Alkaline Phosphatase Activity from Human Osteosarcoma Cell Line SaOS-2: an Isoenzyme Standard for Quantifying Skeletal Alkaline Phosphatase Activity in Serum

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Earlier we described a kinetic assay for quantifying skeletal alkaline phosphatase (ALP) isoenzyme activity in serum. The precision of the assay depends on including ALP standards for the skeletal, hepatic, intestinal, and placental isoenzymes. We wondered whether human osteosarcoma cells could provide an efficient alternative to human bone or Pagetic serum as a source of the skeletal ALP standard. ALP activities prepared from five human osteosarcoma cell lines were compared with a bone-derived ALP standard with respect to heat stability and sensitivity to chemical effectors. Two of the cell lines (SaOS-2 and TE-85) contained ALP activities that resembled the bone-derived standard. We selected SaOS-2 cells for additional evaluation (as a potential source of isozyme standard), because they contained 40–50 times more ALP activity than did the TE-85 cells. To include the SaOS-2 cell-derived ALP activity in the quantitative isozyme assay, we diluted the enzyme in a solution containing heat-inactivated (i.e., ALP-negative) human serum. Surprisingly, this dilution caused a 60–125% increase in maximum enzyme activity. In the quantitative assay of ALP isozyme in serum, the SaOS-2-derived ALP was indistinguishable from the serum skeletal ALP standard, with respect to the above criteria and assay variations. Evidently ALP from SaOS-2 cells is suited as a standard for measuring skeletal ALP activity in this assay.

Additional Keyphrases: kinetic enzyme assay · standardization · effect of matrix on activity · index to bone formation

Ordinarily, human serum contains a variable mixture of ALP isozyme activities, derived from skeletal, intestinal, hepatic, and placental tissues (1). These circulating ALP isoenzymes can be distinguished by their different sensitivities to inhibitors by L-phenylalanine, L-homoarginine, levamisole, heat, and urea (2–5).

Because the skeletal ALP isozyme is a product of osteoblasts (i.e., the bone-forming cells), and because correlations have been shown between skeletal ALP activity in serum and (or) bone and the rate of bone formation, both in vitro (9) and in vivo (10–13), measurement of skeletal ALP activity in serum has been suggested as a useful index of the rate of bone formation. In examining that hypothesis, we adapted a kinetic assay methodology (5) to the quantification of skeletal ALP activity in the mixture of circulating isoenzymes. Initial applications of this assay showed that the skeletal ALP activity concentration in serum (and presumably, the rate of bone formation) was increased in osteoporotic subjects, after treatments with fluoride (14, 15) and stanozolol (14), and in pregnant women, particularly during the third trimester (15).

Applications of this assay have been limited, however, because the precision of the method depends on inclusion of isozyme standards in each assay to minimize interassay variation (6, 7, 14). In previous studies (14, 16), we prepared isozyme standards from tissues (duodenum and bone), from bile (6, 7, 17, 18), and from the serum of subjects with increased concentrations of single isoenzymes in serum—obstructive jaundice and Paget's disease of bone for the hepatic and skeletal ALP standards, respectively. Recent studies have identified ALP activities in human osteosarcomas (19) and in transformed human cell lines derived from osteosarcoma cells (20–24). The current studies were intended to determine whether ALP activities prepared from human cell lines could be used as isozyme standards for quantifying skeletal ALP activity in human serum.

Materials and Methods

Chemicals and Supplies

Purified human placental ALP, NaHCO3, Na2CO3, and p-nitrophosphoyl phosphate (PNP) were from Sigma Chemical Co., St. Louis, MO. Type III "Aquacide" (flake polyethylene glycol, for use in dehydrating and concentrating aqueous samples) was from Calbiochem, San Diego, CA. Pooled human serum (mycoplasma-free) was from Flow Laboratories, McLean, VA. We used an automatic motorized microwell plate reader (Model MR-600; Dynatech, Richmond, VA) for colorimetry of ALP activity. Tissue-culture materials—plates, Dulbecco's Modified Essential Medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, and trypsin/EDTA—were from Grand Island Biological Co., Grand Island, NY. The following human osteosarcoma-derived cell lines were from the American Type Culture Collection (cat. nos. in parentheses): SaOS-2 (ATCC HTB 85), TE-85 (ATCC CRL 1543), TE-89 (ATCC CRL 7738), and U-2 (ATCC HTB 96). The SK-HEP-1 cell line (ATCC HTB 52) was isolated as an ascites cell line from a human hepatic adenocarcinoma. The G-2 cell line, which was isolated from a human giant cell tumor (25), was a generous gift of Dr. Seiichi Iahii, Hokkaido University School of Medicine, Sapporo, Hokkaido, Japan.

