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Modulation of Cultured Chicken Growth Plate Chondrocytes by Transforming Growth Factor-β_1 and Basic Fibroblast Growth Factor

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Abstract Expression of several cellular and matrix proteins which increase significantly during the maturation of growth plate cartilage has been shown to be affected by various endocrine and autocrine factors. In the studies reported here, transforming growth factor-β (TGF-β_1) and basic fibroblast growth factor (bFGF) were administered to primary cultures of avian growth plate chondrocytes at pre- or post-confluent stages to study the interplay that occurs between these factors in modulating chondrocytic phenotype. Added continuously to *pre-confluent* chondrocytes, TGF-β_1 stimulated the cells to produce abundant extracellular matrix and multilayered cell growth; cell morphology was altered to a more spherical configuration. These effects were generally mimicked by bFGF, but cell shape was not affected. Administered together with TGF-β_1, bFGF caused additive stimulation of protein synthesis, and alkaline phosphatase (AP) activity was markedly, but transiently enhanced. During this pre-confluent stage, TGF-β_1 also increased fibronectin secretion into the culture medium. Added to *post-confluent* cells, TGF-β_1 alone caused a dosage-dependent suppression of AP activity, but bFGF alone did not. Under these conditions, TGF-β_1 and bFGF had little effect on general protein synthesis, but TGF-β_1 alone caused large, dosage-dependent increases in synthesis of fibronectin, and to some extent type II and X collagens. Given together with bFGF, TGF-β_1 synergistically increased secretion of fibronectin. These findings reveal that regulation of phenotypic expression in maturing growth plate chondrocytes involves complex interactions between growth factors that are determined by timing, level, continuity, and length of exposure.

Key words: chondrocytes, TGF-β_1, bFGF, collagen, fibronectin, alkaline phosphatase

Many physiological factors are required for normal proliferation and differentiation of chondrocytes. Among these are transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF), mitogenic factors that stimulate the synthesis of DNA and proliferation of chondrocytes in monolayer cultures (Kato et al., 1983, 1987; O'Keefe et al., 1988; Ellingsworth et al., 1986; Sandberg et al., 1988). TGF-β was first identified from its ability to stimulate anchorage-independent growth in rat fibroblasts (Roberts et al., 1983). Since then it has been shown that TGF-β can modulate growth and differentiation in many cell types. In general, TGF-β inhibits growth of epithelial, endothelial, and lymphoidal cells, and stimulates growth of cells of mesenchymal origin (Roberts et al., 1988; Sporn et al., 1986; Centrella et al., 1988). TGF-β also stimulates synthesis of collagen and the deposition of extracellular matrix proteins by fibroblasts (Fine and Goldstein, 1987; Ignotz and Massagué, 1986); this growth factor also plays a regulatory role in the synthesis of matrix proteins by articular chondrocytes (Skantze et al., 1985). TGF-β is most abundant in bone and is homologous with cartilage-inducing factor A (CIF-A), a growth factor isolated from bovine bone which causes primitive mesenchymal cells to differentiate into chondrocytes (Seyedin et al., 1985, 1986). Although bone is a rich source, articular and epi-

Abbreviations used: AP, alkaline phosphatase; AP-IF, alkaline phosphatase-induction factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CIF-A, cartilage-inducing factor A; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PG, proteoglycan; PS, penicillin/streptomycin; SCL, synthetic cartilage lymph; TCA, trichloracetic acid; TGF-β_1, transforming growth factor-beta 1; TMS, 50nM TES, pH7.5, containing 1.5 mM $MgCl_2$ and 10% (W/V) sucrose; TMT, 0.1% Triton x-100 buffer, pH 7.5, containing 10mM Tris and 0.5mM $MgCl_2$.

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physeal growth plate cartilage also have been found to produce low levels of TGF-β (Sandberg et al., 1988). bFGF, homologous with cartilage-derived growth factor, is present in cartilage and supports proliferation and differentiation of chondrocytes (Kato and Gospodarowicz, 1985).

