Characterization of a New Human Osteosarcoma Cell Line OHS-4

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Abstract. We present a new human osteosarcoma cell line designated OHS-4. These cells showed a high alkaline phosphatase activity that is not regulated by 1,25 dihydroxyvitamin D_3. They exhibited a sensitive adenylate cyclase response to parathyroid hormone but not to prostaglandin E_2 or human calcitonin. By Northern blot analysis we could detect type I collagen mRNA but none for type III collagen. The cells were able to produce human osteocalcin at a maximum level of 35 ng per million cells when exposed to 2.4 nM 1,25-dihydroxyvitamin D_3 for 96 h. We purified this protein from conditioned media using successive chromatography and assessed its identity by partial amino acid sequencing. When injected into nude mice, the cells retained their osteogenic activity and developed calcified tumors. After Von Kossa staining, we observed nonmineralized osteoid deposits and mineralized deposits with a structure similar to that of trabecular bone by light microscopy. On the basis of its osteoblastic characteristics, this new osteosarcoma cell line may represent the human counterpart of the ROS 17/2 cell line. This cell line represents a valuable model for the isolation and characterization of human bone specific proteins.

STUDIES on osteoblasts in vitro have been conducted largely on the rat osteosarcoma cell lines ROS 17/2 and UMR-106 (24, 27). Several arguments prompted us to attempt to establish and characterize a human osteosarcoma cell line. Differences in hormonal regulation between species have been described (6). Several human osteosarcoma cell lines have been studied (3, 11, 18, 32, 33) but none of them showed all the osteoblastic features described in the well-characterized rat osteosarcoma cell lines ROS 17/2.8. Among the human osteosarcoma cell lines studied so far, two have been more extensively characterized in terms of their osteoblastic features: the SAOS-2 (26) and the MG-63 (19), and these cells are now quite widely used. The SAOS-2 cells display many osteoblastic characteristics such as high alkaline phosphatase activity, parathyroid hormone (PTH)-sensitive adenylate cyclase, but they do not produce the bone specific protein: osteocalcin also called bone gla protein (BGP).[1] On the contrary, the MG-63 cells described by Lajeunesse et al. (19) produce some BGP but they have very little alkaline phosphatase activity and no PTH-sensitive adenylate cyclase activity; secondly, they are not able to form bone when injected into nude mice. Finally, the need for a source of osteoblast-specific proteins coming from a well-characterized human osteoblastic cell line was a decisive argument. In this paper, we describe a new human osteosarcoma cell line, designated OHS-4, with the following osteoblastic characteristics: high alkaline phosphatase activity, PTH-responsive adenylate cyclase, synthesis of type I collagen, synthesis of BGP regulated by 1,25(OH)$_2$D$_3$, and the ability to produce mineralized tissue when injected into athymic animals.

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1. *Abbreviations used in this paper:* BGP, bone gla protein; CT, calcitonin; NCS, newborn calf serum.

Materials and Methods

The human osteosarcoma cell line MG-63 was obtained from the American Type Culture Collection (Rockville, MD), RPMI 1640 medium, newborn calf serum (NCS), antibiotics, and trypsin solution were obtained from Irvine Scientific (Irvine, CA). 1,25(OH)$_2$D$_3$ was generously provided by Dr. M. Uskokovik Hoffmann-La Roche (Nutley, NJ). Tissue culture dishes were purchased from Falcon Labware (Oxnard, CA). Human PTH(1-34), was from Bachem (Torrance, CA). Prostaglandin E$_2$ (PGE$_2$), phosphoenolpyruvate, pyruvate kinase, p-nitrophenylphosphate, cAMP, and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). Human calcitonin was provided by Ciba-Geigy Ltd. (Basel, Switzerland). α[^{32}P]ATP, [8-^{3}H]cAMP, and [α^{32}P]dCTP were purchased from Amersham Chemicals (Arlington Heights, IL). Random primed DNA labeling kits were obtained from Boehringer (Mannheim, FRG). All reagents were analytical grade unless otherwise stated.

Cloning of the Human Osteosarcoma Cell Line

Clones were isolated from a human osteosarcoma cell line OHS-50, which was a generous gift from Dr. Fodstad (12) (Dethorske Radium Hospital, Norway) by distributing trypsin-treated cells in microtiter plates (0.4 ml capacity; Falcon Labware) at an average dilution of one cell/well. Wells containing only one cell (established by microscopic observation) were maintained until a monolayer was formed, then the contents were transferred to 60-mm culture dishes. An alternative procedure using cloning cylinders was used after distributing trypsin treated cells at decreasing dilutions of 100:5 cells per 100-mm plate.

