L-Ascorbic Acid 2-Phosphate Promotes Osteoblastic
Differentiation of MC3T3-E1 Mediated
by Accumulation of Type I Collagen

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(Received January 10, 1994)

Summary L-Ascorbic acid 2-phosphate (Asc-P: a stable ascorbic acid
derivative) markedly stimulated synthesis of marker proteins for osteo-
blastic differentiation such as alkaline phosphatase and osteocalcin in a
murine osteoblastic cell line, MC3T3-E1, suggesting that Asc-P could
promote osteoblastic differentiation. L-Azetidine 2-carboxylate (AzC)
diminished the stimulatory effects of Asc-P on the synthesis of alkaline
phosphatase and osteocalcin due to its inhibitory effects on mature colla-
gen secretion. Growing cells on the dishes coated with type I collagen
resulted in an increased expression of osteoblastic phenotypes even in the
presence of AzC. Coating with fibronectin, however, failed to promote
differentiation. These results suggest that the promotion of cell differen-
tiation caused by Asc-P is mediated by the accumulation of mature
collagen.

Key Words ascorbic acid, osteoblast, collagen, alkaline phosphatase,
osteocalcin

For normal bone formation, ascorbic acid (AsA) is known to be essential since
scurvy causes a bone defect (1). AsA has a well documented role in collagen
synthesis as a direct requirement for prolyl and lysyl hydroxylases (2). AsA also
increases the mRNA level for procollagen, and this effect is the result of the
stimulation of collagen gene transcription and the decreased degradation of pro-
collagen mRNA (3). Ascorbate treatment was shown to enhance the levels of
alkaline phosphatase (ALPase) activity in osteoblast cell line (4) and type X
collagen mRNA and calcium deposition as well as ALPase in hypertrophic chon-
drocytes (5).

Osteoblasts play a key role in the bone formation by secreting and assembling
bone matrix components. After mitogenic growth as osteoblast precursor cells,
they differentiate to mature cells with specific functions. Although osteoblastic
differentiation is under the control of various hormones and growth factors, AsA

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appears to be necessary for both growth and maturation of osteoblasts (6). During differentiation, osteoblasts synthesize sequentially ALPase and osteocalcin (OC). These are used as marker proteins to confirm osteoblastic differentiation (7,8). The temporal expression of these proteins is required for in vivo bone formation by forming a mineralized extracellular matrix. The MC3T3-E1 mouse calvaria-derived cell line has been used as a model system to investigate osteoblastic differentiation, since these cells express osteoblast marker proteins and form a mineralized extracellular matrix (9). The presence of AsA is required for differentiation of MC3T3-E1 cells (10).

L-Ascorbic acid 2-phosphate (Asc-P), a stable AsA derivative, was utilized instead of AsA, since Asc-P is as effective as AsA in the enhancement of collagen synthesis and has the advantage of being a long-acting (11). In this study, to clarify further the role of AsA in osteoblastic differentiation, the expression of phenotypes in cultured MC3T3-E1 cells was investigated in the presence or absence of Asc-P. Furthermore, we examined the effects of L-azetidine 2-carboxylate (AzC), an inhibitor of collagen synthesis, on differentiation during the growth of the cells in a normal dish and dishes coated with extracellular matrix components such as type I collagen. Asc-P promoted osteoblastic differentiation due to enhanced accumulation of mature collagen molecules.

EXPERIMENTAL

Cell culture. A mouse calvaria-derived osteoblast cell line, MC3T3-E1, provided by Dr. M. Kumegawa was grown at 37°C in Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (Whittaker Bioproducts Inc., Walkersville, MD), 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified 5% CO₂ atmosphere. L-Azetidine 2-carboxylate (Aldrich, Milwaukee, WI), an inhibitor for synthesis of procollagen, was dissolved in phosphate buffered (pH 7.4) saline (PBS) and included at various concentrations in the medium. The medium with or without 0.2 mM L-ascorbic acid 2-phosphate (L-ascorbic acid 2-phosphate magnesium salt: C₆H₆O₉PMg₃/2, Wako Pure Chemical Industries, Osaka, Japan) was changed every 3 days. When cells were cultured in dishes coated with various proteins, dishes were prepared as follows: 3 mg/ml pepsin-solubilized bovine skin type I collagen in 0.5 N acetic acid (Koken, Tokyo, Japan) or 0.2 mg/ml bovine serum albumin (BSA) in PBS were added to culture dishes at a concentration of 100 μg/cm² and air-dried at room temperature. Human plasma fibronectin (Koken) dissolved at 20 μg/ml in DMEM was adsorbed at 37°C for 10 h to culture dishes at 10 μg/cm². These coated dishes were rinsed twice with DMEM prior to use.