Cell Culture Methods

Routine maintenance of cultures. All cell lines were maintained at subconfluent densities, in DMEM containing penicillin/streptomycin plus FCS, 100 mL/L. Cells were passaged by treatment with trypsin/EDTA, and seeded (in dishes of 100 mm diameter) at initial densities of 4 × 104 to 7 × 104 cells per millilitre of medium, 10 mL per dish (i.e., 4
\( \times 10^4 \) to \( 7 \times 10^5 \) cells per 100-mm dish). The culture media were changed at 48- to 72-h intervals.

**Growth and extraction of cells for initial characterizations of ALP activities.** Replicate cultures (n = 6) of each cell line (SaOS-2, TE-85, TE-89, G-2, U-2, and SK-HEP-1) were prepared by seeding cells at an initial density of \( 1.4 \times 10^6 \) cells/cm\(^2\) in DMEM plus FCS, 100 mL/L. After 72 h the media were removed from the confluent cultures, and the cell layers were rinsed with PBS (to ensure the removal of bovine serum ALP activity, from the FCS) and extracted with a solution of 10 mL of Triton X-100 per liter of Tris/NaHCO\(_3\) buffer (12.5 mmol/L each), pH 6.8. Protein concentration, DNA concentration, and ALP activity were determined for each extract. These methods were used to obtain the data summarized below in Table 1.

**Growth and extraction of cells for identification of ALP isoenzymes.** Although Triton X-100 extracts of the cells were used for our initial characterizations of ALP activities (described above), the Triton X-100 interfered in our serum isoenzyme assay. Therefore, we also assessed ALP activities from SaOS-2 and SK-HEP-1 cells after extraction with butanol. Cells were grown to confluence in DMEM containing FCS (10 mL/L) in dishes 100 mm in diameter, and rinsed three times with PBS. We then scraped the cell layers from the dishes with a rubber "policeman" and NaHCO\(_3\) buffer (25 mmol/L, pH 7.2, containing 10 mg of sodium azide per liter). We used one 5 mL volume of buffer to remove the cell layers from three to five plates, applying the liquid to each plate in succession. After rinsing the plates with an additional 5-ml of buffer (used on six to 10 plates in succession), we combined the cell suspensions. Butanol was added to give a final concentration of 200 mL/L, and the suspensions were stirred, overnight, at 4 °C. The resulting solutions were centrifuged, to separate the butanol (upper) phases from the aqueous (lower) phases, and the aqueous phases were dialyzed extensively against 25 mmol/L NaHCO\(_3\) buffer, pH 7.2, containing 0.01 g of azide per liter, for three to five days, with one or two buffer changes per day, to remove the butanol. The resulting carbonate-buffered solutions were either diluted (in the same 25 mmol/L NaHCO\(_3\) buffer) or concentrated (with use of Type II Aquacide) and redialyzed, to yield solutions with ALP activities of 15 to 25 U/L. We used these solutions to compare the ALP activities derived from SaOS-2 and SK-HEP-1 cells with that of the serum ALP isoenzyme standards, as described below.

**Initial Characterizations of ALP Activities in Cell Extracts**

ALP activities of the replicate Triton X-100 extracts of each cell line were determined with 15 mmol/L PNPP in 0.15 mol/L Na\(_2\)HPO\(_4\) buffer, pH 10.3, containing 1 mmol of MgCl\(_2\) per liter, in (non-sterile) 96-place multi-well microtiter plates, at ambient temperature (22 °C). The total reaction volume was 0.3 mL per well. Two or three replicate wells were used for each sample. Reactions were initiated by addition of PNPP and monitored by the time-dependent increase in absorbance at 404 nm, reflecting the production of \( p \)-nitrophenolet. ALP activities were calculated as micromoles of product formed per minute per unit volume of extract, and normalized for DNA (26) and protein (27) in the extracts. Additional replicate wells were used to measure \( \lambda \)-phenylalanine-insensitive, \( \lambda \)-homocarnosine-insensitive, and levamisole-insensitive ALP activities in each extract (i.e., activity remaining in the presence of 100 mmol/L concentrations of each effector). Heat-insensitive ALP activities were determined after 15- and 90-min incubations at 54 °C.

