The Mechanism of Ascorbic Acid-Induced Differentiation of ATDC5 Chondrogenic Cells

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Keywords: ATDC5 cells, chondrocyte differentiation, ascorbic acid, collagen, extracellular matrix
Abstract

The ATDC5 cell line exhibits a multistep process of chondrogenic differentiation analogous to that observed during endochondral bone formation. Previous investigators have induced ATDC5 cells to differentiate by exposing them to insulin at high concentrations. We have observed spontaneous differentiation of ATDC5 cells maintained in ascorbic acid-containing αMEM. A comparison of the differentiation events in response to high dose insulin versus ascorbic acid showed similar expression patterns of key genes, including collagen II, Runx2, Sox9, Indian Hedgehog and collagen X. We took advantage of the action of ascorbic acid to examine signaling events associated with differentiation. In contrast to high dose insulin, which downregulates both IGF-1 and insulin receptors, there were only minimal changes in the abundance of these receptors during ascorbic acid-induced differentiation. Furthermore, ascorbic acid exposure was associated with Erk activation, and Erk inhibition attenuated ascorbic acid-induced differentiation. This was in contrast to the inhibitory effect of Erk activation during IGF-1-induced differentiation. Inhibition of collagen formation with a proline analog markedly attenuated the differentiating effect of ascorbic acid on ATDC5 cells. When plates were conditioned with ATDC5 cells exposed to ascorbic acid, ATDC5 cells were able to differentiate in the absence of ascorbic acid. Our results indicate that matrix formation early in the differentiation process is essential for ascorbic acid-induced ATDC5 differentiation. We conclude that ascorbic acid can promote the differentiation of ATDC5 cells by promoting the formation of collagenous matrix and that matrix formation mediates activation of the Erk signaling pathway, which promotes the differentiation program.
Introduction

Endochondral bone growth occurs in a predictable pattern that is reflected in the complex molecular events that regulate chondrocyte proliferation and maturation (6; 20). The sequential processes of mesenchymal condensation, chondrocyte proliferation, and chondrocyte hypertrophy involve the choreographed expression of many genes and the induction of numerous transcription factors, matrix proteins, and cell-matrix interaction mediators (46). These events occur in a spatially-defined relationship within the growth plate. As round, proliferative chondrocytes form a columnar layer; they stop proliferating and become prehypertrophic chondrocytes, which subsequently differentiate into post-mitotic hypertrophic cells. This is followed by apoptosis of hypertrophic chondrocytes, blood vessel invasion and, finally, the replacement of the cartilaginous matrix by bone (6). In recent years the molecular process of chondrogenesis within the growth plate has been characterized in detail, utilizing various in vitro and in vivo model systems (41).

The ATDC5 cell line is a well characterized chondrogenic cell line derived from mouse teratocarcinoma cells (4). It exhibits a multistep process similar to that observed during chondrocyte differentiation, making it a useful model for in vitro studies. ATDC5 cells are widely used to study chondrocyte growth and differentiation. To date, there are over two hundred publications utilizing ATDC5 cells. Atsumi et al. (4) originally described these cells and demonstrated that insulin at high concentration could effectively induce their chondrogenic differentiation. We previously demonstrated that ATDC5 differentiation could be induced not only by high concentrations of insulin but that physiological concentrations of IGF-I and insulin can do so (28; 29). We (16), along with others (38), have also observed ATDC5 cell differentiation upon exposure to ascorbic acid-containing αMEM medium without any requirement for growth factors. We speculated that this observation would be potentially useful
in studying growth factor signaling during ATDC5 differentiation. The ability to induce ATDC5
cells without growth factors might eliminate the confounding effects of continuous growth factor
exposure. The present studies were undertaken to characterize the mechanisms by which
ascorbic acid induces ATDC5 cell differentiation.

Ascorbic acid is a critical factor in the processes of cartilage and bone development. In humans,
ascorbic deficiency results in scurvy. At the growth plate, ascorbic acid deficiency can result in
decrease chondrocyte proliferation, impaired matrix synthesis and a reduction in osteoblast cell
number (17; 47). This is likely due to the changes in collagen metabolism that accompany
ascorbate deficiency. Ascorbic acid is an essential cofactor for prolyl lysyl hydroxylase (33; 35;
36) a key enzyme in collagen biosynthesis. In addition, ascorbic acid is necessary for the
differentiation of many mesenchymal-derived cell types, including adipocytes(43), osteoblasts
(3; 9; 26; 40; 42), myoblasts (15; 18) and chondrocytes (12; 21). In ATDC5 cells, ascorbic acid
has been shown to enhance the differentiation process in the presence of high dose insulin (2).
In the MC3T3-E1 osteoblastic cell line, exposure to ascorbic acid in the presence of a collagen
synthesis inhibitor not only blocks type 1 collagen synthesis but also decreases ascorbic acid-
induced osteoblastic differentiation (9). The importance of collagen in mesenchymal cell
differentiation is further supported by the observation that collagen deficiency result in an
inability of embryonic avian muscle myoblasts to form myotubes in vitro (25). Taken together,
these observations indicate that ascorbic acid has an important role in mesenchymal
differentiation that is, at least in part, accounted for by its role in collagen synthesis.

