Thyroxine Is the Serum Factor That Regulates Morphogenesis of Columnar Cartilage from Isolated Chondrocytes in Chemically Defined Medium

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Abstract. Epiphyseal chondrocytes cultured in a medium containing 10% serum may be maintained as three dimensional aggregates and differentiate terminally into hypertrophic cells. There is an attendant expression of genes encoding type X collagen and high levels of alkaline phosphatase activity. Manipulation of the serum concentration to optimal levels of 0.1 or 0.01% in this chondrocyte pellet culture system results in formation of features of developing cartilage architecture which have been observed exclusively in growth cartilage in vivo. Cells are arranged in columns radiating out from the center of the tissue, and can be divided into distinct zones corresponding to the recognized stages of chondrocyte differentiation. Elimination of the optimal serum concentration in a chemically defined medium containing insulin eliminates the events of terminal differentiation of defined cartilage architecture. Chondrocytes continue to enlarge into hypertrophic cells and synthesize type X collagen mRNA and protein, but in the absence of the optimal serum concentration, alkaline phosphatase activity does not increase and the cells retain a random orientation. Addition of thyroxine to the chemically defined medium containing insulin and growth hormone results in dose-dependent increases in both type X collagen synthesis and alkaline phosphatase activity, and reproduces the optimal serum-induced morphogenesis of chondrocytes into a columnar pattern. These experiments demonstrate the critical role of thyroxine in cartilage morphogenesis.

TERMINAL differentiation of chondrocytes into hypertrophic cells is an obligatory step in the endochondral ossification pathway that occurs during embryonic bone development, longitudinal bone growth, and fracture healing. This terminal differentiation process is marked by a several-fold increase in cell volume (11), synthesis of type X collagen, and high levels of alkaline phosphatase activity. The net result of this developmental process is an increase in length of the growing bone and the mineralization of the surrounding cartilage matrix. This mineralized extracellular matrix of cartilage provides the scaffold for the deposition of new bone matrix by invading osteoblasts.

The identification of factors regulating this critical developmental pathway of the skeleton has been hampered by lack of a suitable in vitro model. It is well-established that chondrocytes lose their polygonal morphology and dedifferentiate when placed in traditional monolayer cultures in vitro on tissue culture plastic, a problem which is exacerbated by low cell density or multiple passages (4–6, 25). Although suspending chondrocytes in culture media or agarose stabilizes the chondrocyte phenotype and permits terminal differentiation into hypertrophic cells (3, 6, 25), the lack of a three-dimensional tissue architecture prevents morphogenesis of growing cartilage and its study as an organized tissue.

We have resolved this dilemma by modification of a simple method of chondrocyte culture which maintains cells as an aggregated cell pellet, thereby more closely approximating the three-dimensional environment of developing cartilage in vivo under strict control of culture conditions (13). Although this aggregation of cells is not distinguished immediately following centrifugation, after 21 days in culture a sizeable cartilage nodule develops (Fig. 1). When cultured in medium containing 10% serum, chondrocytes maintained as cell pellets terminally differentiate into hypertrophic cells, express genes encoding type X collagen, and exhibit high levels of alkaline phosphatase activity, all of which can be reversibly inhibited by exogenous addition of TGF-β1 (2).

The potential utility of this model to investigate events linked to chondrocyte differentiation is limited due to the presence of serum and precludes the identification of specific factors responsible for the regulation of key steps of this sequential differentiation process. We and others have therefore attempted to establish culture conditions which will allow chondrocyte differentiation to proceed in chemically defined media (7, 19, 23). The present investigation demon-

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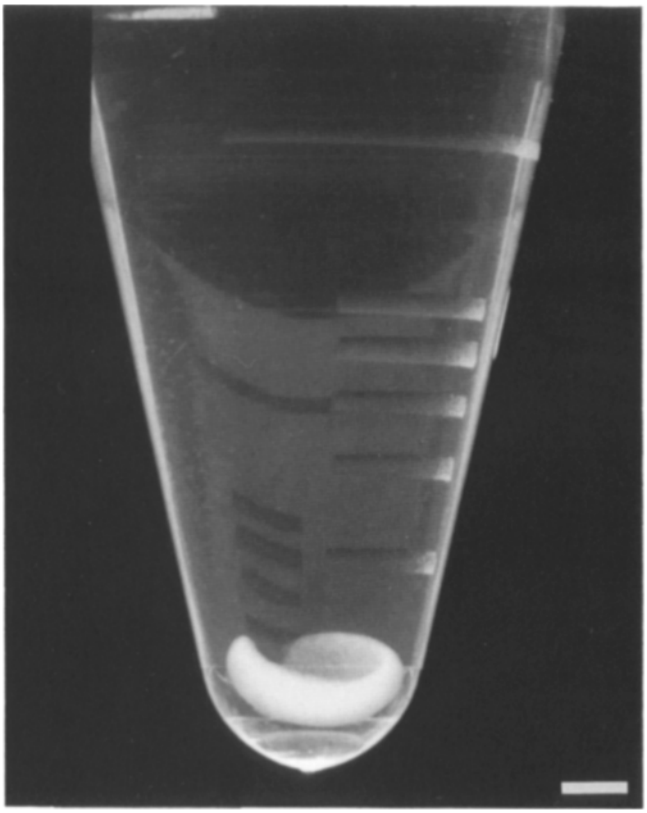

Figure 1. Gross appearance of chondrocyte cell pellet after 21 days of culture in chemically defined DME:F12 medium supplemented with insulin (5 μg/ml). The original cell pellet at the beginning of the culture period was barely visible on gross inspection. Bar, 1 mm.