Another trademark long associated with chondrocyte differentiation and skeletal mineralization is alkaline phosphatase (AP) (Robison, 1923). The enzyme appears to play a multifactorial role in mineralization (Wuthier and Register, 1985), and is known to be vital to normal bone formation (Weiss et al., 1989). In the growth plate, levels of AP in chondrocytes increase dramatically in progressing from the zone of proliferation to the zone of hypertrophy (Follis, 1949) where mineralization first occurs. AP may be involved in tyrosine kinase–based cellular signalling to regulate cell division or maintenance of the differentiated phenotype (Burch et al., 1985). The factors involved in controlling AP activity in growth plate chondrocytes are not well understood, but appear to involve both humoral and nutritional components.

In this report, we describe the interaction that occurs between two primary modulating factors (TGF-β_1 and bFGF) in the regulation of cell growth, synthesis of matrix proteins (collagens and fibronectin), and levels of AP activity at different stages of development in this serum-free, primary cell culture system.

MATERIALS AND METHODS

Chondrocyte Isolation

Chondrocytes were isolated from the hypertrophic region of epiphyseal growth plate cartilage of the tibiae of 8–10-week-old hybrid broiler-strain chickens as previously described (Wuthier et al., 1985), with the following modifications. The cartilage was cut into 3–4-mm cubes and digested with 0.25% trypsin in 10 ml of synthetic cartilage lymph (SCL) (Majeska and Wuthier, 1975) at 37°C for 15 min. The majority of the trypsin solution was removed using a Pasteur pipette, then 10 ml of a collagenase (Worthington, CLS II, 126 units/mg, 0.03% final concentration) solution in SCL was added and digestion continued at 37°C for 30 min. The digesting solution was removed, 15 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), penicillin/streptomycin (PS) (DMEM/FBS/PS) and 0.03% collagenase was added, and the digestion continued at 37°C overnight. The cells were isolated the following day as follows: after replacing the digesting medium with fresh DMEM/FBS/PS, the tissue suspension was vortexed three times, 1 min each, followed by centrifugation in a clinical centrifuge for 5 sec to remove undigested tissue, and then for 10 min at 3,000 rpm to sediment the released cells and mineralized debris. This pellet was demineralized with isotonic pH 6.0 buffer (80.5 mM Na_3 citrate, 8.0 mM citric acid) for 10 min at 37°C, the solution being removed after centrifugation at 3,000 rpm for 15 min. The cell pellet was then washed with DMEM/FBS/PS and resuspended in the same medium. Chondrocytes were plated at a density of 4×10^5 cells per 35-mm dish, and cultured in 2 ml of DMEM/FBS/PS at 37°C under an atmosphere of 95% air/5% CO_2 (Wuthier et al., 1985). The culture medium was changed every 3 days. For serum-free media, on day 6, cells were switched to a medium composed of a 1:1 mixture of DMEM/FBS/PS and serum-free HL-1; from day 9 onward cells were given only the HL-1 medium. Ascorbate was given from day 3 onward at a concentration of 50 μg/ml (Wu et al., 1989).

Growth Factors

TGF-β_1 was diluted in 4 mM HCl containing 1 mg/ml of bovine serum albumin (BSA) and added to the cultures at a final concentration of 0.01–5.0 ng/ml medium. Control cultures were given the same solution minus TGF-β_1. bFGF was dissolved in sterile 0.15 M NaCl and added to the culture at 10 ng/ml after proper dilutions as indicated in the figure legends. For studying the effect of TGF-β_1 and/or bFGF at different stages of development of the chondrocytes, these factors were added for various time periods ranging from days 6, 14, 18, and 21, up to day 27. Effects were monitored at various times (days 14, 18, 22, or as indicated) either during, at the end of, or after the treatment period. [^{45}CA] incorporation into the cell/matrix layer was measured after incubation with 0.5 μCi $^{45}CaCl_2$ for 24h before harvest. The culture media and the cell/matrix layer were harvested; protein and AP activity were measured; and in addition, synthesis of collagen into the culture medium was analyzed by SDS-PAGE (see below).

