Monoclonal Antibody to Human Osteosarcoma: A Novel M, 26,000 Protein Recognized by Murine Hybridoma TMMR-2


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ABSTRACT

Three murine hybridomas (TMMR-1-3) were developed by repeated immunizations of mice with four different human osteosarcoma cell lines in an alternating sequence of inoculations. The monoclonal antibodies were screened for reactivities to cultured cell lines and tissue sections of osteosarcomas using flow cytometry and immunohistochemical techniques. TMMR-2 is a highly specific antibody (IgG,) that reacted with all 14 osteosarcoma tumors and eight human osteosarcoma cell lines tested, including the established human osteosarcoma cell lines HOS and Saos-2. Benign neoplastic cells from two osteoblastomas, osteoblasts from regions of reparative osteoid formation and neonatal new bone, are also reactive with TMMR-2. TMMR-1 has mesenchymal specificity whereas TMMR-3, although reactive with osseous differentiated cells, also reacted with mitotic cells of all cell types. Characterization of antigen structure by Western immunoblotting revealed that TMMR-2 reacted with a 100°C heat labile mercaptoethanol-sensitive M, 26,000 protein, and TMMR-3 recognized a mercaptoethanol-resistant M, 97,000 protein whereas TMMR-1 reacted with a series of bands from 65,000 to 85,000 molecular weight, all of which were mercaptoethanol sensitive. TMMR-1 and TMMR-2 monoclonal antibodies showed complement-independent inhibition of H3Hymidine incorporation into DNA, but did not exhibit cytotoxic activity. The results suggest that TMMR-2 is a specific antibody that recognizes an osteoblast/osteocyte surface antigen present in normal, reactive, and neoplastic disorders of bone. The inhibitory effects on DNA synthesis in cultured osteosarcoma cells by TMMR-2 indicate an important cell growth/proliferation role of this surface antigen. These monoclonal antibodies, in combination with other known antibodies, can be used to characterize mesenchymal cell surface antigenic structure and differentiation.

INTRODUCTION

Murine monoclonal antibodies to surface antigens of human hematopoietic cells such as T- and B-lymphocytes and myelomonocytic cells at various stages of differentiation and maturation have been successfully produced and characterized (1). These MoAbs have played an important role in the basic and clinical study of the normal biology and neoplastic growth of hematopoietic cells. Clinical applications of these antibodies have made a great impact in many areas of medicine especially in immunology, hematology, and transplantation. Parallel development in the field of solid tissue neoplasms with mesenchymal and epithelial cell differentiation has had more limited success.

There have been several reports of monoclonal antibodies specific to osteosarcoma. Emberton and coworkers (2) developed an antibody which reacted with four of 12 human osteosarcomas, but not with homologous fibroblasts. This antibody was used for in vivo localization of lesions and for conjugation to drugs or toxins (3-7). However, the antibody reacted with only a portion of the human osteosarcomas tested, and also with several carcinomas. Hosoi and coworkers (8) also produced monoclonal antibodies to osteosarcoma-associated antigens from fresh tumors, but these antibodies did not react with osteosarcoma cell lines. In addition, they cross-reacted with chondrosarcoma cells and with normal chondrocytes. Bruland et al. produced monoclonal antibodies from a human osteosarcoma that showed strong reactivities to 15 osteosarcomas, but was also reactive to many other soft tissue tumors including MFH, synovial sarcoma, and chondrosarcoma (9), and appeared to react with alkaline phosphatase (10). Tsang et al. (11) obtained MoAbs using partially purified osteosarcoma-associated antigen from spent culture medium of osteosarcoma. Their antibodies reacted with most osteosarcomas in both tissue and cultured cell lines, a colon cancer line, and a fibrosarcoma cell and tissue. Brown produced two MoAbs that reacted with both osteosarcoma and MFH using fresh MFH tumor tissue membrane preparation (12). These MoAbs also exhibit a moderate level of bindings to melanoma, colorectal carcinoma, and first trimester fetal membrane (12). Recently, Wada and coworkers (13) generated two antibodies that reacted with different epitopes of the same cell surface antigen present on osteosarcoma tissues and cell lines but not other tumors. The proteins recognized by these MoAbs to osteosarcoma cells have been characterized by surface iodination, immunoprecipitation, and SDS-PAGE as M, 72,000 (14, 15), 75,000 (13), 80,000 (10), 92,000 (11), and 102,000 (12). All of these apparently different bands were unaffected by mercaptoethanol reduction suggesting the presence of a single antigenic polypeptide.

This paper reports the production and characterization of three MoAbs to human osteosarcoma cell lines, their specificity, biological function on cultured osteosarcoma cells, and reactivity to various human osteosarcomas and other soft tissue tumors. Comparison was also made to the reactivities in benign and reactive osteoblastic lesions and to normal and neonatal bones. One of the MoAbs, TMMR-2, recognized a new M, 26,000 protein present on osteosarcoma cells.

