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In Serum-Free Culture Thyroid Hormones Can Induce Full Expression of Chondrocyte Hypertrophy Leading to Matrix Calcification

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ABSTRACT

A serum-free culture system has been developed to examine the biologic factors involved in the regulation of cellular maturation, extracellular matrix assembly, and calcification in the physis of the bovine fetal growth plate. Isolated prehypertrophic chondrocytes in high density culture undergo a process of cellular maturation whereby full expression of the hypertrophic phenotype is characterized first by type X collagen synthesis followed by matrix calcification. Using this culture system, we compared the capacity of tri-iodothyronine (T_3) with thyroxine (T_4) to stimulate expression of the hypertrophic phenotype and matrix calcification in three (B, C, and D) maturationally distinct prehypertrophic chondrocyte subpopulations. The B cell subpopulation was the most mature followed by C and D subpopulations in order of decreasing maturity. Comparisons were made to cultures in fetal calf serum (FCS). In Dulbecco's modified Eagle's medium supplemented with insulin, transferrin, and selenium, both hormones (T_3/T_4) separately induced, in a dose-dependent manner, chondrocyte maturation to the hypertrophic phenotype characterized by increased type X collagen mRNA and induction of protein synthesis of this molecule, together with increased alkaline phosphatase activity, and eventually calcification of the extracellular matrix. Such cellular maturation to the hypertrophic phenotype was not observed in the absence of T_3 or T_4 with subpopulations C and D. Only in older fetuses (>210 days) was this observed and then only in the B subpopulation. Furthermore, T_3 was at least 50-fold more potent than T_4. The effects of T_3 were most pronounced with the most immature cells (subpopulations C and D) where, in the case of the subpopulation C, in contrast to 0.5 nM T_3 50 nM T_4 was unable to induce expression of the hypertrophic phenotype. Alkaline phosphatase activity was also increased in the C cell subpopulation treated with 1 nM T_3 (35.5 U/μg of DNA) over that supplemented with 50 nM T_4 (7.8 U/μg of DNA). Furthermore, matrix calcification, measured by the incorporation of $^{45}Ca^{2+}$ into the cell layer, always occurred earlier in cells cultured with T_3 compared with T_4. Cellular maturation to the hypertrophic phenotype was not accompanied by significant changes in DNA content; this ordinarily increases during culture in the presence of serum. Compared with cells cultured in the presence of serum, either thyroid hormone more potently induced cellular maturation. This study demonstrates that the most immature chondrocytes at the prehypertrophic stage are direct targets for T_3 and T_4 and, to a much a lesser degree, that either hormone is able to induce full chondrocyte hypertrophy from an early maturational stage leading to matrix calcification. But T_3 is much more potent than T_4. These studies also offer a new serum-free chemically defined medium containing T_3 or T_4 for the culture of defined prehypertrophic chondrocytes that supports matrix assembly, hypertrophic expression, followed by matrix calcification. (J Bone Miner Res 1996;11:105–113)

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INTRODUCTION

The growth plates of mammalian bones are extremely well organized growth centers in which chondrocytes of the physis synthesize a cartilage matrix and then calcify it in a carefully controlled manner as part of the process of endochondral ossification. The extracellular matrix undergoes a series of changes in structure and composition through the resting, proliferative, maturing, and hypertrophic zones before calcification occurs.[1-3] Prior to calcification of the matrix, chondrocytes of the proliferative zone enlarge and become hypertrophic.[4,5] They start to synthesize type X collagen[6,7] and produce increased amounts of alkaline phosphatase,[1,8] degrade extracellular type II collagen,[1] and concentrate the proteoglycan aggrecan in the extracellular matrix[1,9] prior to and as calcification occurs.[2,3] However, the regulation of cellular maturation and phenotypic expression of hypertrophic chondrocytes and the biochemical and physical alterations involved in matrix mineralization are not fully understood.

Alterations in the secretion of thyroid hormones leading to hypothyroidism and hyperthyroidism are associated with dramatic changes in skeletal growth and osseous maturation.[10-12] Synthesis of thyroid hormone (T_3) and thyroxine (T_4) occurs in the thyroid gland. It follows a metabolic pathway that depends on the presence of thyroglobulin and inorganic iodine. The major thyroid hormone secreted by the cell of the thyroid follicle is T_4. Tri-iodothyronine (T_3) accounts for only 20% of the total circulating thyroid hormones.[13] However, T_4 is later converted to T_3 in the peripheral blood, increasing the proportion of available T_3.[14] In vitro thyroid hormones can stimulate alkaline phosphatase activity in fetal pig scapulae tissue explants (T_3)[15] and in rat epiphyseal (T_3)[16] and chick embryo sternal chondrocytes (T_4).[17] Type X collagen, a definitive marker of hypertrophy, is induced by T_4[17,18] and T_3[19] in chick embryo chondrocytes. Clearly thyroid hormones stimulate the growth and maturation of the skeleton. However, no study has shown progression to full expression of the hypertrophic phenotype leading to matrix calcification. Moreover, the relative potencies of these hormones have never been carefully examined. Information about potency could be of clinical relevance in the treatment of thyroid diseases where there is still disaggreement as to which hormone and dose should be used for a particular thyroid pathology.[13]