Determinations of ALPase activity and collagen synthesis. Cell layers formed under various growth conditions were rinsed with cold calcium, magnesium-free PBS, incubated for 30 min at room temperature with ALPase assay buffer (1.5 mM Tris-HCl (pH 10.0), 1 mM ZnCl₂, and 1 mM MgCl₂) containing 1% Triton X-100.
The cell extracts were removed from the dishes, centrifuged (15,000 × g for 10 min at 4°C) and used to assay the ALPase activity. The insoluble materials which remained on the dishes were used for determination of collagen. ALPase activity was determined in ALPase assay buffer with 5 mM p-nitrophenylphosphate as a substrate as described (4) and expressed as μmol of products formed/min/mg protein. Protein concentration was determined by means of a dye-binding assay (Bio-Rad, Richmond, CA) with bovine γ-globulin as a standard. The amount of collagen was determined as follows: the insoluble materials were washed further twice with cold calcium, magnesium-free PBS, digested for 2 h at room temperature with 0.2% (w/v) pepsin in 0.5 M acetic acid and centrifuged (15,000 × g for 10 min at 4°C) (12). Pepsin-resistant mature collagen in the supernatant was determined for its components by SDS-polyacrylamide (7%) gel electrophoresis in the presence of β-mercaptoethanol. After electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue G-250.

Northern blot analysis. Total RNAs (20 μg each) isolated by the AGPC (acid-guanidium-phenol-chloroform) method (13) were denatured, electrophoresed on formaldehyde-agarose (1%) gels and then blotted to Hybond-N+ membranes (Amersham Co., Arlington Heights, IL). The filters were prehybridized, hybridized, and washed according to standard procedures prior to autoradiography. RNAs for ALPase and OC were hybridized with random primed radioactive probes: human liver/bone/kidney type ALPase cDNA (14) and rat OC cDNA (15), respectively. β-Actin RNA, used as an internal standard, was rehybridized with a random primed radioactive mouse β-actin cDNA. When rehybridized, the filters were washed with 0.1× SSC-0.5% SDS for 20 min at 100°C to strip the first probe, and was then reprobed with the second probe.

RESULTS

Effects of Asc-P on proliferation and differentiation of MC3T3-E1 cells

MC3T3-E1 cells, a murine osteoblastic cell line, proliferated with a doubling time of 20 h under the growth conditions used here. At an initial plating density of 10^4 cells/cm², the cells reached confluence within 5 days. Asc-P at a final concentration of 0.2 mM stimulated slightly the growth rate and the confluence was attained within 4 days (Fig. 1A). Proliferation in the presence of Asc-P, however, continued for up to 10 days accompanied by the formation of multiple cell layers.

The effect of Asc-P on the synthesis of ALPase, a marker protein for osteoblastic differentiation, was determined. Under the normal culture conditions, the level of ALPase activity remained relatively low throughout the culture periods, whereas the activity in the cells cultured with Asc-P started to increase on day 5 and reached a maximum level on day 10, approximately 20 times as much as that cultured in the Asc-P free medium (Fig. 1B). This was also the case when the transcript specific for ALPase gene was analyzed by Northern blotting. Only a basal level of RNA for ALPase was detected when cultured without Asc-P, whereas
Fig. 1. Effects of Asc-P on proliferation and induction of ALPase. The MC3T3-E1 cells were inoculated on day 0 at an initial density of 10^4 cells/cm^2 into 60 mm dish and cultured in the presence (■) or absence (□) of 0.2 mM Asc-P. On the days indicated, the number of the cells detached with trypsin was determined (A). Then, cells were processed for determination of ALPase activity as described under EXPERIMENTAL. ALPase activity is expressed as μmol of products formed/min/mg of cell extract protein (B). Each symbol and associated bar represent the M±SD for triplicate samples.

the transcript in the cells cultured with Asc-P began to increase significantly on day 5, reached a maximum level on day 10 and then decreased to the same level as that of day 5 (Fig. 2). Furthermore, mRNA for OC, another marker protein for mature osteoblast, was also markedly induced under the growth condition with Asc-P and reached a maximum level on day 10, as in the case of ALPase mRNA. Only a low level of OC mRNA was, however, detected in the cells cultured without Asc-P.

