CHONDROGENIC GENE EXPRESSION IN ATDC5 MONOLAYER AND PELLET CULTURES IN RESPONSE TO AN INSULIN SUPPLEMENT

INTRODUCTION:
ATDC5, a murine chondrocyte cell line, is widely used as a monolayer culture system to study chondrogenic differentiation. In monolayer culture in the presence of insulin, ATDC5 cells exhibit properties of early chondroprogenitors and with time in culture will form cartilage nodules. The use of ATDC5 cells has been primarily restricted to monolayer culture systems where matrix accumulation is limited. In cartilage, however, the three-dimensional environment may play an important role in promoting chondrogenesis for acquisition of a characteristic extracellular matrix (ECM) of type II collagen and aggrecan. In pellet culture chondrocytes isolated from hyaline cartilage have been tested for effects of growth factors and cytokines, including BMPs on chondrocyte phenotype; properties of ECM; bioenergetics of chondrocytes; and histological, immunohistochemical, and ultrastructural properties of the ECM. It has been reported that pellet cultured chondrocytes share similarities in cellular distribution, matrix composition and density, and tissue ultrastructure with native cartilage. Real-time PCR has enabled the quantification of mRNA with high accuracy, reproducibility and sensitivity in a wide dynamic range, without the need of post-PCR processing. In this study, real-time PCR assays were applied to quantify chondrogenic gene expression in ATDC5 cells in monolayer and pellet culture during cartilage generation in response to insulin supplementation.

METHODS:
Cultures of undifferentiated ATDC5 cells were maintained in DMEM/F12 (a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium) supplemented with 5% FBS (fetal bovine serum), 100 µg/ml penicillin, 100 µg/ml streptomycin. For monolayer culture, once the cells reached confluency, the medium was changed to DMEM/F12 with FBS and antibiotics (as described above), additionally supplemented with ITS (10 µg/ml insulin, 5.5 µg/ml transferring and 6.7 ng/ml sodium selenite) and the cells were cultured for 24 days to induce cartilage generation. For pellet culture, after expansion of the ATDC5 in monolayer culture, cells were trypsinized, washed in serum-containing medium, and cells were centrifuged at 400 g for 10 minutes and resuspended in the serum-free medium. Aliquots of 0.5 x 10^6 cells suspended in 0.5 ml were distributed to sterile 0.5-ml polypropylene tubes. These tubes were put into 15-ml conical centrifuge tubes in a sterile manner and centrifuged at 400 g for 10 minutes to produce micromass pellets. The medium was made up of DMEM/F12 with 5 %FBS and antibiotics, additionally supplemented with ITS. Cells from monolayer and pellet cultures were harvested at day 0, 6, 12, 18, and 24 to examine gene expression. Total RNA was extracted by Trizol Reagent (Sigma) followed by removal of gDNA (Qiagen). Gene expression levels were quantified by real-time RT-PCR (ABI Prism 7900HT Sequence Detection System, Applied Biosystems). Relative expressions of aggrecan, type II collagen, SOX9 were compared with 18s as an internal control. All experiments were carried out as three independent trials with each culture condition tested in triplicate. The data represent the average of the mean for each independent experiment as mean and standard deviation. Statistical analysis include analysis of variance with post-hoc correction for multiple comparisons (Turkey’s, SPSS).

RESULTS:
ATDC5 cells in monolayer and pellet culture exhibited a time-dependent acquisition of cartilage generation that was associated with a differential expression of matrix protein gene expression (Figures 1-3). At day 6 of culture, aggrecan and type II collagen mRNA levels in the pellet cultures were increased by 5- and 3-fold, respectively, when compared to mRNA signal levels in monolayer cultures (Fig. 1,2). With increased time in culture, type II collagen gene expression in the pellet cultures was downregulated compared to monolayer cultures at day 12, 18 and 24 (Fig. 2). Sox9 gene expression in the pellet cultures was downregulated at day 6, 12, 18 and 24 when compared to levels in the day 0 cultures (Fig. 3). In contrast, Sox9 gene expression in monolayer cultures was almost constant during chondrogenic differentiation when compared to the day 0 cultures (Fig. 3).

DISCUSSION:
In addition to various chondrocytic cell lines that provide model systems for chondrocyte biological studies, a number of cell culture methods have been created to investigate chondrocytes in various environments. Monolayer cultures, three-dimensional culture systems, implant models for tissue engineering, and organ culture of cartilage slices have all been studied. In the ATDC5 monolayer culture system, cartilage nodules enlarge while chondrocytes proliferate, lasting about 2 weeks in culture. When chondrocytes cease to grow after 3 weeks in culture, cells at the center of the cartilage nodules become hypertrophic, which is characteristic of the late phase differentiation of endochondral bone formation. After a further 2 weeks, mineralization could be observed. The imperative beholding culture chondrocytes in a three-dimensional environment is to stabilize their phenotype for subsequent applications. In three-dimensional culture system, chondrocytes may proliferate without phenotype loss. High cell density is another critical requirement for chondrocyte phenotype maintenance. Chondrocyte pellet culture leads to extremely high cell density and provides an in vitro model of chondrocyte differentiation. As demonstrated here, pellet culture of the ATDC5 increases gene expression for aggrecan and type II collagen within the first 6 days of culture. The increased gene expression was associated with formation of a compact, uniform, and highly condensed ECM (data not shown). In contrast, in monolayer culture, type II collagen gene expression was not increased until day 12 of culture. Interestingly, Sox9 was decreased in the pellet culture over time but in the monolayer cultures was increased as aggrecan and type II collagen mRNA increased. These data demonstrate that the timing of chondrogenic gene expression can be modulated by cell morphology and possibly by cell-cell interactions, as demonstrated by the differential gene expression profiles under these two culture conditions.

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