To address fully the roles played by thyroid hormones in chondrocyte hypertrophy and their respective potencies, we used high density cultures of fetal bovine growth plate chondrocytes separated into maturationally distinct prehypertrophic subpopulations by a method recently developed in this laboratory.[8] We show here that under serum-rich culture conditions these chondrocytes can synthesize an extracellular matrix, undergo cellular maturation, and express a hypertrophic phenotype (synthesis of type X collagen, and cell enlargement); they finally calcify this matrix. These events occur in a sequential manner for each subpopulation, the timing being dependent upon the maturational state (size and density) of the freshly isolated cells.[8] Moreover, we show that this maturation can occur in the presence of T_3 or T_4.

We also demonstrate that T_3 is a much more potent (at least 50-fold) inducer of chondrocyte hypertrophy than T_4 and that the cellular response is most pronounced in the most immature chondrocyte subpopulations.

MATERIALS AND METHODS

Bovine fetuses

The sources of fetuses and their ages and handling have been described.[8] In the present study, the fetal ages ranged from 160 to 230 days. A total of 10 fetuses were analyzed.

Chondrocyte culture

Multiple growth plate physis removal by dissection and isolation of maturationally distinct growth plate chondrocyte subpopulations has also been described.[8] Briefly, after overnight digestion of the tissue with a mixture of collagenase, hyaluronidase, and DNAse, 1 ml of the cell suspension at a cell density of 2.5–3.0 $\times$ 10^7/ml was carefully layered on the top of discontinuous Percoll density gradients as previously described.[8] Gradient tubes were centrifuged at 400g for 30 minutes at room temperature. This resulted in the formation of five different cellular bands at the solution interfaces. Cells from band A (least dense) to E (most dense) were collected, pooled, and washed several times with Dulbecco's modified Eagle's medium (DMEM). In this study we analyzed three prehypertrophic subpopulations (B, C, and D) of the five previously described.[8]

The different cell subpopulations were cultured at a density of 2 $\times$ 10^5 cells in 200 μl per well of gelatin-coated flat-bottomed 96-well plates in DMEM containing 50 mg/ml of ascorbic acid, 5 mM sodium β-glycerophosphate (both additives were freshly added at each medium change), supplemented with 10% fetal calf serum (FCS, serum present) or, in serum-free medium, with 1 mg/ml of bovine serum albumin (BSA) (fatty acid free; Sigma Chemical Co., St. Louis, MO, U.S.A.). The serum-free medium was supplemented with insulin, transferrin, and selenite (ITS) alone (Boehringer Mannheim, Canada) at two different concentrations as indicated in the Results (at high dose 1 μg/ml of insulin, 1 μg/ml of transferrin, and 1 ng/ml of sodium selenite, or at physiologic (low) dose 75 ng/ml, 75 ng/ml, and 75 pg/ml, respectively) in combination with T_4 (5–200 nM) (Sigma), or T_3 (0.05–50 nM) (Sigma). Media were changed every second day.

Due to the low cell number collected in subpopulation D (compared with B and C subpopulations) it was not possible to study in the same experiment all the different concentrations of T_3 and/or T_4 for the various determinations (DNA, ALP, $^{45}Ca^{2+}$, and type X collagen synthesis). In general, in addition to FCS culture condition, two to three other culture conditions could be analyzed for a particular experiment for the cell subpopulation D.

Cell proliferation

DNA was measured after proteinase K (Sigma) digestion of cell layers as previously described.[20]

Collagen biosynthesis

^{3}H-proline, 25 μCi/ml, (Amersham, Canada Inc., Ontario, Canada) was added to the media for 24 h in the presence of 70 μg/ml of β-aminoproprionitrile. Collagens from media were precipitated by the addition of $(NH_4)_2SO_4$ to a final concentration of 33% (of saturation) for 24 h at 4°C. After centrifugation, pellets were washed twice with 70% ethanol at 4°C. The dry pellets were analyzed directly by SDS–PAGE[21] using 7.5% gels followed by fluorography.[22]

Alkaline phosphatase activity

Cell layers were extracted with 0.1% Triton X-100, in 10 mM Tris HCl, pH 7.4 (200 μl/well) for 60 minutes at 4°C on a gyratory shaker.[8] Alkaline phosphatase (ALP) activity was assayed in 100 μl aliquots by measuring the release of p-nitrophenol from the substrate p-nitrophenyl phosphate disodium (Sigma, technical bulletin 104). One unit of activity is expressed as 1 nmol of substrate hydrolyzed in 30 minutes.

