Institute for Virus Research, University of Kyoto). Human epidermoid carcinoma strain KB cells were cloned and have been cultured continuously in our laboratory (Institute for Virus Research). All of these cell lines were maintained in Eagle's minimal essential medium.

Immunization. Biopsy tissue from an untreated patient with osteosarcoma was minced and passed through stainless steel mesh. RBC were removed by hemolysis, and the osteosarcoma cells were washed with PBS 3 times. Two BALB/c mice were immunized i.p. with about 1 x 10^9 osteosarcoma cells. After 3 weeks, a booster dose of 1 x 10^9 cryopreserved osteosarcoma cells was given i.p. Three days later, spleen cells were collected.

Production and Screening of Hybridomas. Cell fusion was performed essentially as described by Oi and Herzenberg (15). Briefly, spleen cells (2.5 x 10^6) were fused with NS1 cells (2.0 x 10^8) in 1 ml of 50% Polyethylene Glycol 1500 for 1 min at 37°. One ml of RPMI 1640 without serum was added over 1 ml. This step was repeated with another 1 ml of the medium, and finally, a further 7 ml of the medium were added over 2 min. The cells were centrifuged, resuspended in RPMI 1640 with serum, and distributed in 456 wells of microtiter plates. RPMI 1640 containing hypoxanthine:aminopterin:thymidine, 0.1 ml, was added on Day 1, and one-half of the medium was replaced with fresh medium on Days 2, 3, 5, 8, 11, and 14. The production of antibodies was tested by indirect immunofluorescence assay. Cryostat sections of the osteosarcoma tissue used for immunization and tissue culture cells on coverslips were used without fixing. They were washed 3 times with PBS and covered with each culture supernatant of hybrids. After 1 hr, they were washed 3 times with PBS and covered with properly diluted FITC-conjugated rabbit anti-mouse IgG. After 1 hr, they were washed 3 times with PBS and examined with a fluorescence microscope. The hybrids which produced antibodies binding to the osteosarcoma tissue and not to the KB cells or the fibroblasts were subsequently cultured in 24-well multiwell plates with 1 ml of medium and cloned by limiting dilution.

Direct Immunofluorescence and Reciprocal Inhibition Test. The hybrids which produced antibodies which seemed specific to osteosarcoma-associated antigens were injected i.p. in pristane-primed mice. Ascitic fluid was collected after about 3 weeks. After ammonium sulfate precipitation, γ-globulin fractions of the ascitic fluid were conjugated with FITC. To ascertain the specificity of the antibodies, the osteosarcoma tissue sections were preincubated with unconjugated monoclonal antibodies for 1 hr and without washing were covered with FITC-conjugated monoclonal antibodies. After 1 hr of incubation, the

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Received June 8, 1981; accepted October 26, 1981.
sections were washed 3 times with PBS and examined with a fluorescence microscope.

RESULTS

Isolation of Hybridoma Lines. From the fusion between spleen cells from 2 mice immunized with osteosarcoma cells and the mouse myeloma cell line NS1, we obtained a total of 276 growing hybrids (60% of all the wells). In the initial screening test, 93 of them (34%) were found to produce antibodies which bound to the immunizing osteosarcoma cells or the KB cells, as determined by indirect immunofluorescence assay. After being transferred to 24-well multwell plates with 1 ml of culture medium, the 93 hybrids were tested again with immunizing osteosarcoma cells, fibroblasts obtained from the same patient, and KB cells. Twenty of them produced antibodies which bound to the osteosarcoma cells but did not bind to the KB cells or to the fibroblasts. After 10 days, 20 hybrids were tested with another osteosarcoma tissue and immunizing osteosarcoma tissue. Eight of them had lost the ability to produce antibodies. Five of them produced antibodies which bound to the immunizing osteosarcoma tissue but not to other osteosarcoma tissue. Seven of them produced antibodies which bound to both osteosarcoma tissues. Since one of them was proved to produce antibodies which also bound KB cells, 6 hybrids which produced antibodies seemingly specific for osteosarcoma were cloned. After being cloned, one hybrid line lost the ability to produce antibodies. Five cloned hybrid lines were designated OST2, OST4, OST6, OST7, and OST15, and they were analyzed further. Antibodies produced by OST2, OST4, OST6, and OST7 were IgG1, and that produced by OST15 was IgG2a.