MATERIALS AND METHODS

Tissue Culture. Eight cell lines derived from human osteosarcoma were established at St. Louis University Medical Center. Cells were obtained from freshly resected tumors by 0.1% collagenase digestion and filtered through a 100-μm filter. The cells were washed and then assayed for surface antigens. For comparison, lines were generated

Received 5/4/89; revised 9/27/89; accepted 10/3/89.

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1Supported by a Grant from the Orthopaedic Research and Education Foundation, Chicago, IL 60611 and by a Grant from the American Cancer Society (Institutional Grant IN-124), Atlanta, GA 30329. Presented in part at the Annual Meeting of the International Academy of Pathology, Chicago, IL, 1987; at the 34th Annual Meeting of the Orthopaedic Research Society, February 1-4, 1988, Atlanta, GA; at the 35th Annual Meeting of the Orthopaedic Research Society, February 6-9, 1989, Las Vegas, NV; and at the 73rd Annual Meeting of the Federation of American Societies for Experimental Biology, March 19-23, 1989, St. Louis, MO 63104.

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4 The abbreviations used are: MoAb, monoclonal antibody; MFH, malignant fibrous histiocytoma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ATCC, American Type Culture Collection; FCS, fetal calf serum; PBS, phosphate buffered saline.

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from two giant cell tumors, two malignant fibrous histiocytomas, three chondrosarcomas, a neuroblastoma, several leukemia/lymphoma lines of B, T, or myelomonocytic lineage. These eight osteosarcoma cell lines (see Table I) have been maintained in continuous culture for as long as 3 years, and some have also been passed through nude mice. These cell lines provide the model for our study of osteosarcoma and its growth regulation. Additionally, HOS and Saos-2, two well-characterized lines from ATCC, have been used as positive human osteosarcoma cell line controls and WIDR, a colon cancer line, as a negative control. The cells were maintained in growth media consisting of Dulbecco's modified Eagle's medium (Hazelton, Lenexa, KS) with 10% heat-inactivated FCS, 1% glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were grown in 75-cm² plastic T-flasks (Corning) and maintained at 37°C in a humidified incubator with 5% carbon dioxide. The cells were periodically checked for alkaline phosphatase production and by flow cytometric DNA analysis for aneuploidy and DNA index.

Nude mice were injected s.c. with 0.5 ml of 5 × 10⁶ cells/ml of cultured osteosarcoma cells. Tumor formation occurred in about 5 to 6 weeks. These tumors were then removed for testing the reactivity with the MoAbs by immunocytochemistry and flow cytometry, as described, and for reculture and reinjection into nude mice.

Hybridoma Production. BALB/c mice were immunized with 5 × 10⁶ osteosarcoma cells once a week for 12 weeks. Four different osteosarcoma cell lines (OSEV, OSSM, OSLF, OSPR) were used in an alternating sequence of inoculations with 10 i.p. followed by two i.v. injections through the tail vein. The spleen from two mice were removed 4 days after the last injection. The splenocytes were fused with SP2/0 AG14 cells by the SLU/VITEK Hybridoma Laboratory. Growing hybridomas were cloned and the supernatant screened for antibody production against osteosarcoma culture cells by flow cytometry and ABC-immunohistochemistry as described below. The murine immunoglobulin type was determined by single immunodiffusion using a mouse immunoglobulin kit from Miles Scientific (Lisle, IL). Quantitation of IgG₂ in the cell supernatants or ascites fluid was determined by an enzyme-linked immunosorbent assay sandwich assay (16) using purified MOPC/21 IgG₂ as a standard.

Flow Cytometry. The stained cells were analyzed by indirect immunofluorescence flow cytometric methods using either a Becton-Dickinson FACScan or an Ortho Spectrum III modified for two-color fluorescence and fitted with the Model 2140 computer.

Flow cytometric studies were carried out by using freshly harvested cells from cultures with or without trypsin digestion or tumor suspensions from human and nude mice obtained by collagenase-DNase digestion. Surface antigen(s) were assayed by incubating 3 × 10⁶ cells (0.1 ml) with the monoclonal antibodies (1–2 µg/ml) for 30 min at 4°C, washing two times, and the incubating with fluorescein isothiocyanate-labeled F(ab)₂ goat-anti-mouse IgG (Cooper, Malvern, PA) at 4°C for 30 min. Forty µl of FCS per tube was added to block nonspecific binding. Polyclonal mouse antiosteosarcoma antiserum served as a positive control. Monoclonal mouse IgG₂, (Sigma, St. Louis) and IgG₂ anti-TMA hapten (gift of Dr. C. Bellone) were used as negative controls. A channel setting producing fluorescence of 1 to 2% reactivity with a negative control was used for the other antibodies.