Calcium ($^{45}Ca^{2+}$) incorporation

At the indicated times, cell layers were labeled with 1.25 μCi/ml of $^{45}Ca^{2+}$ (Amersham Canada Inc.) for 24 h, washed twice with DMEM alone, and dried under vacuum for 1 h. Residues were solubilized at 70°C by the addition of 200 μl/well 90% formic acid for 45 minutes before liquid scintillation counting.[8]

RNA extraction and Northern blot analysis of type X mRNA

Total RNA was isolated from chondrocyte cell layers using a modified acid guanidine-phenol-chloroform method (Wu, Poole, and Alini, unpublished data). Briefly, cell layers were homogenized in solution D[23] and precipitated overnight by the addition of 1 vol of cold isopropanol at −20°C. The resulting pellet was digested with proteinase K (molecular biology grade; Gibco BRL, Grand Island, NY, U.S.A.) for 2 h at 65°C. Thereafter, 1 vol of solution D (at double the normal concentration) was added, and then the usual guanidine-phenol-chloroform extraction procedure was followed.[23] The contaminating glycosaminoglycan chains (which inhibit ethidium bromide staining of mRNA) were removed from the RNA molecules by washing the pellet, obtained from the ethanol precipitation step, in 4 M LiCl as previously described.[24] For Northern blot analysis, equal amounts (5 μg) of total RNA were electrophoresed on 1.0% agarose-formaldehyde gels and transferred to nylon membranes (Hybond-N$^+$, Amersham). Equivalent loading was assured by ethidium bromide staining of the gel to visualize the ribosomal RNA. The membranes were prehydridized for 3 h at 42°C in hybridization buffer (as described by the manufacturer) and hybridized overnight at 42°C in the same buffer with ^{32}P-labeled probe (random priming kit, Stratagene, La Jolla, CA, U.S.A.). Following hybridization, the membranes were washed as described by the manufacturer and exposed to Kodak (Rochester, NY, U.S.A.) XAR-5 fibrous at −70°C using an intensifying screen. The type X collagen bovine cDNA probe was generated (based on the published bovine cDNA sequence[25] by RT-PCR using primer 21-mer and 24-mer oligonucleotides corresponding to amino acids 542–548 (5′-GGAATGCCTGT GTCTGCTT CA-3′) and 645–652 (5′ GCCTGCATTGGG CAGCTGGAGCCA-3′), respectively. The specificity of the cDNA probe was confirmed by sequencing (sequenase reagent kit; US Biochemicals Corporation, Cleveland, OH, U.S.A.) the PCR product after cloning it into the pCRII vector (TA cloning vector, Invitrogen Corporation, San Diego, CA, U.S.A.).

Histological analysis of chondrocyte cell layers

Cell cultures were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, overnight at 4°C. Tissue was dehydrated with graded ethanols and embedded in Spurr resin (Polysciences Inc., Washington, PA, U.S.A.). Semithin sections (1–2 μm) were stained with Von Kossa's reagent (for mineral) and counterstained with toluidine blue (for proteoglycan) as described previously.[8,29]

RESULTS

Chondrocytes cultured in serum-containing DMEM

As observed previously,[8] type X collagen synthesis was first detected in serum-containing cultures in the B cell subpopulation in both cell layers and medium followed in order, after progressively longer periods of culture, in the C and D subpopulations. Alkaline phosphatase also progressively increased in activity and reached a maximum first in the cell layer of B subpopulations and later in C and D. Again, with serum present in the culture medium, cells from B subpopulations exhibited the earliest detectable accumulation of $^{45}Ca^{2+}$ into the cell layer, followed by C and later by D chondrocyte subpopulations.[8]

Chondrocytes cultured in serum-free DMEM supplemented with ITS do not mature

Cellular division was always observed when cells were cultured with 10% FCS, but in serum-free DMEM supplemented with ITS (at low: 75 ng/ml insulin, 75 ng/ml transferrin, and 75 pg/ml sodium selenite; or high doses: 1 μg/ml of insulin, 1 μg/ml of transferrin, and 1 ng/ml of sodium selenite) there was little or no cell division (data not shown). Without addition of ITS to the serum-free culture medium, a progressive drop in the DNA content occurred after approximately 10 days of culture (data not shown). Thus, in all the subsequent experiments, ITS was always added at the lower concentration (unless otherwise indicated) to the serum-free DMEM to ensure cell viability.

In the absence of serum, prehypertrophic C and D chondrocytes subpopulations were unable to mature and express

the hypertrophic phenotype, signified by lack of type X collagen synthesis and no increase in alkaline phosphatase activity (Figs. 1a, 2a,b,c). Moreover, mineralization reflected by $^{45}Ca^{2+}$ accumulation into forming mineral was not detected in subpopulations C and D in ITS (low: 75 ng/ml of insulin, 75 ng/ml of transferrin, and 75 pg/ml of sodium selenite; and high doses: 1 μg/ml of insulin, 1 μg/ml of transferrin, and 1 ng/ml of sodium selenite) supplemented medium (Figs. 3b and 3c).