strates that optimization of the serum concentration to 0.1 or 0.01% in this three-dimensional culture system results in morphogenesis of developing cartilage architecture which have been observed previously only in growth cartilage in vivo. Cells are arranged in columns radiating out from the center of the tissue, and can be divided into distinct zones corresponding to the recognized stages of chondrocyte differentiation. Elimination of serum in this chemically defined medium containing insulin as the sole regulatory factor results in the blockade of terminal differentiation. Although chondrocytes continue to enlarge into hypertrophic cells and synthesize type X collagen mRNA and protein, in the absence of serum alkaline phosphatase activity does not increase and the cells retain a random orientation. Addition of thyroxine to chemically defined medium containing insulin and growth hormone, however, results in a dose-dependent increase in both type X collagen synthesis and alkaline phosphatase activity, and morphogenesis of chondrocytes into a columnar pattern reminiscent of the in vivo growth plate.

Materials and Methods

Materials

Fitton-Jackson modified BGJb medium, CMRL 1066 medium, DME, a combination of DME: Ham's F12 medium (1:1), MEM, Ham's F12 medium, penicillin-streptomycin, PBS, sodium pyruvate, FBS, and recombinant human insulin-like growth factor I and II (IGF-I and IGF-II) (all from GIBCO, Grand Island, NY); Collagenase P and purified bacterial collagenase (Boehringer Mannheim, Indianapolis, IN); L-ascorbic acid phosphate (Wako Biochemicals, Richmond, VA); ITS + Premix (Collaborative Research, Bedford, MA); L-thyroxine, beta-aminoproprionitrile (BAPN)[1], pepsin, *p*-nitrophenol phosphate, and Folin's reagent (all from Sigma Chemical Co., St. Louis, MO); [^{32}P]dCTP and [U-^{14}C]-proline (Amersham International, Amersham, UK); EN^{3}HANCE (New England Nuclear, Boston, MA); rat growth hormone was a gift from the National Hormone and Pituitary Program (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases and National Institutes of Health, Bethesda, MD); recombinant porcine TGF-β1 was a gift from Michael Sporn and Anita Roberts (National Cancer Institute, and National Institutes of Health, Bethesda, MD); bone morphogenetic protein-3 (BMP-3) was purified from demineralized bovine matrix as previously described (17, 24); recombinant human BMP-4 was a gift from Dr. Glenn Hammonds (Genentech, South San Francisco, CA); recombinant human OP-1 (BMP-7) was a gift from T. Kuber Sampath (Creative Biomolecules, Hopkinton, MA).

Chondrocyte Isolation

Chondrocytes were isolated from the resting zone of the distal femoral growth cartilage of two-d-old neonatal Sprague-Dawley rats by collagenase digestion. The distal femora were exposed by dissection under loupe magnification. Adherent soft tissues were stripped away and the reserve zone of the epiphysis removed by dissection. The cartilage fragments containing the reserve zone chondrocytes were washed three times in PBS containing 1% penicillin/streptomycin and digested for 4 h in a 0.3% solution of Collagenase P in PBS in a shaking water bath at 37°C. The solution containing the isolated cells was filtered through 70-μ mesh and the cells recovered by centrifugation at 1,000 rpm at 4°C and resuspended in culture medium at a final concentration of 160,000 cells/ml.

Three-dimensional Pellet Culture

Chondrocytes were cultured as a three-dimensional cell pellet as previously described (2). Briefly, 1-ml aliquots containing 160,000 cells each were added to 15-ml conical polypropylene centrifuge tubes and the cells pelleted by centrifugation at 1,000 rpm for 5 min at 4°C. The cultures were then maintained at 37°C in 95% oxygen and 5% CO_2 in a humidified incubator. Medium was changed every other day after the third day.

Pellets were maintained up to 21 days in DME:F12 medium supplemented with 50 μg/ml L-ascorbic acid phosphate (10), 100 μg/ml sodium pyruvate, 1% (vol/vol) penicillin-streptomycin, and either heat-inactivated FBS or a defined media supplement yielding a final concentration of 6.25 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml bovine serum albumin, and 5.35 μg/ml linoleic acid. To study the effect of the composition of the chemically defined media on chondrocyte differentiation, pellets were also maintained in the following five culture media supplemented as described above: Fitton-Jackson modified BGJb medium; CMRL 1066 medium; Ham's F12 medium; DME alone; and MEM.

In some experiments, the following factors were substituted one at a time for insulin and/or added to insulin-containing media for the entire 21-d culture period: recombinant human IGF-I (20 ng/ml); recombinant human IGF-II (20 ng/ml); bovine insulin (100 ng/ml); rat growth hormone (50 ng/ml); recombinant porcine TGF-β1 (10 ng/ml); L-thyroxine (1–100 ng/ml); purified bovine BMP-3 (10 ng/ml); recombinant human BMP-4 (10 ng/ml); recombinant human BMP-7 (10 ng/ml); and BAPN (100 μg/ml). These factors were re-added with each change of the culture medium.

Tissue Morphology

Pellets were fixed in 10% neutral buffered formalin, dehydrated through graded alcohols, and embedded in glycol methacrylate. 3-μ sections perpendicular to the short axis of the pellets were cut on a glass knife and stained with toluidine blue for light microscopy.

