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A LONG-LASTING VITAMIN C DERIVATIVE, ASCORBIC ACID 2-PHOSPHATE, INCREASES MYOGENIN GENE EXPRESSION AND PROMOTES DIFFERENTIATION IN L6 MUSCLE CELLS

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SUMMARY: Hormones and growth factors are important regulators of myogenic cell differentiation, but little is known about the effect of vitamins on muscle differentiation and development. We recently showed that L-ascorbic acid 2-phosphate, a stable form of vitamin C, increased the expression of muscle-specific glucose and ion transporters. We now show the effect of L-ascorbic acid 2-phosphate on the kinetics of myogenin expression at both the mRNA and protein levels during differentiation of L6 muscle cells. At the fully differentiated stage, control and L-ascorbic acid 2-phosphate treated cultures showed the same degree of cell fusion, but L-ascorbic acid 2-phophate treated myotubes had a larger diameter than control myotubes. During L6 cell differentiation, the amount of both myogenin mRNA and protein reached a maximal level on day 4 before full myotube formation and then declined. L-ascorbic acid 2-phosphate treated cells expressed a higher amount of myogenin at both the mRNA and protein levels on day 4 compared to untreated cultures. Ethyl-3,4-dihydroxybenzoate, an inhibitor of collagen synthesis, prevented expression of myogenin mRNA and protein in both the control and L-ascorbic acid 2-phosphate treated cells. These results demonstrate that vitamin C can promote muscle differentiation likely through the increase of myogenin expression in myogenic cells, which may in turn regulate muscle differentiation *in vivo*.

The recent cloning of the MyoD family of myogenic regulator including MyoD, Myogenin, Mef5 and MRF4, which can directly activate skeletal muscle-specific genes, has revealed mechanisms that regulate muscle-specific gene transcription (1, 2). These proteins have also been shown to convert cells into the myogenic lineage (3). The putative mechanism by which these basic helix-loop-helix proteins act involves the formation of heterodimers with ubiquitous basic helix-loop-helix proteins, which then interact with muscle-specific genes that contain the consensus sequence CANNTG (E box) to activate their transcription (1-4). The expression of these myogenic determination genes is regulated by hormones and growth factors (5). For example, insulin-like growth factor-I (IGF-I) is known as a potent regulator that induces expression of myogenin in L6

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muscle cells (6-8). Thyroid hormone increases the expression of MyoD1 at both the protein and mRNA levels during differentiation of C2C12 muscle cells (9).

Growing evidence suggests that the extracellular matrix plays an important role in promoting myogenesis. Indeed, deposited collagen substrate enhances the differentiation of primary muscle cell cultures (10); disruption of hydroxylation and glycosylation of procollagen by cis-4-hydroxy-1-proline blocks the formation of the triple helical structure of the collagen molecule and prevents myogenesis in muscle cells in culture (11, 12). Ascorbic acid is a co-factor of proline hydroxylase, essential for collagen processing, which increases both procollagen gene transcription and procollagen mRNA stability (13, 14). Recently, a long-lasting vitamin C derivative, L-ascorbic acid 2-phosphate (Asc 2-P), was shown to induce the fusion of BC3H-1 cells, which are normally nonfusing myocytes, presumbly through promotion of collagen I biosynthesis and deposition; further, the fusion event could be prevented by cis-4-hydroxy-L-proline (15).

We have recently reported that treatment with Asc 2-P specifically increases the expression of muscle-specific functional proteins such as GLUT4, SR Ca^{2+}-ATPase and the dihydropyridine receptor of the voltage-sensitive Ca^{2+} channel during differentiation of L6 cells (16). Theses cells, originated from one day-old rat thigh muscle, undergo the myogenic process in vitro and fuse from myoblasts into myotubes. Therefore, we raise the hypothesis that the stimulatory effect of Asc 2-P on the expression of muscle-specific proteins could be mediated by the myogenic regulator, myogenin. We now show that Asc 2-P can promote the differentiation through the increase myogenin expression in L6 muscle cells. The results suggest that vitamin C may be an important factor for embryonic muscle development and regeneration of satellite myoblasts *in vivo*.

