

Communication

Alkaline Phosphatase Measurements of Paired Normal and Osteosarcoma Tissue Culture Lines Obtained from the Same Patient

William Thorpe,¹ Walter Loeb, Cornelia Hyatt, and Steven A. Rosenberg

Surgery Branch, National Cancer Institute, NIH, Bethesda, Maryland 20014

ABSTRACT

Markedly different levels of alkaline phosphatase production have been found in paired osteosarcoma cells and normal skin fibroblasts grown in tissue culture from 12 patients with osteosarcoma. In all cases, the osteosarcoma line contains significantly more alkaline phosphatase than does the paired normal line from that same patient. These criteria may be useful in distinguishing osteosarcoma from normal fibroblast overgrowth in tissue culture.

INTRODUCTION

We have established pairs of tissue culture lines originating from normal skin fibroblasts and osteosarcoma cells from 12 different patients. These tissue culture lines have been used for immunological studies to attempt to define tumor-specific antigens on osteosarcoma cells (12, 13, 20)² and are currently being used for a variety of immunological and metabolic studies comparing normal and malignant cells. These comparative immunological and metabolic studies depend on the accurate identification of the cells growing in culture as being truly osteosarcoma cultures. In this study, we have attempted to take advantage of the high levels of the enzyme alkaline phosphatase in osteosarcoma cells (8, 16-18) to distinguish osteosarcoma from normal fibroblasts *in vitro*. It appears that osteosarcoma cells in tissue culture maintain high levels of alkaline phosphatase and that this characteristic can be used to help distinguish these cells from normal fibroblasts.

These tissue culture lines have all been cryopreserved in the first several tissue culture passages and are available to interested investigators.³

MATERIALS AND METHODS

Tissue Culture of Skin and Osteosarcoma. Normal skin and osteosarcoma were obtained from 12 patients at the time of amputation and placed in tissue culture by techniques previously described (12, 13, 20). A careful attempt was made to obtain pure tumor, free of surrounding connective tissue. Skin was obtained from the operative specimen far from the tumor. Tissues were extensively washed

in Hanks' balanced salt solution and then minced with a scissors into fragments of approximately 2 cu mm. These fragments were briefly exposed to a solution containing kanamycin (5 mg/ml) (Flow Laboratories, Rockville, Md.). The fragments were then suspended in Eagle's minimal essential medium supplemented with 20% fetal calf serum (Flow Laboratories) containing both kanamycin (50 μ g/ml) and chlortetracycline (50 μ g/ml) and Fungizone (1 to 5 μ g/ml) (referred to hereafter as complete medium). Multiple tissue pieces suspended in 5 to 10 ml of complete medium were then placed in plastic tissue culture flasks (No. 3042; Falcon Plastics, Oxnard, Calif.). The tissue culture flasks with caps slightly loosened were incubated at 37° in an atmosphere of 5% CO₂ and air. Medium was supplemented weekly or biweekly. After approximately 3 to 5 weeks, both skin and tumor cell cultures became confluent and were serially passaged. Most tissue culture generations were cryopreserved.

Measurement of Alkaline Phosphatase and Protein. Osteogenic sarcoma and normal skin fibroblast lines were treated in parallel and were detached from confluent monolayer cultures using 0.25% trypsin. The cells were washed with Hanks' balanced salt solution, counted, and pelleted by centrifugation. The samples were then coded, and alkaline phosphatase measurements were performed blindly on the coded samples. Cells that could not be immediately processed were frozen at -20°.

One ml of 0.85% sodium chloride solution was added for each sample of cells. Cells were sonicated for 2 min at 20 kHz at 0 to 4° using a Bronson W140 Sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) equipped with an L converter and microtip. Sonicates were clarified by centrifugation at 1000 \times g for 10 min.

Alkaline phosphatase determinations were performed kinetically on a Centrifichem 300 analyzer (Union Carbide Corp., New York, N. Y.) by the modification of Morgenstern *et al.* (9) of the method of Bessey, Lowry and Brock. Alkaline phosphatase activity was expressed as milliunits/ml or milliunits/mg protein at 37°. Protein determinations were performed by the phenol method (3).