Our laboratory has focused on the signal transduction events involved in the chondrogenic
process and, in particular, the differentiation of ATDC5 cells (16; 27-29). We have found that
IGF-I and insulin can promote differentiation via their cognate receptors (28). We further
demonstrated the critical role of the mitogen-activated protein (MAP) kinase pathway involving
Erk (Erk1 and Erk2) kinases in chondrocyte differentiation. IGF-I-induced differentiation was enhanced by inhibition of Erk signaling (29), indicating that the pro-proliferative activity of this pathway attenuated differentiation. However, other studies on the role of Erk signaling in chondrocyte growth and differentiation showed that inhibition of Erk signaling with U0126 blocked insulin induced chondrogenesis (24). This was further supported by our own work demonstrating that even low concentrations of insulin that are specific for the insulin receptor can induce Erk phosphorylation, proliferation and chondrocyte differentiation in ATDC5 cells (28).

Erk activation can be mediated by the cross-linking of extracellular matrix with integrins (32; 39). In human chondrocytes, the interaction of collagen II with the \( \beta_1 \) integrin receptor has been shown to activate Erk (37). In the present series of experiments, we hypothesized that Erk signaling is involved in ascorbic acid-induced ATDC5 differentiation. To test this hypothesis, we first characterized the differentiation process that ATDC5 cells undergo in response to the traditional condition for ATDC5 cell induction, high dose insulin, versus ascorbic acid in the absence of insulin or IGF-I. We went on to explore the relationship between the role of ascorbic acid in matrix protein synthesis and its ability to modulate Erk activity.
Materials and Methods

Materials. Standard αMEM, which contains ascorbic acid (Asc-αMEM), αMEM without ascorbic acid (heretofore referred to as αMEM), and DMEM-nutrient mixture F-12 (DMEM/F12) were obtained from Invitrogen (Carlsbad, CA), as were reverse transcriptase PCR reagents, Taq DNA polymerase and custom primers. Holotransferrin and sodium selenite were purchased from Sigma (St Louis, MO). Purified porcine insulin was obtained from Elanco Products (Indianapolis, IN). L-Ascorbic acid was obtained from Wako Pure Chemical Industries (Osaka Japan). 3, 4 dehydro-L-proline (DHP) was purchased from Sigma (St Louis, MO). Electrophoresis reagents and PVDF membrane were obtained from BioRad (Hercules, CA). Tri-Reagent was obtained from Molecular Research Center (Cincinnati, OH). Western immunoblotting was performed using Enhanced Chemiluminescence (ECL) Plus reagents from Amersham (Piscataway, NJ). Antibodies directed towards the β-subunit of the insulin receptor (βIR; antibody C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Anti-phospho Erk1/2 was obtained from Cell Signaling Technology (Beverly, MA) and anti-Erk1/2 was obtained from Upstate Biotechnology (Lake Placid, NY). U0126 was from Calbiochem (La Jolla, CA).

Cell culture conditions and biochemical analyses. The ATDC5 cell line was obtained from the Riken Cell Bank (Tsukuba, Japan). Cells were cultured as described previously (4; 29). To study hypertrophic cells, chondrogenesis was first induced by plating the cells at 3.4 x 10^3 cells/cm² and allowing them to proliferate for 3 days until they reached confluence. At confluence, cells were induced to differentiate with Asc- αMEM or DMEM/F12 supplemented with 1,600 nM porcine insulin. Both media (pH 7.45) were supplemented with 5% FBS, 10 µg/ml human transferrin, 30 nM sodium selenite and 2% antibiotic-antimycotic solution. Media were replaced every other day for the duration of the experiment.
To assess cell proliferation, cells were grown to confluence in DMEM/F12 using 6-well plates. Cells were induced to differentiate in Asc-αMEM or DMEM/F12 containing 1,600 nM insulin. At indicated times, cells were released from the plate by trypsinization and counted.