For monoclonal antibody screening of well supernatants, lymphocytes from the original patients or other persons were added to the same tube containing the osteosarcoma cells to serve as controls for major histocompatibility and other common cross-reactivities. Two computer-defined regions separating the lymphocytes from osteosarcoma cells by the forward and right angle light scatter were used simultaneously (see Fig. 1A).

Immunohistochemistry. Fresh human tissues were snap frozen in isopentane and stored at −70°C for future uses. Many types of primary bone tumors, carcinomas, and lymphomas were included for study. One breast and one colon tumor were metastatic to bone. Frozen sections (7µm thick) were cut in a cryostat, air dried, and fixed in acetone for 15 min and stored at 4°C until they were used for staining.

Avidin-biotin complex immunoperoxidase techniques (ABC-PAP) were used to demonstrate the reactivities of MoAbs to various tissue sections and cells from culture. The reagents for staining were obtained from the BioGenex (Dublin, CA) and Vector Laboratories (Burlingame, CA). Cell slides were prepared by growing culture cells in a chambered Lab-Tek slide (Miles, Naperville, IL) for 48 h at 37°C and 5% CO₂. The slides are then washed, fixed in 100% acetone for 20 min at room temperature, and then rehydrated in phosphate buffered saline. The slides are treated with 3% H₂O₂, then with nonimmune serum from the animals providing the linking antibody, incubated at room temperature for 30 min with 1–2 µg/ml primary antibody, and stained as described (17). Tissue from carcinomas or lymphomas were used as negative tissue controls. A rat osteosarcoma cell line (gift of Dr. N. C. Partridge) served as nonhuman control, as did NIH-3T3 mouse fibroblast and normal rat kidney cells (from ATCC).

Slides were tested with 10% formaldehyde, 2% glutaraldehyde, 10% alcohol, and 100% ether at room temperature to compare with the acetone fixation in order to determine the sensitivity of the antigen.

Growth Inhibition Study. The in vitro inhibition of proliferation of osteosarcoma cells by monoclonal antibodies was carried out using [³H]thymidine incorporation assays in microtitr plates. Wells were seeded with 4 × 10⁴ cells and incubated with varying dilutions of antibody, as indicated in Fig. 5. After incubation for 18 h, cells were pulsed with [³H]thymidine at 6.7 Ci/mm, 0.5 µCi/well (NEN, Boston, MA) for 6 h, harvested in a MASH III (Microbiological Associates, Walkersville, MD), and counted in a liquid-scintillation counter. This inhibition was complement independent as only heat-inactivated FCS was used in the culture. Trypan blue exclusion was used to assess viability.

Preparation of Cell Lysates. Osteosarcoma cells from fresh tumor or tissue culture were centrifuged and washed twice in PBS before lysing in lysis buffer (0.01 M Tris, pH 8.0, 1.5 mM magnesium-acetate, 1% NP-40, 0.08 µg/ml aprotinin, 1 mM EDTA-Na₂, 0.5 µg/ml leupeptin, 50 µg/ml soybean trypsin inhibitor, and 0.2 mM phenylmethylsulfonyl fluoride added immediately prior to use). The five protease inhibitors were included to prevent degradation of the labile antigen. After incubation the lysate was centrifuged 3000 × g for 10 min and the supernatant used immediately, frozen at −20°C, or treated with SDS-PAGE sample buffer. The lysate was stable for less than 7 days at −20°C.

SDS-PAGE and Immunoblotting. Samples were prepared by adding lysate to 3X sample buffer (final concentrations 0.0625 M Tris, 10% glycerol, 2.3% SDS, 0.33% bromophenol blue, and if reduced 5% β-mercaptoethanol). Electrophoresis was performed using the methods of Laemmli (18) in a Bio-Rad Protein II (Richmond, CA) with 7% stacking and 15% separating polyacrylamide gels (37.5:1 acrylamide: bis-acrylamide) at 4°C and 20-40 mA constant current for 45 min. Prestained standards were used from Bio-Rad.

A Genie Electrophoretic Immunoblotter (Idea Scientific, Corvallis, OR) was used to transfer proteins from the gel to nitrocellulose membrane at a constant voltage of 11 V at 4°C in transfer buffer (20 mM Tris, 150 mM glycine, and 20% methanol at pH 8.9) (19). After transfer, the nitrocellulose (S & S) was submerged in a blocking solution (2% bovine serum albumin, 0.25% gelatin, 50 mM Tris, and 0.154 M NaCl at pH 7.4) for 1 h at room temperature. First antibodies were diluted in PBS with 0.05% Tween-20 to a concentration of 1 µg/ml, added to the nitrocellulose, and incubated for 1 h at room temperature with constant agitation. The nitrocellulose membrane was washed twice for 5 min in PBS-Tween before alkaline phosphatase-labeled goat anti-mouse IgG (gamma chain specific, diluted in PBS-Tween as directed by manufacturer's instructions) was incubated for 30 min at room temperature. The nitrocellulose was then washed twice in PBS-Tween and then once in barbital buffer (0.025 M, 0.243 mM sodium acetate, pH 9.6) for 5 min each following which the alkaline phosphatase substrate was added (final concentrations, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.1 mg/ml nitro blue tetrazolium, 0.04 mM MgCl₂ in barbital buffer) and were incubated at room temperature until developed, usually 10–30 min (20). Following development the nitrocellulose was dried and stored protected from light.

