Introduction

The ATDC5 mouse embryonal carcinoma-derived cell line used as an in vitro model that exhibits the multistep chondrogenic differentiation observed during endochondral bone formation [1]. The process of differentiation is begun with 2 weeks of expansion culture, with 5% fetal bovine serum (FBS), transferrin and selenium, followed by the addition of insulin to promote the differentiation into type II collagen-expressing chondrocytes, via the cellular condensation stage with formation of cartilaginous nodules. To induce hypertrophy and mineral deposition, the medium is switched from DMEM to α-MEM, the CO₂ level is switched from 5% to 3%, and cells are incubated in this condition for a further 21 days [2]. We sought to modify these conditions to produce a consistent, reproducible chondrogenesis with hypertrophic differentiation in a more practical time period. α-MEM contains ascorbate, whereas DMEM does not. Other studies have shown that ascorbate increases the mRNA expression levels of chondrogenic markers including collagen type II and aggrecan [3], so we experimented with ascorbate supplementation as a means of producing a reliable system of complete chondrogenesis.

Materials and Methods

Cell culture: ATDC5 cells were plated at 12 x 10⁴ cells/35 mm well and first cultured in expansion medium, consisting of a 1:1 mixture of DMEM and Ham’s F-12 medium containing 5% FBS, 10 µg/ml human and 3 X 10⁻¹⁰ M sodium selenite as previously described [2]. The cells were grown for 14 days and then chondrogenesis was induced by addition of 10 µg/ml insulin during a further 21 days of culture. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. The medium was replaced every other day. In the experimental group, 37.5 µg/ml ascorbate-2-phosphate was supplemented in both the expansion and the differentiation media.

Alcian Blue staining: ATDC5 cells were fixed with 3.7% formaldehyde in PBS, stained with 0.1% Alcian blue (Wako Pure Chemicals Co.) in 0.1 N HCl overnight, and rinsed with distilled water.

RNA extraction and RT-PCR: Total RNA was extracted from ATDC5 cells at different time points. The average yield per 35mm well was 15-20 µg of total RNA. cDNA was then synthesized and used for real-time quantitative PCR using the Biorad ICycler IQ Real Time PCR Detection System. PCR primers for collagen type II: TTG-AGA-CAG-CAC-GAG-GTG-GAG forward and AGC-CAG-GTT-GCC-ATC-GCC-ATA backward, for aggrecan: AGG-ACC-TGG-TAG-TGC-GAG-TG forward and GCG-TGT-GGC-GAA-GAA backward, and for Sox9 ATC-GGT-GAA-CTG-AGC-GAC-forward and GCC-TGC-TGC-ACC-GAC-ATA-C forward, were designed with mouse sequences using oligo 6.8 software. Collagen type X primers sequences were obtained from [4]. For each mRNA, a standard curve was constructed with plasmids having TA-cloned PCR inserts generated using the primers. The results were normalized to 18s rRNA.

Results

We optimized the ATDC5 chondrogenic system by addition of ascorbate throughout the culture period. We found that the cultures supplemented with ascorbate contained cartilaginous nodules that were greater in number and larger in size (figure 1). They were also more intensely stained, indicating greater matrix elaboration. Upon examination of the mRNA expression of chondrogenic markers, it was noted that all the chondrogenic markers were increased when compared with the control (−ascorbate) cultures. Sox9 increased in expression by 10 fold at beginning of differentiation, collagen type II increased by 200 to 250 fold, and aggrecan by 150 to 200 fold. Moreover, we found that collagen type X, the hypertrophic marker, was increased in the mRNA expression level by 6 to 10 fold, in contrast to controls in which it did not increase.

Discussion

ATDC5 cells provide an excellent chondrogenic model by exhibiting all the cartilage developmental stages starting with condensation followed by proliferation and differentiation, ending with hypertrophy. These cells have been used extensively to investigate the biochemical and developmental mechanisms occurring during chondrogenesis. However, to achieve this, investigators previously needed to grow ATDC5 cells in three different media, for extended time, with switches in CO₂ levels. With addition of ascorbate, alcian blue staining indicated that chondrogenesis was more extensive in ATDC5 cells. Furthermore, the real time quantitative PCR results indicated that ascorbate addition to ATDC5 cell cultures increased the mRNA expression level of all chondrocyte markers including sox9, collagen type II and aggrecan. The increase was many-fold higher than was seen in the control culture conditions. In the control cultures, the level of collagen type X mRNA did not significantly change with time. In contrast, addition of ascorbate promoted a detectable increase in collagen type X mRNA expression. It did not increase to the levels of collagen type II or aggrecan (2-300 fold increase), but was consistent and reproducible. In conclusion, addition of ascorbate to ATDC5 cells was found to increase the expression of the differentiated chondrogenic markers. Hypertrophic differentiation was noted to occur in these conditions, abrogating the need for a switch in culture conditions and extended culture time.

References


ASCORBATE-ENHANCED CHONDROGENESIS OF ATDC5 CELLS

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Figure 1: Alcian Blue stained ATDC5 cells at day 14 of chondrogenic culture (post-insulin addition). (A) without ascorbate and (B) with ascorbate.

Figure 2: Quantitative real-time PCR results for mRNA expression levels of Sox9, collagen type II, aggrecan and collagen type X. Day 0 is the first day of insulin addition.