Increase in the Activity of Alkaline Phosphatase by L-Ascorbic Acid 2-Phosphate in a Human Osteoblast Cell Line, HuO-3N1

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Summary The activity of alkaline phosphatase (ALPase) was significantly enhanced in a human osteoblast cell line, HuO-3N1, when it was cultured in the presence of L-ascorbic acid 2-phosphate (ASA-P; a stable ascorbic acid derivative). With ASA-P in the culture, the level of ALPase activity increased approximately 3-fold without any effect on either the morphology or growth rate. This increase was dependent on the ASA-P concentration in the range of 0.2–2 mM and required at least 48 h incubation with ASA-P. The ALPase mRNA level, however, remained rather constant irrespective of the enzyme activity. Removal of ASA-P from the precultured medium decreased the stimulatory effect of ascorbic acid on the ALPase activity, indicating that the effect was reversible. Dexamethasone, an inducer for osteoblastic differentiation, enhanced the level of ALPase activity irreversibly, in parallel with the increase in the level of its mRNA. The enhancement of the ALPase activity by ascorbic acid in this cell line appeared to be independent of cell differentiation.

Key Words ascorbic acid, alkaline phosphatase, osteoblast

Ascorbic acid (AsA) is a well known vitamin distributed in most tissues, but little is known about its precise roles in the body. It is well documented that AsA is a cofactor for the enzymatic hydroxylation of prolyl and lysyl residues in procollagen (1-3), and of ß-monoxygenase in adrenal glands (4,5).

Recently, AsA was suggested to play an important role in either transcription or translation. AsA increases the mRNA levels for procollagen (6-8) and acetylcholine receptor (9-11) in fibroblasts and muscle cells, respectively. Furthermore, AsA elevates the levels of both alkaline phosphatase (ALPase) and collagen (X) mRNA in primary chick chondrocytes (12-14). In an attempt to elucidate the regulatory mechanism of AsA-dependent induction of functional proteins such as ALPase, we found, after extensive examination of various cell lines, an osteoblast

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cell line, HuO-3N1 established by Yamane (15), in which the ALPase activity was markedly increased in the presence of AsA.

ALPase has been used as a marker enzyme during osteoblastic differentiation (16). In a primary culture of osteoblast, the ALPase activity is enhanced dramatically when osteoblastic differentiation is induced with vitamin D$_3$ and dexamethasone (Dex) (17). However, in many transformed osteoblast cell lines derived from osteosarcoma, a low level of ALPase is constitutively expressed.

In this study, the effects of AsA on both transcription and translation of the ALPase gene in HuO-3N1 were investigated by using cells cultured with various concentrations of a stable AsA derivative, L-ascorbic acid 2-phosphate (AsA-P), for different periods. AsA-P increased the level of the ALPase activity by approximately 3 times as much as that synthesized in AsA-P-free medium, while the level of mRNA for the enzyme remained unaltered. However, Dex enhanced the levels of both the activity and mRNA of ALPase, indicating that AsA increased the ALPase activity in HuO-3N1 independently of osteoblastic differentiation.

EXPERIMENTAL

An L-ascorbic acid 2-phosphate derivative (L-ascorbic acid phosphate magnesium salt; C$_6$H$_6$O$_9$P Mg$_{3/2}$) from Wako Pure Chemical Industries Co. (Osaka), RPMI1640 from Nissui Pharmaceutical Co. (Tokyo), fetal calf serum from M. A. Bioproducts (Walkerville, MD) and culture plates from Nunc (Denmark) were used for cultures. The other chemicals used were of reagent grade and obtained from Wako Pure Chemical Industries Co.

Cell culture. HuO-3N1 cells provided by the Japanese Cancer Research Resources Bank (JCRB) were cultured in RPMI1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C under a humidified 5% CO$_2$ atmosphere.