Limited type X collagen synthesis in B cell subpopulations in the presence of ITS (at both doses) was observed (between days 4–6 of culture) only in cells isolated from fetuses older than about 210 days (Fig. 1b) and it decreased with time in culture (Fig. 1b). These type X collagen positive cultures also exhibited matrix calcification measured by $^{45}Ca^{2+}$ incorporation (Fig. 3a) although there was no increase in alkaline phosphatase activity (Fig. 2a). Type X collagen synthesis and calcification were never detected in B cell subpopulations obtained from younger fetuses (<210 days) and cultured in serum-free conditions only in ITS (either dose; data not shown).

Thyroid hormones (T_3 and T_4) induce chondrocyte hypertrophy

The addition of T_4 (5–200 nM) or T_3 (0.05–50 nM) to serum-free DMEM supplemented with ITS induced, in a dose-dependent manner, the B, C, and D chondrocyte subpopulations to mature and to express the hypertrophic phenotype characterized by expression and synthesis of type X collagen (Figs. 1a and 4), increase in alkaline phosphatase activity (Fig. 5a), induction of matrix calcification (Fig. 5b), and cell hypertrophy (Fig. 6). The expression of the hypertrophic phenotype induced by T_3 or T_4 also occurred when ITS was omitted from the serum-free DMEM, although a progressive decrease in cell viability was observed after approximatively 10–15 days of culture (data not shown). In the case of the B chondrocyte subpopulations that were already synthesizing type X collagen in cultures supplemented only with ITS there was an increase in these hypertrophic markers in the presence of T_3 or T_4 (Figs. 1b, and 3a).

In contrast to the serum-containing cultures, cellular maturation in the presence of T_3 or T_4 occurred without significant cell proliferation. B cells were again the first to mature and express the hypertrophic markers followed by the C and then the D chondrocyte subpopulations (data not shown).

T_3 is much more potent than T_4 at inducing synthesis of markers of hypertrophic phenotype and mineral formation

T_3 used at much lower concentrations than T_4 induced hypertrophy. Such differences, which were time-dependent, were noted in all B, C, and D prehypertrophic subpopulations but were most pronounced in the least mature C and D subpopulations. This is illustrated in Fig. 1a, where concentrations of T_3 as low as 50 pM were able to induce similar type X collagen synthesis as that produced by 10 nM