**Preparation of Heat-Inactivated (ALP-Negative) Human Serum**

Pooled normal human serum (100-mL aliquots, stored frozen) was heated for 44 h (55 °C, in a water bath) and 0.25-mL aliquots were removed at 0, 1, 2, 4, 20, and 44 h to determine the ALP activity remaining. We found the ALP activity was decreased by 80% at 1 h, 99% at 20 h, and 99.8% at 44 h. This last value corresponded to an ALP activity of 0.08 U per liter of serum, or an absorbance change (i.e., at 404 nm) of less than 0.002 A in 150 min, for a 25-μL sample of the heated serum. (For comparison, our standard isoenzyme assay, described below, measures an average absorbance change of 0.30 A during a 40-min incubation, with use of a fourth as much serum.)

**Preparation of ALP Isoenzyme Standards**

(a) Skeletal ALP isoenzyme standards were prepared by dialuting serum from patients with Paget's disease (obtained, with consent, by Dr. Frederick Singer, University of Southern California), in the heat-inactivated human serum, to yield a final activity of 30 to 50 U/L. We assume that, for subjects with Paget's disease and total serum ALP activity >800 U/L, total serum ALP = skeletal ALP.

(b) Hepatic ALP isoenzyme standards were prepared by dialuting serum from patients with obstructive jaundice (obtained with their consent), in the heat-inactivated human serum, to yield a final activity of 30 to 50 U/L. We assume that, for subjects with obstructive jaundice and total serum ALP activity >500 U/L, total ALP = hepatic ALP.

(c) Human intestinal ALP activity, prepared from butanol extracts of (pooled) duodenal tissue (obtained at autopsy), was dialyzed at 4 °C against NaHCO\(_3\) buffer (25 mmol/L, pH 8.0, containing 100 mg of sodium azide per liter) and diluted in the same buffer to yield a final activity of 15 to 25 U/L.

(d) Human placental ALP isoenzyme standard was prepared from a highly purified commercial preparation by dissolving the lyophilized activity (500 U) in 5 mL of NaHCO\(_3\) buffer (25 mmol/L, pH 8.0, containing sodium azide, 0.1 g/L) and then dialyzing the enzyme (in more of the same NaHCO\(_3\) buffer) to yield a final activity of 15 to 25 U/L.

**Quantitative Serum ALP Isoenzyme Assay**

ALP activities were measured on an automatic microtitr plate spectrophotometer-printer, in a total reaction volume of 0.3 mL per 96-place immunotiter-plate well (15). Reaction mixtures contained, per liter, 0.15 mol of carbonate buffer (pH 10.3), 1 mmol of MgCl\(_2\) 10 mmol of PNPP, 10 mmol of \( \lambda \)-phenylalanine or water, and 25 μL of enzyme as the solution in serum. Serum solutions were prepared by mixing 75 μL of sample serum with 75 μL of NaHCO\(_3\) buffer (25 mmol/L, pH 8.0, containing 0.5 g of sodium azide per liter) and 15 μL of de-ionized water, in 12 mm × 75 mm test tubes. Equivalent solutions of the isoenzyme standards in serum were prepared by appropriate dilutions, to yield final test solutions containing 7 to 13 U of ALP activity per liter. (The ALP isoenzyme standards that had been prepared by diluting patients' sera with heat-inactivated human serum were further mixed with NaHCO\(_3\) buffer and de-ionized water, and the isoenzyme standards that had been prepared as NaHCO\(_3\) buffer solutions were mixed with heat-inactivated human serum and de-ionized water.)

