Characterization of a Human Osteosarcoma Cell Line (Saos-2) with Osteoblastic Properties

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ABSTRACT

This study examines the osteoblastic properties of the established human osteosarcoma cell line Saos-2. Saos-2 cells inoculated into diffusion chambers, which were implanted i.p. into nude mice, produced mineralized matrix in 4 of 6 chambers at 8 weeks. In 5 of 6 chambers there was a strong positive alkaline phosphatase reaction. In culture the alkaline phosphatase levels increased with time and cell density, reaching very high levels at confluence: 4-7 nmol/mg protein/min. The presence of osteonectin was visualized with a monoclonal antibody which revealed a reticular pattern on the cell surface. Osteonectin was also detected in the medium by Western blots, migrating at around M, 40,000 in nonreduced gels and M, 44,000 in reduced gels. The Saos-2 cells thus possess several osteoblastic features and could be useful as a permanent line of human osteoblast-like cells and as a source of bone-related molecules.

INTRODUCTION

Malignant cells often express differentiated features of the tissue of origin (1) along with cellular immortality. This combination of properties makes it possible to establish cell lines in which tissue-specific products and phenotype-related cellular functions can be studied. The rat osteosarcoma-derived cell lines UMR and ROS have been used in this fashion (2, 3). The object of this study was to characterize a human cell line with osteoblastic properties, since cellular products (peptides/proteins) as well as cellular responses (4) are sometimes species specific. Studies of isolated cells from embryonic calvaria, osteosarcoma, and other types of bone, along with histological and histochemical studies, have helped to establish a set of properties believed to be associated with the osteoblastic phenotype (5). These include elevated alkaline phosphatase, parathyroid hormone-stimulable adenylate cyclase, synthesis and secretion of type I collagen, and production of mineralized matrix in Millipore filter chambers. Other features include the expression of bone γ-carboxyglutamyl acid-containing protein, fibronectin, osteonectin, sialoprotein, proteoglycans, collagenase and others, and the modulation of the above properties by 1,25(OH)2D3,2-PTH, glucocorticoids, prostanoids, growth factors, and so forth. Saos-2 is an established human osteosarcoma cell line which has been partially characterized (5, 6). We show in this investigation that several of the osteoblastic features listed above are present in this human cell line.

MATERIALS AND METHODS

Materials. RPMI powder, FBS, and kanamycin were from Grand Island Biological Co. (Grand Island, NY). Cells were grown in tissue culture dishes purchased from Costar (Cambridge, MA). [3H]Adenine (17 Ci/molm) was from New England Nuclear (Boston, MA). [3H]-1,25(OH)2D3 (180 Ci/molm) was obtained from Amersham Corp. (Arlington Heights, IL). Dexamethasone, isobutylmethylxanthine, adrenine, cAMP, and BSA were from Sigma Chemical Co. (St. Louis, MO). Synthetic human PTH was purchased from Bachem Biochemicals (Torrance, CA). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat antimouse IgG were purchased from Cappel Laboratories (Cochrane, PA). The radioimmunoassay kit for osteocalcin was purchased from Immunonuclear Corporation (Stillwater, MN). 1,25(OH)2D3-treated medium from human bone cells was generously provided by Dr. B. Sacktor (National Institute of Aging, Baltimore, MD). 1,25(OH)2D3 was a gift of Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ). Monoclonal anti-bovine osteonectin antibody was developed and characterized in the laboratory of Dr. Kenneth Mann. Saos-2 cells were obtained from Dr. H. Harris of the University of Pennsylvania. This cell line as well as Saos-1 were established in 1973 (7). Labtek culture chamber/slides were purchased from Miles Scientific (Naperville, IL). Lucite rings with holes and thread were from Millipore Corp. (Bedford, MA).

Cell Culture. Saos-2 cells were cultured in RPMI medium supplemented with 10% FBS, 2 mm l-glutamine, and 0.01% kanamycin in a humidified atmosphere of 95% air-5% CO2 at 37°C. The cells were passaged using 0.01% trypsin-1 mM EDTA every 10 days. For CAM assay, cells were seeded into 6 × 35-mm-well culture dishes.

Chromosomal analysis carried out by Dr. Warren Nichols (Merck Sharp and Dohme Research Laboratories, West Point, PA) showed that this is a heteroid line compatible with human cells.