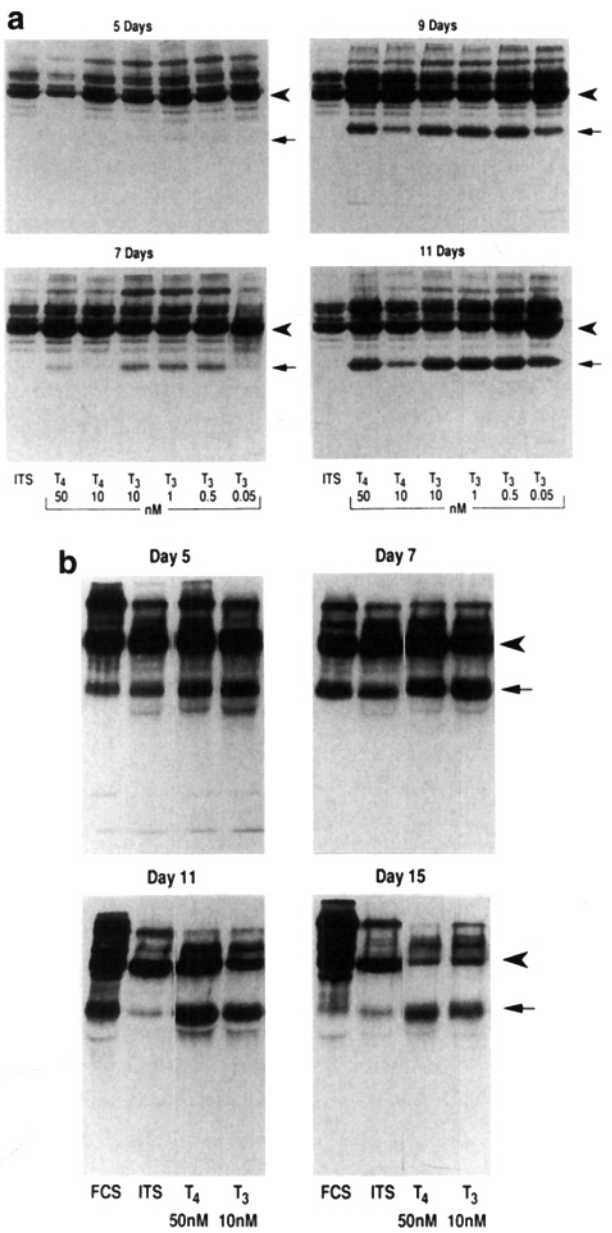

FIG. 1. Induction of type X collagen by T_3/T_4. (a) Dose response in the C subpopulation cultured with DMEM-ITS (low dose; 75 ng/ml of insulin, 75 ng/ml of transferrin, and 75 pg/ml of sodium selenite) ± increasing concentrations of T_3 or T_4. Culture media from two wells for each condition were analyzed at 5, 7, 9, and 11 days in culture after a 24 h labeling with ^{3}H-proline. Exposure time = 20 h. Fetal age = 210 days. Synthesis of type II (arrowhead) and type X (arrow) collagens are shown (b). B subpopulation cultured with DMEM-ITS (high dose; 1 μg/ml of insulin, 1 μg/ml of transferrin, and 1 ng/ml of sodium selenite) ± T_3 (10 nM), T_4 (50 nM), or 10% FCS. As standards, radiolabeled type II and type X collagens, purified by differential salt precipitation from bovine growth plate chondrocyte culture media, were run in separate lanes. Media from two wells for each culture condition were analyzed at 5, 7, 11, and 15 days after 24 h labeling period (^{3}H-Pro). In this particular experiment, $^{45}Ca^{2+}$ was first detected in B cell layers on day 7 in FCS. Exposure time = 20 h. Fetal age = 230 days.

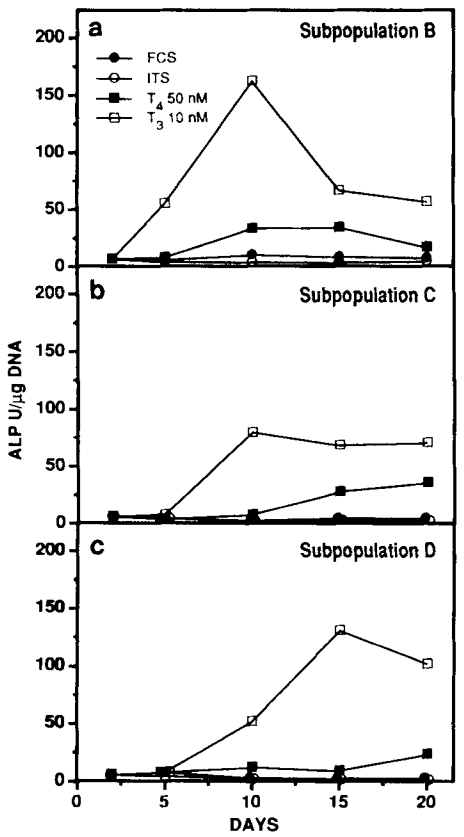

FIG. 2. Stimulation of total alkaline phosphatase activity by T_3/T_4. Activity is expressed per microgram of DNA in the cell layers. Only single concentrations for T_3 and T_4 in DMEM-ITS (high dose; 1 μg/ml of insulin, 1 μg/ml of transferrin, and 1 ng/ml of sodium selenite) and DMEM-ITS (high dose) alone, and 10% FCS are shown. The mean results of triplicate wells are shown. The standard deviation was less than 15% for each triplicate determination. Fetal age = 210 days. Same experiment as Fig. 3.

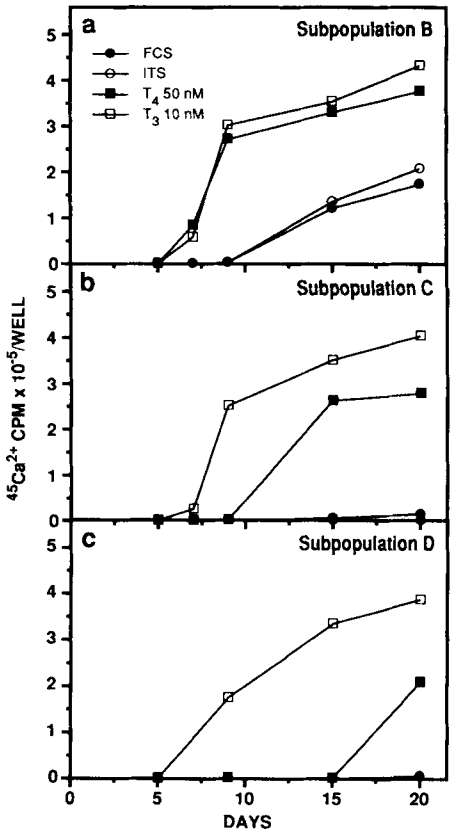

FIG. 3. Stimulation of matrix mineralization by T_3/T_4. $^{45}Ca^{2+}$ accumulation in the cell layer of isolated growth plate chondrocyte subpopulations, showing the greater potency of T_3 compared with T_4, especially in the most immature D cells subpopulation. Cells were cultured in T_3, or T_4 in DMEM-ITS (high dose; 1 μg/ml of insulin, 1 μg/ml of transferrin, and 1 ng/ml of sodium selenite), or DMEM-ITS (high dose) alone, or 10% FCS. The mean results of a single concentration of T_3/T_4 in triplicate wells are presented. The standard deviation was less than 15% for each determination. Fetal age = 210 days. Same experiment as Fig 2.