Preparation of cell extract and measurement of the ALPase activity. Cells were detached with trypsin from a T-flask, washed twice with Tris-HCl buffered (pH 7.0)-saline (TBS) and then solubilized by the addition of 2 ml of TBS containing 1% Triton X-100, with vigorous mixing. After centrifugation, an aliquot of the solubilized cell extract was assayed for ALPase activity using 5 mM p-nitrophenyl phosphate (PNPP) as a substrate in an assay buffer of 1.5 M Tris-HCl (pH 9.0) containing 1 mM ZnCl$_2$, as described by Leboy et al. (12). The reaction was allowed to proceed for 15 min at 37°C and then stopped by adding an equal volume of 0.5 M NaH$_2$PO$_4$. The amount of the product in the enzyme reaction was calculated assuming that the value of 1.0 in absorbance at 410 nm is equivalent to 64 nmol of p-nitrophenol (product) formed. The ALPase activity in the extract was expressed as nmol of product formed/min/mg protein. Protein concentrations were measured by means of a dye-binding assay (Bio-Rad) with bovine γ-globulin as a standard.

Analysis of the type of alkaline phosphatase. The type of isozyme of ALPase was determined by specific inhibitors, L-homoarginine, L-phenylalanine, and L-
leucine. Each compound was added at a final concentration of 10mM in the reaction mixture and the activity was measured under the same condition as described above.

Statistical significance of the difference between values was analyzed by one-way analysis, and subsequently analyzed by Duncan's multiple range test.

Northern blot analysis. Total RNA was isolated by the AGPC (acid-guanidium-phenol-chloroform) method (18), denatured, electrophoresed on a 1% formaldehyde-agarose gel and then blotted onto a Biodyne A membrane (PALL Ultrafine Filtration Co.). The filter was subsequently prehybridized, hybridized with a radioactive probe and then washed according to a standard method prior to autoradiography.

The radioactive probes were prepared by random priming from cDNA coding for human alkaline phosphatase (bone/kidney/liver type) (19) and from a human mitochondrial DNA fragment coding for mitochondrial ribosomal RNA. The former cDNA was provided by JCRB and the latter DNA fragment by Dr. Kitagawa (Nogoya Univ.).

RESULTS AND DISCUSSION

Effects of L-ascorbic acid 2-phosphate on cell growth and the level of alkaline phosphatase activity

HuO-3N1, a human osteoblast cell line derived from an osteosarcoma, proliferated with a doubling time of 40 h under the growth conditions used here. At an initial plating density of 2×10^5 cells/25 cm^2, the cells reached confluence within 6–7 days. The effect of ascorbic acid on cell growth was investigated using L-ascorbic acid 2-phosphate (ASA-P), which had the advantage of being long-acting (20).

ASA-P addition to the medium to a final concentration of 2 mM, had no effect on growth (Fig. 1A). The spindle-shape morphology also remained unaltered. When grown with ASA-P, the enzyme activity started to increase on day 3 and reached the maximum level on day 6, approximately 3 times as much as that cultured in the ASA-P-free medium (Fig. 1B). No significant ALPase activity was detected in either the culture supernatant or the extracellular matrix fraction.

The dose-dependency of ASA-P as to the increase in the ALPase activity was then examined (Fig. 2). The level of ALPase was slightly enhanced at 0.05 mM ASA-P and then increased linearly depending on the concentration of ASA-P up to 2 mM. At higher concentrations ranging from 4 to 16 mM, ASA-P caused no further elevation of the level of ALPase activity. Therefore, ASA-P was used at a concentration of 2 mM in subsequent experiments.

Dexamethasone (Dex) caused a prominent increase in the ALPase level together with a clear morphological change at 100 nM (Fig. 1B). The level of ALPase started to increase rapidly on day 2 and reached the maximum within 6 days (Fig. 1B). The time required for enhancement of ALPase by Dex was shorter (2 days) than that of ASA-P (3 days). Furthermore, the maximum level of ALPase
Fig. 1. Effects of L-ascorbic acid 2-phosphate on cell growth and the level of alkaline phosphatase. Cells were inoculated on day 0 at an initial density of $2 \times 10^5$ cells into a 25 cm$^2$ T-flask containing 10% FCS RPMI with either 2 mM AsA-P (●) or 100 nM Dex (■) and without addition of any compound (○). On the days indicated, the number of cells detached with trypsin was determined. Then, cells were solubilized with a buffer containing 1% Triton X-100 and the ALPase activity in cell extract was determined as described under EXPERIMENTAL. Effects on cell growth and the ALPase activity are shown in A and B, respectively. The ALPase activity level is expressed as nmol of product formed/min/mg of cell extract protein. Each symbol and its associated vertical bar represent the mean ± SD for triplicate samples.
in Dex-treated cells was 3 times as much as that in AsA-P-treated cells. ALPase was drastically increased in a primary osteoblast culture by treatment with Dex as the result of osteoblastic differentiation (17). Therefore, the enhancement of Dex-dependent ALPase in HuO-3N1 was due to the result of differentiation.

