Isolation and Partial Purification of Plasma Membrane-associated Antigens from Human Osteosarcoma (TE-85) Cells in Tissue Culture

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SUMMARY

The plasma membrane of a cloned line of TE-85 (human osteosarcoma) cells subcultured for the last 4 years was isolated. The isolation was by hypotonic swelling, cell homogenization, and discontinuous sucrose gradient ultracentrifugation for 16 hr. Tumor-specific water-soluble antigens were identified by limited papain digestion of the isolated plasma membrane. Fractionation by diethylaminoethyl ion-exchange column chromatography yielded antigens identified by limited papain digestion of the isolated plasma membrane. Fractionation by diethylaminoethyl ion-exchange column chromatography yielded antigens that inhibited the reaction of immune serum (from an unrelated patient with osteosarcoma) with the plasma membrane of TE-85 cells in tissue culture by indirect immunofluorescence test. Microimmunodiffusion confirmed the specificity of the isolated antigen against the sera of other patients with osteosarcoma. The definition of the antigen fraction may permit evaluation of antigen-antibody interaction in tumor immunity.

INTRODUCTION

A cloned line of human osteosarcoma (TE-85) cells from a biologically proven osteosarcoma (by histology and autopsy findings) maintained in the authors' laboratory was used in this investigation to isolate and partially purify the plasma membrane-associated antigens.

In animal models, tumor-specific antigens which are absent in normal tissues (of the host) have been identified on the cell surface of living or fixed cells, by indirect immunofluorescent techniques (13, 14, 16, 17). Recent improvements in cell fractionation have permitted isolation of plasma membrane fragments from HeLa cells by discontinuous sucrose gradient and continuous sucrose gradient ultracentrifugation (6, 7). By electron microscopy, the isolated plasma and smooth membrane fractions appeared to be free of contamination (7).

A soluble extract with a major antigenic fraction has been reported by Baldwin and Glaves (2), using limited papain digestion of rat hepatoma plasma membrane. The antigenicity of the fraction was determined by its ability to elicit tumor-specific antibody response in syngeneic rats.

The present investigation was undertaken to evaluate the nature of the antigens in a spontaneously arising human osteosarcoma and to determine the antigen-antibody relationship to tumor immunity against human osteosarcoma.

MATERIALS AND METHODS

TE-85 human osteosarcoma cells were cultivated, cloned, and cultured by McAllister et al. (17). TE-32 cells (rhabdomyosarcoma cell line) were obtained from R. M. McAllister. WI-38 cells (embryonic human lung) were obtained from the Grand Island Biological Co. (Grand Island, N. Y.). All cell lines were maintained as continuous cell lines in the authors' laboratory, as previously described (22). The TE-85 cells were tested for antigens against isoantibodies in human sera by using anti-A, anti-B, anti-D, and anti-AB blood-grouping sera. The results of these tests were negative against all the sera.

Approximately 2.5 x 10^9 cells were washed and ruptured to isolate the membrane fraction according to the technique described by Bosmann et al. (7). Following rupture and centrifugation at 4000 x g for 10 min at 4°C, the supernatant (S) was decanted and saved. A discontinuous sucrose gradient was made with the supernatant by equilibration with 45% sucrose (w/v); 13.5 ml of this equilibrated 45% sucrose was placed in a cellulose nitrate centrifuge tube (38.7 ml). Then, 8.6 ml of 35% sucrose were carefully layered on this, followed by 8.6 ml of 30% sucrose, over which was layered 6 ml of 25% sucrose. Finally, 2 ml of 0.05 M Tris buffer, pH 7.4, were added. The discontinuous gradient was then centrifuged in a Beckman L3-50 ultracentrifuge (Rotor SW 27) at 23,000 rpm (70,000 x g) for 16 hr at 4°C. The resulting fractions, including the membrane pellet, were identified in 7 separate layers. The membrane pellet (S7) was resuspended in 0.05 M Tris-HCl, pH 7.4, and ultracentrifuged in a Beckman L3-50 ultracentrifuge (SW 50.1) at 23,000 rpm (70,000 x g) for 1 hr. The pellet thus obtained was resuspended in a discontinuous gradient (as above), and ultracentrifugation for 16 hr was repeated, following which the plasma membrane fraction (S7-7) at the bottom of the cellulose nitrate tube was removed.

The isolated plasma membrane fraction S7-7 was subjected to the following studies: (a) enzyme assay; (b) estimation of DNA; (c) estimation of RNA; (d) ferritin conjugation and electron microscopy; (e) membrane immunofluorescent test; (f) microimmunodiffusion; (g) DEAE-cellulose an-
ion-exchange chromatography; and (h) antigen assay.