Thermostability was determined by heating a portion of the sonicate to 56° for 10 min (4). The percentage of thermostable alkaline phosphatase was defined as the alkaline phosphatase activity of the heated aliquot divided by the activity of the unheated aliquot times 100.

RESULTS

The levels of total alkaline phosphatase per cell in osteosarcoma and normal skin fibroblasts obtained from 12

¹ To whom requests for reprints should be addressed, at Surgery Branch, National Cancer Institute, Building 10, Room 10N116, Bethesda, Md. 20014.

² W. Thorpe and S. A. Rosenberg, submitted for publication.

³ For further information about these lines or to obtain these tissue culture lines, please contact Dr. Steven A. Rosenberg, Chief, Surgery Branch, National Cancer Institute, Bethesda, Md. 20014.

Received July 24, 1978; accepted October 13, 1978.

patients are shown in Chart 1 and Table 1. In all cases, the alkaline phosphatase levels in osteosarcoma cells were greater than in skin fibroblasts. The ratio of alkaline phosphatase in osteosarcoma cells to alkaline phosphatase in skin cells was at least 3 and was usually greater. Similar

results were obtained when total alkaline phosphatase was measured in comparison to cellular protein (Chart 1; Table 1). In all but one patient (L. W.) the alkaline phosphatase level in osteosarcoma cells greatly exceeded alkaline phosphatase levels in skin cells from the same patient. Virtually identical results were observed when heat-labile alkaline phosphatase was measured either on an alkaline phosphatase per cell or per mg protein basis. It thus appeared that the production of this enzyme by cultured sarcoma cells consistently exceeded that in normal skin fibroblast obtained simultaneously from the same patient and cultured under identical conditions.

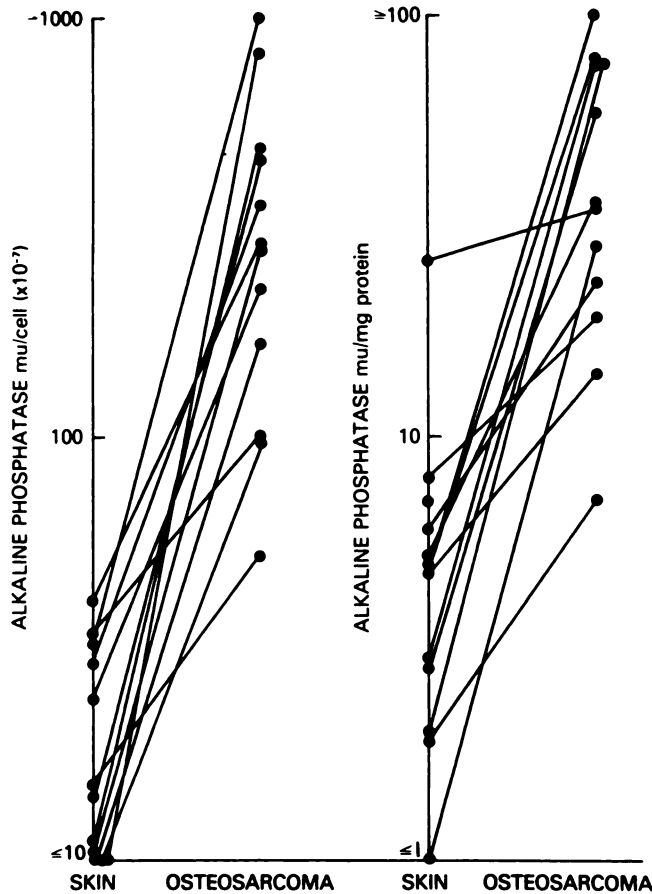


Chart 1. Alkaline phosphatase measurements on paired skin and osteosarcoma tissue cultures from the same patient.

DISCUSSION

Alkaline phosphatase is an enzyme which catalyzes the hydrolysis of phosphoric esters (7). In osseous tissue, it has been reported to be involved with both the calcification of bone matrix (11) and with protein synthetic activity associated with bone matrix production (6).

Bone tumors have been found to exhibit high levels of alkaline phosphatase. Alkaline phosphatase levels in cultured osteosarcoma cells have been previously investigated by Singh et al. (16-18) who established that cultured osteosarcoma was associated with marked alkaline phosphatase production. High levels of alkaline phosphatase have also been found in tissue culture lines of murine osteosarcoma cells (1, 2, 5, 10).