Cell Harvest

Chondrocytes were harvested from the 35-mm culture dishes after removal of the media. The cell layer was rinsed twice with 1 ml of TMS (50

mM TES, pH 7.5, containing 1.5 mM $MgCl_2$ and 10% (w/v) sucrose) and was gently scraped from the culture vessel after addition of 2 ml TMS. The procedure was repeated twice to ensure removal of all cells. The cell suspension was sedimented at 1,000*g* for 25 min and resuspended in 1 ml of 0.1% Triton X-100 buffer (pH 7.5) containing 10 mM Tris and 0.5 mM $MgCl_2$ (TMT), frozen, and later dispersed using sonication for 10 sec. Cellular AP activity (Ishikawa et al., 1991) and total cellular protein (Lowry et al., 1951) were determined on these preparations.

Analysis of Collagen Synthesis

The amount of collagen production, in the presence or absence of modulators, was monitored in both the media and the cell/matrix fraction (Schmid and Linsenmayer, 1983). For isolation of soluble collagen, the medium (2 ml) were removed and pooled, protease inhibitors (5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine, final concentrations) were added, and the solution centrifuged at 13,000*g* for 20 min. A 2-ml portion of the resulting supernatant was removed, $(NH_4)_2SO_4$ was added to 30% saturation, and after 16 h incubation at 4°C, the media collagen was sedimented as above. For isolation of the cell/matrix collagen, the cellular layer was extracted for 3 h at 4°C with 2 ml of 150 mM potassium phosphate buffer (pH 7.6) containing the above inhibitors using slow oscillation to maintain fluid movement. The buffer extract was collected, the fluid clarified by centrifugation, and the cell/matrix collagen precipitated with 30% $(NH_4)_2SO_4$ as above.

Both the media and cell/matrix collagens were dissolved in 0.5 ml of 0.5 M acetic acid containing protease inhibitors. In some cases, to verify specific collagen labeling, the acetic acid extracts were digested with porcine pepsin as previously described (Wu et al., 1989). Aliquots of the acetic acid extracts or pepsin digests of both sources of collagen were analyzed by SDS-PAGE.

SDS-PAGE Analysis

Secreted media proteins and cell/matrix collagens equivalent to 0.1 ml original volume were analyzed by SDS-PAGE (Laemmli, 1970) after trichloroacetic acid (10%, w/v) precipitation or 30% ammonium sulfate saturation. In some instances, [^{35}S]methionine/cysteine and [^{3}H]proline labeled proteins were visualized by SDS-PAGE and fluorography after incubation in ^{3}H-Enhance.

Proteoglycan Synthesis

Proteoglycan (PG) synthesis into the medium and cell/matrix layer was analyzed by incorporation of radiolabeled sulfate. Chondrocyte cultures were labeled for 24 h on the specified day by addition of 10 μCi of [^{35}S]$SO_4^=$ to 2 ml media/35-mm dish. After the labeling period, the culture medium was collected, centrifuged for 10 min in a clinical centrifuge, and a 100-μl sample of the supernatant added to 1 ml of ethanol and allowed to precipitate at 4°C overnight. The precipitate was collected by centrifugation at 10,000*g* for 10 min; the pellet was washed once with ethanol and then dissolved in Laemmli sample buffer and counted. For analysis of the synthesis of matrix PG, the radiolabeled cell/matrix layer was rinsed twice with TMS buffer, the rinses being discarded. To solubilize the cell/matrix PG, 2 ml of 4 M guanidine hydrochloride, 75 mM sodium acetate buffer (pH 5.8) containing 5 mM benzamidine, and 15 mM EDTA was added and allowed to incubate at 4°C overnight with gentle agitation (Heinegård and Sommarin, 1987). The contents of the dishes were transferred to test tubes and centrifuged for 10 min in a clinical centrifuge. A 100-μl sample of the supernatant was added to 1 ml of ethanol, precipitated, redissolved, and counted as above.

Materials

Alkaline phosphatase substrate *p*-nitrophenylphosphate (pNPP), TES (N-tris[hydroxymethyl]methyl 2-aminoethane sulfonic acid), Trizma base, Triton X-100, trypsin (type III from bovine pancreas), Dulbecco's modified Eagle's medium (DMEM), and ascorbic acid were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from Hyclone (Logan, UT), and antibiotic (100×) (penicillin G, sodium 10,000 units, and streptomycin sulfate, 25 μg/ml normal saline) were purchased from GIBCO (Grand Island, NY). HL-1 serum-free medium was from Ventrex (Portland, ME); collagenase (CLS II) was from Worthington Biochemicals (Freehold, NJ). Basic fibroblast growth factor (bFGF) was from either Boehringer Mannheim (Indianapolis, IN) or United States Biochemicals (Cleveland, OH); transforming growth factor-β_1 (TGF-β_1) was a gift from Drs. Michael B. Sporn and Anita B. Roberts, National Cancer