Adenylate Cyclase Assay. cAMP was measured in whole cells as previously described (8) by the conversion of [3H]ATP to [3H]cAMP. Briefly, the cells were incubated with 1 μCi [3H]adenine for 2 h at 37°C. Cells were washed, preincubated with 1 mM isobutylmethylxanthine for 10 min, and incubated with hormones for 5 min. [3H]cAMP was isolated by the method of Salomon et al. (9). Alkaline Phosphatase Assay. Alkaline phosphatase activity was measured as previously described (10). The assay mixture contained 0.1 μM 2-amino-2-methyl-1-propanol (pH 10.5), 2 mM MgCl2, and 2 mM 4-nitrophenylphosphate in 0.2 ml.

Protein Determination. Protein was measured by the method of Spector (11) using BSA as standard.

Immunofluorescence Microscopy. Cells were plated onto Labtek culture chamber/slides at a density of 50,000/cm2 in RPMI medium supplemented with 10% FBS and 2 mM glutamine. After 24 h of incubation, the medium was removed and serum-free medium was added to the cells for another 24 h. All of the staining procedures were done at room temperature. Cells were washed twice with calcium-magnesium-free Hanks' balanced salt solution, fixed with 1% paraformaldehyde in PBS for 20 min, and permeabilized with 0.01% Nonidet P-40 for 20 min. At each subsequent step the cells were washed with PBS. Monoclonal antibody against osteonectin (1:100 dilution in PBS) was added to each slide for 1 h. Cells were incubated with a 1:200

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2The abbreviations used are: 1,25(OH)2D3, 1-25-dihydroxyvitamin D3; PTH, parathyroid hormone; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; KTED buffer, 300 mM KCI, 10 mM Tris-HCl, 1.5 mM EDTA, 5 mM dithiotreitol, pH 7.4; SDS, sodium dodecyl sulfate.
Preparation of Cell Cytosol and[^3H]25(OH)2D3 Binding Assay. Cells were incubated with 0.01% trypsin-1 mM EDTA, collected by centrifugation at 800 x g, washed twice in cold Dulbecco's PBS containing Ca and Mg (137 mM NaCl, 2.68 mM KCl, 8 mM Na2HPO4, 2.68 mM KH2PO4) containing 10 mg/ml BSA, then rinsed 3 times in cold PBS, resuspended in 500 mM KCl, 10 mM Tris-HCl 7.5, 1.5 mM EDTA, 5 mM diethiothreitol, pH 7.4 (KTED buffer), homogenized, and kept on ice for 30 min. The homogenates were centrifuged at 40,000 x g for 15 min, the supernatant was kept on ice, and the pellet was taken up in KTED buffer for an additional 30 min on ice. After additional centrifugation, the 2 supernatants were pooled and aliquots (220 µl containing cytosol derived from 5 x 10^6 cells) were combined and assayed for[^3H]25(OH)2D3 binding at 4°C for 18 h. Parallel incubations contained[^3H]25(OH)2D3 plus 100-fold excess nonradioactive hormone to assess nonspecific binding. Bound ligand was recovered using the hydroxyapatite method.

Sucrose Gradient Analysis of[^3H]25(OH)2D3 Binding Moteity. Cell extracts were incubated with 1 nM[^3H]25(OH)2D3 ± 10 nM nonradioactive hormone, layered on linear gradients of 5-20% sucrose in KTED buffer, and centrifuged at 4°C for 20 h in a Beckman L3-50 ultracentrifuge (SW 50.1 rotor) at 50,000 rpm (13). Fractions of 500 µl were collected and counted. Sedimentation coefficients were estimated by comparison with protein markers (BSA, 6.0 S; ovalbumin, 3.7 S; and chymotrypsinogen, 2.54 S) that were centrifuged in the same experiment.

Preparation and Implantation of Diffusion Chambers. Lucite rings with holes and thread were glued to 0.45-µm pore size membrane filters and polyethylene tubing to make chambers with a volume of 200 µl. The chambers were sterilized by ethylene oxide. Cell suspensions (about 1 x 10^7 cells) were injected into the chambers, which were then implanted i.p. in athymic nu/nu (14) mice, 2 per animal. Some of the animals were implanted with 1 chamber containing Saos-2 and 1 containing ROS 17/2.8 cells as positive controls. The chambers were harvested after 8 weeks.