Northern Blotting

20–30 pooled samples per group were snap frozen and total cellular RNA extracted by homogenization in 6 M guanidine HCl, followed by cesium chloride gradient centrifugation and sequential lithium chloride and lithium chloride/ethanol precipitation. 5 μl of total cellular RNA per lane were

1. *Abbreviations used in this paper*: BAPN, beta-aminoproprionitrile; BMP, bone morphogenetic protein; GAPDH, glyceraldehyde phosphate dehydrogenase.

separated by electrophoresis through 1% agarose gels. The separated RNA was transferred to nylon membranes and crosslinked to the filter by exposure to ultraviolet light. Radiolabeling of cDNA probes with [^{32}P]dCTP was performed using the random priming technique. Membranes were prehybridized, hybridized, and washed by the method of Church and Gilbert (8), and exposed to radiographic film at −70°C using intensifying screens.

To examine the endogenous expression of BMP genes in intact growth cartilage, total cellular RNA was extracted from the entire growth cartilage of pooled distal femoral epiphyses obtained from 2-d-old neonatal Sprague-Dawley rats by the above method.

cDNA Probes

The following cDNA sequences were used to examine gene expression by Northern hybridization: pRC2, encoding 600 bp of the amino terminus of the rat alpha I(II) collagen gene (14); pSAM10h, encoding 600 bp of exon 3 and the 3′ untranslated region of the mouse type X collagen gene (1); and a PstI fragment of the rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA for assessment of equivalence of RNA loading.

Collagen Labeling and Extraction

Cultures were treated with 100 μg/ml of BAPN for 20 min before labeling with 5 μCi/ml of [U-^{14}C]-proline for the final 48 h of the culture period. Labeled collagens were extracted according to previously published methods (6, 12). Briefly, labeled pellets were homogenized in 0.5 N acetic acid and digested with 1 mg/ml pepsin in 0.5 N acetic acid for 24 h at 4°C before inactivation of the pepsin by the addition of Tris to 50 mM and titration to neutral pH with concentrated NaOH using phenol red as an indicator. Pepsin-resistant material was extracted for an additional 24 h in 0.15 M NaCl containing 20 mM DTT and 50 mM Tris-HCl at pH 7.4 in the presence of protease inhibitors (5 mM EDTA, 1 mM PMSF, 1 mM PABA, and 5 mM NEM). Pepsin digests and DTT extracts were combined and concentrated in a Centricon-30 concentrator with a molecular weight cutoff of 30,000. Aliquots of labeled collagen (10,000 cpm per sample) were analyzed by SDS/PAGE on 4–20% gradient gels under reducing conditions. Some samples were digested with 2.5 units of purified bacterial collagenase for 1 h at 37°C prior to gel electrophoresis. Gels were processed for fluorography with EN^{3}HANCE and exposed to radiographic film at −70°C.

To examine collagen synthesis in explant cultures of intact growth cartilage, the hypertrophic zones of both the distal femoral ephiphyses of 2-d-old neonatal rats were removed and cultured for 3 d in DME:F12 medium containing 10% FBS. Collagens were then labeled and extracted exactly as described above.

Alkaline Phosphatase Activity

Pellets were homogenized in 2 ml of ice-cold 0.15 M NaCl containing 3 mM $NaHCO_3$ (pH 7.4) with three 10-s bursts using a Polytron homogenizer and centrifuged at 20,000 *g* for 30 min at 4°C. The supernatants were assayed for alkaline phosphatase activity in 0.1 M sodium barbital buffer (pH 9.3) using *p*-nitrophenyl phosphate as a substrate as previously described (21). The amount of protein contained in the enzyme extracts was determined (16), and the enzyme activity expressed as units of alkaline phosphatase activity per mg protein. 1 unit of alkaline phosphatase was defined as the enzyme activity that liberated 1 μmol *p*-nitrophenol/30 min at 37°C per mg protein.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to assess the effect of the measured variables on alkaline phosphatase activity. Statistical significance between pairs of means within a group was determined by multiple *t* tests after adjusting the significance level for multiple comparisons by the Bonferroni method.

Results

Reducing the Serum Concentration in the Media Facilitates Morphogenesis of Columnar Cartilage Architecture

Chondrocytes cultured in DME:F12 medium supplemented with 10% serum acquired a hypertrophic morphology by day 21 of the culture period, as previously demonstrated (2) (Fig. 2 *a*). It is noteworthy that perichondrium-like layer of flattened proliferating cells surrounded the tissue. Cells cultured in DME:F12 containing insulin in place of serum also acquired a hypertrophic morphology by day 21, but in the absence of serum, no perichondrium-like layer of cells formed on the outside of the pellet (Fig. 2 *f*).

To determine the minimal concentration of serum which would support full terminal differentiation of chondrocytes in vitro, cells were cultured in DME:F12 with serum concentrations of less than 10%. Surprisingly, histologic analysis of pellets maintained for 21 d in minimal serum concentrations of 0.1 or 0.01% revealed that chondrocytes became organized in a columnar pattern, with the columns of cells radiating outward from the center of the pellet (Fig. 2, *c* and *d*). This columnar architecture remarkably resembled the appearance of developing growth plate cartilage in vivo. The columns consisted of at least two discrete zones of differentiating cells, with the hypertrophic zone located on the inner aspect of the pellet and the proliferative zone located in the middle region. This columnar architecture produced by culturing cells under optimal serum conditions was disrupted by withholding ascorbic acid from the medium (Fig. 2 *e*), or by inhibiting collagen crosslinking by addition of beta-aminoproprionitrile (Fig. 2 *g*).

In the pellets maintained in 10% serum, an envelope of flattened perichondrium-like cells ~3–5 cells thick was observed around the exterior of the pellet (Fig. 2 *a*). As the serum concentration decreased to 1%, the thickness of this perichondrium-like layer decreased to 2–3 cell layers (Fig. 2 *b*). At serum concentrations of 0.1 and 0.01%, this flattened layer of cells could no longer be identified (Fig. 2, *c* and *d*).