RESULTS

Selection of MoAb. The hybridoma flow cytometry screening procedure yielded three MoAbs named TMMR-1 through

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MONOCLONAL ANTIBODIES TO OSTEOSARCOMA

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MONOCLONAL ANTIBODIES TO OSTEOSARCOMA

Fig. 1. Flow cytometric monoclonal screening procedure using a mixture of osteosarcoma cells and lymphocytes. Hybridoma supernatants were incubated with the cell mixture for 30 min at 4°C, washed, and stained with fluorescein isothiocyanate-anti-mouse immunoglobulin antibody. Cells were analyzed in the Spectrum III flow cytometer. A, forward (abscissa) and right angle scatter (ordinate) showing region 1 lymphocytes and region 2 osteosarcoma; B and C, fluorescence histograms of cells from regions 1 and 2 using TMMR-2 antibody, respectively; D and E, fluorescence histograms of cells from regions 1 and 2 using TMMR-1 antibody, respectively; F and G, fluorescence histograms of cells from region 1 and 2 using isotype control anti-TMA antibody, respectively; H, fluorescence histograms of cells from region 2 cells using polyclonal antiosteosarcoma serum; B-H, abscissa is channel number, ordinate is cell number except for A, where intensity of scatter is displayed in arbitrary units.

TMMR-3 which initially reacted more strongly against osteosarcoma cells than against lymphocytes. This procedure uses the forward and right angle light scatter to distinguish cells by size and granularity, respectively. Fig. 1A shows the light scatter properties of an admixture of lymphocytes (region 1) and osteosarcoma cells (OSPR) (region 2). The cell surface staining using a TMMR-2 supernatant is shown in Fig. 1, B and C, where no fluorescence staining is observed on lymphocytes and 100% staining on osteosarcoma cells, respectively. Staining with TMMR-1 showed weak reactivity on nearly all lymphocytes and very strong reactivity on osteosarcoma (Fig. 1, D and E, respectively). Fig. 1, F and G, demonstrate absence of binding by two different isotype monoclonal mouse IgG controls on osteosarcoma cells. Fig. 1H illustrates the intense staining with the polyclonal mouse antiosteosarcoma serum on osteosarcoma cells.

Specificity Studies of TMMR-2. The monoclonal antibodies were then tested against other normal and neoplastic human cells to determine tumor specificity. The reactivities of various human neoplasia including soft tissue sarcomas, carcinomas, and lymphoma leukemia cells with the three MoAbs (TMMR-1-3) are summarized in Table 1. Binding of these MoAbs to normal human cells assayed by either flow cytometry or ABC-PAP is shown in Table 2. The results suggest that TMMR-2 is a highly specific antibody (IgG) that reacted with all 14 fresh osteosarcoma tumors tested and eight of eight human osteosarcoma cell lines in addition to ATCC osteosarcomas, HOS and Saos-2 (see Table 1). One chondrosarcoma cell line (HCS-1) which had a histological appearance of dedifferentiated cells showing osteoid differentiation was strongly positive, whereas a conventional cell line, HCS-2, was only weakly positive (5-19%). TMMR-2 did not stain any of the lymphomas or leukemias of either T- or B-cell lineage (n = 20), nor did it stain any acute myeloblastic of 15 or 5 myelomonocytic leukemias tested.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Method</th>
<th>TMMR-1</th>
<th>TMMR-2</th>
<th>TMMR-3</th>
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<tr>
<td>Osteosarcoma tumors (14)</td>
<td>B</td>
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<td>++++</td>
<td>+</td>
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<tr>
<td>Osteosarcoma cell lines</td>
<td>B</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>HOS</td>
<td>+++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Saos-2</td>
<td>+++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>OSPR</td>
<td>+++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>OSSM</td>
<td>+++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
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<tr>
<td>OSFL</td>
<td>+++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
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<tr>
<td>OSEV</td>
<td>+++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
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<tr>
<td>OSON</td>
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<td>+++++</td>
<td>+</td>
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<tr>
<td>OSWO</td>
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<td>+++++</td>
<td>+</td>
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<tr>
<td>OSFN</td>
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<td>+++++</td>
<td>+</td>
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<tr>
<td>OSSK</td>
<td>+++++</td>
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<td>++++</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td>Chondrosarcoma cell lines (2)</td>
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<td>++++</td>
<td>+++++</td>
<td>+</td>
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<tr>
<td>HSC-1 (dedifferentiated)</td>
<td>++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
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<tr>
<td>HCS-2</td>
<td>++++</td>
<td>+++++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Malignant fibrous histiocytoma (4)</td>
<td>IP</td>
<td>++++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma (1)</td>
<td>B</td>
<td>++++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ewing's sarcoma (4)</td>
<td>B</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Synovial sarcoma (2)</td>
<td>IP</td>
<td>++++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Leukemias</td>
<td>FCM</td>
<td>++++</td>
<td>--</td>
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<tr>
<td>Lymphocytic</td>
<td>T/B (20)</td>
<td>+++++</td>
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</tr>
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<td>Myelocytic (15)</td>
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<td>Monocytic (5)</td>
<td>+++++</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Carcinomas</td>
<td>IP</td>
<td>++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Colon (3)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Prostatic (1)</td>
<td>--</td>
<td>--</td>
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<td>--</td>
</tr>
<tr>
<td>Breast (2)</td>
<td>--</td>
<td>--</td>
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<td>--</td>
</tr>
<tr>
<td>Dysgerminoma (1)</td>
<td>IP</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Giant cell tumor (5)</td>
<td>IP</td>
<td>++++</td>
<td>--</td>
<td>--</td>
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</tbody>
</table>