Recently several groups have attempted to establish multiple lines of human sarcomas in tissue culture (14, 15, 19), although distinguishing normal from malignant cells has been a major problem. A comparative study of the relative production of alkaline phosphatase in osteosarcoma versus normal control fibroblasts in the human has not been reported.

Our studies indicate that cultured osteosarcoma cells are associated with elevated alkaline phosphatase levels compared to levels found in normal fibroblasts from the same

Table 1
Alkaline phosphatase measurements on normal and osteosarcoma tissue culture lines obtained from the same patient

All measurements performed at least twice on separate cultures.

Patient	No. of determinations	Alkaline phosphatase (mu/cell × 10 ⁻⁷)		Ratio (osteosarcoma/normal)	Alkaline phosphatase (milliunits/mg/protein)		Ratio (osteosarcoma/normal)
		Osteosarcoma	Normal fibroblasts		Osteosarcoma	Normal fibroblasts	
C. B.	2	454 ± 213 ^a	14 ± 6	32	76 ± 13	3 ± 3	25
R. C.	2	352 ± 79	29 ± 6	12	79 ± 51	5 ± 0	16
L. S.	2	100 ± 24	34 ± 20	3	18 ± 8	8 ± 1	2
R. H.	2	1024 ± 960	33 ± 33	31	122 ± 75	5 ± 5	24
L. W.	2	289 ± 165	41 ± 3	7	34 ± 1	26 ± 18	1
J. G.	2	225 ± 130	24 ± 14	9	23 ± 0	6 ± 1	4
K. C.	2	166 ± 159	3 ± 2	55	76 ± 72	2 ± 1	38
P. S.	2	99 ± 28	6 ± 2	17	58 ± 14	3 ± 1	19
R. W.	3	283 ± 117	10 ± 7	28	35 ± 15	5 ± 3	7
J. B.	3	823 ± 408	2 ± 0	412	14 ± 2	7 ± 1	2
J. A.	5	483 ± 426	1 ± 1	483	28 ± 17	0.2 ± 0	140
D. W.	2	53 ± 15	15 ± 15	3	7 ± 1	2 ± 2	4
Mean ± I S.D.		363 ± 298	18 ± 14		48 ± 34	6 ± 7	

^a Mean ± S.E.

patient. The variability of these levels is large, however, and may be due to obtaining cultures at slightly different stages of growth. The mean alkaline phosphatase level of the tissue culture lines was $362 \pm 298 \times 10^{-7}$ milliunits/cell for the osteosarcoma lines and 17.6 ± 14 milliunits/cell for the normal skin lines. In each case, however, osteosarcoma-associated alkaline phosphatase was far greater than was the corresponding autologous normal cell line.

The consistent elevation in alkaline phosphatase levels observed in these osteosarcoma cell lines helps differentiate these lines from normal fibroblasts but does not of itself prove the malignant nature of these lines. Multiple other criteria such as saturation density, cell morphology, growth pattern, growth on contact inhibited monolayers, growth in immunosuppressed mice, and growth in soft agar have been used to differentiate normal from malignant cells. Many of these criteria, however, have proved ambiguous in studies of human osteosarcoma cells in tissue culture. Levels of alkaline phosphatase in tissue culture cells appear to be an additional criterion that is simple to perform and that can help to distinguish osteosarcoma from normal fibroblastic cells in tissue culture.