Type X collagen gene expression was detectable by day 14 and persisted through day 21 in 10% serum-treated cultures (Fig. 3). In cultures maintained in chemically defined media containing insulin in place of serum, type X collagen gene expression was present as early as day 14, but did not reach levels comparable to 10% serum-treated specimens until day 21. Reducing the serum concentration modestly increased the level of type X collagen gene expression by day 21 of the culture period (Fig. 4).

Synthesis of type X collagen protein in the presence of 10% serum was also apparent by day 14, but was only faintly detectable by day 21 (Fig. 5, lane *5*). The ratio of type X collagen to type II collagen protein was increased in the insulin-treated cells compared to the 10% serum-treated cells by day 21 (Fig. 5, lanes *1* and *5*). Reducing the serum concentration appeared to increase the level of type X collagen protein synthesized by the cells at day 21 (Fig. 5, lanes *1–5*).

Alkaline phosphatase activity increased significantly in 10% serum-treated cultures compared to insulin-treated controls by day 14 ($p < 0.01$), and remained significantly elevated over controls by day 21 ($p < 0.001$) (Fig. 6). Alkaline phosphatase activity reached maximum levels in cultures treated with 10% serum, decreased moderately as the serum concentration was reduced to 0.01%, then fell precipitously as serum was completely removed and replaced with insulin (Fig. 7).

One-way analysis of variance (ANOVA) confirmed a significant effect of serum concentration on resulting alkaline phosphatase activity ($p < 0.001$). Pairwise comparisions of means confirmed that the insulin-treated group exhibited sig-

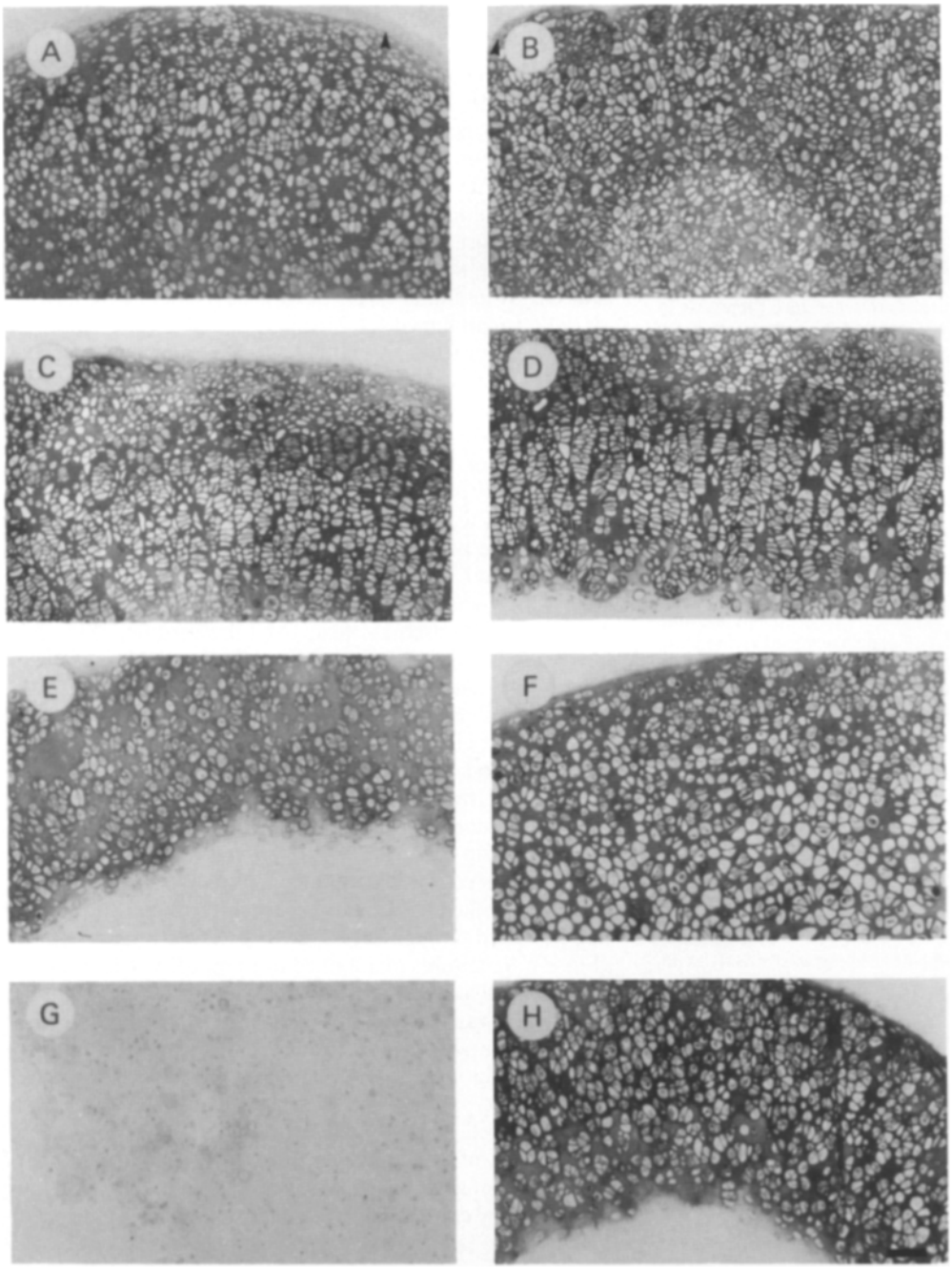

Figure 2. Morphologic appearance of chondrocytes maintained in pellet cultures for 21 days in DME:F12 media supplemented with decreasing concentrations of serum: (*A*) 10% FBS; (*B*) 1% FBS; (*C*) 0.1% FBS; (*D*) 0.01% FBS; (*E*) 0.1% FBS minus L-ascorbic acid phosphate; (*F*) insulin alone (5 μg/ml); (*G*) 0.1% serum plus beta-aminoproprionitrile (100 ng/ml); (*H*) insulin (5 μg/ml) minus L-ascorbic acid phosphate. The arrowheads in *A* and *B* identify the perichondrium-like layer of cells surrounding the cell pellet. Bar, 100 μm.

nificantly less alkaline phosphatase activity at day 21 than all other serum-treated specimens ($p < 0.01$).