Three structurally distinct ALPase isoenzymes (placenta, intestine, and bone/kidney/liver types) have been well characterized. The type of these enzymes can be determined by specific inhibitors (21). The bone/kidney/liver type are known to be inhibited by L-homoarginine drastically and by L-phenylalanine or L-leucine slightly. In contrast, both of the placenta type and intestine type are inhibited by L-phenylalanine or L-leucine modestly.

The type of the basal and enhanced ALPase activities determined here was investigated with these inhibitors (Table 1). ALPase in extracts from cells cultured under different conditions was inhibited by L-homoarginine. The two other inhibitors, which inhibited calf intestine ALPase singificantly, reduced slightly the enzyme activities in cell extracts. These results indicates that HuO-3N1 contains only a bone-type ALPase. In preliminary work, AsA-P was found to enhance the level of placenta-type ALPase activity in MKN45 cell extract (K. Hitomi, unpublished data). Although the mechanism underlying AsA-P-dependent elevation of the enzyme level is not known at present, it is possible that all ALPase isozyme
Table 1. Inhibition of the alkaline phosphatase activity of HuO-3N1 by specific inhibitors.

HuO-3N1 was cultured for 6 days in the basal medium and basal medium supplemented with 2 mM AsA-P or 100 nM Dex. Then, cell extracts were prepared as described in the legend of Fig. 1. The ALPase activity was determined as described under EXPERIMENTAL in the presence of inhibitors at a final concentration of 10 mM. The enzyme activity of calf intestine ALPase (intestine-type) as an authentic enzyme was also determined under the same conditions. Inhibition is expressed as the mean percentage of original activity remaining (±SD) in the presence of the inhibitor for triplicate samples.

| Inhibitors         | ALPase in cell extract of HuO-3N1 | Calf intestine ALPase (intestine-type) |
|--------------------|-----------------------------------|---------------------------------------|
|                    | Basal | AsA-P | Dex    | 100±5^a |
| No addition        | 100±3^a | 100±3^a | 100±3^a | 100±5^a |
| l-Homoarginine     | 39±5^b  | 29±4^b  | 27±5^b  | 95±3^a  |
| l-Phenylalanine    | 84±6^c  | 87±7^d  | 91±5^e  | 41±8^b  |
| L-Leucine          | 89±5^e  | 90±4^e  | 90±5^e  | 53±4^b  |

Means within a column not followed by the same letter are significantly different (p < 0.05).

types are enhanced in a similar manner.

Northern blot analysis of the ALPase mRNA from cells treated with L-ascorbic acid 2-phosphate or dexamethasone

Total RNA was prepared from cells grown for 2, 4, and 6 days in the presence or absence of AsA-P (2 mM) or Dex (100 nM) and then used to analyze the amounts of the transcripts from the ALPase gene. When the radioactivity of each ALPase mRNA was normalized as to that of mitochondrial ribosomal RNA, no specific increase in the ALPase mRNA was observed in cells grown for various periods with AsA-P (Fig. 3). These data suggested that AsA-P did not enhance either stabilization of transcripts or rate of transcription from the ALPase gene. In contrast, Dex increased the level of the ALPase mRNA as the incubation time increased. The amount of the mRNA appeared to correspond roughly to that of ALPase.

Although it is not known why the level of ALPase mRNA remained unaltered in spite of the enhancement of the enzyme activity in cells cultured with AsA-P, the two following possibilities could be considered: (1) the translation rate from ALPase mRNA is enhanced and/or (2) the ALPase itself is stabilized in the presence of AsA-P.