**Enzyme Assays.** For verification that the plasma membrane fraction (S7-7) in all 3 cell lines was free of contamination, the pellet obtained after the homogenization, the supernatant fraction (S7) after the 1st ultracentrifugation and that (S7-7) after the 2nd ultracentrifugation were subjected to enzyme analyses. The following enzymes were tested.

Esterase (EC3.1.1.1) was tested for microsomes by the Bier (5) method, using p-nitrophenyl acetate as substrate; UDPase (EC 3.6.1.6) was tested by the Plaut (19) method for Golgi and smooth membrane; succinic dehydrogenase (EC 1.3.99.1) was tested by the method of Slater and Bonner (32) for mitochondria; glucose-6-phosphatase (EC 3.1.3.9) was tested by the Swanson method (24) for microsomes; and 5-nucleotidase (EC 3.1.3.5) was tested by the method described by Heppel and Hilmoe (12) for the plasma membrane, using 5'-AMP as substrate.

Fraction S-7 and Fraction S7-7 were also checked for RNA contamination by the orcinol reaction (9). DNA contamination in these fractions was determined by Burton’s technique (8).

**Ferritin Conjugation and Electron Microscopy.** The immunoglobulin fraction was precipitated from the sera of osteosarcoma patients with sodium sulfate. The coupling of ferritin to the precipitated globulin was performed according to the method described by Singer and Shick (21). Normal globulin was conjugated in a similar manner and served as a control.

An equal volume of the ferritin-conjugated immunoglobulin was added to the plasma membrane fraction (S7-7), and the mixture was gently shaken for 30 to 45 min at 37°C. The ferritin-treated membrane fraction was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 2 hr and was postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for 1 to 2 hr. Following dehydration in graded ethanol and acetone, the specimen was embedded in Epon 812 resin. Thin sections were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Phillips 300 electron microscope.

**Membrane Immunofluorescence Test.** Aliquots of between 3 to 5 x 10^6 viable cells were incubated at 37°C for 20 min in a humid chamber, with 0.1 ml of undiluted serum from patients with osteosarcoma. After incubation, the cells were sedimented by centrifugation (300 x g for 5 min) washed 3 times with Medium 199, and reincubated for 20 min with 0.1 ml of fluorescein-conjugated goat anti-human polyvalent γ-globulins (Grand Island Biological Co.) in 1:20 dilution. The cells were then washed 3 times in Medium 199 and resuspended in glycerol:phosphate-buffered saline (1:1 v/v; NaCl, 8.5 g; KCl, 0.2 g; NaHPO₄, 1.15 g; KH₂PO₄, 0.2 g; in 1 liter, pH 7.3), and examined for membrane fluorescence under a Zeiss UV microscope using excitor Filter I/BG 12 and barrier Filters 53 and 44. Sera from patients with adenocarcinoma of the breast with extensive osseous metastases and liposarcoma were used and normal human serum served as control.

**Papain Digestion.** The isolated plasma membrane (S7-7) was suspended in 5 mM Tris-phosphate buffer, pH 7.8, containing 5 mM cysteine, and the membrane was partially digested with papain (Sigma Chemical Co., St. Louis, Mo.) (1 mg of papain per 30 mg of membrane protein) by stirring for 30 min at 37°C. The undegraded membrane material was removed by centrifugation at 78,000 x g for 1 hr. The supernatants were removed, pooled, and dialyzed overnight against 5 mM Tris-phosphate, pH 7.8, at 4°C. The dialyzed supernatant was concentrated before use.

**Microimmunodiffusion.** Immunodiffusion was performed in microimmunodiffusion plates (Hyland Div., Costa Mesa, Calif.). In 1 set of experiments, the papain-digested plasma membrane solution (TE-85, TE-32, WI-38) was placed in the center well separately, and in the surrounding wells were placed sera from 5 patients with osteosarcoma, 1 with liposarcoma, and 1 with adenocarcinoma of the breast with osseous metastases; normal human serum and papain solutions were used as a control. In another set of experiments, serum from an osteosarcoma patient was placed in the center well and in the surrounding wells were placed plasma membrane solution isolated from TE-85 cells, TE-32 cells, WI-38 cells, papain solution, and 0.9% NaCl solution. The plates were incubated in a moist chamber at room temperature for 2 to 3 days.

**DEAE-Cellulose Anion-Exchange Column Chromatography.** Preswollen DEAE-cellulose was packed in a 2.5- x 40-cm column. The papain-digested, solubilized (dialyzed overnight) plasma membrane (S7-7) was eluted with a starting buffer of 5 mM Tris-phosphate, pH 7.8, which was used as a continuous gradient with a limiting buffer of 0.5 mM Tris-phosphate, pH 4.5. The flow rate was adjusted to 0.5 ml/min and 10-ml fractions were collected at 4°C in a Buchler fraction collector. The method of Lowry et al. (15) was used to determine the protein content of the fractions.