Processing for Light Microscopic Examination. The chambers were removed from the animals immediately at sacrifice and placed intact in 70% ethanol for the fixation of cells and matrix. The chambers were impregnated for 48 h and embedded in glycol methacrylate under vacuum. Sections, 4 µm thick, were removed from the chambers in a transverse plane. Multiple levels were sectioned in each chamber. The sections were stained with von Kossa for mineral deposits and counterstained with toluidine blue and for alkaline phosphatase (Sigma). All sections were examined under bright light microscopy.

Preparation of Cell-conditioned Media for Western Blot Analysis. Saos-2 cells were grown to confluence and maintained in serum-free medium for the last 24 h prior to harvesting. The medium was centrifuged to remove cellular debris and concentrated 100-fold by centrifugation at 4000 x g in a Centrificon-10 tube. One half of the concentrated medium received an equal volume of double-concentration SDS cocktail (0.125 M Tris-HCl, pH 6.8, 4% SDS, and 20% glycerol), containing 1.46 M beta-mercaptoethanol; the other half received the same solution without beta-mercaptoethanol. Samples were then fractionated on SDS-12.5% polyacrylamide gels according to Laemmli (15). Protein bands were electrophoretically transferred to nitrocellulose membranes according to Towbin et al. (16) for 14 h at 4°C and 0.2 amperes. Following transfer, the filter was blocked in a solution of PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 1% BSA for 1 h at room temperature. The paper was then incubated for 2 h in 6 ml of PBS-1% BSA containing 250 µg of antihuman osteonectin monoclonal antibody. The blot was then washed 3 times with PBS and incubated for 1 h in 10 ml of PBS-1% BSA containing 300 µg of peroxidase-conjugated goat antimouse IgG and washed 3 times with PBS. The blot was developed for 10 min in a mixture of 3 ml of 3 mg/ml 4-chloro-1-naphthol in methanol and 15 ml of 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.001% H2O2.

RESULTS

Growth Rate. Fig. 1 shows the growth rate of Saos-2 cells in 10% fetal bovine serum. Cells seeded at 5000 cells/cm² exhibited logarithmic growth for 7 days with a doubling time of 37 h. Cells reached the density of 300,000 cells/cm².

Alkaline Phosphatase. The specific activity of alkaline phosphatase was about 4 µmol/mg protein/min 24 h after plating. It then dropped to 1.8 µmol/mg protein/min at 4 days of culture and rose to 6.5 µmol/mg protein/min at confluence (Fig. 2).

Parathyroid Hormone-sensitive Adenylate Cyclase. Fig. 3 shows the dose-response curve for PTH (1-34) stimulation of in situ adenylate cyclase measured by the conversion of endogenously labeled[^3H]ATP to[^3H]cAMP. Statistically significant stimulation (about 50%) is seen at 0.3 nM hormone. Half...

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Fig. 3. PTH stimulation of adenylate cyclase in Saos-2 cells. Adenylate cyclase (cpm/10^6 cells/5 min) was estimated in whole cells (1.65 × 10^6/well) as described in "Methods." Values are the mean ± SD from triplicate wells.

### Table 1 PTH stimulation of adenylate cyclase as a function of cell density in Saos-2 cells

<table>
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<th>PTH (nm)</th>
<th>0.5 × 10^-8</th>
<th>0.7 × 10^-8</th>
<th>1.4 × 10^-8</th>
<th>3.0 × 10^-8</th>
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<tr>
<td>0</td>
<td>3825 ± 135</td>
<td>1656 ± 20</td>
<td>842 ± 62</td>
<td>564 ± 15</td>
</tr>
<tr>
<td>1</td>
<td>3371 ± 343</td>
<td>2036 ± 174</td>
<td>1260 ± 150</td>
<td>1679 ± 24</td>
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<td>3434 ± 61</td>
<td>2834 ± 173</td>
<td>4455 ± 50</td>
</tr>
<tr>
<td>100</td>
<td>8423 ± 309</td>
<td>3624 ± 285</td>
<td>3605 ± 172</td>
<td>4644 ± 50</td>
</tr>
</tbody>
</table>

* Number of cells/cm².

maximal stimulation occurred around 2.2 nm. This dose-response curve of the PTH-stimulatable adenylate cyclase was obtained at 1.65 × 10^6 cells/well. Table 1 shows the responsiveness to PTH as a function of cell number. The baseline adenylate cyclase activity decreased and the sensitivity to PTH increased with cell density.