Institute, Bethesda, MD. Rabbit anti-human polyclonal antibody to fibronectin was from Upstate Biotechnology (Lake Placid, NY). [^{3}H]Proline (35 Ci/mmol), methyl [^{3}H]thymidine, and ^{3}H-Enhance were from NEN (Boston, MA); [^{35}S]Na_2SO_4 (carrier-free, ~43 Ci/mg S), and Tran^{35}S-label™ ([^{35}S]methionine + [^{35}S]cysteine, >1000 Ci/mmol) and $^{45}CaCl_2$ (23 Ci/g) were from ICN Biomedicals (Costa Mesa, CA). Sterile culture dishes were obtained from Corning Glass Works (Corning, NY).

RESULTS

Both TGF-β_1 and bFGF have been implicated in repair processes in connective tissues (Centrella et al., 1988; Kato and Gospodarowicz, 1985), bone fracture healing (Joyce et al., 1990), and initiation of chondrogenesis (Seyedin et al., 1985, 1986). Accordingly, we investigated their influence, alone or in combination, on growth plate chondrocytes grown in the presence of ascorbate in vitro to provide insight into the complex mechanisms of the above-mentioned processes. All cultures were provided with ascorbate in the media since this vitamin is known to be essential for maintaining normal cellular growth and matrix synthesis (Wu et al., 1989). TGF-β_1 and/or bFGF were added to the cultures either at a later confluent stages, or at an earlier pre-confluent stage, to explore a more complete spectrum of their effect on cellular development and differentiation.

Effect of TGF-β_1 and bFGF on Post-Confluent, Mature Chondrocytes

Figure 1 shows that TGF-β_1 strongly inhibited AP activity in confluent chondrocytes grown continuously in serum-free, *ascorbate-containing* HL-1 medium. Inhibition by TGF-β_1 was profound (65% reduction at 2.5 ng/ml); half-maximal inhibition occurred at about 0.4 ng/ml TGF-β_1. From numerous experiments, inhibition of AP activity of confluent chondrocytes by TGF-β_1 ranged from 35% to 65%. However, TGF-β_1 exerted little influence on total cellular protein in these mature, differentiated chondrocytes, except when very high levels were used. TGF-β_1 also caused inhibition of AP activity in *serum-containing* DMEM media, demonstrating that the effect of TGF-β_1 was not overridden by other serum factors (Fig. 2). Since AP activity was higher in 10% FBS– than in 2% FBS–containing media, AP expression also must be governed by a factor or factors in FBS (e.g., fetuin). Figure 3 shows that in post-confluent cells bFGF alone had no effect on AP activity; added together with TGF-β_1, bFGF ameliorated the inhibitory effects of 5.0 ng/ml TGF-β_1 on AP expression.

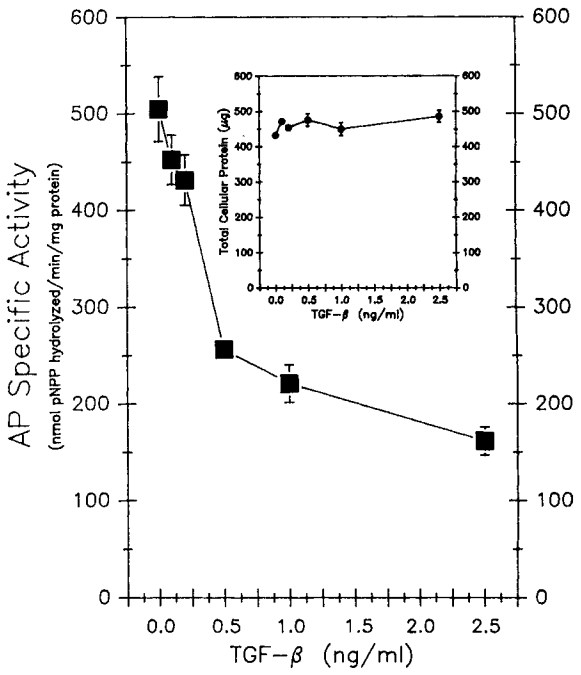


Fig. 1. Effect of TGF-β_1 on cellular AP activity and protein levels in *post-confluent* primary cultures of growth plate chondrocytes grown in serum-free HL-1 medium in the presence of ascorbate. Cells were grown in HL-1 + ascorbate (50 μg/ml) from day 3 onward as described in Materials and Methods. On day 24 they were exposed to the indicated concentrations of TGF-β_1 and harvested after 72 h as described previously. ■, AP activity; ●, protein levels. Values are the mean ± SE of four samples.