Histochemical quantitation of chondrocyte differentiation was assessed as proteoglycan accumulation as measured by staining of cell monolayers with Alcian Blue and Neutral Red (29). Cells were first rinsed with PBS three times, then fixed with 100% methanol for 10 min at -20 °C. Staining was accomplished by applying a solution of 0·1% Alcian Blue 8 GX in 0·1 M HCl to the cells for 2 h at room temperature. To quantify the intensity of the staining, the stained culture plates were rinsed with PBS three times and each well extracted with 1 ml 6 M guanidine/HCl overnight at room temperature. The optical density of extracted dye was measured at 650 nm. Total cellular material was quantified in parallel by staining with Neutral Red, which is a weak base that is taken up by viable cells. The detection of cellular Neutral Red content was measured as optical density at 550 nm of the same extracts used for Alcian Blue quantification. This method was used because it allowed us to combine analyses for an indicator of cell growth (proliferation and/or hypertrophy) with quantification of an indicator of chondrocyte differentiation.

Western immunoblotting of cell lysates was carried out using previously described methods (29). Where indicated, blots were stripped according to the ECL Plus protocol prior to reprobing (29). Immunoblotting results were quantified using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

Determination of gene expression by PCR. To determine the expression levels of collagen II, BMP2, collagen X, Indian hedgehog (Ihh), p21, Sox9, β-actin, Runx2 in ATDC5 cells, total RNA was prepared from triplicate plates using TRIzol reagent. Semi-quantitative PCR was performed
as described previously (29). Primer sequences used for detection of collagen II, collagen X, BMP2, Ihh, β-actin were those used by Phornphutkul et al. (27). Primers used for Sox9 were those used by Kojima et al. (19), and primers used for detection of p21 and Runx2 were those used by Chen et al. (7). After reverse transcription, PCR was performed as previously described (28). The optimal cycle number was determined empirically for each primer set to maximize the sensitivity of quantification. PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide. Resulting photographs were quantified as described above for Western blots.

Preconditioning of tissue culture plates. ATDC5 cells were plated in DMEM/F12 medium using 6-well, tissue-culture treated polystyrene (Fisher scientific, Pittsburgh, PA). At confluence, cells were allowed to differentiate in αMEM or Asc-αMEM. Cells were maintained in this medium for 5 days after which they were removed from the plates by incubation for 1 hr at 37°C with hypotonic buffer (water with 0.5% Triton X-100). The plates were rinsed 6 times with Hank's buffered salt solution (HBSS) and maintained in HBSS at 4°C until use.

Statistical analyses. Except where noted, the significance of differences between groups was determined by one-way ANOVA followed by a Tukey post hoc test using GraphPad Prism software (GraphPad Software, San Diego, CA). Differences were considered significant at $P < 0.05$. 

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Results

A comparison of ATDC5 cell differentiation induced by ascorbic acid versus high-dose insulin.

We previously observed that ATDC5 cells can differentiate in Asc-αMEM without the addition of insulin or IGF-I (16). This was in contrast to DMEM/F12, which is routinely used to differentiate these cells (4). When comparing the components of Asc-αMEM to DMEM/F12, Asc-αMEM contains 50 μg/ml of ascorbic acid, approximately twice the concentration of calcium, and higher concentrations of several non-essential amino acids.

In order to determine which components in Asc-αMEM could support ATDC5 cell differentiation, we tested eight different conditions in which calcium, non-essential amino acids, and ascorbic acid were added alone or in combination to DMEM/F12 at concentrations equal to those found in Asc-αMEM. We studied a duration of incubation of 14 days. At the end of this time, total RNA was isolated and analyzed by semi-quantitative RT-PCR for three markers of chondrocyte differentiation, collagen II, Runx2 and collagen X. ATDC5 cells grown in Asc-αMEM were used for comparison. Expression levels were normalized to the expression of β-actin, which remained stable throughout the experiment. Results (Fig. 1, Panels A through D) showed that the expression of all three differentiation markers was increased only in DMEM/F12 media to which ascorbic acid was added. A parallel experiment was done in which cells were stained for proteoglycan accumulation using Alcian blue. Similar results were obtained (Fig. 1E). We also noted that cells cultured in the presence of ascorbic acid maintained a round shape, characteristic of chondrocytes, and formed cell clusters during the culture period. Controlling for calcium and essential amino acid concentrations did not suffice to induce differentiation, though these factors may have enhanced the effect of ascorbic acid. We concluded that ascorbic acid is the key component in the standard αMEM formulation that allows for differentiation of ATDC5 cells in the absence of insulin or IGF-I.
We next examined the concentration dependence of the differentiation process on ascorbic acid. Ascorbic acid, 0-100 μg/ml, was added to DMEM/F12 and differentiation was assessed by measuring proteoglycan accumulation. Results (Fig. 2A), showed a dose-dependent effect up to 100 μg/ml, twice the concentration in the Asc-αMEM medium.