Thyroxine Is the Serum Factor That Regulates Morphogenesis of Columnar Cartilage

Addition of rat growth hormone (50 ng/ml) to insulin-containing media inhibited differentiation of these cells into hypertrophic chondrocytes and did not alter the random orientation of cells previously observed in the absence of serum (Fig. 8 *a*). Growth hormone treatment also did not result in a noticeable increase in pellet size, type X collagen synthesis, or alkaline phosphatase activity compared to insulin-containing medium alone. When thyroxine was added to chemically defined media containing growth hormone and insulin, however, the inhibitory effect of growth hormone on chondrocyte hypertrophy was reversed. At a concentration of 100 ng/ml, thyroxine administration reproduced the serum-induced organization of chondrocytes into longitudinal columns (Fig. 8 *b*). Likewise, thyroxine effected dose-responsive increases in both type X collagen synthesis (Fig. 5, lanes *10–12*) and alkaline phosphatase activity (Fig. 9). One-way ANOVA confirmed that thyroxine concentration significantly increased resulting alkaline phosphatase activity ($p < 0.001$).

Chondrocyte Hypertrophy in the Absence of Serum Is Dependent on the Formulation of the Culture Media

The acquisition of the hypertrophic morphology by chondrocytes cultured in insulin-supplemented medium was de-

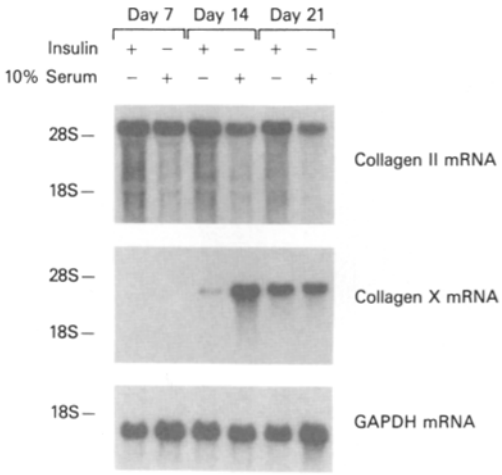

Figure 3. A. Expression of genes encoding type II collagen and type X collagen by chondrocytes in pellet cultures maintained for 7, 14, and 21 days in the presence of 10% FBS or insulin (5 μg/ml).

pendent on the formulation of the culture medium used. Only cells cultured in DME or a 1:1 combination of DME:F12 medium demonstrated widespread morphologic evidence of hypertrophy (Fig. 10, *c* and *d*). Cells cultured in insulin-containing Fitton-Jackson modified BGJb medium, CMRL 1066 medium, MEM, and Ham's F12 medium did not show consistent morphologic evidence of cellular hypertrophy (Fig. 10, *a*, *b*, *e*, and *f*).

Similarly, only cells cultured in DME:F12 or DME alone displayed evidence of type X collagen gene expression by day 21 (Fig. 11). Unexpectedly, this level of type X gene expression was noticeably greater in DME:F12 medium than DME alone. Cells cultured in insulin-containing Fitton-Jackson modified BGJb medium, CMRL 1066 medium, MEM, and Ham's F12 medium demonstrated no type X collagen gene expression by day 21, even on overexposed autoradiographs.

Although alkaline phosphatase activity was significantly elevated in DME or DME:F12 medium compared to the other formulations ($p < 0.001$) (Fig. 12), this activity remained at essentially baseline levels compared to serum-treated or thyroxine-treated cultures.

Insulin/IGF Is Required for Continued Chondrocyte Survival, but Does Not Stimulate Terminal Differentiation

In the absence of serum, replacement of insulin with recombinant human IGF-I (20 ng/ml), IGF-II (20 ng/ml), or a lower dose of insulin (100 ng/ml) did not result in a deleterious effect on cell survival, but chondrocyte hypertrophy did not occur (data not shown). Attempts to substitute rat growth hormone (50 ng/ml), recombinant porcine TGF-β1 (10 ng/ml), purified bovine BMP-3 (10 ng/ml), recombinant human BMP-4 (10 ng/ml), and recombinant human OP-1 (BMP-7) (10 ng/ml) for insulin uniformly resulted in failure of cells to survive and proliferate within the first few days of the culture period.

Of interest is the observation that addition of 10 ng/ml of BMP-4 or BMP-7 to insulin-containing DME:F12 medium effected a striking increase in pellet size by day 21 (data not shown). Histologic examination revealed large areas of hypertrophic cells in the center of the pellet, indicating an amplified anabolic response to BMP-4 and BMP-7 without inhi-

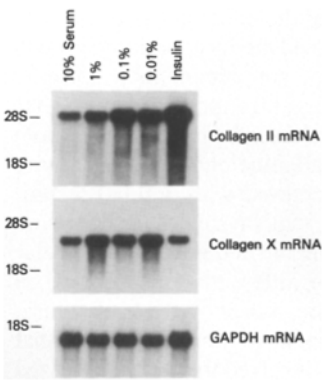

Figure 4. Expression of genes encoding type II collagen and type X collagen by chondrocytes in pellet cultures maintained for 21 days in the presence of 0.01% to 10% FBS or insulin (5 μg/ml).