Replicate 25-μL aliquots of the sample solutions (i.e., the diluted serum samples and the isoenzyme standards) were transferred to 96-place immunotiter plates containing 30-μL aliquots of NaHCO\(_3\) buffer (pH 10.3) with or without 100
mmol of L-phenylalanine per liter (i.e., to yield 10 mmol of L-phenylalanine per liter in the final 0.3-mL reaction volume) for measurement of total serum ALP activity and L-phenylalanine-resistant ALP activity. The test tubes (containing the rest of the serum solutions) were transferred to a heated water bath for a 10-min incubation at 54 °C and then to an ice-water bath to stop the heat inactivation. Replicate 25-μL aliquots of the heated samples were transferred to an additional set of 96-place immunotiter plates (containing 30-μL aliquots of NaHCO₃ buffer (pH 10.3, with the 100 mmol/L L-phenylalanine) for measurement of heat-resistant ALP activity. Reactions were initiated by the addition of 245 μL of reaction mixture to the buffered enzyme samples. (Total reaction volume = 0.3 mL per well, containing, per liter, 0.15 mol of NaHCO₃ buffer, pH 10.3, 1 mmol of MgCl₂, and 10 mmol of PNPP.) Controls (containing heat-inactivated human serum without added enzyme) were included on each microtiter plate to correct for nonspecific hydrolysis of PNPP. The standard assay ran for 40 min, at ambient temperature (22 °C), but we used shorter and longer incubation to measure high and low activities, respectively. Although the reaction rate was linear for longer incubations (up to 70 min), we selected 40 min so as to provide an average absorbance change of 0.3–0.4 A (i.e., in our microtiter plate reader assay) for total ALP activity in the (diluted) serum solutions.

ALP isoenzyme activities were calculated by the simultaneous solution of multiple equations. For sera from nonfasted subjects, sera assumed to contain three unknowns (i.e., skeletal, hepatic, and intestinal ALP activities), we used three equations: (a) total ALP activity = B + H + I; (b) L-phenylalanine-resistant ALP activity = aB + bH + cI; (c) heat-resistant ALP activity = eB + fH + gI, where B is skeletal (bone) ALP, H is hepatic ALP, and I is intestinal ALP.

The isoenzyme activity factors (a, b, c, etc.) were determined from the isoenzyme standards in each assay (e.g., if 11 min at 53 °C decreased the activity of the skeletal ALP standard by 65%, the factor "e" would be equal to 0.35 for that assay).

For serum samples from fasted subjects, sera assumed to contain only two unknowns (i.e., skeletal and hepatic ALP activities), we used only two equations (i.e., the equations for total ALP activity and heat-resistant ALP activity were simplified by assuming that I = 0). This simplification was applied only when intestinal ALP activity in the samples constituted <10% of the total. Samples that contained less than 75% L-phenylalanine-resistant ALP activity were assumed to contain more than 10% intestinal ALP, and were analyzed by simultaneous solution of the three equations shown above. Samples that contained at least 75% L-phenylalanine-resistant ALP activity were assumed to be composed of two components (the skeletal and hepatic isoenzymes) and were analyzed accordingly. (In our assay, 10 mmol/L L-phenylalanine inhibits the skeletal and hepatic ALP isoenzymes by 15–20% and the intestinal isoenzyme by 60–65%. Therefore, we assume that an isoenzyme distribution of at least 90% skeletal + hepatic ALP and less than 10% intestinal ALP would contain more than 75% L-phenylalanine-resistant ALP activity.)

All ALP activities are reported as U/L (of serum). ALP activities determined by this method must be multiplied by a factor of 2.7 for comparison with values obtained by measurements of ALP activity in 2-amino-2-methyl-1-propanol buffer at 30 °C.

Identification and Characterization of ALP Isoenzyme Activities in Cell Extracts

Butanol extracts of SaOS-2 and SK-HEP-1 cells were concentrated and dialyzed (at 4 °C, against 25 mmol/L NaHCO₃ buffer, pH 8.0, containing 100 mg of sodium azide per liter) to yield ALP activities of 15–25 U/L. Aliquots of these solutions were mixed with heat-inactivated human serum and with de-ionized water (in a volume ratio of 1/1/2 for comparisons of thermostability with serum-derived isoenzyme standards: (a) as a function of time at 53–54 °C, and (b) as a function of temperature between 47 °C and 58 °C. The SaOS-2 cell-derived ALP activity was also compared with the serum-derived skeletal ALP standard with respect to reproducibility (intra- and inter-assay variations), and stability (during storage at 22 °C, 4 °C, and −20 °C, and repeated freeze–thaw cycles). We noted that the cell-derived ALP activities were always greater after dilution with heat-inactivated serum, so we also assessed the effects of that dilution on ALP activity and on the kinetics of the ALP reaction (i.e., effects on Vmax and Km).

Statistical Analyses

All data are shown as the mean of duplicate values or the mean and standard deviation of replicate values, except where otherwise noted. Comparisons were made by Student's two-tailed t-test, analysis of variance, and linear regression, as appropriate.