The effect of ascorbic acid on ATDC5 cell differentiation was further supported by a direct comparison of ATDC5 cells maintained in αMEM versus Asc-αMEM. Only Asc-αMEM medium was able to induce ATDC5 cell differentiation, as measured by collagen II, Ihh and collagen X mRNA expression (Fig. 2B).

We went on to assess the temporal aspects of ATDC5 cell proliferation and differentiation in Asc-αMEM relative to the process induced by high dose insulin (1,600 nM) in DMEM/F12. ATDC5 cells were grown to confluence and then cultured under these two conditions for up to 14 days. At various time points, cells were collected by trypsinization and counted. Results (Fig. 3A) showed that cells maintained in DMEM/F12 containing high dose insulin proliferate at a significantly higher rate than cells maintained in Asc-αMEM. In a parallel experiment, mRNA levels of chondrogenic differentiation markers, collagen II, Sox9, Ihh, p21 and BMP2, were measured at intervals throughout the experiment. Results (Fig. 3B-F) showed that all of these markers were induced in cells cultured in both Asc-αMEM and DMEM/F12 with high concentration insulin. The pattern and level of induction were similar with the exception of collagen II, which was more potently induced by Asc-αMEM.

A parallel experiment was done in which differentiation was assessed as proteoglycan accumulation (Fig. 4A). Again, induction of differentiation was similar for the two culture conditions. Additional analyses for collagen X and Runx2 mRNA expression (Fig. 4B and 4C), both markers of chondrocyte hypertrophy, showed induction that occurred more rapidly and was
greater in magnitude in Asc-αMEM than in DMEM/F12 with high concentration insulin. We concluded that ascorbic acid initiates a differentiation program in ATDC5 cells that mimics the pattern observed using the standard method of exposure to high concentration insulin.

Growth factor signaling in ATDC5 cells induced to differentiate by ascorbic acid versus high-dose insulin. The use of insulin at high concentration would be expected to induce down-regulation of insulin and IGF-I receptors through ligand-induced endocytosis (28). We therefore took advantage of the ability of ascorbic acid to induce differentiation to study growth factor and insulin signaling during the differentiation process. Cells were cultured in Asc-αMEM or 1,600 nM insulin in DMEM/F12 for up to 14 days. Insulin and IGF-I receptor content were analyzed by direct Western immunoblotting. Results (Fig. 5A) showed a rapid and persistent downregulation of insulin and IGF-1 receptor β subunits in the presence of insulin. Levels persisted at pre-differentiation levels in the presence of ascorbic acid. A modest upregulation of the content of both receptors was seen on day 6 in cells cultured in Asc-αMEM. Total-Erk content was constant throughout the differentiation period.

To examine the role of the Erk pathway in Asc-αMEM -induced ATDC5 cells differentiation, we assessed Erk activity at various stages of the differentiation process (Fig. 5B). Erk phosphorylation, an indicator of Erk activity, was increased in the presence of Asc-αMEM relative to DMEM/F12 maintenance medium. A sharp increase in Erk phosphorylation occurred on day 2 and day 6, coincident with the changes in IGF-1 receptor content.

The role of collagen synthesis in ascorbic acid-induced ATDC5 cell differentiation. Given that ascorbic acid is required for the formation of collagen triple helices, we hypothesized that the ability of ascorbic acid to induce ATDC5 cell differentiation depends on its ability to promote synthesis of collagen matrix. To test this hypothesis, we analyzed the effect of an inhibitor of
collagen triple helix formation, 3, 4 dehydro-L-proline (DHP), on Asc-αMEM-induced ATDC5 cell differentiation. Cells were maintained in Asc-αMEM in the presence or absence of DHP for 10 days. Total RNA was collected and analyzed for collagen X expression. Results (Fig. 6A) showed that DHP at 0.4 mM completely inhibited the induction of collagen X. The effect of DHP on proteoglycan accumulation was assessed using Alcian blue staining with normalization to the intensity of Neutral Red staining, a measure of cell content (Fig. 6B). In this experiment, DHP was added at different stages of the differentiation process (day 0, 3 or 5).

Cells were cultured for up to 14 days. Proteoglycan accumulation was completely inhibited relative to the level observed with Asc-αMEM when DHP was added on day 0 or day 3. Only partial inhibition of proteoglycan accumulation was observed when DHP was added on day 5. As noted above (see Fig. 3A), when ATDC5 cells at confluence are induced to differentiate in the presence of ascorbic acid, they go through several cycles of cell proliferation prior to undergoing hypertrophy. We observed twenty percent inhibition of cell proliferation in the presence of DHP (data not shown), indicating that collagenous matrix can modulate both chondrocyte proliferation and differentiation.