in T_4. At higher concentrations of T_3 (1–10 nM) type X collagen synthesis was induced earlier in culture than that induced by similar or higher concentrations of T_4 (10–50 nM) (Fig. 1b). Similar differences between T_3 and T_4 were also observed for type X mRNA content (Fig. 4). Moreover, alkaline phosphatase activity and $^{45}Ca^{2+}$ accumulation were also examined in the cell layers of the different chondrocyte subpopulations. At day 10 of culture, alkaline phosphatase activity was higher in the C cell subpopulation treated with 1 nM T_3 (35.5 ALP U/μg of DNA) than that supplemented with 50 nM T_4 (7.8 ALP U/μg of DNA) (Fig. 5a). Furthermore, matrix calcification, measured by $^{45}Ca^{2+}$ incorporation into forming mineral in the cell layer, always occurred earlier in cells cultured with T_3 than those treated with T_4 (Fig. 5b). The most immature subpopulation D exhibited the greatest differences, showing a lack of response to T_4 for up to 15 days of culture, whereas cells responded to T_3 at 9 or 10 days (Figs. 2c and 3c). Morphological evidence for cell hypertrophy was also more pronounced in cells cultured with T_3 than those supplemented with T_4 (Fig. 6).

In general, either T_3 or T_4 was more potent than serum in inducing the above hypertrophic markers. This is shown in Figs. 1, 2, and 3, where both hormones induced increased synthesis of type X collagen, and strongly increased alkaline phosphatase activity at an earlier time point than in FCS. Moreover, calcification occurred earlier in cultures containing either T_3 or T_4. However, this marked difference between these thyroid hormones and FCS to stimulate chondrocyte maturation was not always observed. Depending upon FCS batches, similar or slightly lesser activity than thyroid hormones could be found (data not shown). This is probably due to the different concentrations of the biological factors present in the various preparations of serum used. In particular, thyroxine (T_4) content in fetal bovine blood has been shown to vary with fetal age.[27]

Furthermore, as previously described,[8] type X collagen synthesis in chondrocytes cultured with 10% FCS was rapidly arrested once calcification was established. In contrast,

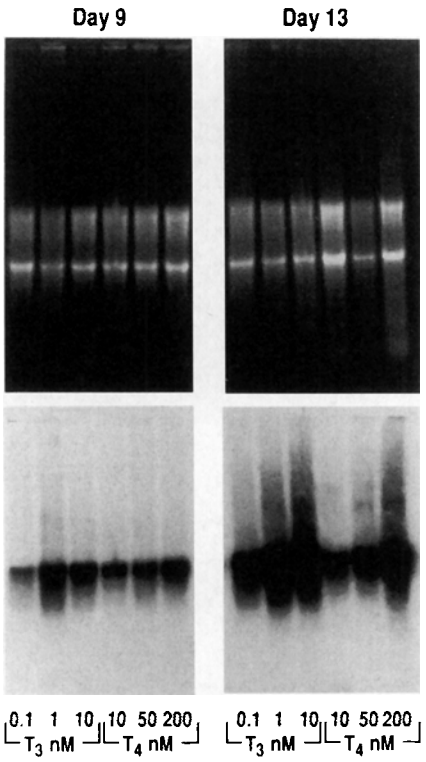

FIG. 4. Induction of type X collagen mRNA by T_3/T_4: dose response. (a) Total RNA (5 μg/track) from the B subpopulation in a formaldehyde-agarose gel (1%) stained with ethidium bromide. (b) Northern blot analyses for type X collagen mRNA of the same RNA as in (a). B chondrocyte subpopulation was cultured with DMEM-ITS alone, and with different concentrations of T_3 or T_4. Total RNA was extracted at 9 and 13 days after culture as described in Materials and Methods. Similar results were obtained with the other two subpopulations C and D (data not shown). Exposure time = 8 h. Fetal age = 220 days.

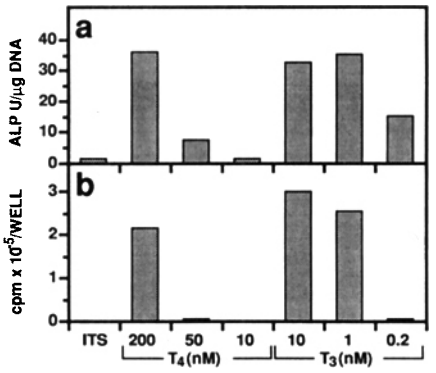

FIG. 5. Stimulation of (a) alkaline phosphatase activity and (b) induction of matrix mineralization by T_3/T_4: dose response. Results show (a) alkaline phosphatase activity in cell layer expressed per microgram of DNA and (b) counts per minute of $^{45}Ca^{2+}$ accumulation in cell layer of subpopulation C cultured with DMEM-ITS alone ± different concentrations of T_3 or T_4. The mean ± SD of triplicate wells are shown. The standard deviation was less than 15% for each triplicate determination. Fetal Age = 210 days.

chondrocytes cultured with T_3 or T_4 continued to synthesize type X collagen when calcification was initiated. It reached its maximum within 2–4 days of the initiation of mineral formation, and then it progressively decreased over a period of 7–10 days (Fig. 1b). Type II collagen synthesis, which in the presence of serum only showed a slight reduction[8] (Fig. 1b), exhibited a marked drop when thyroid hormones were present (Fig. 1b). This occurred soon after calcification started and before type X collagen synthesis began to decrease. In the experiment, shown in Fig. 1a, $^{45}Ca^{2+}$ accumulation into the cell layers was first detected at day 7.