Dexamethasone was shown to enhance PTH stimulation of adenylate cyclase in osteoblastic calvaria and ROS 17/2.8 cells (17, 18). Fig. 4 shows the effect of dexamethasone on the time course of PTH (10 nM)-dependent cAMP accumulation in Saos-2 cells. Maximal cAMP levels in dexamethasone-treated and -untreated cells were seen at about 7–10 min, but the extent of PTH stimulation was enhanced about 2.5-fold in dexamethasone-treated cells.

### 1,25(OH)_2D_3 Receptors

1,25(OH)_2D_3 receptors were quantitated in 40,000 × g supernatants prepared from Saos-2 cells when the cells reached a density of 50,000 cells/cm². Fig. 5
Fig. 7. Immunocytochemical demonstration of osteonectin in Saos-2 cells. Cells exposed to antisteonectin antibody: A, permeabilized with 0.01% Nonidet P40; B, unpermeabilized. C, cells exposed to preimmune serum.

This study describes a human osteosarcoma cell line with osteoblastic properties. The production of mineralized matrix presents a binding curve of [3H]1,25(OH)2D3 as a function of hormone concentration. Scatchard analysis of the data (Fig. 5, inset) indicates an apparent Kd of 0.21 nM and a receptor abundance of 3,300 binding sites per cell. At a higher density of 280,000 cells/cm², the number of sites dropped to 1,800/cells, with no change in the apparent Kd. Fig. 6 shows the sedimentation properties of Saos-2 cell cytosol that had been labeled with 1.0 nM [3H]1,25(OH)2D3 in the presence and absence of 100 nM unlabeled 1,25(OH)2D3. Receptors for 1,25(OH)2D3 sediment at 3.2 S on sucrose gradients.

Immunofluorescence and Western Blot Analysis of Osteonectin. Immunocytochemical visualization of osteonectin in permeabilized cells revealed a reticular pattern. The staining intensity was highest in the perinuclear region, possibly corresponding to the Golgi apparatus (Fig. 7). This staining was not seen in nonpermeabilized cells.

Since osteonectin is a secreted protein (19), the presence of osteonectin in cell media was examined by Western blot analysis. As seen in Fig. 8, the media contained an immunoreactive band which migrated on SDS-polyacrylamide gel electrophoresis at M, 40,000 (lane 1) and was retarded by β-mercaptoethanol reduction (lane 2).

Histological Analysis of Saos-2 Cells in Millipore Chambers Implanted in Nude Mice. Eight weeks after i.p. implantation of diffusion chambers in nude mice, 4 of 6 chambers examined showed positive staining for calcified matrix with the von Kossa method. The calcified matrix was invariably localized within 1 mm of the filters (Fig. 9B). No cells or calcified matrix was found in the center of the chambers.

Five of 6 chambers and all 4 chambers which contained calcified matrix stained positively for alkaline phosphatase activity (Fig. 9A).

DISCUSSION

This study describes a human osteosarcoma cell line with osteoblastic properties. The production of mineralized matrix
is the major phenotypic characteristic of osteoblastic cells. Similar to bone marrow cells (20), calvaria cells (21), and osteoblastic rat osteosarcoma cell lines 17/2 and 17/2.8 (14), the Saos-2 cells form a calcifying matrix in diffusion chambers. Light microscopy examination revealed production of a mineralized matrix typical of woven bone, similar to that produced by ROS 17/2.8 cells. Cell growth, matrix production, and mineralization were less extensive than for 17/2.8 cells, which were strongly positive in 6 of 6 chambers in this series. There are several possible reasons for this difference. Unlike the 17/2.8 cells, the Saos-2 cells do not form tumors when injected s.c. in nu/nu mice (data not shown) and may grow less well in the diffusion chambers. Some murine growth or differentiation factors may be species specific and act better on rat than on human cells. Nonetheless, a majority of the implanted Saos-2 cells scored positively in this assay, a strong indication of their osteoblastic nature.

Biological mineralization has long been associated with elevated alkaline phosphatase levels (22). The Saos-2 cells reach very high levels of alkaline phosphatase, 4-6 μmol/mg protein/min, about 5- to 10-fold higher levels than calvaria cells (2) or osteoblastic rat osteosarcoma cells (2, 23). Similar to ROS 17/2.8 cells the alkaline phosphatase-specific activity in Saos-2 cultured cells decreased after seeding at low density and increased thereafter, peaking at confluence. The density- and/or growth-dependent expression of this phenotypic property in the 2 different cell lines may be a feature of osteoblastic maturation. Unlike in ROS 17/2.8 cells (24), dexamethasone had only a very small effect on alkaline phosphatase activity in Saos-2 cells, increasing its level by about 30% after 4 days in culture (data not shown), possibly due to the very high level of alkaline phosphatase expression in these cells.