Characterization of 5 Monoclonal Antibodies. The specificity patterns of 5 monoclonal antibodies are shown in Tables 1 and 2. OST2 bound to 4 of 5 osteosarcomas and to some other tumors and normal tissues. OST4 bound to all 5 osteosarcomas, to almost all other tumors, and to all normal epithelial cells. OST6, OST7, and OST15 had almost the same characteristics. As shown in Fig. 1, they bound to all 5 osteosarcomas (to one of them very weakly) and to one chondrosarcoma. They did not bind to 2 fetal mesenchymal cells or to the cranial and vertebral bones of 2 neonates. Interestingly, they did not bind to 2 cell lines (TE 85, MG 63) derived from human osteosarcomas. Other malignant and benign tumors were all negative. With one exception, normal tissues or cells were all negative.

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Bindings of hybridoma antibodies to tumor tissues and cell lines by immunofluorescence assay</th>
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<tbody>
<tr>
<td>Tumor</td>
<td>OST2</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>4/5</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Giant cell tumor of bone</td>
<td>1/1</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>0/1</td>
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<tr>
<td>Wilms' tumor</td>
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<tr>
<td>Hepatoma</td>
<td>0/1</td>
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<tr>
<td>Neuroblastoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>0/3</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>2/7</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>1/5</td>
</tr>
<tr>
<td>Uterine myoma</td>
<td>0/2</td>
</tr>
<tr>
<td>Hemangiomoma</td>
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**Table 2**

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Bindings of hybridoma antibodies to normal tissues and cells by immunofluorescence assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>OST2</td>
</tr>
<tr>
<td>Vertebra of neonate</td>
<td>0/2</td>
</tr>
<tr>
<td>Cranial bone of neonate</td>
<td>0/1</td>
</tr>
<tr>
<td>Fetal mesenchymal cell</td>
<td>2/2</td>
</tr>
<tr>
<td>Articular chondrocyte b,c</td>
<td>0/2</td>
</tr>
<tr>
<td>Decalcified bone</td>
<td>0/1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0/1</td>
</tr>
<tr>
<td>Peripheral blood lymphocyte</td>
<td>0/1</td>
</tr>
<tr>
<td>Fibroblast of skin</td>
<td>0/2</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>0/2</td>
</tr>
<tr>
<td>Colon mucosa</td>
<td>1/2</td>
</tr>
<tr>
<td>Cerebrum</td>
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<tr>
<td>Thyroid</td>
<td>0/1</td>
</tr>
<tr>
<td>Liver</td>
<td>1/1</td>
</tr>
<tr>
<td>Kidney</td>
<td>1/2</td>
</tr>
</tbody>
</table>

* ND, not done.
* b From patients with osteosarcoma.
* c One was decalcified.
* d A few layers of chondrocytes near the subchondral bone were bound weakly.
* e Tubular epithelium was bound.

DISCUSSION

In the present study, 3 hybrid cell lines which secrete monoclonal antibodies binding to autologous osteosarcoma tissues,
but not to autologous skin fibroblasts, were generated by cell fusion between spleen cells from mice immunized with freshly obtained osteosarcoma cells and a mouse myeloma cell line, NS1. Five osteosarcoma tissues all reacted positively with these antibodies, but one of them was only very weakly positive; this osteosarcoma tissue was morphologically immature and contained few osteoids; the serum alkaline phosphatase level of this patient was within normal limits. Even in one section of osteosarcoma tissue, there were differences in the intensity of fluorescence (Fig. 1). This suggests that antigen expressions appear to be related to the degree of differentiation of tumor cells and to several factors that are not well understood. All of the other tumor tissues tested were negative.

Our results indicate clearly that the antigens of osteosarcomas were not expressed on 2 established tissue culture lines derived from osteosarcomas (TE 85 and MG 63). Variability in the expression of tumor cell surface antigens has been reported. The oncofetal antigen described by Irie et al. (8) was not present on normal biopsy cells, but it appeared on the membranes of normal skin and muscle during culture. The loss of tumor antigens during the course of cell culture has been detected in human melanoma cell lines. However, these cells still express another tumor antigen (22). The expression of tumor-related antigens by tissue culture cell lines appears to be related to several factors. Therefore, it is necessary to obtain monoclonal antibodies against freshly obtained tumor cells for complete understanding of tumor-associated antigens. However, the possibility that these antibodies may be looking at polymorphic antigenic determinants, preferably expressed in Oriental people, cannot be excluded.