MATERIALS AND METHODS

Materials: Asc 2-P was kind gift from Dr. S. Ohkuma (Faculty of Pharmaceutical Sciences, Kanazawa University). Plasmid pUC65-2 containing the complete coding sequence for myogenin and anti-myogenin monoclonal antibody (F5D) were the kind gift from Dr. W. Wright (Department of Cell Biology, University of Texas Southern Medical School). [α-^{32}P] dCTP was purchased from Amersham. Affinity purified goat anti-mouse IgG (H+L) alkaline phosphatase conjugate was purchased from BIO-RAD. Glutaraldehyde and Giemsa stain were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl-3,4-dihydroxybenzoate (EDHB) was purchased from Aldrich (Milwaukee, WI).

Cell Culture and Quantitation of Myotube Formation: A subclone of the rat skeletal muscle L6 cells (17), selected for high fusion potential, was used in this study. The cells were differentiated in α-MEM containing 2% fetal bovine serum in an H_2O-saturated 5% CO_2 atmosphere at 37 °C. Incubations were done in 100 mm diameter tissue culture dishes, and approximately 1.5 x 10^4 cells/cm^2 were plated. To promote myogenesis, Asc 2-P was added at a concentration of 100 μM on day 2. This concentration produces a maximal effect on collagen synthesis in *in vitro* cell cultures (18, 19). In all experiments, the cells were fed fresh medium in the presence or absence of

Asc 2-P every 48 h after seeding. EDHB was dissolved in ethanol at a concentration of 40 mM and the stock was added to the culture medium at 0.5% (v/v) (final concentration 200 μM). Quantitation of myotube formation was measured as previously described (16).

RNA Isolation and Northern Blot Hybridization: Total RNA was isolated from the cells after culture in the indicated conditions, using the single-step extraction into acid guanidium thiocyanate-phenol-chloroform (20). RNA concentrations were measured at 260/280 nm with a double beam spectrophotometer (Hitachi U-2000). For Northern blot analysis, 20 μg of total RNA were electrophoresed under denaturing conditions in 1-2% (w/v) agarose gels containing 8% (v/v) formaldehyde. The integrity and relative amounts of RNA were confirmed by visualization of ethidium bromide-stained ribosomal RNA under UV light. The RNA was then transferred onto nylon membranes. A complementary DNA probe for myogenin was labeled using random primers to 1.8 x 10^9 cpm/μg. The membranes were prehybridized, hybridized and washed as previously described (16). Quantitative analysis of autoradiograms was carried out by laser scanning densitometry using a PDI model DNA 35 scanner with version 1.3 of the discovery series one-dimentional gel analysis.

Immunoblot Analysis: After washing with phosphate-buffered saline, the cells were gently scraped with a rubber policeman into Hepes-buffered saline (250 mM sucrose, 5 mM NaN_3, 2 mM EGTA, 100 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, 10 μM E-64, 20 mM HEPES pH 7.4) and homogenized with a teflon-glass homogenizer. Protein content was determined by the BioRad Bradford procedure (21). Total cell extracts (75 μg protein) were heated at 100 °C for 2 min and then were subjected to SDS-PAGE on 10% polyacrylamide mini gels essentially according to the method of Laemmli (22). Myogenin protein was detected by immunoblotting analysis using anti-myogenin monoclonal antibody (1:10) and alkaline phosphatase-conjugated secondary antibody. The blots were developed as previously described (23). The protein was quantitated by laser scanning densitometry using a PDI model DNA 35 scanner with version 1.3 of the discovery series one-dimentional gel analysis software.

RESULTS

Effect of Asc 2-P on the Morphology of L6 Cells

L6 myoblasts spontaneously differentiate in the presence of 2% fetal bovine serum and achieve a fully differentiated phenotype on day 7, as determined by morphological and biochemical criteria. These include the appearance of insulin-stimulated glucose transport and creatine phosphokinase activity (16, 24, 25). Treatment of L6 myoblasts with 100 μM Asc 2-P, a concentration at which the vitamin derivative significantly stimulates the expression of muscle-specific proteins such as GLUT4, SR Ca^{2+}-ATPase and voltage-sensitive Ca^{2+} channels (16), promoted myotube formation. Interestingly, although both cultures showed the same degree of fusion content at the fully differentiated stage (95% at day 7), Asc 2-P-treated myotubes had larger diameter than control myotubes (Fig. 1). Thus, Asc 2-P appear to promote further maturation of myotubes suggesting that the vitamin C derivative has a hypertrophic effect on skeletal muscle cells.