These results were interpreted as consistent with cell:matrix interactions that are stimulatory for chondrocyte differentiation. In order to further assess this, culture plates were conditioned by culturing ATDC5 cells for 5 days in the presence of Asc-αMEM. Cells were then removed using hypotonic buffer. ATDC5 cells were cultured on the conditioned plates for 7 days in αMEM or Asc-αMEM. Results (Fig. 7) showed that on conditioned plated, the preformed matrix was sufficient to induce differentiation of ATDC5 cells as assessed by collagen II, Ihh and collagen X expression. Conditioning of the tissue culture plate induced collagen X expression in the absence of ascorbic acid to a level similar to that seen in cells cultured in Asc-αMEM without tissue culture plate conditioning. The addition of ascorbic acid to cultures on conditioned plates
enhanced collagen X expression. There was no effect on Ihh. At 7 days, cells cultured in Asc-αMEM showed a lower level of collagen II induction relative to cells cultured in pre-conditioned plates. We interpreted this as consistent with an acceleration of the differentiation process with a concomitant, late decrease in collagen II expression.

**Role of Erk signaling in matrix-induced ATDC5 cell differentiation.** It is well established in a number of cell systems that Erk activity is stimulated by cell:matrix interactions (32; 37; 39). As noted above, the Erk pathway has an important role in the chondrocyte differentiation process. To examine the role of Erk signaling in ascorbic acid-induced ATDC5 cell differentiation, we studied the effect of U0126 on Erk phosphorylation and chondrocyte differentiation. ATDC5 cells grown in Asc-αMEM were incubated with U0126 or DMSO for up to 14 days. Cultures were assessed for effect of the inhibitor on Erk phosphorylation and markers of chondrogenic differentiation and hypertrophy. Results (Fig. 8) confirmed that U0126 at a concentration of 10 µM abolished Erk phosphorylation at all experimental time points used to assess chondrocyte differentiation. The presence of U0126 was associated with a marked reduction in the accumulation of sulfated glycosaminoglycans (Alcian blue staining) relative to control at 6, 9 and 14 days. U0126 also inhibited the induction of collagen II, Runx2 and collagen X. A different pharmacological inhibitor of MEK 1, PD98059, was able to inhibit both ascorbic acid induced ERK phosphorylation and ATDC5 cell differentiation. However, PD98059 was unable to suppress Erk activation and ATDC5 cell differentiation as effectively as U0126 (data not shown).

To further test our hypothesis that the activation of Erk is likely mediated by cell:matrix interaction, we assessed Erk phosphorylation in cells that were induced to differentiate by preconditioning of the tissue culture plates. On day 3 and 6, basal Erk phosphorylation was markedly higher in cells grown on plates preconditioned by cells grown in Asc-αMEM relative to plates conditioned by cells maintained in αMEM without ascorbic acid (Fig. 9A). We examined
the induction of several markers of differentiation by preconditioning of the tissue culture plates, with or without Erk pathway inhibition by U0126. Results (Fig. 9B and 9C) demonstrated that the differentiation of ATDC5 cells culture on preformed matrix is markedly attenuated when Erk is inhibited by U0126.
Discussion

It is well established that ascorbic acid is necessary for the differentiation of mesenchymal cell types, including adipocytes, osteoblasts, myoblasts and chondrocytes (21; 43-45). While insulin at high concentration has been routinely used to induce differentiation of ATDC5 cells, we found previously that ascorbic acid-containing αMEM alone was sufficient to induce differentiation in this cell line (16). The present studies were aimed at characterizing ascorbic acid-induced ATDC5 cell differentiation and investigating the mechanisms of ascorbic acid action. Our results show that ascorbic acid treated cells produce a pattern of gene expression similar to that seen in insulin-induced ATDC5 cell differentiation. This observation provided the basis for further studies to characterize growth factor signaling during ATDC5 cell differentiation in the absence of tonic growth factor exposure.

We previously demonstrated the effect of high dose insulin on insulin and IGF-1 receptors (28). Those studies showed a rapid, marked and persistent downregulation of receptor abundance. In contrast, such changes were not seen during ascorbic acid-induced ATDC5 cell differentiation. This result indicates that the differentiating effect of ascorbic acid probably does not depend on induction of IGF-I. In addition, it appears that insulin and IGF-I receptors are persistently expressed during ATDC5 cell differentiation and that their downregulation in the presence of high concentration insulin is a consequence of ligand-induced receptor endocytosis and degradation. Thus, ascorbic acid may be a useful agent for studies on the modulation of growth factor signaling during ATDC5 cell differentiation.