Results

Initial Characterizations of ALP Activities in Cell Extracts

Table 1 summarizes initial characterizations of the ALP activities prepared from human cell lines. Of the five osteosarcoma cell lines we tested, ALP specific activity was highest in SaOS-2 and G-2 cells. TE-85 cells contained 40-fold less ALP activity per milligram of cell protein, and both the TE-89 cells and the U-2 cells contained less than that. The observed effects of heat, L-phenylalanine, and levamisole strongly suggested that SaOS-2 cells and TE-85 cells contained only skeletal ALP. The ALP activity prepared from TE-89 cells was more thermostable than the bone-derived skeletal ALP standard, and more sensitive to inhibition by L-phenylalanine—a pattern of response characteristic of intestinal ALP (14, 16). The ALP activity prepared from U-2 cells was entirely heat-stable (i.e., no effect at 54 °C for 90 min) and sensitive to L-phenylalanine—a pattern of response characteristic of placental ALP (16, see reference 1 for a review). The ALP activity prepared from G-2 cells may represent an isoenzyme mixture, because it did not show a pattern of response that was characteristic of any single isoenzyme. [Previous studies (28) indicate that human osteosarcoma cell lines may contain multiple ALP activities.] Together, these data suggested that both SaOS-2 cells and TE-85 cells could provide skeletal ALP standards. We chose the SaOS-2 cells because of the difference in ALP activity per milligram of cell protein.

The SK-HEP-1 cell line (which was derived from ascites tissue and, presumably, hepatic) contained 1% as much ALP activity per milligram of cell protein as the SaOS-2 cells. The SK-HEP-1 cell-derived ALP activity was more sensitive to inhibition by L-phenylalanine, and less sensitive to inhibition by levamisole than was the bone-derived skeletal ALP standard. This pattern of response was not characteristic of the hepatic ALP isoenzyme (14, 16), so our data suggest that the SK-HEP-1 cell line would probably not
provide an hepatic ALP isoenzyme standard.

To examine this conclusion, and to assess the behavior of the SaOS-2 cell-derived ALP activity as a skeletal ALP standard, we needed to test the cell-derived ALP activities in our quantitative isoenzyme assay. Because Triton X-100 interfered with that assay, ALP activities were prepared from SaOS-2 cells and SK-HEP-1 cells by butanol extraction and dialysis (at 4 °C, in 25 mmol/L NaHCO3 buffer, pH 8.0, containing 100 mg of sodium azide per liter). The diazlyzed solutions were diluted (in the same NaHCO3 buffer) to yield ALP activities of 15 to 25 U/L, then mixed with heat-inactivated human serum and de-ionized water for inclusion in the isoenzyme assay. The ALP activities prepared in this manner from SaOS-2 cells and from SK-HEP-1 cells were consistently increased after dilution in the heat-inactivated human serum.

Effect of Heat-Inactivated Human Serum to Increase Cell-Derived ALP Activities

Although the heat-inactivated human serum did not contain significant ALP activity (i.e., <0.08 U/L), it significantly increased the ALP activity in dialysed butanol extracts of SaOS-2 cells and SK-HEP-1 cells. Kinetic analyses showed that the heat-inactivated serum was increasing the V_max of the reactions without affecting the value of K_m. In other words, the heat-inactivated human serum was behaving like a non-competitive activator of the extracted ALP activities. Figure 1 shows this effect for SaOS-2 cell-derived ALP activity, at pH 9.3. (The effect was similar at pH 10.3.) A parallel dilution in heat-inactivated human serum had no effect on a skeletal ALP standard prepared from Pagetic human serum. Preliminary studies indicate that the activating factor in the (heated) serum was decreased (by 40% to 55%) during a 72-h dialysis against NaHCO3 buffer (25 mmol/L, pH 8.0, containing 100 mg of sodium azide per liter), at 4 °C, in a dialysis membrane with a molecular-mass cutoff of 6 to 8 kDa.

Time- and Temperature-Dependent Heat-Inactivation of ALP Activity from SaOS-2 Cells

As shown in Figure 2, we found that, after dilution in heat-inactivated human serum, we could not distinguish SaOS-2 cell-derived ALP activity from a skeletal ALP standard, with respect to time-dependent heat inactivation at 53–54 °C. Consistent with previous findings (1, 5–8, 14, 16), hepatic ALP was less thermolabile than skeletal ALP, and placental ALP was not affected. The time-dependent decrease in SK-HEP-1 cell ALP activity was between the hepatic and skeletal ALP standards (data not shown).