Effect of Asc 2-P on Myogenin mRNA and protein Levels During Differentiation of L6 Cells

To explore the potential role of Asc 2-P on myogenin expression, we examined the kinetics of accumulation of myogenin mRNA during differentiation of L6 cells (Fig. 2). The accumulation

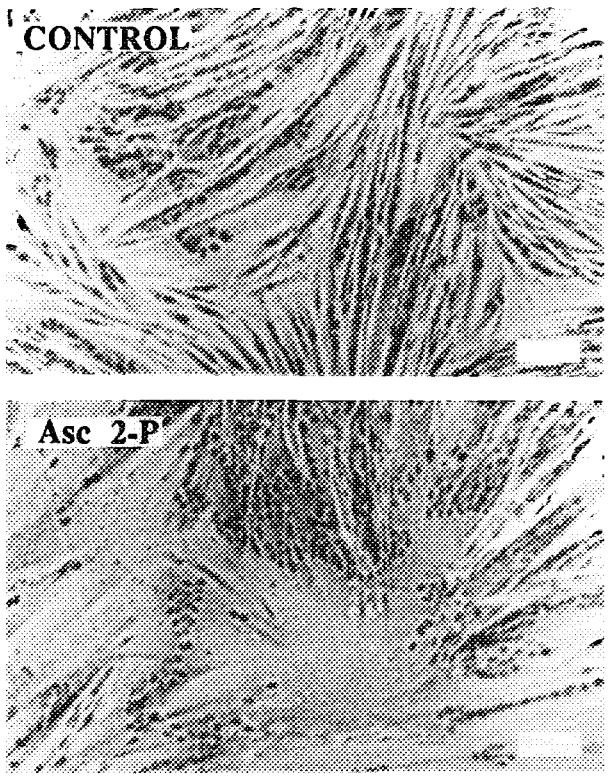

Figure 1. Effect of Asc 2-P on the morphology of L6 cells: L6 myoblasts were cultured for 2 days and transferred to culture medium containing 2% fetal bovine serum in the absence or presence of 100 μM Asc 2-P. The cells were refed every 2 days with fresh medium. At day 7 the cells were stained with Giemsa stain following glutaraldehyde fixation and photographed with a Nikon Inverted Microscope DIAPHOT-TMD using x100 magnification. Scale bar, 100 μm.

of myogenin mRNA preceeded the full myotube formation and the expression of creatine kinase activity in these cells. Myogenin mRNA peaked at day 4 to 5, by 9.8- and 11.7-fold relative to day 2 myoblasts, in control and Asc 2-P treated cultures. At day 7 myogenin mRNA decreased and reached 68% and 58% of the peak level in both control and Asc 2-P treated cells, respectively. At day 4 Asc 2-P significantly increased the myogenin mRNA by 54% compared to the control cells (Fig. 2B).

At the protein level, myogenin was detected on immunoblots as a protein with an apparent molecular size of 40 kDa and a minor band of 42 kDa (Fig. 3). Myogenin was barely detectable in day 2 myoblasts but its cellular amount increased markedly by day 4, then decreased for the next 24 h and was maintained at a constant level until day 7. At day 4 Asc 2-P significantly increased the amount of myogenin protein by 42% compared to control cells (Fig. 3B). The amount of protein per culture (113 μg protein/DNA) did not change during differentiation from day 1 to day 7.