Osteosarcoma Cell Culture

MG-63 cells and OHS-4 clones were grown in RPMI 1640 medium with 10% NCS, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution. The cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. The medium was changed three to four times weekly and the cells were subcultured once a week.

For measurement of BGP production, cells were grown to confluency in 35-mm culture dishes. These cultures were then placed in 2 ml fresh medium containing 2% NCS for 24 h and then into 1.2 ml fresh serum-free

medium. At this point, the cells were treated with 1,25(OH)₂D₃ diluted in absolute ethanol to the concentration of 2.4 nM or with 10 μl of absolute ethanol/ml medium as the control. Every subsequent 24 h, the conditioned media was removed and the cultures were given fresh serum-free media with the same amount of 1,25(OH)₂D₃ or absolute ethanol. For each day over 7 d, two plates were counted. Cell counts were performed on trypsinized cell suspensions using a Coulter counter (Coulter Electronics Inc., Hialeah, FL).

Growth Properties

Cells were plated at different densities: 5,000, 10,000, and 20,000 cells/cm² in microtiter plates. Medium was changed every 48 h. Growth was monitored over a period of 8 d.

RNA Isolation and Blot

RNA was extracted from cell cultures by the guanidium isothiocyanate method (7). 20 μg of RNA were electrophoresed on a 0.8% agarose gel containing 2.2 M formaldehyde and blotted onto a Hybond sheet. The filters were hybridized at 42°C with the appropriate probe. cDNA for procollagen α1(I) and α1(III) were a kind gift from Dr. Ramirez (8, 9).

Identity of the Protein Measured by Human BGP Radioimmunoassay

80 100-mm plates of OHS-4 were grown to confluency and treated with vitamin D3 during 4 d as described above. Conditioned media from the last 3 d was collected. This media was passed over an affinity column with antibodies directed against calf BGP. The peak containing the immunoreactivity was eluted through a reverse phase HPLC column as already described (23). The peak collected was subjected to automatic Edman degradations using a gas phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA).

Radioimmunoassay

The procedure used for human BGP radioimmunoassay has been described elsewhere (25). Results reported in this paper are averages of three determinations.

Alkaline Phosphatase Determination

Cells were seeded at a density of 40,000 cells/cm² in microtiter plates. After 24 h, the medium was replaced with one containing 2% heat-inactivated FBS. The cells were incubated for 72 h with daily change of medium. After 72 h, the cells were washed with PBS, pH 7.6, trypsinized and collected. The cells were then suspended in TMX buffer (10 mM Tris HCl, pH 7.4, 1 mM MgCl₂, 20 μM ZnCl₂, 0.02% (wt/vol) NaN₃, and 0.1% Triton-X 199), and sonicated for 30 s. The sonicate was centrifuged 30 min at 9,000 g at 4°C. Alkaline phosphatase was routinely determined by measuring the release of p-nitrophenol from p-nitrophenylphosphate spectrophotometrically (410 nM at 37°C) by the method of Lowry et al. (20). Protein was measured by the Pierce BCA protein assay kit (Pierce Chemical Co., Rockford, IL) (31). Results were expressed in micromoles/minute/milligram protein.

Adenylate Cyclase Activity

Adenylate cyclase assays were carried out on cells at confluency. The cells were rinsed with 10 ml of homogenizing medium consisting of 0.25 M sucrose, 1 mM DTT, and 1 mM Na₂ EDTA in 25 mM HCl, pH 8.2. Cells were scraped from the surface of the flask with a plastic policeman and homogenized in an all-glass, handheld tissue homogenizer (10 strokes) in ice. The reaction mixture consisted of 25 mM Tris HCl, pH 7.4, 0.1 mM cAMP, 5 mM phosphoenol pyruvate, 3 U pyruvate kinase, 30 mM KCl, 9 mM MgCl₂, 0.1 g/liter BSA, 0.1 mM [α³²P]ATP and hormone as indicated in 100 μl reaction volume. The reaction was initiated by addition of cell homogenates (50–100 μg protein) and allowed to proceed for 10 min at 37°C. The reaction was stopped by addition of 10 mM ATP followed by boiling for 3 min. The cAMP formed in the reaction was then separated from the other labeled molecules in the mixture by chromatography on columns of Dowex AG 50 W × 8 and alumina as described by Salomon et al. (28). The [³²P]cAMP was counted after addition of 10 ml Instagel in a scintillation counter (model LS 233; Beckman Instruments, Fullerton, CA).