**Antigen Assay.** The antigenic activity was determined by the ability of the isolated fraction of the eluate to inhibit cellular membrane immunofluorescence of the TE-85 osteosarcoma cells (maintained in tissue culture) by the antibody from the sera of patients with known osteosarcoma. The antigenicity of the fraction was determined by its capacity to neutralize the reaction of the specific human antibody in the serum of the patient (using the indirect immunofluorescence test) and was indicated by a reduction in the number of fluorescent-stained cells by a patient’s immune serum neutralized with antigenic fractions from the eluate. This was compared with the same immune serum diluted with an equivalent volume of 0.1 mM phosphate-buffered saline, pH 7.4. The proportion of fluorescent-stained cells was calculated by examining 150 cells. The fluorescence indices were calculated as the proportion of negative TE-85 cells in the sample exposed to normal human serum minus the proportion of the negative cells in the sample exposed to the test serum and the eluted fractions neutralized with an equal volume of test serum. The percentage of inhibition of the fluorescence index with the absorbed serum was used as a measure of the antigenic activity, as described by Baldwin et al. (3).

**RESULTS**

The results of the 1st ultracentrifugation of the supernatant obtained following homogenization of the cells are
shown in Chart 1. Seven distinct fractions were recognized. Fraction S-7 represented the plasma membrane with some subcellular contaminants. Following resuspension and the 2nd ultracentrifugation in a discontinuous gradient, Fraction S7-7 was isolated as contaminant-free plasma membrane.

**Enzyme Assay.** The results of the enzyme analyses are summarized in Table 1. It was noted that Fraction S-7 after the 1st ultracentrifugation was contaminated with microsomes and mitochondria, as indicated by the enzyme activity. After resuspension and centrifugation, the membrane pellet was ultracentrifuged in a 2nd discontinuous gradient. Enzyme analysis of this fraction indicated absence of contamination by other subcellular material. Furthermore, the 5-nucleotidase activity (a plasma membrane marker) was highest in this fraction as expressed in moles per mg protein per hr (Table 1). Enzyme assay was performed only on the 1st ultracentrifugation was contaminated with microsubcellular contaminants. Following resuspension and the centrifugation, the membrane pellet was ultracentrifuged in a 2nd discontinuous gradient. Enzyme analysis of this fraction indicated absence of contamination by other subcellular material. Furthermore, the 5-nucleotidase activity (a plasma membrane marker) was highest in this fraction as expressed in moles per mg protein per hr (Table 1). Enzyme assay was performed only on TE-85 cell plasma membrane. The fractionation technique appeared to be satisfactory.

The orcinol test for RNA was negative. The diphenylamine reaction indicated an absence of DNA in all 3 plasma membrane (S7-7) fractions.

**Ferritin Conjugation and Electron Microscopy.** As shown in Fig. 1, granules of ferritin were observed around the plasma membrane fraction (S7-7) on electron microscopy. This positive result was observed only when the ferritin conjugated with the immunoglobulin from the sera of patients with osteosarcoma was used to incubate the TE-85 cell plasma membrane. The fractionation technique appeared to be satisfactory.

The antiserum to ferritin was used to react with sera from the same osteosarcoma patient.

**Microimmunodiffusion.** The microimmunodiffusion test showed precipitate lines (Fig. 3) only against the sera of patients with osteosarcoma. All other sera, and the papain, failed to form any precipitate lines against the antigen extracted from the TE-85 cell plasma membrane. No positive reaction was seen when plasma membrane of TE-32 cells and WI-38 cells was used. When serum from patients with osteosarcoma was placed in the center well and plasma membrane from TE-85, TE-32, and WI-38 cells was placed in the surrounding wells, precipitate lines were seen only with the TE-85 cell plasma membrane (Fig. 4).

**DEAE-cellulose Chromatography.** The amount of protein per tube was as shown in Charts 2 to 4. It was observed that traces of protein were detectable immediately after the void volume (50 ml). The antigenicity of the protein fractions eluted (by DEAE column chromatography) is also shown in Charts 2 to 4 and is expressed as a percentage of inhibition of the fluorescence index against TE-85 osteosarcoma cells maintained in tissue culture in our laboratory. The maximum antigenicity appears to be between Fractions 33 and 52, as 3 peaks of activity. No inhibition of fluorescence indices against TE-85 cells was observed when plasma membrane fractions of TE-32 cells and WI-38 cells were used for neutralization of the immune serum from a patient (Charts 2 to 4).