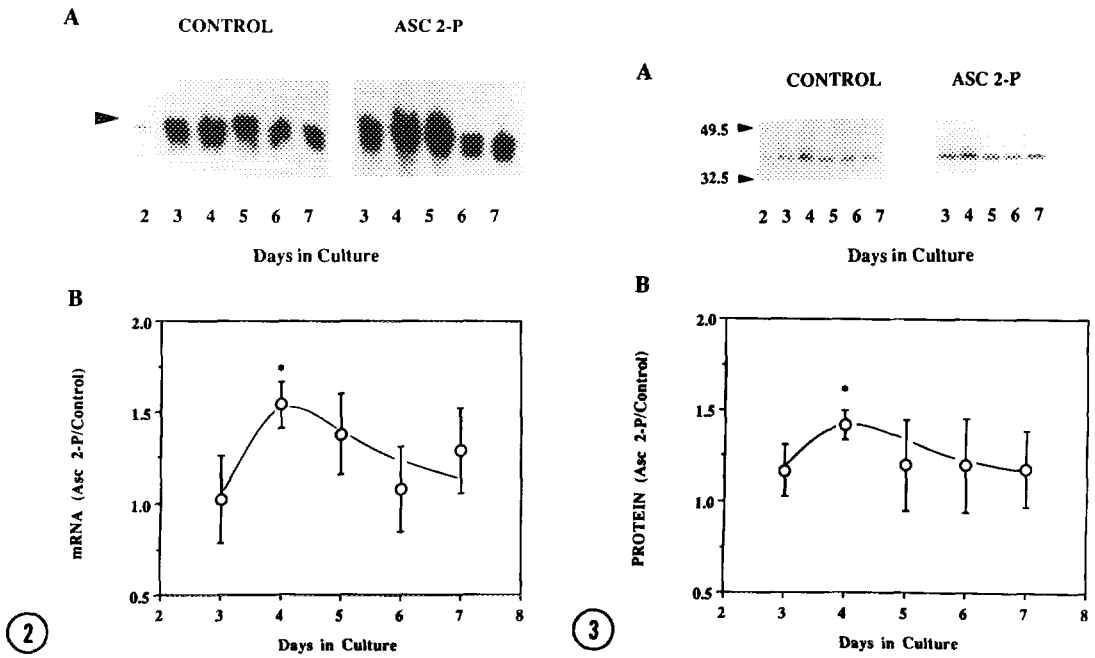

Figure 2. Effect of Asc 2-P on the time course of myogenin mRNAs level during differentiation of L6 cells: L6 myoblasts were cultured as described in the legend to Fig. 1, and RNA was isolated and analyzed by Northern blots as described under Materials and Methods. **A,** Total RNA (20 µg) was probed with labeled myogenin cDNA probe. The arrowhead indicates the position of 18S ribosomal RNA. **B,** Quantitative representation of the three independent experiments, expressed as the mean ± s.e.m. Values are given in arbitrary units, relative to the value in control cultures. * Quantitative values were significantly different from control (Student's t-test) at $P<0.05$.

Figure 3. Effect of Asc 2-P on the time course of myogenin protein level during differentiation of L6 cells: L6 myoblasts were cultured as described in the legend to Fig. 1, and total cell extracts were prepared and analyzed under Materials and Methods. **A,** Immunoblots were probed with anti-myogenin monoclonal antibody (F5D). The blots were developed by the alkaline phosphatese detection system as described under Materials and Methods. Molecular weight marker positions ($x10^{-3}$) are indicated on the left. **B,** Quantitative representation of the three independent experiments, expressed as the mean ± s.e.m. Values are given in arbitrary units, relative to the value in control cultures. * Quantitative values were significantly different from control (Student's t-test) at $P<0.05$.

Therefore, the change in myogenin protein during differentiation is maintained whether the results are expressed per protein or per DNA. It should be noted that Asc 2-P increased both DNA and protein content during differentiation (data not shown).

EDHB Blocked Myogenin mRNA and Protein levels in both control and Asc 2-P treated cells

To elucidate the importance of collagen symthesis and extracellular matrix formation in the Asc 2-P-induced increase of myogenin expression of L6 cells, we tested the effect of a specific collagen synthesis inhibitor. EDHB, a structural analog of ascorbic acid, diminishes the synthesis and secretion of both type I and type III procollagens as the result of direct inhibition of prolyl and lysyl hydroxylase activity (26). We used L6 cells at an early differentiation stage when Asc 2-P

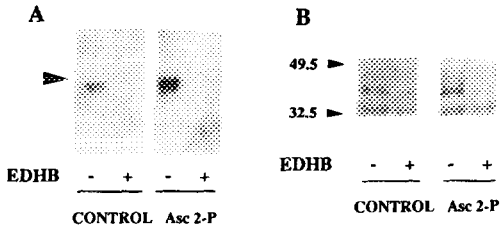

Figure 4. Effect of EDHB on myogenin mRNA and protein levels in L6 cells: L6 myoblasts were cultured as described in the legend to Fig. 1. EDHB was added at final concentration of 200 μM in the culture medium in the absence or presence of Asc 2-P at day 2. After 48 h culture (at day 4), total RNA (A) and total cell extracts (B) were prepared and analyzed as described under Materials and Methods. The figure illustrates one representative experiment of two independent ones for mRNA and of four independent ones for protein. Quantitative results are shown in the text. The arrowhead in A indicates the position of 18S ribosomal RNA.

produced its maximum effect on the expression of myogenin (Figs. 2, 3). Fig. 4 shows that 200 μM EDHB completely prevented the expression of myogenin mRNA in both the control and Asc 2-P treated cells (-EDHB, 1.0; +EDHB, 0.0; Asc-EDHB, 1.9; Asc+EDHB, 0.2 in relative units, n=2). Myogenin protein content was also diminished by the compound in both the control and Asc 2-P treated cells (-EDHB, 1.0; +EDHB, 0.1; Asc-EDHB, 1.7; Asc+EDHB, 0.1 in relative units, n=4).