Our results also indicate that ascorbic acid induction of ATDC5 cell differentiation is dependent on the synthesis of collagenous extracellular matrix and that this effect requires Erk signaling. Ascorbic acid stimulates procollagen hydroxylation and processing, and is required for collagen
fibril assembly and collagen secretion (33-35). The rate-limiting step in this overall process is
the hydroxylation and secretion of unprocessed procollagen chains that accumulate in the
endoplasmic reticulum of ascorbic acid deficient cells (22).

The requirement of ATDC5 cell differentiation for intact collagen production and secretion is
supported by the observation that chondrocyte hypertrophy was inhibited by a collagen
synthesis inhibitor. Maximal inhibition occurred only when collagen synthesis was inhibited at
the start of the differentiation process, suggesting that collagen matrix formed before day 5 was
sufficient to induce an intermediate degree of differentiation. Similar observations have been
reported for osteoblasts, in which inhibition of collagen I synthesis inhibited ascorbic acid
induction of osteoblast proliferation (13) and differentiation (9). The reduction of proliferation in
the presence of DHP is unlikely to be the factor accounting for a 90% decrease in chondrocyte
differentiation measured by collagen X expression. In fact, we (29) demonstrated that inhibition
of ATDC5 cells proliferation by the addition of Erk inhibitor PD98059 resulted in enhanced
differentiation.

In an extension of our studies showing that collagen synthesis was critical to the induction of
ATDC5 cell differentiation in response to ascorbic acid, we were able to demonstrate that
extracellular matrix synthesized by cells exposed to ascorbic acid was sufficient to promote
differentiation in the absence of ascorbic acid. Given that the collagenous matrix was effective
when produced by cells early in their differentiation program, and given that the inhibitor of
collagen synthesis could only partially inhibit differentiation when added several days after
initiation of chondrocyte differentiation, it is likely that collagens expressed early in the
differentiation process, such as collagen type II, IX and IV, are playing a major role in the
induction process. The important role of early collagen in chondrocyte differentiation is
supported by observations made by several groups. Bosnakovski et al. (5) showed that
collagen II hydrogels could stimulate the differentiation of bone marrow mesenchymal stem cells in the absence of growth factors. Qi et al. (31) observed an increase in bovine articular chondrocyte proteoglycan synthesis in a dose dependent fashion in the presence of collagen II in the extracellular matrix.

Growth plate cartilage is an avascular tissue that consists of chondrocytes and extracellular matrix. The importance of interactions between extracellular matrix and growth factors are supported by a number of studies. Extracellular signaling involves the interaction of cell surface receptors with soluble cytokines and growth factors, with extracellular matrix constituents such as collagen and proteoglycans, or with the cell surface proteins of neighboring cells. Extracellular matrix has other physiological functions, such as promoting the availability of nutrients and mediating cellular functions through direct interaction with cell surface receptors (1; 10; 11). Integrin family members are a family of receptors that transmit information from the matrix to the cells. In the growth plate, the interaction of integrins with matrix protein has various effects on cell proliferation and differentiation of cells via the activation of a variety of signal transduction pathways (14). The Erk pathway has a central role in transducing integrin signals, in particular those that regulate gene expression through activation of transcription factors(8; 11; 23). We previously made the observation that collagen X expression and glycosaminoglycan accumulation were enhanced in ATDC5 cells by inhibition of the Erk pathway when IGF-I is used to promote differentiation (29). More recent work using metatarsal explants from E15.5 mouse embryos (30) resulted in the converse observation. Inhibition of Erk signaling using U0126, attenuated the differentiation process with no subsequent effect on chondrocyte proliferation. In the latter model, chondrocyte proliferation and differentiation are occurring simultaneously in separate zones within the metatarsal growth plate. The results of the present
studies support a pro-differentiation role for the Erk pathway, one that is downstream from extracellular matrix biosynthesis and, possibly, integrin signaling.

In summary, we have shown that we can induce ATDC5 chondrocyte to differentiation in ascorbic acid containing media and that the resulting pattern of gene expression closely mimics that seen with the standard, insulin-mediated induction of ATDC5 cell differentiation. This activity of ascorbic acid is dependent on collagen synthesis and Erk activation. Furthermore, the extracellular matrix produced by ATDC5 cells exposed to ascorbic acid is sufficient to promote differentiation in an Erk-dependent manner. Our findings support a critical role for matrix proteins in the early signaling events that mediate chondrogenesis. Furthermore, our results indicate that ascorbic acid-containing growth media can be used to study chondrogenic signaling events in the absence of the confounding effects of tonic, pharmacologic growth factor stimulation.

Acknowledgments

C. Phornphutkul was funded by the Department of Pediatrics, Rhode Island Hospital, Research Fund and National Center for Research Resources Grant No. P20 RR024484 (PI Q.Chen). This work was also supported by National Institutes of Health Grant R01 HD24455 (to P. A. Gruppuso).