SDS-PAGE was used to examine more closely the effect of TGF-β_1 and bFGF on the composition of proteins secreted into the *culture medium* and the *extracellular matrix* of chondrocytes grown in ascorbate-containing HL-1 media. Figure 4A shows that TGF-β_1, administered for a 3-day period to either 18- or 21-day post-confluent cultures, caused an increase in the levels of a 250 kDa protein, and less obviously in levels of type II and X collagens present in 30% ammonium sulfate precipitates of the culture medium.

Using a more sensitive Ag-staining method (Fig. 4B) it is evident that levels of both type II and X collagens were increased by the higher (2.5 ng/ml) dosage of TGF-β_1 treated from days 21 to 24, using pepsin digestion of 30% ammonium sulfate precipitates shown in Figure 4A to verify that the effect was on collagen. To exam-

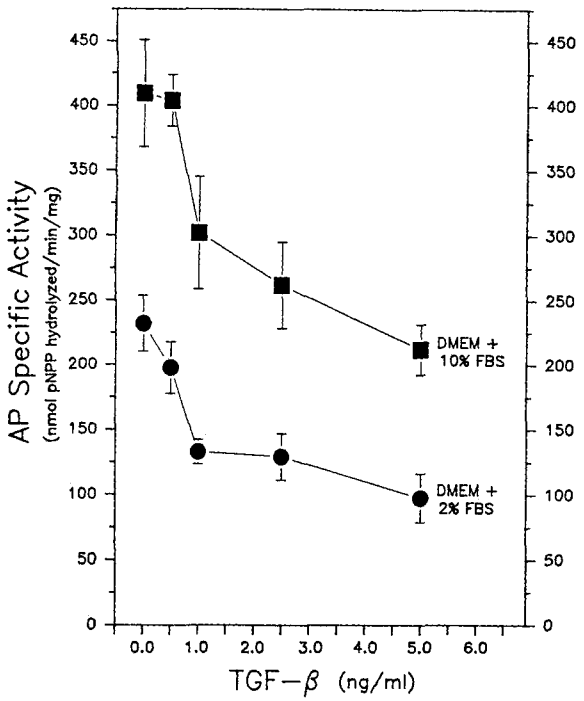


Fig. 2. Effect of TGF-β_1 on cellular AP activity of *post-confluent* primary cultures of growth plate chondrocytes grown in the presence of ascorbate in serum-containing media. Cells were grown in DMEM containing either 2% or 10% FBS. Ascorbate (50 μg/ml) was supplied to all cultures from day 3 onward as described in Materials and Methods. On day 21, cells were exposed to TGF-β_1 at the indicated concentrations for 72 h, and then harvested as described previously. ■, DMEM + 10% FBS; ●, DMEM + 2% FBS. Values are the mean ± SE of four samples.