DISCUSSION

Ascorbic acid has multiple important biological activities, notably promoting the formation of collagen matrix and proteoglycan (27). These two classes of extracellular matrix proteins play major roles in determining the properties and functions of many vital structures, including blood vessels, granulation tissue, and basament membrane. In spite of these important activities of ascorbic acid, it has not been possible to observe long-term effects with this compound, possibly because it is very unstable under normal culture conditions of neutral pH and 37 °C. Recently it was reported that Asc 2-P, a phophate derivative of L-ascorbic acid, has cofactor activity for collagen biosythesis in cells in culture, and this activity is retained for 1week when the derivative is dissolved in culture medium (18, 19). Matsuda et al. (15) showed in a preliminary communication that this compound induced the fusion and increased the expression of myogenin in BC3H-1 cells. This is a very exciting observation, because BC3H-1 cells, which express many biochemical characteristics of skeletal muscle (28), are non-fusing myocytes under normal culture conditions. Furthermore, we previously reported that Asc 2-P selectively elevates the expression of muscle-specific proteins such as GLUT4, voltage-sensitive Ca^{2+} channels and SR Ca^{2+}-ATPase during

differentiation of L6 cells (16). Therefore, it was important to investigate whether Asc 2-P played a significant role in the expression of myogenic regulators in skeletal muscle cells.

In this study we show that Asc 2-P increases the diameter of myotubes in fully differentiated L6 cell cultures (Fig. 1). Interestingly, it has been reported that the insulin and IGF-I induced hypertrophy in cultured myofibers is accompanied by an increase in the number of myonuclei per unit myofiber length (29). Florini and Magri (30) also reported that insulin and IGF-I stimulate myoblast proliferation and fusion into new myofibers. Asc 2-P may have a similar stimulatory effect to that of insulin and IGF-I on both myoblast proliferation and differentiation. Like Asc 2-P, IGF-I stimulates myogenin expression in L6 cells (7).

Asc 2-P increased myogenin content at both mRNA and protein levels during differentiation of L6 cells (Figs. 2 and 3). We observed the myogenin protein as doublet 40 and 42 kDa consistent with the results previously observed on a subclone of L6 and C2C12 muscle cells (31, 32). The temporal profile of myogenin mRNA in both the control and Asc 2-P treated cells is very similar to that previously reported in another subclone of L6 cells (7, 33) and in BC3H-1 myocytes (34). On the other hand, Trudel and Holland showed that myogenin protein level raised steadily until day 8 of differentiation of L6 cells (31). This difference may reflect differential abilities of each cell line to differentiate.

The mechanism whereby Asc 2-P increases the expression of myogenin is unknown. It is established that ascorbic acid increases collagen biosynthesis and deposition (13, 14). Saitoh et al. (12) proposed that commitment to terminal differentiation and activation of myogenin regulatory genes requires active synthesis of the extracellular matrix component collagen. Their experimental results showed that two distinct inhibitors of collagen synthesis, cis-4-hydroxy-L-proline and EDHB prevented myotube formation and expression of sarcomeric myosin heavy chain as well as myogenic regulatory genes, MyoD1 and myogenin in C2C12 mouse muscle cells. Interestingly, it has been reported that Asc 2-P stimulates type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells (35). Taken together, these results suggest that the stimulatory effect of Asc 2-P on the expression of myogenin may be mediated by the production of the extracellular matrix component collagen. In fact, we showed that 200 μM EDHB completely inhibited the expression of myogenin mRNA and protein in Asc 2-P treated cells as well as control cells (Fig. 4). In contrast, EDHB did not affect the expression of the α1 subunit of the Na^+/K^+-ATPase protein (data not shown), which is expressed throughout the culture of these cells (16). However, it still

remaines to be elucidated how the extracellular matrix regulates the expression of the muscle determination factor, myogenin.

In conclusion, Asc 2-P promotes maturation and induces hypertrophy of myotubes in L6 cells. Asc 2-P increases the expression of myogenin at both the protein and mRNA levels throughout the culture. The extracellular matrix component collagen may contribute these effects of Asc 2-P since specific inhibitor of collagen synthesis and deposition blocked the action of the vitamin C derivative. We propose that vitamin C is an important factor during muscle regenaration and development in embryonic stage and may play an important role in muscle tissue regeneration after muscle injury.

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