Transplantation of Clonal Osteogenic Sarcoma Lines

OHS-4 cells were detached from the plates by trypsinization, then collected and resuspended in a small volume (5–6 × 10⁶ cells/1 ml media). These suspensions were injected subcutaneously into six nude mice (three male/ three female).

Biochemical Analysis of the Tumors

Half of the tumors were dissected to dissociate soft tissue from mineralized tissue. Mineralized tissue was washed with PBS saturated with hydroxyapatite. The mineralized tissue was freeze dried and weighed before acid extraction using a 10-fold (vol/wt) excess of 10% formic acid. The amount of phosphorus present in the supernatant was determined by the colorimetric method of Chen et al. (5). Calcium was determined by atomic absorption spectrophotometry. Human BGP was measured by radioimmunoassay on freeze-dried samples of 20–50 μl of formic acid extract.

Light Microscopy

The tumors were transferred directly from the host animal into 70% ethanol and fixed for 48 hours. They were then embedded in glycol methacrylate and sectioned. The sections (8 μm) were stained with hematoxylen eosin and by the method of Von Kossa.

Bone Formation In Vitro

The cells were plated in 100-mm plates in normal culture medium. At confluency, the medium was replaced by RPMI 1640 containing 10% NCS, 2 mM L-glutamine, 1% antibiotic/antimycotic solution with the addition of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate as described by Bellows et al. (2). The media were changed every second day. The cells were maintained in these conditions for 30 d. At the end of the experiment, the medium was removed, the cells washed with distilled water and fixed overnight in neutral buffer formalin, and stained with Von Kossa technique. After staining, the cells were observed by light microscopy.

Results

Cloning and Growth Properties

We obtained ∼20 clones from the OHS-50 cells by the method described above. Surprisingly, most of the clones underwent terminal differentiation. We were able to maintain eight viable clones and made our selection for further study on the basis of BGP production. The cells selected were called OHS-4 and presented a quite homogeneous population with cuboidal shape. Growth properties are illustrated in Fig. 1.
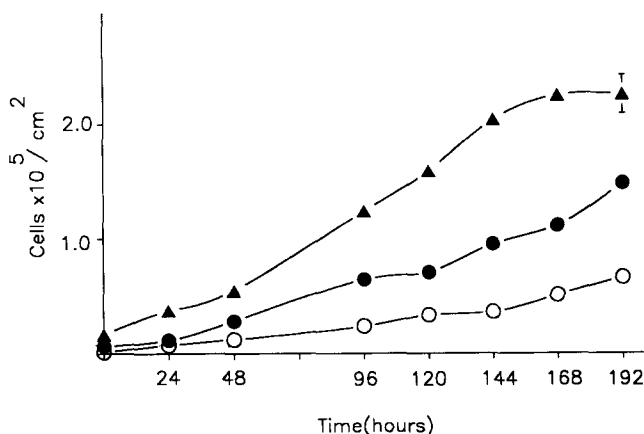

Figure 1. Growth curve for OHS-4 cells. The cells were plated at different densities. (O) 5,000; (●) 10,000; and (▲) 20,000 cells/ cm². Cell numbers were then determined as number of cells/cm².

Table I. Alkaline Phosphatase Activity in Human Osteogenic Sarcoma Cell Lines

Cell line	Alkaline phosphatase activity
	μmol/min per mg protein
MG-63	0.02 ± 0.05
SAOS-2	2.4 ± 0.25
OHS-4	2.5 ± 0.14

Cells were maintained for 72 h in medium containing 2% heat-inactivated serum. Cells were harvested after trypsinization. The intracellular alkaline phosphatase was extracted by sonication and centrifugation. Aliquots of the supernatant were assayed for alkaline phosphatase activity by the method of Lowry et al. (19). Another aliquot was assayed for protein. Results were averages of two different wells with duplicate determinations and are expressed ± SE per mg protein.

The cells were confluent at a density of 70,000 cells/cm². The doubling time was calculated from the slopes of the growth curves and corresponded to 39 h, the saturation density was 250,000 cell/cm².