* Cells were stained by the technique as described in "Materials and Methods," FCM (flow cytometry), IP (immunoperoxidase), B (both).

** Positivity is: +++++, 90-100%; ++++, 70-89%; ++, 20-69%; +, 5-19%; -, less than 5%; no symbol, not done.

* Conventional HCS cells were weakly positive (+), whereas one tumor with dedifferentiated HCS areas showing osteoid features was strongly positive (+++).
Table 2  Monoclonal antibodies to human osteosarcoma reactivities to normal human mesenchymal cells

See legend to Table 1 for explanation of symbols. Tissues were obtained from normal persons, for example, osteoid cells were examined in healing fracture material. In addition, tissue was obtained from normal areas of tumor-bearing patients.

<table>
<thead>
<tr>
<th>Cells</th>
<th>TMMR-1</th>
<th>TMMR-2</th>
<th>TMMR-3</th>
</tr>
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<tbody>
<tr>
<td>Osteoblast</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Osteocyte</td>
<td>++++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Osteoclast</td>
<td>++</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>Chondroblast</td>
<td>+</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>Chondrocyte</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Endothelial</td>
<td>++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>++++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Schwann cell</td>
<td>++++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neurons</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lymphocyte T/B</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plasmacyte</td>
<td>++++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Histioyte</td>
<td>++++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Mitosis-activated cell</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
</tbody>
</table>

The other tumors tested are all generally negative or only minimally positive (<5%), e.g., neuroblastoma, WIDR colon cancer line, malignant melanoma, and HeLa cell line. Also negative were a breast and colon carcinoma metastatic to the bone. A rat osteosarcoma cell line, the normal rat kidney cell line, and mouse 3T3 cells did not react with TMMR-2. It is interesting to note, however, that there are a small number (ranging from 5 to 15%) of positive staining cells in three of the three MFH lines, three of three chondrosarcoma tumors, and in each of five giant cell tumors with TMMR-2 (Table 1). As observed with the dedifferentiated HCS-1 cell line, one chondrosarcoma tumor containing dedifferentiated properties was strongly positive with TMMR-2 in these areas. Small numbers of positive cells were observed by immunohistochemistry on the same frozen tumor tissue sections.

TMMR-2 shows a cell membrane as well as cytoplasmic staining pattern in both tissue culture cells (not shown) and in tissue sections of osteosarcoma (Fig. 2A). This section of human osteosarcoma (Fig. 2A) shows extensive staining in all cells including malignant osteoblasts within the osteoid areas (arrow). The reactivity of TMMR-2 to the osteosarcoma cell lines has remained constant and stable over a period of up to 3 years of continuous cell culture and is still present in the ATCCs HOS and Saos-2 lines. The intensity of staining has not diminished with time (data not shown).

Two cases of aggressive osteoblastoma were also found to react strongly with TMMR-2 by both flow cytometry and tissue sections. Continued culture of these cells demonstrated retained antigenicity recognized by TMMR-2 (data not shown). TMMR-2 also reacted with the osteoblasts present in premature newborn bone sections and normal or malignant osteoid tissue (Tables 1 and 2).