Induction of ALPase synthesis by Asc-P dependent collagen accumulation in MC3T3-E1 cells

Both AsA and Asc-P stimulate the synthesis and processing of procollagen in various fibroblasts (11). To determine whether the enhanced synthesis of ALPase in MC3T3-E1 cells cultured with Asc-P was induced due to the accumulation of

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Fig. 2. Northern blot analysis of mRNAs for osteoblastic phenotypes. The MC3T3-E1 cells were split and cultured further for the indicated periods in either the Asc-P-free medium or medium containing 0.2 mM Asc-P. Twenty μg of total RNA was analyzed on Northern blot hybridized with radioactive probes of ALPase and OC cDNAs followed by rehybridization of radioactive cDNA coding for β-actin, as described under EXPERIMENTAL.

Fig. 3. Dose-dependence of AzC-inhibitory effects on the accumulation of collagen and induction of ALPase in MC3T3-E1 cells. Split cells were cultured for 5 days in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of Asc-P with the indicated concentrations of AzC (0, 0.1, 0.3, 1 mM). Collagen was extracted and analyzed on 7% SDS-PAGE (A) as described under EXPERIMENTAL. Type I collagen α1 and α2 chains are indicated. The ALPase activity in the extracts from the cells cultured in the presence (■) or absence (□) of Asc-P was determined and expressed (B) as described in the legend to Fig. 1.
collagen, the processed type I collagen was analyzed by SDS-polyacrylamide gel electrophoresis as described in **Experimental**. Since the level of mRNA for procollagen on day 5 was not affected by the presence of Asc-P when determined by Northern blotting (data not shown), pre-procollagen (posttranslationally un-modified) appeared to be synthesized to a similar degree in the cultured cells with or without Asc-P. However, insoluble type I collagen accumulating in the cell layer increased in the cells cultured with Asc-P for 5 days, while almost no accumulation of insoluble collagen molecules was observed in control cells (Fig. 3A, lanes 1 and 2).

To further examine whether the enhanced accumulation of type I collagen was accompanied by a similar change in the level of ALPase activity, the triple helix formation of procollagen was blocked by using AzC, a proline analogue. At a concentration of 0.3 mM, AzC inhibited the collagen accumulation by more than 90% (Fig. 3A, lanes 5 and 6). Similarly, ALPase activity decreased to the level of control cells depending on the concentration of AzC (Fig. 3B). The decrease in the ALPase activity by AzC coincided well with the reduced accumulation of collagen.

**Increased expression of osteoblastic phenotypes in MC3T3-E1 cells cultured in a dish coated with type I collagen**

To confirm that the accumulation of mature collagen contributes to the development of osteoblastic phenotypes, cells were grown in dishes coated with type I collagen, the processed type I collagen was analyzed by SDS-polyacrylamide gel electrophoresis as described in **Experimental**. Since the level of mRNA for procollagen on day 5 was not affected by the presence of Asc-P when determined by Northern blotting (data not shown), pre-procollagen (posttranslationally un-modified) appeared to be synthesized to a similar degree in the cultured cells with or without Asc-P. However, insoluble type I collagen accumulating in the cell layer increased in the cells cultured with Asc-P for 5 days, while almost no accumulation of insoluble collagen molecules was observed in control cells (Fig. 3A, lanes 1 and 2).

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Fig. 5. Effects of coating with extracellular matrix proteins on the proliferation and induction of osteoblastic phenotypes in MC3T3-E1 cells. The 60-mm dishes coated with bovine type I collagen, human plasma fibronectin, and bovine serum albumin (BSA) were prepared as described under EXPERIMENTAL. Cells were inoculated in these dishes at an initial density of 10^4 cells/cm^2. Cell numbers and the expression of the osteoblastic phenotypes are shown in A and B, respectively. The number of the cells (A) detached with trypsin on day 3 and the ALPase activity (B) in the cell extract prepared after the growth for 7 days were determined as described in the legend to Fig. 1. Data represent the M±SD for triplicate samples. In B, 20 μg of total RNA extracted from each culture on day 10 was analyzed by Northern blot using a radioactive probe for OC cDNA.

I collagen (Fig. 4). In normal dishes, Asc-P increased the level of both ALPase activity and OC mRNA (Fig. 4, lanes A and B), while the Asc-P stimulatory effects were completely blocked by the addition of AzC at 0.3 mM (Fig. 4, lane C). Growth on coated type I collagen also induced increases in both ALPase activity and OC mRNA as observed in the Asc-P containing culture (Fig. 4, lanes B and D). However, these increased levels were not affected by AzC (Fig. 4, lanes D and E).

