Thyroid Hormone, Insulin, and Glucocorticoids Are Sufficient to Support Chondrocyte Differentiation to Hypertrophy: A Serum-free Analysis

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Abstract. Chondrocytes from chicken embryo tibia can be maintained in culture as adherent cells in Coon's modified Ham's F-12 medium supplemented with 10% FCS. In this condition, they dedifferentiate, losing type II collagen expression in favor of type I collagen synthesis. Their differentiation to hypertrophy can be obtained by transferring them to suspension culture. Differentiation is evidenced by the shift from type I to type II and type IX collagen synthesis and the following predominant expression of type X collagen, all markers of specific stages of the differentiation process. To identify the factors required for differentiation, we developed a serum-free culture system where only the addition of triiodothyronine (T_3; 10^{-11} M), insulin (60 ng/ml), and dexamethasone (10^{-9} M) to the F-12 medium was sufficient to obtain hypertrophic chondrocytes. In this hormonal context, chondrocytes display the same changes in the pattern of protein synthesis as described above. For proper and complete cell maturation, T_3 and insulin concentrations cannot be modified. Insulin cannot be substituted by insulin-like growth factor-I, but dexamethasone concentration can be decreased to 10^{-12} M without chondrogenesis being impaired. In the latter case, the expression of type X collagen and its mRNA are inversely proportional to dexamethasone concentration. When ascorbic acid is added to the hormone-supplemented medium, differentiating chondrocytes organize their matrix leading to a cartilage-like structure with hypertrophic chondrocytes embedded in lacunae. However, this structure does not present detectable calcification, at variance with control cultures maintained in FCS. Accordingly, in the presence of the hormone mixture, the differentiating chondrocytes have low levels of alkaline phosphatase activity. This report indicates that T_3 and insulin are primary factors involved in the onset and progression of chondrogenesis, while dexamethasone supports cell viability and modulates some differentiated functions.

In vertebrates, chondrogenesis and subsequent endochondral calcification originate from a complex differentiation process involving the maturation of mesenchymal prechondrogenic cells and the continuous synthesis and remodeling of the surrounding extracellular matrix. It has been postulated that the interrelationship between some environmental factors and various systemic elements present in the serum controls the development and possibly the maintenance of cartilage phenotype expression (Reddi, 1982). Cell-cell and cell-matrix interactions represent the local factors most studied and best understood (Hewitt et al., 1980; Weiss and Reddi, 1980; Solursh, 1982; Solursh et al., 1984). Among the various serum components, endocrine agents are believed to have a major developmental role. In vivo studies and clinical surveys clearly indicate that excess or deficiency in most hormones and/or some growth factors affects statural growth (Silberman, 1983; Underwood and van Wyk, 1985). With the development of various in vitro models including organ culture, primary culture of isolated cells, and established cell lines, most emphasis has been given to factors such as growth hormone, thyroid hormones, glucocorticoids, sex steroids, insulin, and somatomedins (Burch and Lebovitz, 1982; Kato and Gospodarowicz, 1985; Grigoriadis et al., 1989; Maor et al., 1989; Itagane et al., 1991). All these in vitro systems in conjunction with the establishment of better defined culture media and serum substitutes have allowed a more accurate approach to the role of these factors on particular aspects of cartilage metabolism or on the maintenance of particular differentiated functions. However, the factors required for and directly controlling the onset of chondrogenesis have yet to be identified. To address this question, this study was carried out with the system of dedifferentiated chondrocytes (i.e., cartilage cells originally differentiated and modulated by a specific culture regimen) that we have developed in the last few years, which reproduces in vitro the major events of the physiological process of hypertrophic cartilage development (Castagnola et al., 1986). In this system dedifferentiated cells, characterized by a high proliferative rate and the synthesis of type I collagen, mature and reach terminal differentiation passing from stage I chondrocytes (producing type II and type IX collagens) to stage II chondrocytes (characterized by hypertrophy and high expression of type X collagen and Ch21 protein) (Castagnola et al., 1988; Dozin et al., 1992). This differentiation model

originally required the presence of 10% FCS in the medium. We herein demonstrate that serum can be substituted by a defined hormonal mixture in which dedifferentiated cells fully develop, reaching hypertrophy and organizing a cartilage-specific matrix. In this process, triiodothyronine (T_3)[1] and insulin have a predominant differentiative effect, while dexamethasone (Dex) modulates the expression of some acquired functions.