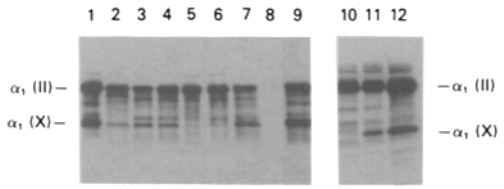

Figure 5. SDS/PAGE (4–20% gradient gel) analysis of [^{14}C]-proline-labeled proteins extracted from chondrocyte pellets as described under Materials and Methods. Unless otherwise indicated, all cultures were maintained for 21 days in DME:F12 medium supplemented with L-ascorbic acid phosphate (50 μg/ml), sodium pyruvate (100 μg/ml) and the following components: 5 μg/ml insulin (lane *1*); 0.01% FBS (lane *2*); 0.1% FBS (lane *3*); 1% FBS (lane *4*); 10% FBS (lane *5*); 5 μg/ml insulin in DME medium alone (lane *6*); 5 μg/ml insulin in DME:F12 medium (lane *7*); same sample as in lane *7*, digested with purified bacterial collagenase (1 mg/ml) at 37°C for 30 min prior to electrophoresis (lane *8*); intact growth cartilage from neonatal rat distal femur maintained as an explanted organ culture for 3 d (lane *9*); 5 μg/ml insulin and 50 ng/ml rat growth hormone (lane *10*); 5 μg/ml insulin, 50 μg/ml rat growth hormone, and 1 ng/ml L-thyroxine (lane *11*); 5 μg/ml insulin, 50 ng/ml rat growth hormone, and 100 ng/ml L-thyroxine (lane *12*).

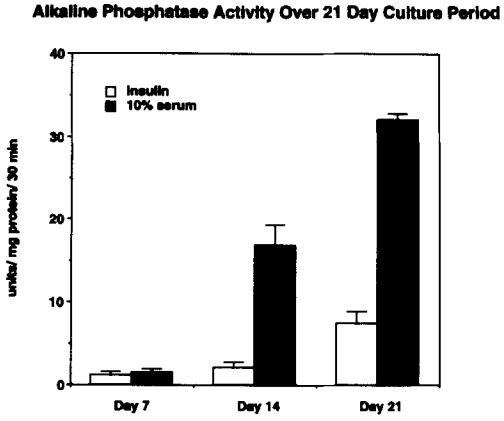

Figure 6. Alkaline phosphatase activity at 7, 14, and 21 d in cultures maintained in chemically defined DME:F12 medium supplemented with insulin (5 μg/ml). Differences between means for serum- and insulin-treated cultures were significant both at Day 14 ($p < 0.001$) and Day 21 ($p < 0.001$).

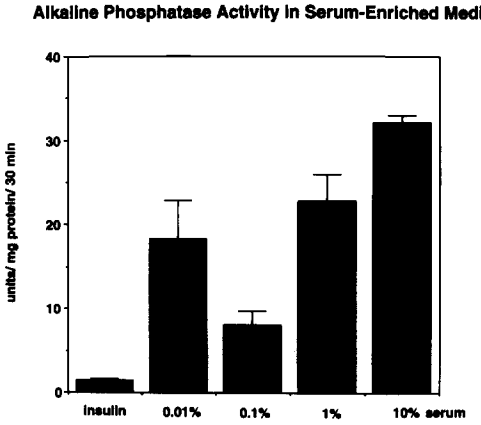

Figure 7. . Alkaline phosphatase activity at day 21 in cultures maintained in DME:F12 media in the presence of 0.01 to 10% FBS or insulin (5 μg/ml). One-way ANOVA confirmed a significant effect of serum concentration on resulting alkaline phosphatase activity ($p < 0.001$). The insulin-treated cultures demonstrated significantly less alkaline phosphatase activity at day 21 than all other serum-treated specimens ($p < 0.001$).

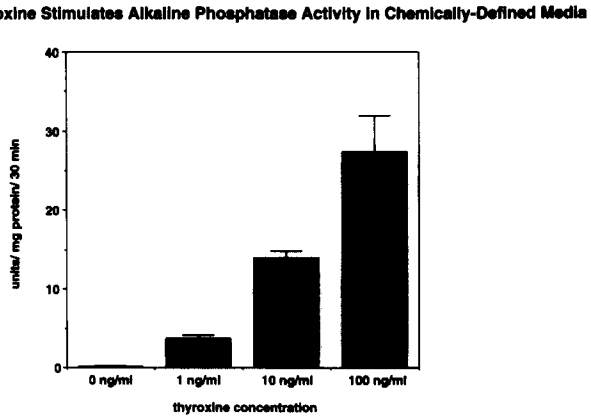

Figure 9. Alkaline phosphatase activity at day 21 in cultures treated with thyroxine in the presence of growth hormone (50 ng/ml) and insulin (5 μg/ml). One-way ANOVA confirmed a significant effect of thyroxine concentration on resulting alkaline phosphatase activity ($p < 0.001$).

bition of terminal differentiation. As reported previously for serum-containing media (2), addition of TGF-β1 (10 ng/ml) to insulin-containing media reduced the final size of the pellet compared to controls, and also inhibited terminal differentiation of these cells.