DISCUSSION

Although serum provides nutrients and factors required for normal growth and the metabolic activities of cultured cells, we know comparatively little about the hormones and growth factors required for the maturation of growth plate chondrocytes. In vitro studies which ordinarily use mixtures of growth plate chondrocytes have produced conflicting results.[2,3] This is because the responses of chondrocytes from different regions of a growth plate are dependent upon their maturational state. Mixed cultures contain varying proportions of cells in different maturational and responsive states. Thus, if we are to study growth plate maturation in vitro, maturationally distinct and defined cell subpopulations (resting, proliferation, maturing, and hypertrophic zones) of the physis must first be separated into more homogeneous populations. This has recently been possible by using discontinuous Percoll gradient centrifugation.[8] In conjunction with high density cell cultures, we have shown that maturationally distinct fetal bovine growth plate chondrocytes can undergo a complete maturation culminating in the expression of a hypertrophic phenotype in medium supplemented with 10% FCS.[8] This occurs after different periods of culture according to the maturational state of the freshly isolated cells.[8]

Here we show that we can establish high density cell cultures under serum-free conditions in DMEM-ITS. Ordinarily these cells when they are of the prehypertrophic phenotype on isolation cannot mature and express hypertrophy. But we found that cells of the prehypertrophic B subpopulation from older fetuses (≥210 days) matured and synthesized type X collagen in medium DMEM-ITS alone (Fig. 1b), indicating that they had reached a maturational stage that no longer required further stimulation for continued maturation to hypertrophy. This behavior may be related to the fact that the mean final cell heights of the hypertrophic cells (and by inference their cellular volume) may decrease progressively with increasing age of the fetuses as previously described in rat growth plates.[28] Consequently, during the separation procedure (Percoll gradient centrifugation) a higher proportion (increasing with increased age of the fetus) of hypertrophic chondrocytes that exhibit a smaller cellular volume may be collected at

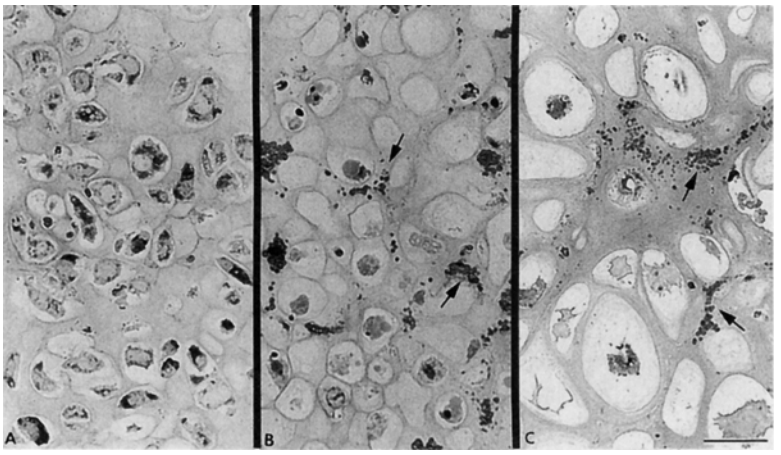

FIG. 6. Chondrocyte morphology and von Kossa's staining of the mineral deposited in the extracellular matrix (arrow) in the presence and absence of T_3/T_4. After 18 days in culture with 50 nM T_4 (B), or 10 nM T_3 (C), B cells have enlarged and differentiated into large hypertrophic chondrocytes which calcify their matrix. Note the more pronounced cellular enlargement (hypertrophy) of cells treated with T_3 (C) than those treated with T_4 (B). Cells cultured with DMEM-ITS alone did not enlarge nor calcify their matrix (A). Fetal age = 210 days. Bar = 25 μm.

the same gradient position as that of prehypertrophic cells from younger fetuses.

To maintain these serum-free cultures we used a mixture of insulin, selenium, and transferrin. Surprisingly and in contrast with previous reports,[14,29] under these conditions, insulin at physiologic (75 ng/ml) or higher (1 μg/ml) doses was only slightly mitogenic over a period of 20 days of culture. This difference could be due to species variation or differences in culture techniques such as the use of monolayers (used here) versus suspension cultures[16] or high density (used here) versus nonconfluent cultures.[29] Furthermore, insulin has been shown to exert a biphasic response on chondrocyte proliferation.[29] Thus the two extreme concentrations used here could indeed be only slightly mitogenic.