Materials and Methods

Reagents

FCS was from Flow Laboratories (Irvine, Ayshire, Scotland). Hybond-N membranes, the random primed labeling system, [^{35}S]methionine (10 mCi/ml), and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK). Ascorbic acid, T_3 and Dex were from Sigma Chemical Co. (St. Louis, MO). Insulin and insulin-like growth factor I (IGF-I) were purchased from Collaborative Research (Bedford, MA). The genomic clone pXCR7 for ribosomal RNAs was a gift from Dr. F. Amaldi (Universita' Tor Vergata, Rome, Italy). The cDNA pCIII8 for α1 type X collagen has been described previously (Castagnola et al., 1987).

Cell Culture

Cell culture methods have been extensively described elsewhere (Castagnola et al., 1986). Briefly, primary cultures of chondrocytes were prepared from 6-d-old chick embryos (Hamburger and Hamilton, 1951; stage 28–30) by trypsin/collagenase digestion of tibiae. Dedifferentiated cells were obtained by culturing these freshly dissociated chondrocytes in adherent conditions on regular plastic dishes for 3 wk. Differentiation was then studied after the transfer of fully dedifferentiated cells to suspension culture on agarose-coated dishes. Differentiating chondrocytes were routinely plated at a starting density of 0.5–1 $\times$ 10^6 cells/ml. Culture medium was Coon's modified Ham's F-12 (Ambesi-Impiombato et al., 1980) supplemented with either 10% FCS (control) or a mixture of T_3 (10^{-11} M), insulin (60 ng/ml), and/or Dex (10^{-9}–10^{-12} M). These values are the effective concentrations resulting after filtration of the medium as estimated by radioimmunoassay. They are also comparable to the hormonal concentrations present in FCS (which are, as kindly communicated by Flow Laboratories: insulin, 0.3 μInt.U/ml with a conversion factor of 26 mU/mg; cortisol, 0.082 μg/dl; T_3, 1.2 ng/ml). In some experiments, insulin was substituted by 4–40 ng/ml of IGF-I, the concentrations of which were not corrected for filtration loss. For reconstitution of hypertrophic cartilage in vitro, cells were grown in suspension culture in medium daily supplemented with 100 μg/ml of ascorbic acid.

Cell Metabolic Labeling and SDS-PAGE of Secreted Proteins

Cultured cells were extensively washed in phosphate saline buffer, transferred to methionine-free Coon's modified Ham's F-12 medium supplemented with 0.1% FCS and 50 μg/ml of ascorbic acid, and incubated for 2 h at 37°C. [^{35}S]Methionine was added at a concentration of 100 μCi/ml and the incubation was resumed for 2 h. Supernatants were collected, clarified by low speed centrifugation, dialyzed against 0.5 N acetic acid, and digested overnight at 4°C with 100 μg/ml of pepsin. The digestion products were analyzed by electrophoresis in reducing conditions on 12.5% polyacrylamide gels.

Northern Blot Analysis

Total RNA was extracted from cultured chondrocytes by the guanidinium isothiocyanate/CsCl method of Chirgwin et al. (1979). Formaldehyde-denatured RNA was electrophoresed through 1% agarose gel and blotted by capillary transfer onto Hybond-N membrane. The blot was hybridized with the cDNA insert of the clone pCIII8 labeled by random priming to a specific activity of 1.5 $\times$ 10^9 cpm/μg DNA. Hybridization and washing conditions were as recommended by Amersham. After hybridization, the same blot was rehybridized with the probe pXCR7 to assess the amount of RNA loaded on each lane.

1. *Abbreviations used in this paper*: Dex, dexamethasone; IGF-1, insulin-like growth factor-I; T_3, triiodothyronine.