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Figure legends

Fig. 1. ATDC5 cell differentiation in various media conditions. Panel A: ATDC5 cells were incubated with DMEM/F12 supplemented with 90 mg/ml calcium, non-essential amino acids or 50 μg/ml of ascorbic acid alone or in combination. Cells were also maintained in Asc-αMEM. Three markers of differentiation, collagen II, Runx2 and collagen X, and a control gene, β-actin, were assessed using semi-quantitative RT-PCR. Triplicate samples were analyzed for each condition. Quantification of these results, calculated as the ratio of collagen II (Panel B), Runx2 (Panel C) and collagen X (Panel D) to β-actin expression is shown as mean ± 1 standard deviation for triplicate determinations. Panel E: Proteoglycan accumulation was assessed using Alcian Blue and normalized for cell content using Neutral Red staining. The dyes were extracted and absorbance determined. The ratio of Alcian Blue to Neutral Red was calculated (n=3 for each condition). Filled bars, DMEM/F12; unfilled bars, Asc-αMEM. *, P<0.05 versus DMEM/F12 alone or **, P<0.01 versus DMEM/F12 alone as determined by ANOVA. A second, replicate experiment gave similar results.

Fig. 2. The effect of ascorbic acid on the induction of differentiation markers in ATDC5 cells. Panel A: ATDC5 cells cultured in DMEM/F12 were incubated with various concentrations of ascorbic acid (0-100 μg/ml) for 14 days. Proteoglycan accumulation was analyzed by Alcian Blue staining and normalized for cell content using Neutral Red staining. Results, expressed as the ratio of Alcian Blue to Neutral Red, are presented as the mean ± 1 standard deviation for triplicate determinations. *, P<0.01 versus control, as determined by ANOVA. An additional experiment gave similar results. Panel B: Cells were grown in αMEM or Asc-αMEM for 10 days. The expression of collagen II, Ihh and collagen X was assessed in triplicate samples by semi-quantitative RT-PCR. β-actin was used as an internal control.
Fig. 3. The time course of the effect of ascorbic acid on cell proliferation and chondrogenic-related gene expression. ATDC5 cells were grown to confluence in DMEM/F12. At confluence (day 0), cells were induced to differentiate for 14 days in the presence of Asc-αMEM (filled circles) or DMEM/F12 with 1,600 nM insulin (unfilled circles). Panel A: At day 0, 1, 2, 4, 7, and 10, cells were counted. Data represents the mean ± 1 SD (n = 3 for each condition). A similar experiment was performed in which cells were lysed and RNA prepared. The expression of collagen II, Sox9, Ihh, p21, BMP2 and β-actin was assessed using semi-quantitative RT-PCR. The ratio of collagen II (Panel B), Sox9 (Panel C), Ihh (Panel D), p21 (Panel E) and BMP2 (Panel F) to β-actin expression is shown as mean ± 1 SD for triplicate analyses. *P<0·05 versus the corresponding time point; **P<0·001 versus the corresponding time point.

Fig. 4. The effect of ascorbic acid on the induction of markers of chondrocyte hypertrophy. ATDC5 cells were grown to confluence and induced to differentiate as for Figure 3 (Asc-αMEM, filled bars or circles; 1,600 nM insulin in DMEM/F12, unfilled bars or circles) for up to 14 days. Panel A: On days 0, 6, 9 and 14, cells were stained with Alcian blue and Neutral Red. Dye was extracted and absorbance measured. The ratio of Alcian Blue to Neutral Red is shown as the mean ± 1 standard deviation for triplicate analyses. Differences between groups were not significant as determined by ANOVA. A parallel experiment was done to assess the expression of Runx2 (Panel B) and collagen X (Panel C) using semi-quantitative RT-PCR. Data are shown as the mean ± 1 standard deviation for triplicate analyses. Additional experiments gave similar results. *, P<0.05 or **, P<0.001 versus corresponding time point by ANOVA.

Fig. 5. The effect of ascorbic acid on insulin and IGF-1 receptor content and Erk activation. ATDC5 cells were maintained for the indicated times in Asc-αMEM or DMEM/F12 containing 1,600 nM insulin. Panel A: At the times indicated, duplicate cell lysates were prepared and...
analyzed by Western immunoblotting for the IGF-I receptor β subunit and the insulin receptor β subunit. Samples were also analyzed for total Erk to assess sample loading and transfer.

Panel B: A similar experiment was performed in which duplicate samples were prepared from cells maintained in DMEM/F12 (0) or Asc-αMEM for a duration of 14 days. At indicated times, these samples were analyzed by Western immunoblotting for the IGF-I receptor β subunit, insulin receptor β subunit, phosphorylated Erk (P-Erk) and total Erk.