The antigen recognized by TMMR-2 is preserved after short periods (3-5 min) of 10% trypsin digestion at room temperature although the mean fluorescence channel decreased somewhat. Treatment for more than 10 min totally abolished the reactivity of TMMR-2. The antigenicity of the cells was then recovered after overnight incubation in culture media in suspension. Fixation of the osteosarcoma cells by alcohol, ether, or acetone did not change the reactivity using immunoperoxidase staining. However, fixation of the cells in formaldehyde or glutaraldehyde abolished the reactivity.

The antigen recognized by TMMR-2 was characterized by Western immunoblotting techniques. TMMR-2 recognizes a 23,000-26,000 molecular weight protein (Fig. 3) using 15% acrylamide gels (26,100 ± 760, mean ± 1 SD, n = 17) which is completely reduction sensitive. Inclusion of 5% mercaptoethanol or 0.01 M dithiothreitol (not shown) in the SDS sample buffer caused complete loss of antigenicity (Fig. 3). The protein was also inactivated completely by boiling in SDS sample buffer but in the NP-40 cell lysate the antigenicity was completely stable at 56°C (30 min) and only partially stable at 85°C (30 min) (not shown). The M, 50,000 band present with all antibodies tested including MOPC-21 IgG, control is a nonspecific
compared to M, 26,000 on 15% gels. The latter value will be used. Cells were untreated, or incubated at pH 6.0 for 30 min at room temperature with no addition, 0.2 unit/ml neuraminidase, or trypsin/EDTA (I/250) (Lanes 2-5, respectively) added before preparation of lysates. Lane 1 is a prestained standard. Note that trypsin treatment resulted in a loss of the M, 50,000 band is present in all blots and is nonspecific.

FIG. 3. Western immunoblotting analysis with TMMR-1, -2, and -3 monoclonal antibodies. Lysate was from osteosarcoma cell line OSWO not boiled with SDS sample buffer. Lanes 1 and 10, prestained standards; Lanes 2, 4, 6, and 8, unreduced; and Lanes 3, 5, 7, and 9, reduced with 5% mercaptoethanol. Nitrocellulose blots from Lanes 1-3, 4-5, 6-7, and 8-10 were cut out and incubated with TMMR-1, TMMR-2, TMMR-3, and MOPC-21, respectively. MOPC-21 is the nonspecific isotype control antibody. The M, 50,000 band is present in all blots and is nonspecific.

FIG. 4. Western immunoblotting analysis of osteosarcoma cell line OSWO with TMMR-2. Cells were untreated, or incubated at pH 6.0 for 30 min at room temperature with no addition, 0.2 unit/ml neuraminidase, or trypsin/EDTA (1/250) (Lanes 2-5, respectively) added before preparation of lysates. Lane 1 is a prestained standard. Note that trypsin treatment resulted in a loss of the M, 26,000 band.

Specificity of TMMR-1 and -3. TMMR-1 (IgG1) reacted with all human cells of mesenchymal origin, including malignant fibrous histiocytomas demonstrated in Fig. 2B and hematopoietic cells, but not with human carcinomas. This suggests that TMMR-1 recognizes a common mesenchymal/sarcoma cell antigen (Table 1). Other than reactivity to a small percent-age of osteosarcoma cells, TMMR-3 also shows a predominant membrane staining pattern with chondroblasts/chondrocytes and in both normal and neoplastic states. TMMR-3 (IgG2a) reacted with greater than 60% of cells in three chondrosarcomas (Fig. 2C, left). In addition, TMMR-3 reacts strongly in the cytoplasm of cells in mitosis in all cell types tested (Fig. 2C, right). This antigenicity is well preserved in paraffin sections fixed in alcohol, formalin, or B5 fixatives (Fig. 2C, right). The antibody seems to react with a common cytoplasmic antigen which is associated with cells in mitosis, a mitosis associated cytoplasmic antigen.

TMMR-3 demonstrated on immunoblotting a M, 97,000 band (range, M, 95,000–97,000, n = 5) which was not effected by reduction (Fig. 3) or boiling in SDS sample buffer. TMMR-1 reacted with a series of bands approximately M, 65,000–85,000 that were all completely sensitive to reduction. Both TMMR-1 and TMMR-3 reacted with antigens stable to boiling in SDS buffer for 3 min, unlike TMMR-2.

DNA Inhibition Studies. Fig. 5, upper, shows the effect of incubation of the culture media of TMMR-1–3 with the HOS cell line for 18 h on the amount of DNA synthesis as measured by [3H]thymidine incorporation in a 6-h labeling period. The results demonstrate that TMMR-1 and TMMR-2 caused a marked reduction of DNA synthesis compared to untreated or TMMR-3-treated cells. Purified TMMR-2 IgG1 inhibited the osteosarcoma cell line HOS compared to isotype control IgG1, (middle) but had no effect on chondrosarcoma cells (lower). Further experiments demonstrated significant inhibition of DNA synthesis in six of six of the human osteosarcoma cell lines and an osteoblastoma cell line, but not a neuroblastoma, MFH, fibroblast, or T-cell leukemia cell line or additional chondrosarcoma by TMMR-2 at 6 μg/ml of purified antibody (data not shown). TMMR-1 also exhibited inhibition of DNA synthesis in one cell line tested. Cytotoxicity measured by trypan blue exclusion did not increase after incubation with the MoAb for 18 h.