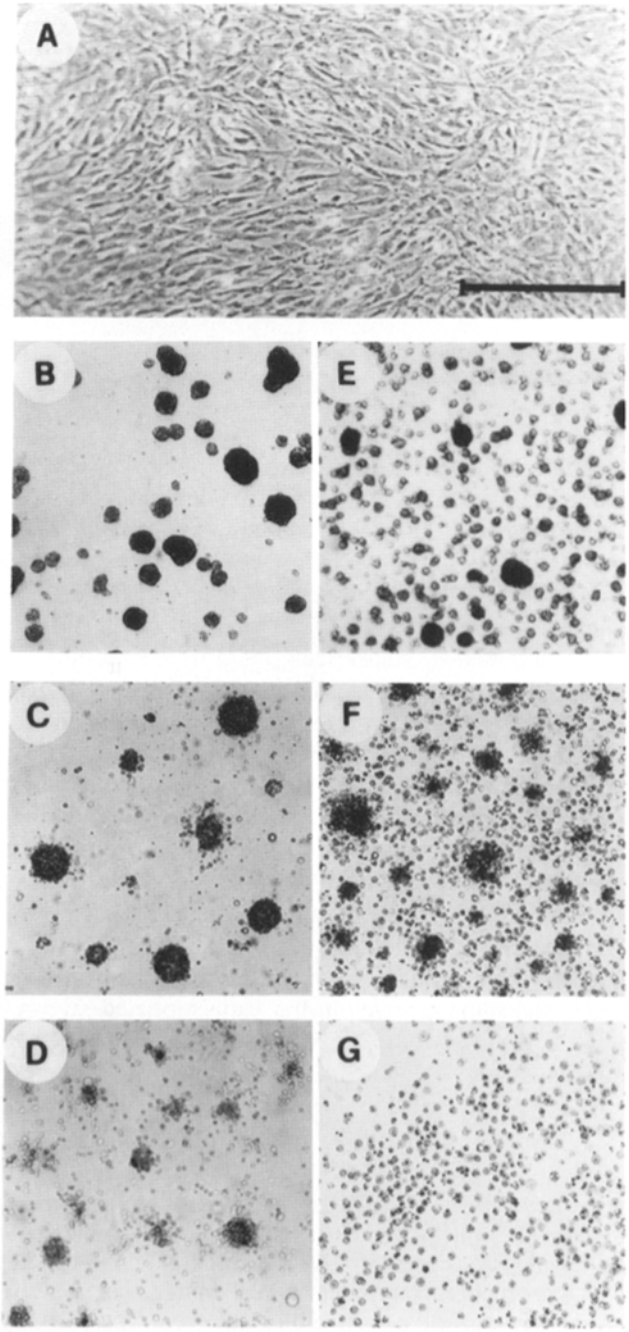

Figure 1. Comparative morphology of tibia chondrocytes differentiating in serum- or hormone-containing medium. (*A*) Starting confluent culture of dedifferentiated cells grown as a monolayer in the presence of 10% FCS. (*B–D*) Differentiation of adherent cells after transfer to suspension culture in the presence of 10% FCS. Times of culture were 24 h (*B*), 1 wk (*C*), and 2 wk (*D*). (*E–G*) Same sequence of cultures as in *B–D*, except that the serum was substituted with T_3 (10^{-11} M), insulin (60 ng/ml), and Dex (10^{-9} M). Bar: (*A*) 200 μm; (*B–G*) 500 μm.

Histology

Histological stainings were performed on paraffin-embedded cellular aggregates obtained by suspension culture in the constant presence of ascorbic acid. Serial 4-μm-thick sections were stained with Toluidine blue for cartilage structure or Alizarine red S for calcium deposition.

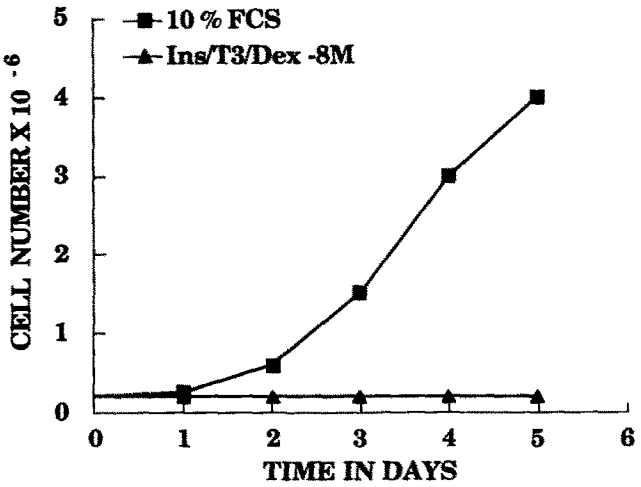

Figure 2. Growth curves of tibia chondrocytes maintained as monolayer in serum- or hormone-containing medium. Primary chondrocytes were expanded for 3 wk as a monolayer in the presence of 10% FCS. They were then either maintained in the same medium or passed to a serum-free medium supplemented with 10^{-11} M T_3, 60 ng/ml insulin, and 10^{-9} M Dex. The growth curves were determined by plating 7×10^3 cells/cm^2 at time 0 and counting the resulting cells at the time intervals indicated. All determinations were done in triplicate.

Determination of Alkaline Phosphatase Activity

Alkaline phosphatase activity was assayed as described previously (Tacchetti et al., 1989) on cells grown as a monolayer or on aggregates maintained in suspension culture in the presence of ascorbic acid.