Fig. 6. The effect of a collagen synthesis inhibitor on ascorbic acid-induced ATDC5 cell differentiation.  Panel A: Cells were grown to confluence in DMEM/F12 then switched to Asc-αMEM.  3, 4-dehydro-L-proline (DHP; 0.4 mM) or vehicle (control) were added to the media.  Cells were lysed on day 10.  Total RNA was extracted and analyzed for the expression of collagen X and β-actin using semi-quantitative RT-PCR.  Data representing the ratio of collagen X to β-actin expression are shown as the mean ± 1 standard deviation for triplicate samples. *, P< 0.001, control versus DHP by unpaired t-test.  The lower panel shows collagen X and β-actin expression.  Triplicate samples were analyzed for each condition.  A replicate experiment gave similar results.  Panel B: Cells were grown to confluence in DMEM/F12 (unfilled bar) then switched to Asc-αMEM (filled bars).  DHP (0.4 mM) was added starting on day 0, 3 or 5.  Cells were maintained until day 14.  At that time, cells were stained for proteoglycan accumulation using Alcian blue and Neutral Red.  The dye was extracted and absorbance determined.  Data representing the ratio of Alcian Blue to Neutral Red are shown as the mean ± 1 standard deviation for triplicate determinations (n= 3 for each condition). *, P<0.01 versus Asc-αMEM/-DHP.

Fig. 7. The effect of extracellular matrix produced by ascorbic acid-induced ATDC5 cells on chondrocyte differentiation.  Plates were conditioned by culturing ATDC5 cells in αMEM (unfilled
bars) or Asc-αMEM (filled bars) for 5 days. Cells were removed and fresh ATDC5 cells were
plated and maintained in αMEM with (+) or without (-) ascorbic acid for 7 days. The expression
of collagen II (Panel A), Ihh (Panel B) and collagen X (Panel C) was determined using RT-PCR.
Data normalized to β-actin expression are shown as the mean ± 1 standard deviation for
triple samples. Letters above the bars indicate groups that differ significantly from one
another based on ANOVA. An additional experiment gave similar results.

Fig. 8. The effect of the Erk pathway inhibitor, U0126, on chondrocyte hypertrophy. Panel A:
ATDC5 cells at confluence were incubated continuously for 14 days with Asc-αMEM medium
containing either 0.1% DMSO vehicle (-) or 10 μM U0126 (+). Levels of phosphorylated and total
Erk were determined by Western blot analysis. Panel B: ATDC5 cells at confluence (grey bar)
were switched to Asc-αMEM in the absence (filled bars) or presence (unfilled bars) of U0126
(10 μM) for up to 14 days. At the times indicated, cells were stained with Alcian Blue and
Neutral Red. Data representing the ratio of Alcian blue to Neutral Red are shown as the mean
± 1 standard deviation for triplicate determinations. Panels C to E: A similar experiment was
performed in which the expression of collagen II (Panel C), Runx2 (Panel D) and collagen X
(Panel E) was analyzed by semi-quantitative RT-PCR. Data represent the ratio of the
expression of each gene to β-actin. Gene expression data are shown as the mean ± 1 standard
deviation for triplicate determinations. *, P<0.01 versus corresponding control. **, P<0.001
versus corresponding control. Additional experiments gave similar results.

Fig. 9. The effect of preconditioned plates and Erk signaling. Panel A: Preconditioned plates
were prepared as in Figure 7 using αMEM without (-Asc) or with (+Asc) ascorbic acid. Fresh
ATDC5 cells were seeded on the preconditioned plates and cultured for up to 15 days in αMEM.
At the time points indicated, cell lysates were prepared and analyzed for phosphorylated and
total Erk by Western immunoblotting. **Panel B**: ATDC5 cells seeded on plates preconditioned by ATDC5 cells grown in the presence of ascorbic acid were maintained in the absence or presence of 10 μM U0126. At the times indicated, total RNA was prepared and analyzed by RT-PCR for collagen II. **Panel C**: The samples from the experiment in **Panel B** were analyzed for collagen X expression. Data representing the ratio to β-actin expression are shown as the mean ± 1 standard deviation of triplicate analyses. *, P < 0.05 or **, P < 0.001 versus corresponding control. An additional experiment gave similar results.
A

Alcian Blue:Neutral Red

Ascorbic Acid, μg/ml

0 25 50 100

B

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A) Alcian Blue:Neutral Red Ratio

B) Runx2/β-Actin Ratio

C) Col X/β-Actin Ratio
**A**

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**B**

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P-Erk

Total Erk

B

Col I/β-Actin Ratio

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C

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