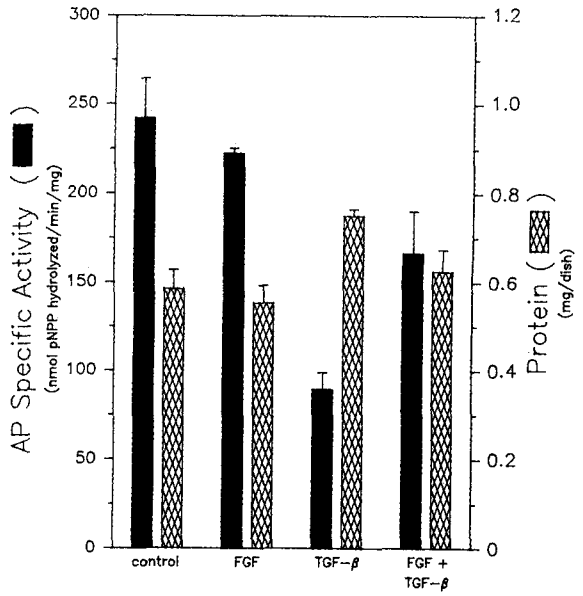


Fig. 3. Effect of TGF-β_1 and bFGF, alone or in combination, on levels of cellular AP activity and protein in *post-confluent* primary cultures of growth plate chondrocytes grown in HL-1 medium with ascorbate. Cells were treated with TGF-β_1 (5 ng/ml) and bFGF (10 ng/ml) alone or in combination on day 21 of culture. After 72 h exposure to the growth factors, cellular AP activity and Lowry protein content were measured as described in Materials and Methods. Values are the mean ± SE of four samples each.

ine collagen biosynthesis per se, the effect of TGF-β_1 on the incorporation of [^{3}H]proline into collagen secreted into the cell/matrix layer and culture medium was also studied. The fluorogram in Figure 5 shows that dosages of TGF-β_1 higher than 1.0 ng/ml stimulated incorporation of [^{3}H]proline into both type II and X collagens secreted into the medium. In the cell/matrix layer, type II collagen was stimulated by TGF-β_1, particularly at levels higher than 2.5 ng/ml. Table I shows that [^{3}H]proline incorporation into newly synthesized cell/matrix layer collagenous proteins was greatly stimulated by TGF-β_1.

We also examined *post-confluent* chondrocytes for the effect of TGF-β_1 and bFGF on synthesis of total media proteins isolated by 10% trichloracetic acid (TCA) precipitation, and on the collagen-enriched 30% ammonium sulfate fraction from culture media (Fig. 6). As before, TGF-β_1 (5 ng/ml) greatly increased secretion of a 250 kDa protein into the medium harvested either by 10% TCA (panel A) or 30% ammonium sulfate precipitation (panel B). In addition, TGF-β_1 increased synthesis of a 22 kDa protein, a 40 kDa protein, and two 33–36 kDa proteins into the TCA-precipitable medium fraction. bFGF (10 ng/ml) also caused increased synthesis of the 250 kDa protein. However, together, TGF-β_1 and bFGF caused even greater increase in the secretion of the 250 kDa, and the 22, 33, and 36 kDa proteins into the culture medium. Analysis of TCA-precipitable media proteins revealed that the 250 kDa band was labeled much more intensely with [^{35}S]methionine/cysteine in the presence of TGF-β_1 and bFGF together than with either one alone (Fig. 6C).

Exposure of the post-confluent cells to TGF-β_1 for a longer period (days 21 to 27) (Fig. 7) caused an even greater stimulation of the secretion of the 250 kDa protein into the TCA-precipitable media fraction (lane 2) and the ammonium sulfate fraction (lane 4). TGF-β_1 also appeared to alter the distribution of type II collagen between the procollagen and the mature processed form (lane 4). Upon pepsin digestion (lanes 5 and 6), the 250 kDa protein was destroyed, but the increased secretion of type II and X collagens into the media caused by TGF-β_1 became more clearly evident. Using this approach, it is evi-