Results

We have previously reported that dedifferentiated chondrocytes from chicken embryo tibia can undergo complete differentiation in vitro when they are cultured in suspension in a medium supplemented with 10% FCS. The following data demonstrate that in serum-free conditions, chondrocyte hypertrophy and cartilage matrix organization can be reached with the addition of T_3, insulin, and Dex.

The starting cell population consisted of dedifferentiated fibroblast-like cells obtained by culturing tibia chondrocytes freshly dissociated from a 6-d-old chick embryo for at least 3 wk in anchorage-dependent conditions (Fig. 1 *A*). To promote differentiation, this population was transferred in suspension culture either in 10% FCS (Fig. 1, *B–D*) or serum-free F-12 medium supplemented with 10^{-11}M T_3, 60 ng/ml insulin, and 10^{-9} M Dex (Fig. 1, *E–G*). Within 24 h, all cells were recruited into aggregates in both culture conditions (Fig. 1, *B* and *E*). Cells then reverted to the chondrogenic phenotype and resumed their differentiation as evidenced by the progressive "flourishing" (i.e., the initial opening) of the aggregates and the release into the medium of single hypertrophic cells. This morphological maturation was clearly detectable within 1 wk of culture (Fig. 1, *C* and *F*) and nearly complete by the end of the second week (Fig. 1, *D* and *G*). The overall process was essentially identical in the two culture media, except that in the hormonal context the size of the aggregates was smaller and complete hypertrophy of the cells was achieved faster.

One may argue that the maturation was not impeded or altered in the presence of the hormones because the starting population of dedifferentiated cells had been grown in 10% FCS and had therefore been exposed to serum factors already committing the cells toward differentiation. This possibility seems unlikely as we observed that dedifferentiated cells maintained in the hormone-containing medium presented an initial fibroblast-like phenotype and a subsequent maturation pattern identical to those illustrated in Fig. 1 (data not shown). Also, the protein profile of the adherent cells was not modified by the substitution of the serum with the hormones. In either medium, the chains α1 and α2 of type I collagen remained the major products of synthesis (Fig. 3, lanes *1* and *2*). Interestingly, the hormonal mixture allowed full survival of the adherent cells but failed to support their growth while the same cells rapidly replicated in the presence of 10% FCS with the characteristic doubling time of 16–18 h (Giaretti et al., 1988) (Fig. 2).

To evaluate the impact of each hormonal factor on the differentiation process, we varied the composition of the supplement and monitored the resulting morphology of the cells and the proteins they expressed. In the latter case, we focused on the expression of type II collagen as an indication

Table I. Morphological and Biochemical Response of Differentiating Chondrocytes to the Hormonal Content of the Culture Medium

	1	2	3	4	5	6	7	8	9	10	11	12	13
Insulin (ng/ml)	60	6	0.6	–	–	60	60	60	60	60	60	60	60
IGF-I (ng/ml)	–	–	–	–	4–40	–	–	–	–	–	–	–	–
T_3 (M)	10^{-11}	10^{-11}	10^{-11}	10^{-11}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	–	10^{-11}	10^{-11}	10^{-11}	10^{-11}
Dex (M)	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	–
Aggregation	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell survival	++	+/–	–	–	–	++	+	+/–	–	++	++	++	+/–
Flourishing aggregates	4 d	7 d	–	–	–	4 d	7 d	10 d	–	4 d	4 d	4 d	7 d
Hypertrophy	++	+	–	–	–	++	+	+/–	–	++	++	++	++
Type II collagen	7 d	10 d	–	–	–	7 d	10 d	15 d	–	7 d	7 d	7 d	10 d
Type X collagen	20 d	–	–	–	–	–	–	–	–	15 d	10 d	10 d	15 d

Aggregation and cell survival were monitored by light microscopy and compared with a parallel suspension culture maintained in 10% FCS. Cell hypertrophy refers to complete opening of the aggregates and release in the medium of single hypertrophic chondrocytes. For flourishing aggregates, type II and type X collagens, the table indicates the number of days necessary after passage of the cells from monolayer to suspension culture to observe maximal detection of these parameters. The levels of synthesis of type II and type X collagens were assessed by SDS-PAGE of the proteins secreted by the chondrocytes at the time indicated.