Because previous studies indicated that variations in the time and temperature of heat-inactivation represented the most substantial sources of variation in our isoenzyme assay (6, 7, 14), we compared the half-lives of ALP activity from SaOS-2 cells and a skeletal ALP standard over the temperature range 47–58 °C. (As for the time-dependent heat-inactivation studies summarized above, the ALP activity from SaOS-2 cells was prepared as a mixture of heat-

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**Table 1. Initial Characterizations of ALP Activities in Cell Extracts**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ALP activity/cell</th>
<th>U/g DNA</th>
<th>Activity remaining, %, after treatment with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>662 000 ± 30 000</td>
<td>3040 ± 310</td>
<td>Heat 10 ± 2% , L-Phe 82 ± 4% , Levam. 4 ± 7%</td>
</tr>
<tr>
<td>TE-85</td>
<td>12 170 ± 3540</td>
<td>77 ± 5</td>
<td>11 ± 5% , 86 ± 4% , 10 ± 7%</td>
</tr>
<tr>
<td>TE-89</td>
<td>75 ± 14</td>
<td>32 ± 1</td>
<td>0 ± 0% , 27 ± 2% , 0</td>
</tr>
<tr>
<td>G-2</td>
<td>359 000 ± 13 000</td>
<td>4040 ± 220</td>
<td>67 ± 4% , 61 ± 4% , 54 ± 4%</td>
</tr>
<tr>
<td>U-2</td>
<td>23 ± 5</td>
<td>20 ± 1</td>
<td>90 ± 1% , 0 ± 0% , 0</td>
</tr>
<tr>
<td>SK-HEP-1</td>
<td>872 ± 96</td>
<td>—</td>
<td>22 ± 3% , 54 ± 2% , 23 ± 2%</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 6 each.

ALP activity, protein, and DNA measured in Triton X-100/NaHCO3 buffer extracts of replicate cell cultures for each indicated cell line (see Materials and Methods for details).

ALP activities were measured after a 15-min incubation at 53–54 °C, or in the presence of 100 mmol/L concentrations of L-phenylalanine or levamisole, and compared with untreated-control activities. A human bone-derived skeletal ALP standard (prepared by comparable extraction with Triton X-100 in NaHCO3 buffer) showed residual activities of 9%, 90%, and 2%, respectively.

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**Fig. 1. Heat-inactivated human serum non-competitively increases maximum ALP activity in butanol extracts of SaOS-2 cells, as shown by reciprocal ALP activity (relative absorbance units)−1 vs reciprocal PNPP concentration (mmol/L)−1 at pH 9.3**

SaOS-2 cell-derived ALP was diluted in 25 mmol/L NaHCO3 buffer, pH 8.0 (A), and in a 250 mL/L solution of heat-inactivated human serum in the same NaHCO3 buffer (B). The 0.3-mL reaction volume contained 25 μL of diluted enzyme solution, so the final concentration of heat-inactivated serum in the treated samples was 25.8 mL/L. Data shown are the averages of duplicates.

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**Fig. 2. Time-dependent heat inactivations of SaOS-2 cell-derived ALP (V) and isoenzyme standards of skeletal (A), hepatic (O), and placental (M) ALPs**

All enzyme solutions were prepared as mixtures of 25 mmol/L NaHCO3 buffer (pH 8.0), heat-inactivated human serum, and water (see text). Data shown are the average of duplicates, reported as percent of values for unheated controls (i.e., percent activity remaining).
inactivated serum, NaHCO₃ buffer, and water.) Figure 3 shows our results. Together, these data suggest that ALP activity from SaOS-2 cells can be used as a skeletal ALP standard in our isoenzyme assay.

Stability and Reproducibility of SaOS-2 Cell-Derived Skeletal ALP Standards

Three consecutive preparations of ALP activity, from confluent cultures of SaOS-2 cells, yielded 0.19 to 0.35 U per milligram of butanol-extracted protein (0.31–0.69 U per 100-mm-diameter culture dish). After dialysis and dilution (in the isoenzyme assay mixture of heat-inactivated human serum, NaHCO₃ buffer, and water), the apparent yield of ALP activity was increased by 1.9 times.