Alkaline Phosphatase Activity

Table I shows intracellular alkaline phosphatase activity produced in different human osteosarcoma cell lines. OHS-4 cells as well as the SAOS-2 cells produced a high alkaline phosphatase activity ~100-fold higher than that of MG-63 cells. When cultivated in the presence of an increased concentration of 1,25(OH)₂D₃, OHS-4 cells displayed very different sensitivity towards vitamin D₃ in comparison to the MG-63 cells (Fig. 2, *a* and *b*). As reported previously (13), we also observed a net decrease in proliferation for the MG-63 and a dose-dependent increase in alkaline phosphatase activity with a maximum reached at 10 nM 1,25(OH)₂D₃. In comparison, the OHS-4 cells showed no significant differences in alkaline phosphatase activity between control and vitamin D₃-treated cells.

Adenylate Cyclase Activity

Table II summarizes the extent of stimulation of the adenylate cyclase activity in the presence of PTH, PGE₂, and calcitonin. The high degree of stimulation of the adenylate cyclase activity by PTH in the OHS-4 cells was of the same order of magnitude as that observed in ROS 17.2/8 cells. Fig. 3 shows a dose-dependent stimulation of the adenylate cyclase activity, the half-maximal stimulation was obtained at 5 × 10⁻⁸ M PTH. In a parallel experiment with MG-63 cells we could not detect any stimulation of the adenylate cyclase activity either by PTH or human calcitonin (results not shown).

Analysis of Type I and III Collagen Expression

The Northern blot analysis of total RNA obtained from OHS-4 cells is shown in Fig. 4. We were not able to detect any collagen type III but we did obtain a good signal for type I collagen. In normal osteoblast cells collagen type III is not coexpressed with collagen type I (29).

Isolation of Human BGP from OHS-4 Media

To prove that the protein measured by RIA was indeed human BGP and not contaminating calf BGP coming from newborn calf serum, we isolated the protein from the conditioned media of OHS-4 cells. After a first step of immuno-
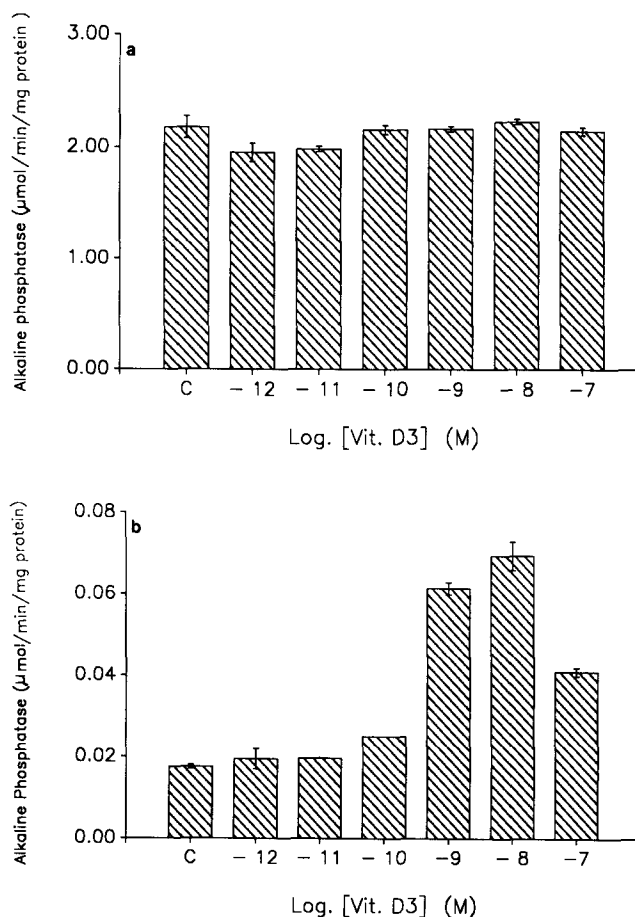

Figure 2. Effects of 1,25(OH)₂D₃ on the activity of alkaline phosphatase in OHS-4 (*a*) and MG-63 (*b*) cells. MG-63 and OHS-4 cells were plated at 20,000 cells/cm² in medium containing RPMI supplemented with 10% NCS. After 24 h the medium was removed and the cells were fed with RPMI supplemented with 2% heat-inactivated denatured NCS and vitamin D₃ (or carrier) at the indicated concentration. The medium was changed every 24 h during 6 d. After 6 d, the cells were treated as described in Materials and Methods. Each point represents the mean of two different wells. Statistically significant differences from control values ($p < 0.05$) were determined by *t* test.