REFERENCES

1. Amitani, K., and Nakata, Y. Establishment and Alkaline Phosphatase Activity of Clonal Cell Lines of Murine Osteosarcomas. A Preliminary Study. *Clin. Orthopaed. Related Res.*, **113**: 164-167, 1975.
2. Amitani, K., and Nakata, Y. Characteristics of Osteosarcoma Cells in Culture. *Clin. Orthopaed. Related Res.*, **122**: 315-324, 1977.
3. Daughaday, W. H., Lowry, O. H., Rosebrough, N. J., and Fields, W. S. Determination of Cerebrospinal Fluid Protein with the Folin Phenol Reagent. *J. Lab. Clin. Med.*, **39**: 663-665, 1952.
4. Fitzgerald, M. X., Fennelly, J. J., and McGeeney, K. The Value of Differential Alkaline Phosphatase Thermostability in Clinical Diagnosis. *Am. J. Clin. Pathol.*, **51**: 194-201, 1969.
5. Ghanta, V. K., Hiramoto, R. N., Weiss, A. B., and Caudill, L. Monitoring of Murine Osteosarcoma by Serial Alkaline Phosphatase Determinations. *J. Natl. Cancer Inst.*, **57**: 837-839, 1976.
6. Kuhlman, R. Phosphatases in Epiphyseal Cartilage. Their Possible Role in Tissue Synthesis. *J. Bone Joint Surg.*, **47**: 545-550, 1965.
7. Kuhlman, R., and Bakowski, M. The Biochemical Activity of Fracture Callus in Relation to Bone Production. *Clin. Orthopaed. Related Res.*, **107**: 265-285, 1975.
8. Lundgren, E., Ponten, J., and Westermark, B. Isozyme Variations in Human Cells Grown *in Vitro*. IX. Enzyme Regulation in Normal and Malignant Cell Lines. *Human Heredity*, **24**: 186-193, 1974.
9. Morgenstern, S., Kessler, G., Auerbach, J., Flor, R. V., and Klein, B. An Automated *p*-Nitrophenylphosphate Serum Alkaline Phosphatase Procedure for the Autoanalyzer. *Clin. Chem.*, **11**: 876-888, 1965.
10. Nakakuki, K., Shimokawa, K., Yamauchi, H., and Akitsugu, O. A Spontaneous Transplantable Osteogenic Sarcoma in AKR/Ms Mice. *Gann*, **67**: 513-521, 1976.
11. Robinson, R. The Possible Significance of Hexosephosphoric Ester in Ossification. *Biochem. J.*, **17**: 286-293, 1923.
12. Rosenberg, S. A. Lysis of Human Normal and Sarcoma Cells in Tissue Culture by Normal Serum: Implications for Experiments in Human Tumor Immunology. *J. Natl. Cancer Inst.*, **58**: 1233-1238, 1977.
13. Rosenberg, S. A., Schwarz, S., Anding, H., Hyatt, C., and Williams, G. M. Comparison of Multiple Assays for Detecting Human Antibodies Directed against Surface Antigens on Normal and Malignant Human Tissue Culture Cells. *Immunol. Methods*, **17**: 225-239, 1977.
14. Saxton, R. E., Irie, R. F., Fenone, S., Pellegrino, M. A., and Morton, D. L. Establishment of Paired Tumor Cell Lines to Refine Humoral Immune Responses in Melanoma and Sarcoma Patients. *Intern. J. Cancer*, **21**: 299-306, 1978.
15. Sethi, J., Hirshaut, Y., Hajdu, S. I., and Clements, L. G. Growing Human Sarcomas in Culture. *Cancer*, **40**: 744-755, 1977.
16. Singh, I., and Tsang, K. Y. An *in Vitro* Production of Bone Specific Alkaline Phosphatase. *Exptl. Cell Res.*, **95**: 345-358, 1975.
17. Singh, I., Tsang, K. Y., and Blakemore, W. S. Isolation and Partial Purification of Plasma Membrane-associated Antigens from Human Osteosarcoma (TE-85) Cells in Tissue Culture. *Cancer Res.*, **36**: 4130-4136, 1976.
18. Singh, I., Tsang, K. Y., and Ludwig, G. D. Alkaline Phosphatase and Ultrastructural Alterations in Human Osteosarcoma Cells in Tissue Culture. *Eur. Surg. Res.*, **6**: 247-263, 1974.
19. Smith, H. S., Owens, R. B., Hiller, A. J., Nelson-Rees, W. A., and Johnston, J. O. The Biology of Human Cells in Tissue Culture. I. Characterization of Cells Derived from Osteogenic Sarcomas. *Intern. J. Cancer*, **17**: 219-234, 1976.
20. Thorpe, W. P., Parker, G. A., and Rosenberg, S. A. Expression of Fetal Antigens by Normal Human Skin Cells Grown in Tissue Culture. *Immunol.*, **119**: 818-823, 1977.