**DISCUSSION**

This investigation indicates that it was possible to isolate a pellet of plasma membrane (free of detectable subcellular contaminants) from TE-85 osteosarcoma cells maintained in tissue culture for over 4 years. It further indicated that the TE-85 (osteosarcoma) cells retained their antigenic characteristics on the surface of the plasma membranes even after prolonged passage in tissue culture. The specific antigen could be rendered water soluble by limited papain digestion and partial purification achieved by anion-exchange chromatography. Baldwin et al. (3) fractionated the "extranuclear" membranes from viable rat hepatoma D23 cells. The antigen inhibition immunofluorescence index on the extranuclear membrane was obtained by using sera from syngeneic rats sensitized to hepatoma D23 cells.

To obviate the pitfalls of artificially induced antibodies in syngeneic animals by sensitization with tumor cells, human subjects were used in this investigation. The osteosarcoma
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osteosarcoma, as evidenced by the fluorescence inhibition index (Charts 2 to 4). A limiting factor in the characterization of the solubilized osteosarcoma antigen is the absence of sensitive in vitro methods for the quantitation and detection of the antigen.

The membrane immunofluorescence test used initially in this investigation only measures the antibody complexing with the antigens expressed on the cell surface. However, in this investigation, a positive reaction was obtained against the TE-85 cells (Fig. 2) with the sera of unrelated patients with osteosarcoma. When the TE-85 cells were reacted against the sera of patients who did not have an osteosarcoma, but had multiple osseous metastases, the reaction was negative. The authors agree with Baldwin and Moore (4) that the immunofluorescence test is relatively insensitive and laborious. Morton and Malmgren (16) have reported cross-reactivity between the antigens and antibodies (in the sera) of patients with various sarcomas, particularly osteosarcoma, liposarcoma, fibrosarcoma, and chondrosarcomas. Their data also indicated a positive reaction against 2 of 4 melanomas, but not against adenocarcinoma of the breast or colon.

Priori et al. (20) reported studies of the fixed indirect immunofluorescence tests on imprints of 8 osteosarcomas and cells of tissue cultures derived from 5 osteosarcomas and suggested the presence of a common antigen in the tumors. These results indicated the presence of a common cross-reacting tumor-associated antigen in sarcomas of the same type, as well as in sarcomas of different histological types. Furthermore, circulating complement-fixing (10, 11) and cytotoxic antibodies against these sarcoma-associated antigens were demonstrated in the sera of patients with these tumors.

In this investigation, electron microscopic examination of ferritin-conjugated antibody confirmed that osteosarcoma antigens are surface bound on the plasma membrane of the TE-85 cells but not on the TE-32 and WI-38 cells. Our results indicate that the plasma membrane-bound osteosarcoma antigens react specifically with the sera of patients with osteosarcoma and not other tumors tested.

The results of microimmunodiffusion in the present investigation indicated positive reaction of the isolated TE-85 cell antigen only to sera from 5 different patients with osteosarcoma and no reaction or precipitation against normal human serum, serum from a patient with adenocarcinoma of the breast with osseous metastases, liposarcoma, and a solution of papain. No positive reaction was observed between sera from patients with osteosarcoma against plasma membrane isolated from TE-32 cells from a rhabdomyosarcoma or WI-38 cells (embryonic lung).

One of the goals of this investigation was the purification of osteosarcoma-specific antigens to permit investigation of the nature of antigenic determinant in spontaneously occurring osteosarcoma in human beings.

The TE-85 osteosarcoma antigen fractions prepared in this investigation were not sufficiently purified to permit characterization of the antigenic determinant in osteosarcoma. Further purification and sophisticated immunological techniques will probably be necessary to achieve this goal.
In relation to tumor immunology for osteosarcoma, the isolation of antigenic fractions will make it possible to study the significance of the antigen in immune competence of patients with the disease. Preliminary data indicate that the solubilized extracted antigen inhibits the cytotoxic effects of lymphocytes on the target cells. These preliminary in vitro studies may determine lymphocyte blocking by free-circulating antigen or antigen-antibody complexes at the target cell or effector cell level in the blocking effect seen in patients with tumors.

REFERENCES

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Fig. 1. Thin section of TE-85 plasma membrane (S7-7) incubated with ferritin conjugated with immunoglobulin from an unrelated patient with osteosarcoma. Note the presence of ferritin granules (arrows) on the plasma membrane fragments.

Fig. 2. Positive membrane immunofluorescence test with serum from a patient (M. Mc.) with osteosarcoma against TE-85 cells harvested from tissue culture. × 800.
Fig. 3. Microimmunodiffusion. A, partially papain-digested TE-85 cell plasma membrane (S7-7); B and C, serum from 2 unrelated patients with osteosarcoma; D, serum from patient with breast carcinoma and osseous metastases; E, papain solution; and F, normal human serum.

Fig. 4. Microimmunodiffusion. A, serum from patient with osteosarcoma; B, TE-85 cell plasma membrane (S7-7); C, TE-32 cell plasma membrane (S7-7); D, WI-38 cell plasma membrane (S7-7); E, papain solution; and F, saline.
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