Table II. Adenylate Cyclase Responsiveness of OHS-4 Cells to PTH, Human Calcitonin (CT), and PGE₂

Treatment	Adenylate cyclase activity	Extent of stimulation (hormone/control)
	pmol/min per mg protein	
Control	5.06 ± 0.17	–
PTH	52.66 ± 8.54	10.5
PGE₂ *(10 nM)*	6.82 ± 0.46	1.3
PGE₂ *(100 nM)*	4.19 ± 0.20	0.8
PGE₂ *(1 μM)*	5.57 ± 1.36	1.1
CT *(100 nM)*	6.46 ± 1.69	1.3

Number of determinations ≥3.

Adenylate cyclase activity was measured on cell homogenates (50–100 μg protein/assay) as described in Materials and Methods. Blanks were prepared by adding cell homogenates after the stop solution. Values are the mean of at least two determinations.
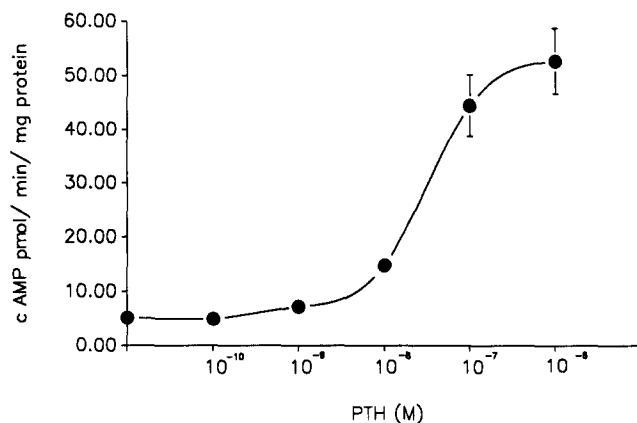
Figure 3. Dose-dependent stimulation of PTH-sensitive adenylate cyclase in OHS-4 cells. Adenylate cyclase was measured for 10 min as described in Materials and Methods. Each point is the result of at least two determinations.

affinity chromatography, the immunoreactive peak was subjected to reverse phase chromatography. Fig. 5 *a* shows a chromatogram of human BGP purified from bone, Fig. 5 *b* shows the peak of human BGP obtained from the media. These two proteins are eluted with the same percentage of acetonitrile. Partial sequencing of the human BGP purified from media identified the first residues of the protein as $Tyr^3-Gln^4-\times^5-Leu^6$. This allowed us to discount contamination by calf BGP since residues 3 and 4 of this BGP are Asp-His.

In OHS-4 cells no basal level of BGP was detectable (<0.67 ng/ml). As shown in Fig. 6, OHS-4 cells treated with 2.4 nM $1,25(OH)_2D_3$ produced BGP with a production of 35 ng/10^6 cells for OHS-4. The OHS-50 cells from which the OHS-4 were subcloned, treated in the same way in a parallel experiment, produced significantly less BGP, illustrating the efficacy of the subcloning process. In a similar experiment using the MG-63 cells treated with vitamin D_3 we could only detect 6 ng BGP/10^6 cells.

Tumor Induction in Nude Mice

8 wk after injection of cells into nude mice five out of six animals had palpable tumors. Table III shows the biochemical composition of the mineralized part of the tumor. The calcium content of these calcified tumors was in the range
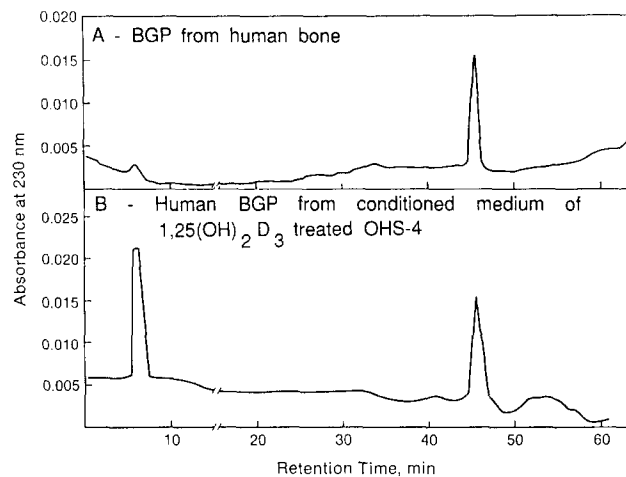