As shown in Table 2, we found that skeletal ALP standards from SaOS-2 cells were stable for 48 h at −20 °C and showed no significant loss of activity after one month and three months of storage at −20 °C. Although we found significant decreases in ALP activity after five cycles of freezing and thawing, the remaining activity still behaved like the skeletal ALP standard.

Consistent with previous data (1, 29) we also observed evidence of enzyme aggregation. ALP activity from SaOS-2 cells was decreased (by 65%) by overnight freezing (−20 °C) in NaHCO₃ buffer (25 mmol/L, pH 8.0, with azide, 100 mg/L), and slowly restored by pre-incubation at 22 °C in the same buffer solution after thawing (e.g., activity increased by 50% after 60 min, compared with a freshly thawed sample). This effect was less apparent when enzyme solutions containing detergent were frozen. ALP activity from SaOS-2 cells was only decreased by 30% after overnight storage at −20 °C, in NaHCO₃ buffer (as above) containing Triton X-100, 0.1 mL/L, and half of the lost activity reappeared during a 60-min pre-incubation at 22 °C. Although previous studies (30) have reported time-dependent increases in ALP activity in serum samples that have been stored frozen, two observations indicate that frozen storage caused insignificant, transient decreases in ALP activity in our serum samples (i.e., ALP solutions containing heat-inactivated human serum, 0.25 L/L). First, we found no difference in enzyme activity between freshly thawed serum solutions (i.e., after a 30–60 min pre-incubation at 22 °C, after overnight storage at −20 °C) and samples stored at 4 °C. Second, we consistently found that ALP activity in freshly thawed serum solutions was linearly related to time for up to 70 min (absorbance at 404 nm recorded at 10-min intervals, maximum absorbance = 0.51 A), indicating that an additional 60 min of pre-incubation at 22 °C (after frozen storage) had no effect on ALP activity. Additional studies will be required to determine if the (heat-inactivated) serum is maintaining activity (i.e., during frozen storage) by preventing enzyme aggregation.

To compare the variations seen with skeletal ALP standards prepared from SaOS-2 cells and from Fagetic serum, we tested both standards in a series of four isoenzyme assays (with triplicate determinations of ALP activities in each). Results of these studies are summarized in Tables 3 and 4. Consistent with previous findings (14, 16) inter-assay variations were consistently greater than intra-assay variations for the isoenzyme standards, and particularly for measurement of heat-stable ALP. This is shown in Table 3. (Heat-stable ALP activities represented 80.2 ± 7.8%, 20.9 ± 4.1%, and 21.3 ± 3.1% of the total activities for hepatic, skeletal, and SaOS-2 cell-derived ALP, respectively.) Analyses of

![Figure 3. Temperature-dependent rate constants for the thermal inactivation of SaOS-2 cell-derived ALP (△) and an isoenzyme standard of skeletal ALP (□), incubated at 47 °C to 58 °C for 1 to 60 min.](image)

Table 2. Stability of Skeletal ALP Isoenzyme Standard Prepared from SaOS-2 Cells

<table>
<thead>
<tr>
<th>Storage temp., °C</th>
<th>Range of ALP activity (% remaining)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td>−20</td>
<td>98–103</td>
</tr>
<tr>
<td>−4</td>
<td>85–93</td>
</tr>
<tr>
<td>22</td>
<td>78–82</td>
</tr>
<tr>
<td>−20 b</td>
<td>—</td>
</tr>
<tr>
<td>−20 c</td>
<td>—</td>
</tr>
</tbody>
</table>

aALP was prepared from a butanol extract of SaOS-2 cells, dialyzed against NaHCO₃ buffer, and diluted in heat-inactivated human serum and water, as described in Materials and Methods. Stored 0.2-mL aliquots were treated as shown, assayed in triplicate.

bStated aliquots subjected to either 4 or 5 freeze–thaw cycles during 48 h.