Discussion

The organization of proliferating chondrocytes into longitudinal columns is a critical event during the development and growth of the skeleton. During the early stages of embryogenesis, the condensation of mesenchyme into cartilage is followed by organization of this tissue into longitudinal arrays of flattened, proliferative cells which impose pattern on subsequent limb and vertebral growth. This columnar architecture is maintained throughout the developmental period and into early adulthood as the growth plates of the vertebrae and long bones. Disorganization of these columns is often seen in association with disorders of longitudinal bone growth (18, 22).

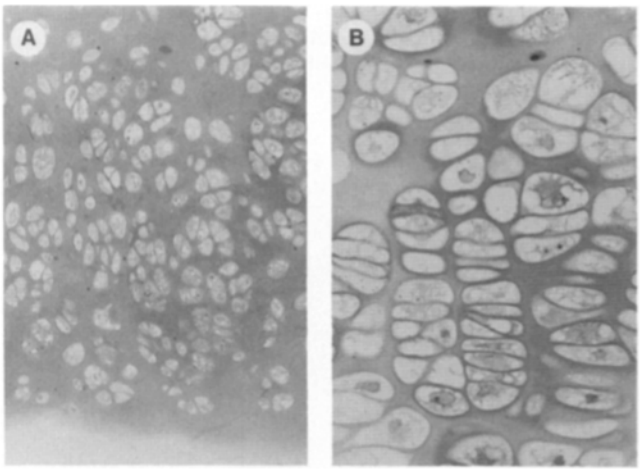

Figure 8. (*A*) Chondrocyte morphology at day 21 in pellet cultures in which rat growth hormone (50 ng/ml) was added to chemically defined media containing insulin (5 μg/ml). Lack of cellular hypertrophy and random orientation of the cells were noted. (*B*) Higher power photomicrograph of pellet cultures in which L-thyroxine (100 ng/ml) has been added to chemically defined media containing both growth hormone (50 ng/ml) and insulin (5 μg/ml). The chondrocytes have organized into columns of flattened cells resembling the proliferative zone of the growth plate in vivo.

Our results indicate that factors present in serum are responsible for organization of this columnar architecture in growing cartilage. The observation that optimal serum concentrations of 0.1 to 0.01% have a profound effect on tissue organization suggests that the responsible serum factors must be active at very low concentrations, and therefore may be hormonal in nature.

It is well known from the early studies of Evans and colleagues that growth hormone is of paramount importance in the regulation of skeletal growth and maturation (15), therefore we initially examined the effect of this anterior pituitary hormone on terminal chondrocyte differentiation. The results of these experiments revealed that growth hormone alone has no pronounced effects on the longitudinal column architecture of chondrocytes. Because thyroxine is known to have synergistic effects with growth hormone during endochondral ossification (20, 21) and has recently been shown to stimulate type X collagen synthesis and alkaline phosphatase activity in suspension cultures of chick embryo chondrocytes (7, 19), we investigated the effect of thyroxine supplementation of chemically defined media containing growth hormone and insulin. Thyroxine supplementation not only recreated the serum-induced organization of chondrocytes into columns, but also produced attendant increases in both type X collagen synthesis and alkaline phosphatase activity to levels equivalent to those achieved with optimal serum. These results strongly suggest that thyroxine is the serum factor responsible for facilitating the cellular and molecular events of terminal chondrocyte differentiation observed in optimal serum cultures, including morphogenesis of columnar cartilage. This observation also illuminates potential pathophysiological mechanisms involved the well-recognized