DISCUSSION

Successful production and characterization of monoclonal antibodies specific to human osteosarcoma cell lines will increase our present understanding of the biology of osteosarcoma and may lead to clinical applications. Monoclonal antibodies to osteosarcoma cells have been used in many studies for immunolocalization of xenograft tumors in mice (5, 9, 21–24) and in patients (3, 6, 9, 25). Conjugates of MoAb and toxins or drugs have been used in an attempt to increase cytotoxicity (7, 26–30).

Our results suggest that TMMR-2 is specific for mesenchymal cells with osseous differentiation, namely the osteoblast and osteocyte. TMMR-2 reacts with normal neonatal osteoblasts and osteocytes at the growth plate, but not with the accompanying chondroblasts or chondrocytes (Tables 1 and 2). The antibody also stains the osteoblastic cell line, but not a neuroblastoma, MFH, fibroblast, or T-cell leukemia cell line or additional chondrosarcoma by TMMR-2 at 6 μg/ml of purified antibody (data not shown). TMMR-1 also exhibited inhibition of DNA synthesis in one cell line tested. Cytotoxicity measured by trypan blue exclusion did not increase after incubation with the MoAb for 18 h.

FIG. 5. DNA Inhibition Studies. Fig. 5, upper, shows the effect of incubation of the culture media of TMMR-1–3 with the HOS cell line for 18 h on the amount of DNA synthesis as measured by [3H]thymidine incorporation in a 6-h labeling period. The results demonstrate that TMMR-1 and TMMR-2 caused a marked reduction of DNA synthesis compared to untreated or TMMR-3-treated cells. Purified TMMR-2 IgG1 inhibited the osteosarcoma cell line HOS compared to isotype control IgG1, (middle) but had no effect on chondrosarcoma cells (lower). Further experiments demonstrated significant inhibition of DNA synthesis in six of six of the human osteosarcoma cell lines and an osteoblastoma cell line, but not a neuroblastoma, MFH, fibroblast, or T-cell leukemia cell line or additional chondrosarcoma by TMMR-2 at 6 μg/ml of purified antibody (data not shown). TMMR-1 also exhibited inhibition of DNA synthesis in one cell line tested. Cytotoxicity measured by trypan blue exclusion did not increase after incubation with the MoAb for 18 h.
cells. TMMR-2 compared to isotype control MOPC on HCS-1 human chondrosarcoma
antibodies. Upper, HOS cells were incubated with varying proportions of culturesupernatants from the different hybridomas as indicated. After 18 h at 37°C in
change the reactivity on cytospin preparations, however, fixa
tion with formalin or glutaraldehyde completely abolishes the
reactivity suggesting the protein nature of the antigen. Treatment with alcohol, ether, or acetone does not
abolishes the reactivity on cytospin preparations, however, fixa
tion with formalin or glutaraldehyde completely abolishes the
antigenicity.

An additional reactivity of TMMR-2 with normal heart,
lymph node, and soft tissues has been noted by the presence of
one to four clearly positive cells per high-power field. This
reactivity was also seen in tumors of mesenchymal origin such
as MFH, synovial sarcoma, and chondrosarcoma. Chondrosar-
comas demonstrated the highest reactivity (up to 20%). The
significance of these findings is unclear but we speculate that
these cells may be the committed osteodifferentiation progeni-
tor cells that are distributed throughout the soft tissues in the
body. These cells may become fully committed osteoblasts and
then osteocytes in response to changes in microenvironment
involving trauma, hormones, growth factors, Ca²⁺, vitamin D₃,
or other factors. These cells might explain the observation of
extraosseous osteogenesis, the possible origin of so-called de-
differentiated chondrosarcoma, or the coexistence of osteosar-
coma and malignant fibrous histiocytoma in the same tumor.
Further study is required to isolate the TMMR-2-positive cells
in MFH or chondrosarcomas by fluorescence-activated cell
sorting and to characterize these cells in order to understand
their growth potential.