The cranial and vertebral bones of 2 neonates did not react with these antibodies, and decalcified normal bone matrix was also negative. Among the tests of various normal tissues, positive results were obtained with articular cartilages from 2 subjects. The antigens recognized by these antibodies in the immunofluorescence assay were expressed exclusively on the chondrocytes in calcified areas of cartilage near the subchondral bone. This suggests that these antibodies recognize some antigens displayed on the surface of a subpopulation of chondrocytes. Thus, these antibodies defined here not only provide a useful tool for studying osteosarcoma but also help to clarify the differentiation of chondrocytes.

Osteosarcoma-associated antigens have been identified with sera from osteosarcoma patients (2, 5, 14, 16–18, 20). The relationship of these antigens, to those recognized by antibodies OST6, OST7, and OST15 is not clear. However, most, if not all, of these antigens are distinct from those identified by the monoclonal antibodies, since several studies have shown that antibodies in sera from patients with osteosarcoma reacted not only with osteosarcoma but also with other sarcomas, such as giant cell tumor of bone, leiomyosarcoma, and rhabdomyosarcoma (2, 14, 16, 17).

The results of reciprocal blocking tests prove that these 3 antigenic determinants are different from each other, but whether these antigenic molecules are the same or not is not clear.

These antibodies were not directed against bone alkaline phosphatase, since alkaline phosphatase activities in the sera of patients with osteosarcoma were not removed by immunoprecipitation with these antibodies and rabbit anti-mouse IgG. Further studies to characterize and purify these antigens are in progress.

ACKNOWLEDGMENTS

We thank Dr. Matsumoto (Division of Surgery, Koga Hospital) for his generosity in providing surgically resected tissues.

REFERENCES

1. Billiau, A., Edy, V. G., Heremans, H., Damme, J. V., Desmyter, J., Geor-
giades, J. A., and Somer, P. D. Human interferon: mass production in a
newly established cell line, MG-63. Antimicrob. Agents Chemother. 12: 11–
immunofluorescence studies of the tumor antigen-bearing cell in giant cell
1975.
human melanoma-associated antigen(s) detected by monoclonal antibodies.
cytotoxicity to human sarcomas: evidence for a cell-mediated host immune
5. Eiras, A. S., Robins, R. A., Baldwin, R. W., and Byers, V. S. Circulating
739, 1980.
antigen stimulated lymphocyte proliferation in osteosarcoma. Cancer
7. Herlyn, M., Steplewski, Z., Herlyn, D., and Koprowski, H. Colorectal carci-
against a human neuroblastoma antigen present on fetal brain. Science
10. Köhler, G., and Milstein, C. Continuous cultures of fused cells secreting
12. Marsh, B., Flynn, L., and Enneking, W. Immunologic aspects of osteosar-
1387, 1972.
and Landing, B. H. Cultivation in vitro of cells derived from a human

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Fig. 1. a, photomicrograph of osteosarcoma tissue of the patient used for immunizing mice. H & E, × 200. b to f, direct immunofluorescence of osteosarcoma tissues from 5 patients (b from the patient used for immunizing mice) with OST6/FITC at 1:100 dilution (except b which was stained at 1:1000 dilution). These indicate that the patterns and the intensities of fluorescence differ from each other and that, even in the same tissue section, there are differences in the intensity of fluorescence. × 200.
Monoclonal Antibodies against Human Osteosarcoma

Fig. 2. Direct immunofluorescence of articular cartilage with OST6:FITC at 1:100 dilution. A few layers of the chondrocytes near the subchondral bone are bound with OST6. X 200.

Fig. 3. Results of absorption tests with lyophilized tumor tissues: a, with OST6:FITC at 1:2000 dilution without absorption; b, absorbed with 8 mg of lyophilized osteosarcoma tissue; and c, absorbed with 80 mg of lyophilized leiomyosarcoma.
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