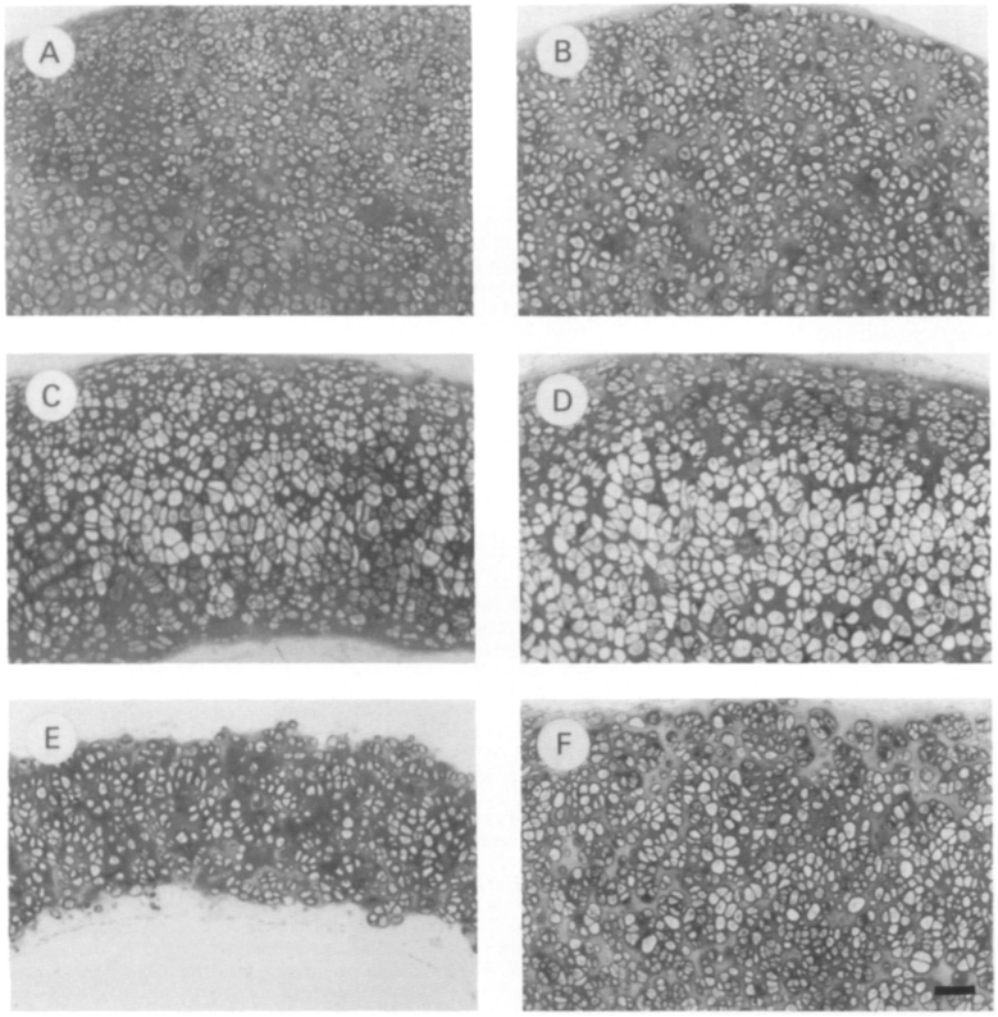

Figure 10. Morphologic appearance of chondrocytes maintained in pellet cultures for 21 days in various formulations of chemically defined media supplemented with insulin (5 μg/ml). (*A*) Fitton-Jackson modified BGJb medium; (*B*) CMRL 1066 medium; (*C*) DME; (*D*) DME:F12 medium (1:1); (*E*) MEM; (*F*) Ham's F12 medium. Bar, 100 μm.

clinical disorder of slipped capital femoral epiphysis which is common among children with hypothyroidism (26).

The mechanism by which chondrocytes form these longitudinal columns of cells is not known, but is likely to be related to interactions between the chondrocyte cytoskeleton and the extracellular matrix proteins surrounding the cell. This mechanism could conceivably involve integrins, since it has recently been shown that the β1 integrin mediates the attachment of chondrocytes to type I and type II collagen (9). Future investigations will likely focus on the specific role

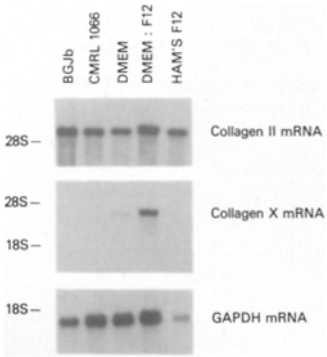

Figure 11. Expression of genes encoding type II collagen and type X collagen by chondrocytes in pellet cultures maintained for 21 d in various formulations of chemically defined media containing insulin (5 μg/ml).

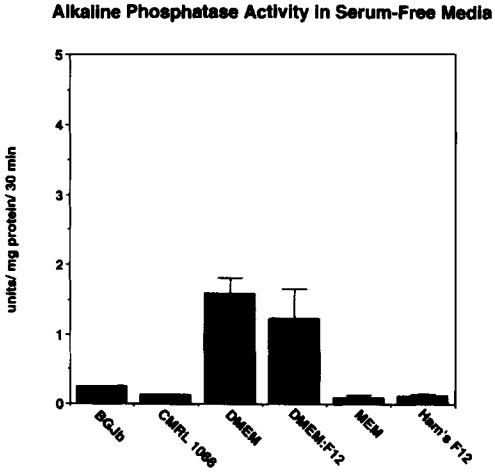

Figure 12. Alkaline phosphatase activity at day 21 in cultures maintained for 21 d in various formulations of chemically defined media containing insulin (5 μg/ml). One-way ANOVA confirmed a significant effect of media formulation on resulting alkaline phosphatase activity ($p < 0.001$). Both DME and DME:F12 media yielded significantly higher alkaline phosphatase activity than other formulations ($p < 0.01$), but were not significantly different from each other ($p > 0.05$).

thyroxine and the DNA-binding thyroid hormone receptor play in regulating type X collagen synthesis, alkaline phosphatase activity, and the morphogenesis of columnar cartilage.

It was of interest to observe the formation of a perichondrium-like layer around the exterior of the cell pellet in cultures maintained in serum concentrations greater than 0.1%. This perichondrial structure may be related to the presence of cell adhesion proteins in the serum. This layer is not necessary for continued growth of the aggregate as elimination of serum effected even larger increases in pellet size despite the absence of this perichondrium.

Current data also demonstrate that the events of terminal chondrocyte differentiation are uncoupled when serum is removed from the culture media. In the presence of insulin alone, chondrocytes maintain the ability to increase their intracellular volume, indicating that chondrocyte hypertrophy is a default pathway that does not require exogenous signals. The long lag period (21 days) between the beginning of insulin treatment and the onset of hypertrophy suggests that the role of insulin in regulating chondrocyte hypertrophy is perhaps permissive rather than instructive. All of the information necessary for cellular enlargement to occur is resident in the resting cells.

At the high doses used in these experiments, insulin has been reported to bind to both the insulin receptor and the IGF-I receptor, suggesting that both pathways might require activation to permit chondrocyte hypertrophy to occur. In this regard, it is noteworthy that when the insulin concentration was reduced or replaced with IGF-I or IGF-II, chondrocyte viability was maintained, but there was no cellular hypertrophy.

The modest elevation in gene expression for type X collagen combined with the noticeable increase in type X collagen protein as the serum concentration decreased suggests the possibility that 0.01 to 0.1% serum represents the optimum culture conditions for chondrocyte differentiation. Although we have not directly evaluated transcriptional or translational regulation of the type X collagen gene in these studies, it is possible that specific factors present in serum may inhibit transcription or translation of this gene when present in larger concentrations at the higher serum levels.

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