Western immunoblotting experiments demonstrated that
TMMR-2 binds a M, 26,000 protein that was also identified in
all six different osteosarcoma cell lines and one fresh osteosar-
coma tested. The M, 26,000 antigen was sensitive to disulfide
reduction with 5% mercaptoethanol or 0.01 M dithiothreitol
(Fig. 4). The antigenic activity was unstable at 100°C (3 min)
in the SDS sample buffer but was stable at 56°C for 30 min in
the absence of SDS. The antigen structure was not stable for
longer than 1 week at -20°C despite the presence of five
protease inhibitors nor was it stable at -20°C if the lysate was
washed with SDS sample buffer (not boiled) in order to inactiv-
ate the proteases. These results suggest a labile antigenic
structure requiring intact disulfide bonds. Further work is in
progress to establish if intra- or interchain disulfides are in-
volved. The M, 26,000 antigen recognized by TMMR-2 is
different than proteins recognized by other investigators based
on considerable difference in molecular weight and disulfide
requirement. The M, 72,000 band recognized by the 791T/36
MoAb described by Baldwin and coworkers (14, 15), the M,
75,000 band of Wada and coworkers (13), the M, 102,000
protein of Brown (12), the M, 80,000 band recognized by TP1
and TP3 of Bruland and coworkers (10), and the M, 92,000
band of Tsang (11) were all unaffected by mercaptoethanol
reduction performed after immunoprecipitation. The Western
immunoblots do not demonstrate the requirement for an
intact disulfide bond for antibody binding.

Previous work (2-6, 10, 13-15) on MoAbs to human osteo-
sarcoma did not investigate any potential biologic functional
activity of the MoAbs. In order to test whether the antigenic
structure recognized by TMMR-2 has any growth regulatory
properties, we studied the effect of TMMR-1-3 MoAbs on
[³H]thymidine DNA incorporation human osteosarcoma cell
lines. The results showed that TMMR-1 and TMMR-2 exert a
strongly inhibitory effect on DNA synthesis in osteosarcoma
cell lines (Fig. 5) and in osteoblastoma but not in control cells
from neuroblastoma, carcinoma, or T-cell lymphomas at a final
antibody concentration of 3-6 μg/ml. It was of interest that DNA
synthesis in HCS-1 cell line (Fig. 5, lower) and one addi-
tional chondrosarcoma cell line was not inhibited, despite
the binding of TMMR-2 for these cells (Table 1). Other matura-
tion-dependent factors may be different between chondrosar-
coma and osteosarcoma cells and may be responsible for the
lack of DNA inhibition by TMMR-2 on the former. The
inhibition of the DNA synthesis by TMMR-2 was complement
independent and was not related to interference in anchoring
of tumor cells. Further work is required to elucidate the mecha-
nism of this inhibition of DNA synthesis and test whether
TMMR-2 MoAb conjugated to isotopes, drugs, or toxins may
have clinical applications.
TMRR-1 reacts with cells of mesenchymal origin (Tables 1 and 2). These include the normal, reactive, and neoplastic states of osteoblast, chondroblast, fibroblast/fibrohistiocyte, neuroblast, smooth muscle, and endothelial cells. Cells of the hematopoietic system, including myelomonocytic cells and both T- and B-lymphocytes of normal and neoplastic states, are also reactive, but weakly (Fig. 1, D and E). This data suggests the existence of an antigen common to mesenchymal cells and the sarcomas from which they arise. A similar common antigen, HL-1, exists for human hematopoietic cells and their neoplastic counterparts on leukemias and lymphomas. A common epithelial membrane antigen has also been described for the carcinomas (31). TMRR-1 may be useful clinically in distinguishing carcinomas from sarcomas.

TMRR-3 recognizes, in addition to a significant cell surface antigen in osseous or cartilaginous cells, all cells in mitosis (Table 2). Occasionally nonmitotic cells presumed to be in the late G2 phase are reactive. This antibody could be used to determine the mitotic index of cells or to separate G2 and M phases in two-color DNA flow cytometric analysis. TMRR-3 recognizes a highly abundant, mercaptoethanol resistant, single polypeptide chain of M, 97,000 (Fig. 3), whereas TMRR-1 detects a series of bands from 65,000 to 85,000 molecular weight that are all mercaptoethanol sensitive. Further work is required to establish if the multiple bands are the result of degradation or alternative processing mechanisms. The relationship of the TMRR-3 M, 97,000 band to the M, 92,000 protein immunoprecipitated by the MoAb OSA-1, generated to antigen isolated from spent culture media (11) remains to be determined.

In summary, TMRR-2 appears to recognize a novel M, 26,000 labile antigenic protein with unusual temperature sensitivity and requirement for intact disulfide bonds. Based on its specificity and ability to inhibit DNA synthesis it may have utility for further study on the biology of osteosarcoma cells and for immunolocalization and therapeutic experiments in the native state or conjugated to toxins, drugs, or isotopes.

ACKNOWLEDGMENTS

We thank Theresa Klein for expertly typing the manuscript.

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Monoclonal Antibody to Human Osteosarcoma: A Novel $M_r$ 26,000 Protein Recognized by Murine Hybridoma TMMR-2


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