To examine the specificity of the response with regard to the species of extracellular matrix, the effect of coating with fibronectin was investigated. Cell proliferation was stimulated significantly in dishes coated with collagen or fibronec-
tin compared to that in non-treated dish or in a dish coated with BSA (Fig. 5A). After confluence was attained in these cultures, the expression of osteoblastic phenotypes was examined. Only collagen increased the levels of both ALPase activity and OC mRNA, while fibronectin and BSA had no stimulatory effects on the expression of those proteins (Fig. 5B). These data clearly indicate that the presence of type I collagen promotes the expression of osteoblastic phenotypes.

**DISCUSSION**

The present study demonstrated that Asc-P promoted osteoblastic differentiation due to increased accumulation of collagen. Inhibition of collagen accumulation either by growing cells in the absence of Asc-P or by utilizing AzC under defined conditions blocked the expression of osteoblast marker proteins. Furthermore, only type I collagen examined so far stimulated differentiation of MC3T3-E1 cells even in the absence of Asc-P.

MC3T3-E1 cells have been used to investigate osteoblast marker expression, collagen matrix production and mineralization (9). MC3T3-E1 cells, as in the case of several previously characterized osteoblast culture systems, express osteoblast markers and form a mineralized matrix when grown with AsA (6, 16, 17). In the absence of Asc-P in the medium, MC3T3-E1 cells proliferate and reach confluence at almost the same rate as the cells in Asc-P-containing medium, but remain undifferentiated for approximately 30 days even after the completion of proliferation. Therefore, MC3T3-E1 cells might require specific signals for the onset of differentiation mediated by AsA in addition to the completion of proliferation.

AsA plays a key role in collagen matrix production where procollagen synthesis, hydroxylation, and secretion are tightly coupled processes. Even in MC3T3-E1 cells, Asc-P stimulated the accumulation of insoluble type I collagen in the extracellular matrix, as in the case of fibroblasts (11). When cells were grown in the presence of AzC with or without Asc-P, AzC blocked Asc-P-dependent accumulation of collagen without affecting basal proliferation rates. Furthermore, AzC suppressed Asc-P-dependent expression of the ALPase and OC genes. This is also the case when other collagen accumulation inhibitors such as 3,4-dehydroproline and cis-4-hydroxyproline were used to disrupt collagen maturation and accumulation (10). It is highly likely that the accumulation of mature collagen is required for the onset of differentiation even after cessation of proliferation.

The mechanisms linking collagen accumulation to osteoblastic differentiation remain to be clarified. Recently, extracellular matrix has been shown to be a potent regulator of proliferation and differentiation of various cells (18, 19). Since type I collagen is the major component of bone matrix (20), MG-63 and Saos-2 (human osteosarcoma cell lines) cells grown on type I collagen are examined for osteoblastic differentiation (21). In these cells, type I collagen alone appears able to increase expression of ALPase, but fails to induce an increase in OC production. In the present study with MC3T3-E1 cells, type I collagen increased the expression of both
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ALPase and OC genes to the same levels as those induced by Asc-P treatment. An receptor molecule for type I collagen as described below is responsible for the attachment of MC3T3-E1 cells to the collagen matrix, which in turn promotes osteoblastic differentiation. MC3T3-E1 cells like MG-63 cells may also have a fibronectin receptor, because these cells proliferated well on dishes coated with fibronectin. Since growing MG-63 on fibronectin resulted in an increased expression of ALPase activity (27), the interaction between fibronectin and its receptor of MC3T3-E1 cells might differ from that of MG-63 cells in that no inducible effects were observed on the phenotypic expression.

Integrins appear to be the major receptor by which cells attach to the extracellular matrix, and some of them also affect cellular events (22). At least four members of the integrin family, \(\alpha_1\beta_1\), \(\alpha_2\beta_1\), \(\alpha_3\beta_1\), and \(\alpha_v\beta_3\), have been shown to interact with collagen (23). It will be necessary to determine the types of integrins responsible for differentiation. The extent to which AsA plays a role in bone formation in vivo is still unclear. We are now in the progress of examining the effects of AsA on the synthesis of other extracellular matrix components as well as phenotypic expression in MC3T3-E1 cells.

We are grateful to Dr. Masayoshi Kumegawa of Meikai University for MC3T3-E1 cells, Dr. Paul Price of University of California, San Diego for rat osteocalcin cDNA, and JCRB (Japanese Cancer Research Resources Bank) for human alkaline phosphatase cDNA.

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