Reversible effect of L-ascorbic acid 2-phosphate on the level of alkaline phosphatase activity

The effect of the incubation time with AsA-P or Dex on the ALPase activity
is shown in Fig. 4. When cells were grown with AsA-P for the last 6 or 24 h of the whole culture of period (144 h), the level of ALPase activity was not affected to any significant extent. Incubation with AsA-P for at least 48 h was required before significant enhancement of the enzyme was detected (Fig. 4A). These results are consistent with those in Fig. 1B. The enhanced ALPase activity by AsA-P, however, diminished when AsA-P was removed from the medium after pretreatment for 48 or 72 h, which was long enough to increase in the ALPase activity (Fig. 4B). The effect of AsA-P on the enhancement of the ALPase activity appeared to be reversible.

Incubation with Dex for the first 24 h of the whole culture period was sufficient to cause a drastic increase in the level of ALPase, which remained unaltered even
Fig. 4. Effect of the incubation period with L-ascorbic acid 2-phosphate or dexamethasone on the alkaline phosphatase activity. The cultured periods with AsA-P (2 mM) or Dex (100 nM) are indicated as an arrow. The culture medium in each case was daily exchanged for the designated medium. In experiment A, AsA-P was added to the AsA-free medium at the indicated times. In experiments B and C, cells were grown first in the medium supplemented with AsA-P or Dex for the indicated periods and then cultured further in drug-free medium for the remaining periods. After 144 h from the start of culture, the cells were harvested and cell extracts were prepared as described under EXPERIMENTAL. The ALPase activity is expressed as described in the legend of Fig. 1. Values shown are the means ± SD for triplicate samples.

| A | Days in Culture | ALPase activity (nmol product/min/mg protein) |
|---|----------------|---------------------------------------------|
| AsA-P Exposure | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Hours | 144 | ← | 120 ± 8 | ← | 100 ± 11 | ← | 78 ± 5 | ← | 44 ± 6 | ← | 41 ± 4 | ← | 42 ± 5 |
| 96 | ← | ← |
| 48 | ← |
| 24 | ← |
| 6 | ← |
| 0 | ← |

| B | Days in Culture | ALPase activity (nmol product/min/mg protein) |
|---|----------------|---------------------------------------------|
| AsA-P Exposure | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Hours | 144 | ← | 112 ± 8 | ← | 104 ± 9 | ← | 82 ± 8 | ← | 44 ± 6 | ← | 42 ± 7 | ← | 42 ± 6 |
| 120 | ← |
| 96 | ← |
| 72 | ← |
| 48 | ← |
| 24 | ← |

| C | Days in Culture | ALPase activity (nmol product/min/mg protein) |
|---|----------------|---------------------------------------------|
| Dexamethasone Exposure | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Hours | 144 | ← | 430 ± 13 | ← | 452 ± 11 | ← | 422 ± 12 | ← | 416 ± 11 | ← | 423 ± 9 | ← | 421 ± 11 | ← | 34 ± 7 |
| 120 | ← |
| 96 | ← |
| 72 | ← |
| 48 | ← |
| 24 | ← |
| 0 | ← |
after removal of Dex from the medium (Fig. 4C). The irreversibility of the enhancement of the ALPase activity by Dex was due to the result of differentiation, as described above.

The mechanism underlying the AsA-P enhanced the ALPase activity remains to be elucidated. ALPase expression in a primary osteoblast culture is accompanied by the onset of the extracellular matrix development (16), suggesting a possible linkage between collagen accumulation and enhancement of the ALPase activity. HuO-3N1 cells in which ALPase was enhanced by AsA-P became rather tightly attached to T-flask probably as a consequence of the accumulation of extracellular matrix with mature collagen molecules. Therefore, the enhancement of the ALPase activity caused by AsA-P might be also mediated by the extracellular matrix around the HuO-3N1 cells.

In conclusion, we demonstrated that AsA-P increased the level of bone-type ALPase in a cultured human osteoblast cell line, HuO-3N1. The AsA-dependent increase in the ALPase activity appeared to be mediated by a mechanism different from that underlying the enhancement of the ALPase activity by Dex.