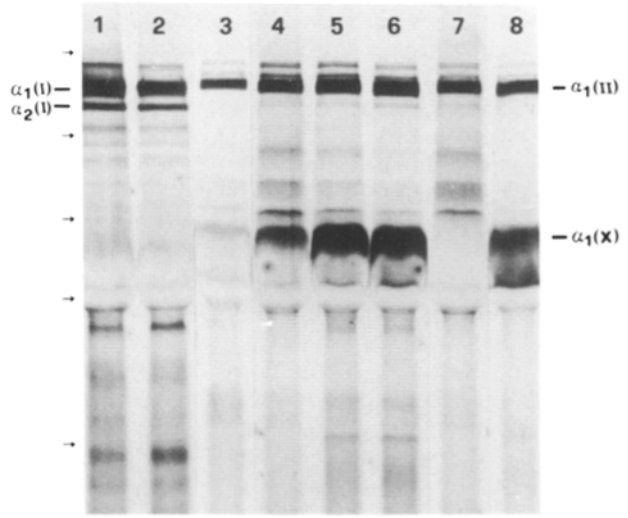

Figure 3. Electrophoretic profile of proteins secreted by dedifferentiated cells and maturating chondrocytes grown in serum- or hormone-containing medium. Proteins metabolically labeled with [^{35}S]methionine were analyzed on 12.5% SDS-PAGE after limited pepsin digestion. Equal amounts of counts were loaded on each lane. Culture conditions: (lanes *1* and *2*) dedifferentiated adherent cells; (lanes *3–8*) differentiating chondrocytes maintained for 15 d in suspension culture. Medium compositions: (lanes *1* and *8*) control cultures in 10% FCS; (lanes *3–6*) cultures in 10^{-11} M T_3, 60 ng/ml insulin, and decreasing concentrations of Dex as 10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12} M, respectively; (lane *7*) same as lane *3*, except that insulin was decreased to 6 ng/ml. Arrows on the left refer to the migration of the molecular mass markers as follows from top to bottom: 200, 92, 69, 46, 30, and 14 kD.

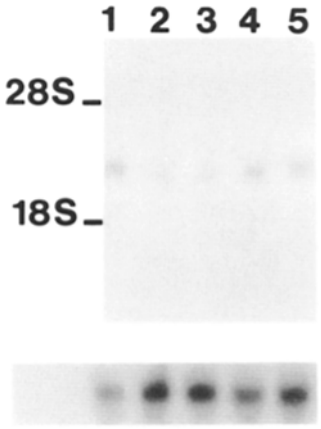

Figure 4. Effect of Dex concentration on the level of type X collagen mRNA in differentiating chondrocytes. Total RNA was extracted from cells maintained in suspension culture for 15 d in medium containing 10^{-11} M T_3, 60 ng/ml insulin, and decreasing concentrations of Dex as 10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12} M (lanes *2–5*), respectively. A control culture grown in 10% FCS is shown in lane *1*. Aliquots of 3 μm RNA were loaded on each lane. Numbers on the left indicate migration of the rRNAs. At the bottom, the 28S rRNA region of the same filter after rehybridization with the probe pXCR7 for rRNA is shown.

of ongoing chondrogenesis and of type X collagen as a marker of cell hypertrophy. Table I summarizes all the parameters considered, while Fig. 3 presents the most significant profiles of protein synthesis. The first column of the table corresponds to the hormone concentrations used in Fig. 1 and serves as a reference. In any culture condition, the initial step of recruitment of the cells into aggregates occurred typically within 24 h. The survival of the culture did not exceed 3–4 d when insulin or T_3 was omitted (columns 4 and 9) or if insulin was substituted by IGF-I (column 5). The cells would start undergoing chondrogenesis, although more slowly, when the concentration of insulin or T_3 was gradually lowered (columns 2, 3, 6–8), but the overall survival was affected and the hypertrophic stage (type X collagen synthesis) would not be reached even in longer-term cultures. A protein profile typical of any of the conditions just described is presented in lane *7* of Fig. 3, where type II, but not type X, collagen was detected. In the absence of Dex, the viability of the cells was reduced right after transfer to suspension culture, but the remaining population eventually matured to hypertrophic chondrocytes producing type X collagen (column 13). The effect of dexamethasone on the expression of this collagen was interesting. As shown in Fig. 3, at 15 d of suspension culture the level of synthesis of the protein was inversely proportional to the concentration of the hormone (lanes *3–6*). However, Dex would not inhibit but rather delay the time of appearance of the protein: indeed, at the highest hormonal dose (10^{-9} M), maximal expression was achieved after 20 d of culture while comparable levels were reached earlier (10–15 d) when lower concentrations of 10^{-11}–10^{-12} M were used (Table I, columns 1 and 10–12). By comparison, the other marker of ongoing differentiation, type II collagen, was not affected by the concentration of the glucocorticoid. It is also worth mentioning that the morphological maturation of the cells to hypertrophy proceeded normally regardless of the dose of Dex used.