Figure 5. HPLC of immunoaffinity-purified BGP on a Vydac C4 column (4.6 × 25 cm). Mobile phase: buffer A, 0.1% trifluoroacetic acid; buffer B, 0.1% trifluoroacetic acid in (60:40) acetonitrile: H_2O. Flow rate, 1.0 ml/min. Temperature: 25°C. Samples in 4 M guanidine HCl were applied in 100% buffer A. After the guanidine had passed through the column a linear gradient from 0 to 100% B was developed. (*A*) 10 μg of human BGP from bone. (*B*) 6.64 μg of immunoaffinity-purified BGP from OHS-4 cultures treated with 2.4 nM $1,25(OH)_2D_3$ in serum-free medium, during 4 d with daily changes.
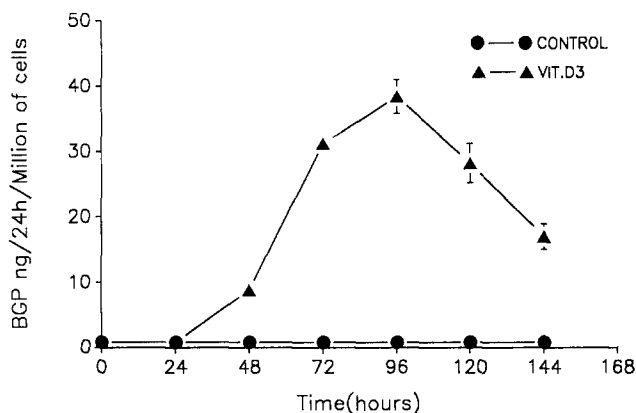

Figure 6. Rate of BGP secretion in $1,25(OH)_2D_3$-treated OHS-4 cells. Cultures received fresh medium containing 2.4 nM $1,25(OH)_2D_3$ or vehicle every 24 h as described in Materials and Methods. Each point represents the average of two culture wells.
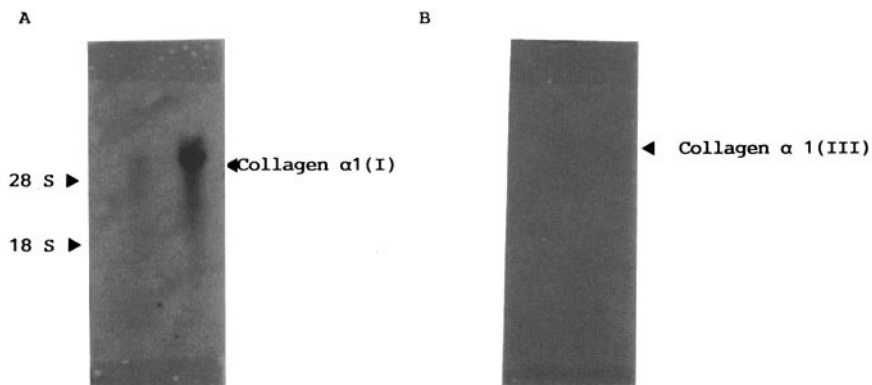

Figure 4. Northern blot analysis of type I and III mRNA collagen expression in OHS-4 cells. RNA was extracted from confluent plates of OHS-4 cells. 15 μg of total RNA was run on an 0.8% agarose gel containing 2.2 M formaldehyde and blotted onto a Hybond sheet. Filters were hybridized using ^{32}P-labeled α1(I) procollagen cDNA and autoradiographed (*A*). After autoradiography the probe was boiled off and the Northern blot was hybridized to a ^{32}P-labeled α1(III) procollagen cDNA and autoradiographed (*B*).

Table III. Biochemical Measurement on the Tissue Formed in Nude Mice after Injection of OHS-4 Cells

Sample	Calcium	Phosphate	Calcium/ Phosphorus	Human BGP
	μg/mg tissue			ng/mg tissue
1	218.2 ± 3.3	312.1 ± 6.1	1.66	3.9
2	244.2 ± 9.7	167.3 ± 1.6	3.47	<2
3	227.2 ± 7.0	59.3 ± 1.4	9.09	<2
4	161.1 ± 4.1	140.5 ± 6.7	2.72	<2
5	178.5 ± 3.0	203.2 ± 17.9	2.09	4.1
Human bone	285.5 ± 3.2	354.9 ± 8.9	1.91	810.0
	264.6 ± 11.0	388.7 ± 3.0	1.61	800.0

of that found in human bone. With the exception of one sample, the Ca/P ratio in the mineralized tumor tissue was close to the value of 1.6, which is usually found in hydroxyapatite. We were able to detect human BGP in only two out of five samples. Histological examination of calcified tumors showed an homogenous cell population with the appearance of sarcoma tissue, some mitotic figures were observed (Fig. 7 *a*). After Von Kossa staining, we observed non-mineralized osteoid and mineral deposits with a structure similar to that of the trabeculae (Fig. 7 *b*). No cartilaginous component could be discerned in any of the samples. In vitro, after 30 d in culture, the cells did not show any nodular structure. These cells did not lay down any calcified extracellular matrix. This discrepancy with the osteogenic ability of these cells found in vivo should be further investigated, in particular by using collagen coated surfaces.