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REFERENCES

1) Myllylä, R., Kuuti-Savolainen, E.-R., and Kivirikko, K. (1978): The role of ascorbate in the prolyl hydroxylase reaction. Biochem. Biophys. Res. Commun., 83, 441–448.
2) Puistola, U., Turpeenniemi-Hujanen, T. M., Myllylä, R., and Kivirikko, K. (1980): Studies on the lysyl hydroxylase reaction. Biochim. Biophys. Acta, 611, 40–50.
3) Myllylä, R., Majamaa, K., Gunzler, V., Hanauske-Abel, H. M., and Kivirikko, K. (1984): Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. J. Biol. Chem., 259, 5403–5405.
4) Levine, M., Morita, K., and Pollard, H. (1985): Enhancement of norepinephrine biosynthesis by ascorbic acid in cultured bovine chromaffin cells. J. Biol. Chem., 260, 12942–12947.
5) Levine, M., Hartzell, W., and Bdolah, A. (1988): Ascorbic acid and Mg-ATP co-regulation dopamine β-monoxygenase activity in intact chromaffin granules. J. Biol. Chem., 263, 19353–19362.
6) Chojkier, M., Houglum K., Solis-Herruzo, J., and Brenner D. A. (1989): Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblast cells. J. Biol. Chem., 264, 16957–16962.
7) Chan, D., Lamande S. R., Cole W. G., and Batemen J. F. (1990): Regulation of procollagen synthesis and processing during ascorbate induced extracellular matrix accumulation. Biochem. J., 269, 175–181.
8) Kurata, S.-i., and Hata, R. (1991): Epidermal growth factor inhibits transcription of type I collagen genes and production of type I collagen in cultured human skin fibroblasts in the presence or absence of L-ascorbic acid 2-phosphate. J. Biol. Chem.,
9) Horovitz, O., Spitsberg, V., and Salpeter, M. M. (1989): Regulation of acetylcholine receptor synthesis at the level of translation in rat primary muscle cells. *J. Cell. Biol.*, **108**, 1817–1822.

10) Horovitz, O., Knaack, D., Podleski, T. R., and Salpeter, M. M. (1989): Acetylcholine receptor α-subunit mRNA is increased by ascorbic acid. *J. Cell. Biol.*, **108**, 1823–1832.

11) Salpeter, M. M., Liu, E., Minor, R. R., Podleski, T. R., and Woton, J. A. M. (1991): Acetylcholine receptor regulation in L5 muscle cells is independent of increase in collagen secretion induced by ascorbic acid. *Am. J. Clin. Nutr.*, **54**, 1184S–1187S.

12) Leboy, P. S., Vaias, L., Uschmann, B., Golub, E., Adams, S. L., and Pacifici, M. (1989): Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. *J. Biol. Chem.*, **264**, 17281–17286.

13) Wu, L. N. Y., Sauer, G. R., Genge, B. R., and Wuthier, R. E. (1989): Induction of mineral deposition by primary cultures of chicken growth plate chondrocytes in ascorbate-containing media. *J. Biol. Chem.*, **264**, 21346–21355.

14) Shapiro, I., Leboy, P. S., Tokuoka, T., Forbes, E., Debolt, K., Adams, S. L., and Pacifici, M. (1991): Ascorbic acid regulates multiple metabolic activities of cartilage cells. *Am. J. Clin. Nutr.*, **54**, 1209S–1213S.

15) Yamane, T. (1985): Establishment and characterization of cell lines derived from a human osteosarcoma. *Clin. Orthopaedics Relat. Res.*, **199**, 261–271.

16) Stein, G. S., Lian, J. B., and Owen, T. H. (1990): Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *Faseb. J.*, **4**, 3111–3123.

17) Leboy, P. S., Beresford, J. N., Devlin, C., and Owen, M. E. (1991): Dexamethasone induction of osteoblast mRNA in rat marrow stroma cell line. *J. Cell. Physiol.*, **146**, 370–378.

18) Chomczynski, P., and Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

19) Weiss, M. J., Henthorn, P. S., Lafferty, M. N., Slaughter, C., Raducha, M., and Harris, H. (1986): Isolation and characterization of a cDNA encoding a human liver/bone/kidney type alkaline phosphatase. *J. Biol. Chem.*, **83**, 7128–7186.

20) Hata, R., and Senoo, H. (1989): L-Ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue-like substrate by skin fibroblast. *J. Cell. Physiol.*, **138**, 8–16.

21) Mulivor, R. A., Hann, V. L., and Harris, H. (1978): Development change in human intestinal alkaline phosphatase. *Proc. Natl. Acad. Sci. USA*, **75**, 3909–3912.