The Northern blot analysis presented in Fig. 4 provides evidence that the modulation of type X synthesis by Dex occurs at a pretranslational level. At lower concentrations of the hormone, higher amounts of type X collagen mRNA were detected by hybridization with the specific cDNA.

In a previous study we demonstrated that dedifferentiated cells cultured in 10% FCS and the constant presence of ascorbic acid, which is an obligatory cofactor of collagen hydroxylases required for the correct tridimensional assembly of collagen fibrils, do not evolve into isolated hypertrophic chondrocytes but develop into a tissue strongly resembling hypertrophic cartilage as seen in vivo (Tacchetti et al., 1987). We also reported that this structure could calcify and show increased levels of alkaline phosphatase activity (Tacchetti et al., 1989). The histological sections presented in Fig. 5, *A* and *C* show that when chondrocytes differentiated in the presence of T_3, insulin, and Dex (Fig. 5 *C*), a cartilage structure similar to the one obtained in serum-supplemented medium (Fig. 5 *A*) was reconstituted. In either culture condition, the hypertrophic chondrocytes appeared embedded in lacunae and surrounded by an Alcian blue positive matrix, denoting a high production of proteoglycans (not shown). As already mentioned in Fig. 1, the hormone culture differed from the control only by the size of the aggregates. When the sections were stained with Alizarine red S, no calcium deposition was detected in the structures formed in the hormone medium (Fig. 5 *D*) while the control aggregates grown in FCS presented a high positive reaction (Fig. 5 *B*). In view of the absence of calcification, the levels of alkaline phosphatase activity were then assessed in both types of aggregates (Fig. 6). As expected, the control aggregates differentiated in FCS presented increasing levels of enzyme activity comparable to those reported previously (Tacchetti et al., 1989). By contrast, the level of alkaline phosphatase detected in the aggregates formed in the presence of the hormones reached at the most 25% of the control