Discussion

This paper reports on the osteoblastic features of a new human osteosarcoma cell line OHS-4 and compares them with the characteristic features of the other human osteosarcoma cell lines, SAOS-2 and MG-63. In the temporal program of bone-specific protein expression associated with mineraliza-
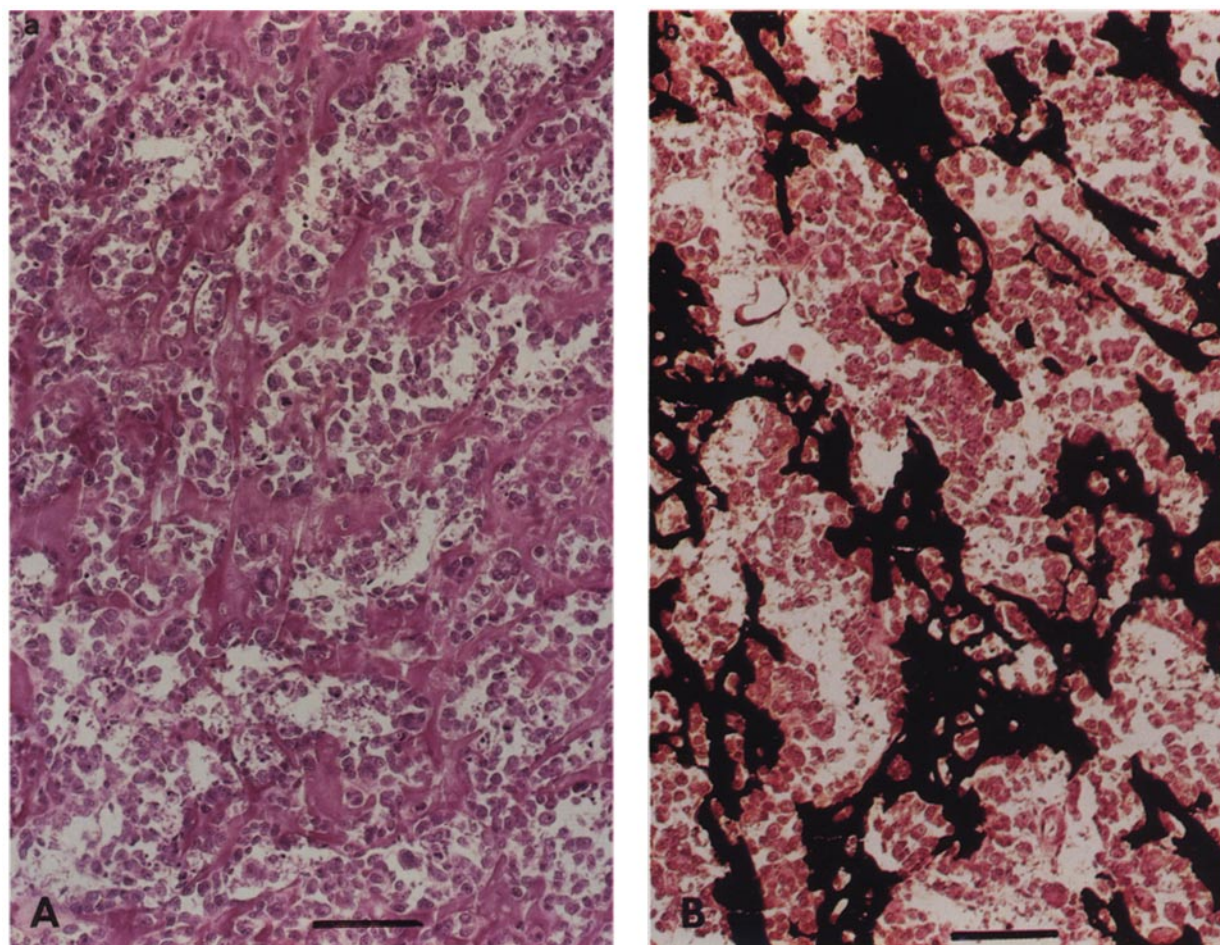