Another osteoblastic feature of Saos-2 cells is the presence of PTH receptors coupled to adenylate cyclase. This property has been observed in osteoblasts in situ and in vitro (25-27). PTH stimulation of adenylate cyclase in Saos-2 cells has been reported by Boland et al. (6) along with vasoactive intestinal peptide and prostaglandin E2 stimulation. The present study provides a detailed dose-response curve for PTH-stimulatable adenylate cyclase and shows that the Saos-2 cells become increasingly sensitive to lower doses of PTH with time in culture, resulting from larger stimulation without changes in the apparent PTH affinity (2.8 NM ± 1.0, N = 6). At the end of logarithmic growth significant stimulation is seen at 0.3 NM PTH. The increased sensitivity to PTH is also partly due to the fall in basal activity with time in culture (Table 1). Maximal PTH stimulation of adenylate cyclase is 6- to 10-fold, as compared to 100-fold in ROS 17/2.8 cells, primarily a reflection of the high basal adenylate cyclase activity of Saos-2 cells. PTH sensitivity was further enhanced by dexamethasone treatment, a feature shared with the ROS 17/2.8 rat osteosarcoma cells (18).

The Saos-2 cells also possess 1,25(OH)2D3 receptors, which are found in fetal bone cells (28), mouse bone cells (29), osteosarcoma cells (30, 31), and many other cells. The 1,25(OH)2D3, Kd (0.21 nM) was comparable to that reported in rat osteosarcoma and bone cells, and the number of sites per cell was almost identical to that found in the human osteosarcoma cell line MG-63 (32). A sedimentation coefficient of 3.2 S for 1,25(OH)2D3 receptors is similar to that reported in bone cells (4, 33) and other cells. Receptor abundance decreased with cell density. There were 3,300 sites/cell when the cells were harvested at 50,000/cm2 and 1,800/cell when the cells were harvested at 240,000 and 280,000/cm2, with no change in Kd. These results are very similar to those observed in mouse bone cells (33) and MG-63 cells (32). The physiological role of 1,25(OH)2D3 in osteoblastic cells remains to be determined. One of the effects of this hormone, documented both in vivo and in vitro, is the enhanced production of osteocalcin (34, 35). This protein may be bone specific, since only bone-derived cells have been shown to synthesize it. Using immunocytochemistry and radioimmunoassay of 1,25(OH)2D3-treated cells, we found no positive reaction for osteocalcin in Saos-2 cells in culture. If this observation is upheld and Saos-2 cells also fail to make osteocalcin in vivo when they produce mineralized matrix, osteocalcin may not be a requisite marker for osteoblastic cells.

Another bone-abundant protein, osteonectin, is present in Saos-2 cells. Osteonectin was visualized by immunocytochemistry and was also shown to be secreted into the medium by Western blot analysis. Osteonectin was first isolated from bone matrix and was shown to be produced by fetal bovine (36), human (37), and porcine (19) bone cells. The SDS-polyacrylamide gel electrophoresis migration of osteonectin immunoprecipitated from the medium of human or porcine cells was around Mr 40,000-45,000, similar to that identified by the Western blot of Saos-2 cell media.

Among the human osteosarcoma cell lines reported so far (38-40) none has been examined for all of the properties reported here. MG-63 (32) and Saos-1 cells (41) have very low levels of alkaline phosphatase activity (2.5 nmol/min/mg protein) compared to Saos-2 cells (4-6 μmol/min/mg protein). In Saos-1 cells, alkaline phosphatase levels did not vary with cell density; however, 1,25(OH)2D3 and hydrocortisone increased alkaline phosphatase activity 3- to 5-fold in these cells. Even after stimulation, alkaline phosphatase levels were less than 1% of these in Saos-2. MG-63 cells have PTH-unresponsive adenylate cyclase (our unpublished observation). As pointed out, the Saos-2 cells share many properties with the rat osteosarcoma ROS 17/2.8 cell line. Since the mechanism for malignant transformation in those 2 tumors from different species is likely to be different, their common properties are probably related.
to their bone origin. This cell line could thus offer a useful experimental model for studying osteoblastic properties and osteoblast-produced molecules in an established human cell line.

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