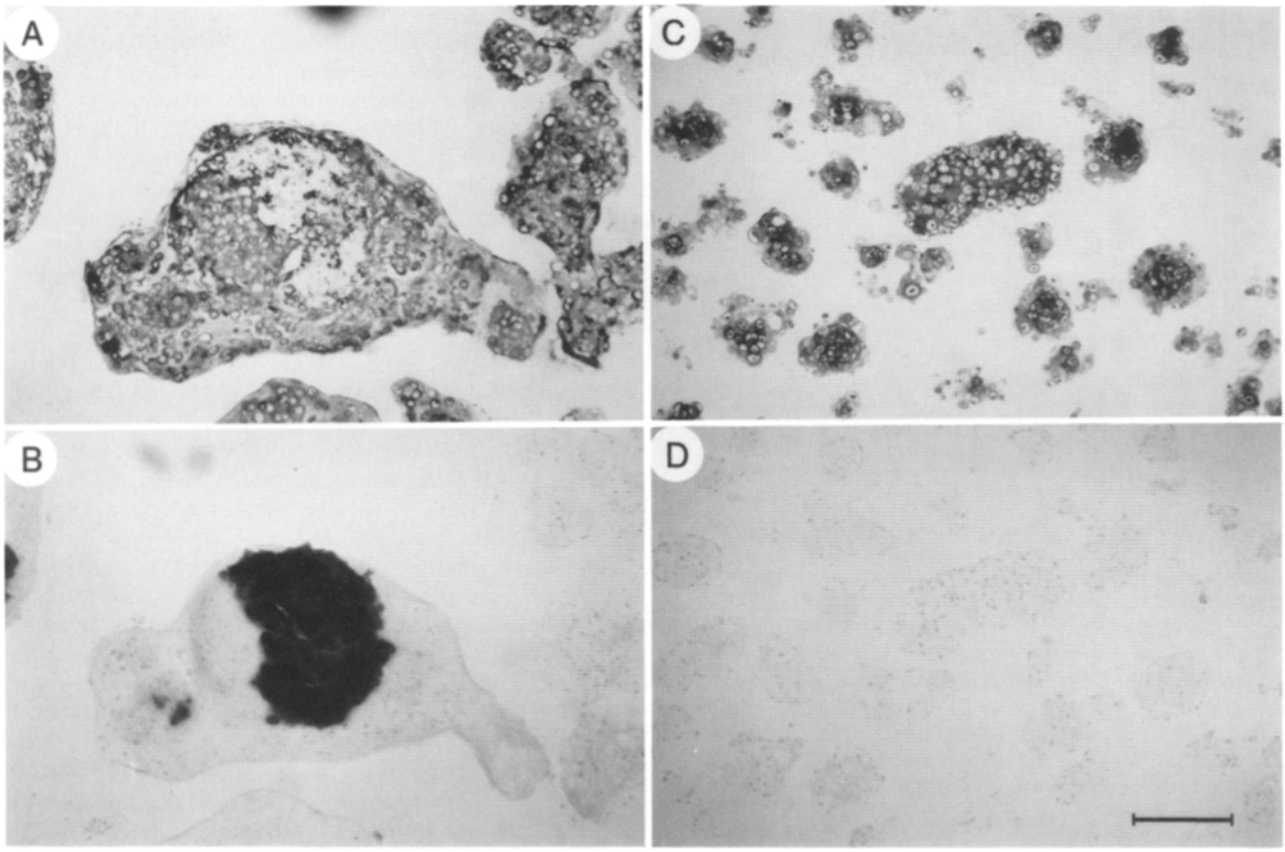

Figure 5. Histological sections of in vitro reconstituted cartilage. Dedifferentiated cells were transferred to suspension culture and maintained for 14 d either in medium containing FCS (*A* and *B*) or in the presence of 10^{-11} M T_3, 60 ng/ml insulin, and 10^{-9} M Dex (*C* and *D*). Ascorbic acid was added daily to the cultures. The cell aggregates were embedded in paraffin and 4-μm serial sections were stained either with Toluidine blue (*A* and *C*) or Alizarine red S (*B* and *D*). Images comparable to those shown in *C* and *D* were obtained when the concentration of Dex was decreased to 10^{-12} M. Bar, 100 μm.

values. Changes in the concentration of Dex did not increase the calcification. The aggregates remained negative with the Alizarine stain (data not shown) and the alkaline phosphatase activity remained close to basal level whether 10^{-9} or 10^{-12} M concentration of Dex was used in the culture medium (Fig. 6).

Discussion

During cartilage differentiation and calcification, a series of morphological and concomitant biochemical events occurs whose sequence is controlled by systemic factors still largely unknown. In a few reports, chemically defined media have been used to approach the basic requirement for chondrocytes to proliferate, mature, and maintain differentiated properties. Glaser and Conrad (1984) succeeded in maintaining chondrocytes in a proliferative state in a defined mixture of hormones and growth factors, but observed that the cells would eventually lose their chondrogenic phenotype. Other authors presented evidence for an autocrine potential that chondrocytes may have on their own growth and differentiation but cell hypertrophy and type X collagen expression could not be obtained without the presence of additional factors provided by the serum (Bruckner et al., 1989; Tschan et al., 1990). A more extended study was performed by Kujawa et al. (1989) starting from chick limb mesenchymal cells where prechondrogenic cells were shown to proliferate and differentiate in a hormonally controlled medium. However, in these conditions differentiation could not proceed beyond the stage of proliferating, type II collagen-synthesizing chondrocytes. Therefore, the purpose of the present work was to develop a chemically defined medium that would support the complete maturation of chicken embryo tibia chondrocytes up to the hypertrophic stage. We here demonstrate that primary cells first dedifferentiated as a monolayer revert to the chondrocyte phenotype when they are transferred to suspension culture in a serum-free medium supplemented with physiological concentrations of T_3 (10^{-11} M), insulin (60 ng/ml), and Dex (10^{-9}–10^{-12} M). Chondrogenesis is evidenced by the initial aggregation of the cells, a process reminiscent of the transient condensation which occurs in vivo in the center of the limb buds (Thorogood and Hinchliffe, 1975), the shift from type I to type II collagen synthesis, the cell maturation to the stage of hypertrophic chondrocytes highly expressing type X collagen, and the organization of the extracellular matrix, in the presence of ascorbic acid, in a structure–rich in proteoglycans, but not calcified–resembling the hypertrophic cartilage in vivo. Among the three hormones, T_3 and insulin are necessary for determining proper chondrogenesis while Dex mostly supports cell viability and modulates the expression of one of the major differentiated functions, the