Table 3. Reproducibility of the Serum ALP Isoenzyme Assay with the Isoenzyme Standards

<table>
<thead>
<tr>
<th>ALP isoenzyme</th>
<th>Source</th>
<th>Total ALP</th>
<th>Heat-stable ALP</th>
<th>Total ALP</th>
<th>Heat-stable ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic</td>
<td>Human serum</td>
<td>2.9</td>
<td>4.6</td>
<td>6.9</td>
<td>13.0</td>
</tr>
<tr>
<td>Skeletal</td>
<td>Human serum</td>
<td>4.2</td>
<td>3.8</td>
<td>8.1</td>
<td>19.6</td>
</tr>
<tr>
<td>Skeletal</td>
<td>SaOS-2 cells</td>
<td>4.5</td>
<td>5.1</td>
<td>5.9</td>
<td>16.5</td>
</tr>
</tbody>
</table>

ALP isoenzyme standards were prepared in a mixture of NaHCO₃ buffer, heat-inactivated human serum, and water (1/1/2 by vol), as described in Materials and Methods. Heat-stable ALP activity was defined as the activity remaining after 10 min (± 0.1 min) at 54 °C (± 1 °C).

The values shown are representative of the variations in the four separate assays used to calculate the inter-assay variations.
Table 4. Reproducibility of the Serum ALP Isoenzyme Assay for Measurements in Artificial Mixtures

<table>
<thead>
<tr>
<th>% of total ALP</th>
<th>CV, %&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Pagetic serum</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>SaOS-2 cells</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>*</sup>n = 4 each. Because these mixtures contained only skeletal and hepatic ALPs, they were analyzed by the simultaneous solution of two equations with two unknowns (as described in Materials and Methods).

Discussion

Our initial studies showed that only two of the five human osteosarcoma cell lines tested (SaOS-2 and TE-85) contained exclusively (or predominantly) skeletal ALP, and that SaOS-2 cells contained 40–50 times more ALP activity per milligram of cell protein than did TE-85 cells. These observations were consistent with previous reports of ALP activities in human osteosarcoma cell lines (19–24).

SaOS-2 cells have been shown to be osteoblastic by the criteria of (a) PTH-sensitive adenylate cyclase activity, (b) specific receptors for 1,25-dihydroxyvitamin D<sub>3</sub>, (c) osteonectin production, and (d) osteogenesis in diffusion chambers (23), and the ALP activity in SaOS-2 cells has been shown to resemble a skeletal ALP standard with respect to inhibitions by L-homoarginine and levamisole, and immunoprecipitation with antibodies recognizing bone/liver/kidney ALP (24). Although previous studies have also examined the thermostability of ALP activity from SaOS-2 cells in a buffered Triton X-100 extract (24), the current studies were needed to compare the thermostability of ALP activity from SaOS-2 cells with a skeletal ALP standard, under the conditions of our isoenzyme assay (i.e., in the specified mixture of heat-inactivated serum, NaHCO<sub>3</sub> buffer, and water that our protocol requires). We focused our attentions on comparisons of time- and temperature-dependent heat inactivations, because that aspect of our method represents the largest source of inter-assay variation (14).

The results of these studies demonstrate that ALP activity from SaOS-2 cells is indistinguishable from a serum-derived skeletal ALP standard with respect to (a) thermostability, (b) inhibition by L-phenylalanine, and (c) variation, in our quantitative isoenzyme assay. Together, these data indicate that ALP activity from SaOS-2 cells is an acceptable alternative to serum and bone-derived skeletal ALP standards in our (kinetic) serum isoenzyme assay. This finding should allow for wider application of our assay: because SaOS-2 cells are more readily available than either Pagetic sera or human bone, the standard should be more abundant. But additional studies will still be required to identify alternative cellular sources for hepatic, intestinal, and placental ALP standards. (The ALP activity extracted from the putative hepatic cell line, SK-HEP-1, did not resemble the hepatic ALP standard in the current studies.) Additional studies will also be required to determine whether ALP activity from SaOS-2 cells can be used as a skeletal ALP standard in alternative assay procedures that depend on electrophoresis (31), isoelectric focusing (32), liquid chromatography (33), lectin affinity chromatography (34), or monoclonal antibody specificity (35, 36) for quantitative measurements of circulating ALP isoenzyme activities.

We thank Susan Linkhart and Sandra Herring for obtaining and establishing the human cell lines used in these investigations, and the secretarial staff of the Mineral Metabolism Unit and the Medical Media staff of the Jerry L. Pettis Memorial Veterans' Hospital for their assistance in the preparation of this manuscript. All studies were performed in the Loma Linda University Dept. of Medicine's Mineral Metabolism Research Unit, which is located in the Jerry L. Pettis Memorial Veterans' Hospital, and directed by David J. Baylink, M.D. This research was supported by the Veterans' Administration, and by Loma Linda University.

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