In our serum-free cultures, either of these hormones (T_3 or T_4) could stimulate maturation of prehypertrophic cell subpopulations (in particular C and D) into a fully hypertrophic chondrocyte subpopulation leading to matrix calcification. This observation confirms and significantly extends previous reports that T_3[15,16,19] and T_4[17,18] can stimulate maturation of growth plate chondrocyte to express type X collagen and alkaline phosphatase. However, unlike in these earlier studies, we now show that cellular maturation was complete in that matrix calcification was also observed. We also show for the first time that T_3 is much more potent than T_4. Such direct comparisons were surprisingly never made previously.

At all the concentrations tested, T_3 was a more potent inducer than T_4 of hypertrophic expression, characterized by earlier and increased expression type X collagen mRNA, type X synthesis, increased alkaline phosphatase activity, and earlier mineral formation in cell layers. These changes occurred in the presence of ITS without cell proliferation, as observed previously.[17]

The low potency of T_4 may be due to its low binding affinity to the nuclear thyroid hormone receptors.[27] Moreover, a significant amount of T_4 has been shown to be metabolically converted to T_3 in peripheral blood tissue cells by deiodination of T_4 in the 5′ position to generate T_3.[14] Although it remains to be established, such a conversion in chondrocytes could explain the delayed effect of T_4 compared with T_3, particularly in the most immature cells subpopulations C and D.

The several-fold increase in the expression of the hypertrophic markers by T_3 and T_4 in serum-free cultures over those observed with serum may be related to the complexity of the biological effectors present within serum compared with the single signal in thyroid hormone serum-free cultures. Recently, Ballock and Reddi[18] reported that by decreasing the concentration of FCS added to the culture medium, chondrocytes became organized in a columnar pattern remarkably similar to that of developing growth plate cartilage in vivo. Furthermore, Tschan et al.[30] have shown that synthesis of type X collagen declines (unlike type II collagen) with increasing chondrocyte density as a consequence of a possible increase in the production of autocrine factors by chondrocytes (in this case TGF-β_2) which interfered with cell hypertrophy. This inhibitory effect of TGF-β on growth plate chondrocyte maturation was earlier reported by Kato et al.[31] Thus the cocktail of factors present in serum, in addition to the ability of cells to produce autocrine factors inhibitory of chondrocyte maturation, such as TGF-β, may negate the stimulatory effect on growth plate chondrocyte maturation of thyroid hormones, which are also present in fetal calfs serum.[19,27] In contrast, in serum-free cultures T_3 and T_4 can induce and accelerate chondrocyte hypertrophy in the absence of exogenous antagonists.

This is also reflected by the different pattern of collagen synthesis observed in thyroid hormone–stimulated cultures compared with that observed in serum where type X collagen synthesis drops soon after calcification is initiated.[8] In contrast, in chondrocytes cultured in serum-free medium with thyroid hormones, calcification was accompanied by increased synthesis of type X collagen, reaching its maximum 2–4 days after first mineral deposition; thereafter it gradually decreased. Also, in serum-free media, thyroid hormones caused type II collagen synthesis to drop soon after calcification started, in contrast to the serum-containing cultures where synthesis persisted. The profiles of collagen type II and collagen type X synthesis observed in thyroid hormone containing serum-free cultures reflect more closely the pattern followed by these proteins in situ.

Type II collagen mRNA is at its highest concentration when cells first synthesize type X collagen in maturing and upper hypertrophic regions, then it is progressively reduced.[32–35] In the same region, initiation of an extensive cleavage of type II collagen occurs.[2] In contrast, at both mRNA and protein levels type X collagen shows no net loss from the hypertrophic zone.[33,34,36–38] Thus, the serum-free conditions generated with thyroid hormones more accurately represent the in vivo situation.

Furthermore, although either thyroid hormone is able to induce prehypertrophic growth plate chondrocyte to express fully the hypertrophic phenotype, the lack of cell division with T_3 or T_4 implies that for this maturation process to occur in vivo additional biological factors are required. In fact in mammalian primary growth plates chondrocyte hypertrophy follows cell proliferation which with cell enlargement is responsible for the longitudinal growth of the long bones. Thus other factors are likely necessary in the early events of the primary growth plate maturation to induce resting chondrocytes to differentiate and proliferate in their typical columnar arrangement. Later, in prehypertrophic development, thyroid hormones possibly arising by diffusion from the invading capillaries of the lower hypertrophic zone could initiate cell hypertrophy.

The establishment of this serum-free culture system containing T_3 or T_4 for the study of chondrocyte maturation, matrix assembly, and calcification offers the possibility to investigate the properties of hormones and cytokines under more defined conditions in a manner not previously possible. Collectively these results demonstrate that T_3 and T_4 are sufficient to drive prehypertrophic chondrocytes at different prehypertrophic stages of maturation into fully differentiated hypertrophic cells leading to matrix calcification in vitro. Furthermore, they demonstrate the much greater potency of T_3 compared with T_4 as an inducer of chondrocyte hypertrophy. This striking distinction in potency between these two thyroid hormones has never before been shown. This information may be of help in evaluating their use in the treatment of thyroid pathology.

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