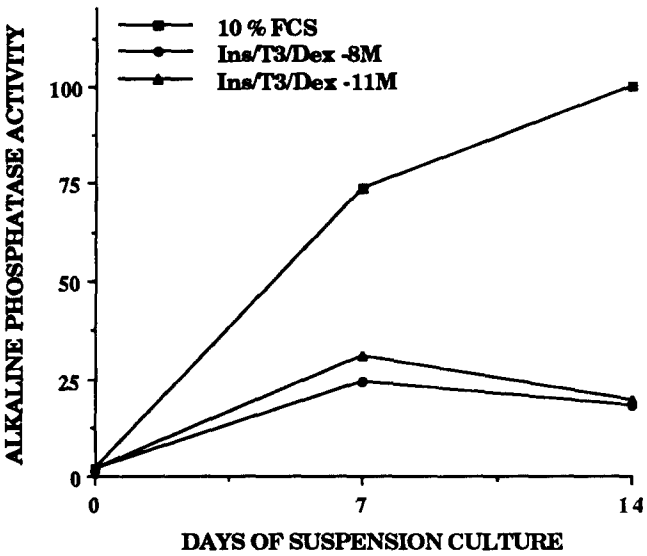

Figure 6. Levels of alkaline phosphatase activity in chondrocytes differentiating in medium containing serum or hormones. The enzyme activity was determined in dedifferentiated adherent cells (starting basal level) and in aggregated chondrocytes differentiated in suspension culture for 7 and 14 d in the different media indicated in the figure. Concentrations of T_3 and insulin were 10^{-11} M and 60 ng/ml, respectively. The suspension cultures were daily supplemented with ascorbic acid. All values were equalized for the content of DNA of the samples and normalized to the 14-d value in FCS taken as 100.

synthesis of type X collagen, without impeding the differentiating cells to fully reach hypertrophy. The question as to whether the influence of Dex on the culture survival affects maturating chondrocytes more than dedifferentiated cells cannot be clearly answered. The latter hypothesis might be preferred since the cartilage-specific phenotype of hypertrophic chondrocytes was obtained and the integrity of the extracellular matrix was preserved regardless of the presence of Dex in the medium.

Thyroid hormones are known to have a wide range of pleiotropic effects on development and cell differentiation and/or metabolism. This work clearly demonstrates that chondrocytes are also direct targets for the hormones that appear to play a determinant role in the onset and progression of chondrogenesis. Without T_3 no differentiation occurs, and as the concentration of T_3 is lowered the overall maturation is delayed and the hypertrophic stage characterized by the synthesis of type X collagen is never reached. Instead, cell differentiation seems to be blocked along the process at the step of stage I chondrocyte producing essentially type II collagen. The mechanisms by which thyroid hormone may affect the maturation of cartilage have long remained obscure. In vivo and in vitro studies have led to controversial conclusions, some proposing an indirect action through either an interference with the synthesis, secretion, or action of growth hormone (Burstein et al., 1979; Lewinson et al., 1989), or through the production of IGF-I (Burstein et al., 1979; Burch et al., 1986), others suggesting instead a direct effect of the hormones on chondrocytes (Burch and Lebovitz, 1982; Burch and Van Wyk, 1987).

Regarding IGF-I and somatomedins in general, much attention has been paid recently to these factors and their potential growth-promoting effect on various tissues. Besides their hepatic origin, they are also believed to be locally produced by target cells in response to growth hormone. Chondrocytes of embryonal rat tibia are among those cells (Schlechter et al., 1986). Since our attempt to reduce the insulin content in the medium or to substitute the hormone with IGF-I failed to support the differentiation of chondrocytes, we believe that insulin can act directly on chondrocyte maturation without undergoing the secondary pathway of binding to the IGF-I receptors, and that any growth-promoting effect of IGF-I must occur when chondrogenesis is already in progress.

Most interesting was our observation of a dose-related modulation of type X collagen by Dex. The level of regulation is surely pretranslational since parallel changes in the cellular amount of the specific mRNA were also detected. Whether Dex acts directly on the rate of transcription of the gene or modulates its expression by a secondary posttranscriptional effect is not known.

A previous report by Habuchi et al. (1985) described a coordinate regulation of type X collagen and alkaline phosphatase levels in differentiating chick embryo chondrocytes. Our data on in vitro reconstituted cartilage in serum-free conditions disagree with this observation, since at any concentration of Dex, and therefore at any level of type X collagen, the activity of the enzyme was consistently low. Consequently, a direct correlation between level of alkaline phosphatase and cartilage differentiation does not seem to exist, as also concluded by Grigoriadis et al. (1989). Moreover, while chondrocyte differentiation is fully supported by the combination of T_3, insulin, and Dex, the complete formation of a cartilage structure calcifying through increased levels of alkaline phosphatase activity apparently requires additional, as yet unidentified factors.

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