Figure 7. Section of the tumor obtained after implantation of OHS-4 cells into nude mice. (*a*) Light microscopy of a hematoxylin and eosin section. 144×. (*b*) Light microscopy of a Von Kossa-stained section. 144×. Bars: (*a* and *b*) 100 μM.

tion, alkaline phosphatase appears first and has a key role in matrix mineralization. The cell line OHS-4 expresses a high alkaline phosphatase level comparable to that expressed in SAOS-2 cells. In both cell lines this alkaline phosphatase activity could not be modulated by 1,25-dihydroxyvitamin D_3 (22). Cell lines other than MG-63 have been shown to display a high sensitivity to vitamin D_3 (13–21). Spiess et al. (31) proposed that the osteoblastic cell response to vitamin D_3 depends on their phenotypic state of differentiation. These differences added to the differences in basal level of alkaline phosphatase activity in MG-63 and OHS-4 cells illustrate that we are dealing with cells which are clearly at a different stage in the osteoblastic lineage. OHS-4 as well as ROS 17.2 and SAOS-2 cells possess a PTH-sensitive adenylate cyclase, but no stimulation was observed when exposed to PGE_2 and calcitonin.

In contrast SAOS-2 cells possess a PGE_2-sensitive adenylate cyclase activity (4). Aubin et al. (1) have already described in their analysis of different bone cell clones the existence of different cell types, one responding to both PGE_2 and PTH and the other to PTH alone. The absence of a response to PTH and calcitonin in the MG-63 cells is puzzling. From other studies (17) we would have expected a change in the adenylate cyclase sensitivity towards calcitonin.

The exclusive expression of type I collagen in OHS-4 is consistent with the fact that osteoblastic cells only synthesize this collagen type (29). In contrast, MG-63 cells produce both types of collagen. The ratio of type I and III collagen has been shown to be regulated by vitamin D_3 in these cells (14). This observation suggests that OHS-4 cells are more mature cells. The production of BGP after treatment with $1,25(OH)_2D_3$ reached a plateau after 96 hours for OHS-4 cells and then decreased over the next few days. This phenomenon has already been described for the ROS 17/2 cells (16). The maximal production of human BGP per million of cells is about 200-fold less than that observed in ROS 17/2. This difference in production in vitro may be related to the 100-fold difference between circulating BGP levels in the rat and in man. This ability to produce BGP was not observed in the SAOS-2 cell line. Fraser et al. (15) described a mutually exclusive production of human matrix GLA protein (MGP) and BGP. Therefore, it is possible that, as in the case of the rat cell lines (ROS 17.2 and UMR-106), the BGP production observed in OHS-4 excludes MGP production and the inverse holds true for SAOS-2 cells. Information on the production of other noncollagenous proteins secreted by these different cell lines will shed some light on the further classification of these cell lines in the osteoblastic lineage.

Finally, the ability of the OHS-4 cells to produce mineralized tumors when injected into nude mice is a very important osteoblastic feature. Whereas Rodan et al. (26) described that SAOS-2 cells produced nonmineralized tumors when injected into nude mice, they were only able to describe production of mineralized matrix typical of woven bone when the cells were put into diffusion chambers. We showed a clear development of mineralized tissue after injection of our cells into nude mice. The measurement of human BGP discounts any possibility that the bone formed arose from the host cells, no cross-reactivity was found between mouse and human BGP. When injected into nude mice, the MG-63 cells were not able to produce calcified matrix, however the manipulation of this cell line by Dedhar et al. (10) led to a more

differentiated cell type (MG-63 3A) which, unlike the parent cell line, was able to produce mineralized matrix.

In conclusion, we present a new human osteosarcoma cell line, OHS-4, exhibiting all the osteoblastic features necessary to fulfill the definition of an osteoblast-like cell. We believe that this cell line will provide a useful good model to study hormone action on human osteoblasts in vitro. We also emphasize the diversity of the osteoblastic phenotype (27) and present a comparison of this new cell line with other human osteosarcoma cell lines.

Through the description of these different phenotypes, we present a panel of different models to study osteoblastic differentiation and in particular to correlate one stage of differentiation to the expression of a specific protein or enzymatic expression.

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