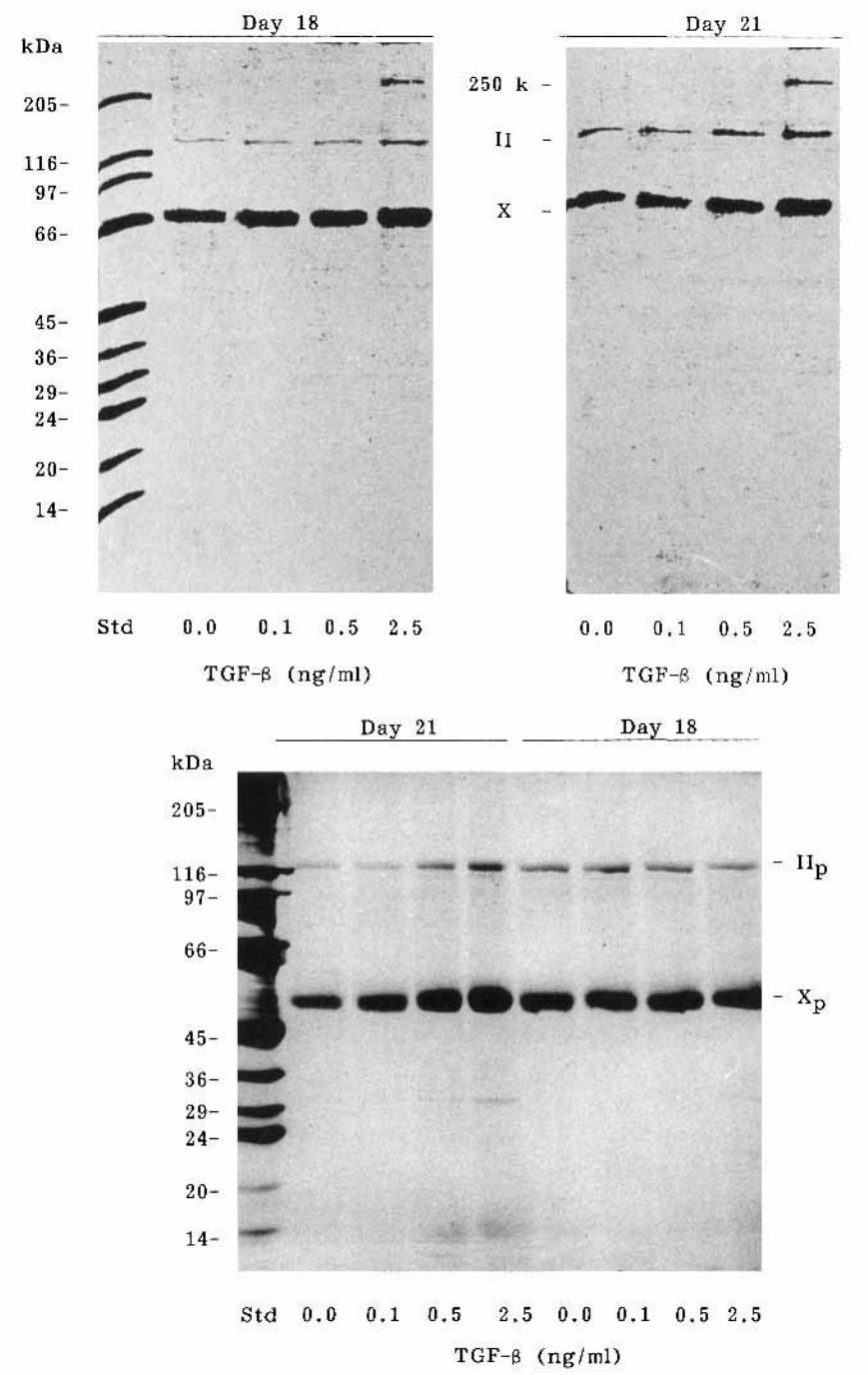


Fig. 4. SDS-PAGE analyses of the dose-dependent effect of TGF-β_1 on collagen and fibronectin synthesis into the media of *post-confluent* growth plate chondrocyte cultures. Cells were grown in the presence of ascorbate (50 μg/ml) in serum-free HL-1 medium, and on either day 18 or day 21 treated for 72 h with 0, 0.1, 0.5, and 2.5 ng/ml of TGF-β_1. **A:** The media were collected and fractionated with 30% ammonium sulfate. Precipitates were dissolved in 0.25 M acetic acid (0.5 ml), and 25 μl was applied to the acrylamide gel after neutralization with NaOH. The gel was stained with Coomassie blue. Note the graded increase in level of the 250 kDa protein (250 k), type II (II) and type X (X) collagen caused by the increasing levels of TGF-β_1. **B:** The media were collected, precipitated with 30% $(NH_4)_2SO_4$ saturation, acidified with acetic acid, and further treated with pepsin to remove noncollagenous proteins as described in Materials and Methods. Aliquots (25 μl) of the pepsin digest were *neutralized with* NaOH, separated by SDS-PAGE, and visualized with Ag stain. Again, note the graded increase in level of pepsinized type II (II_p) and type X (X_p) collagen caused by the increasing levels of TGF-β_1. The 250 kDa protein was destroyed by the pepsin digestion.

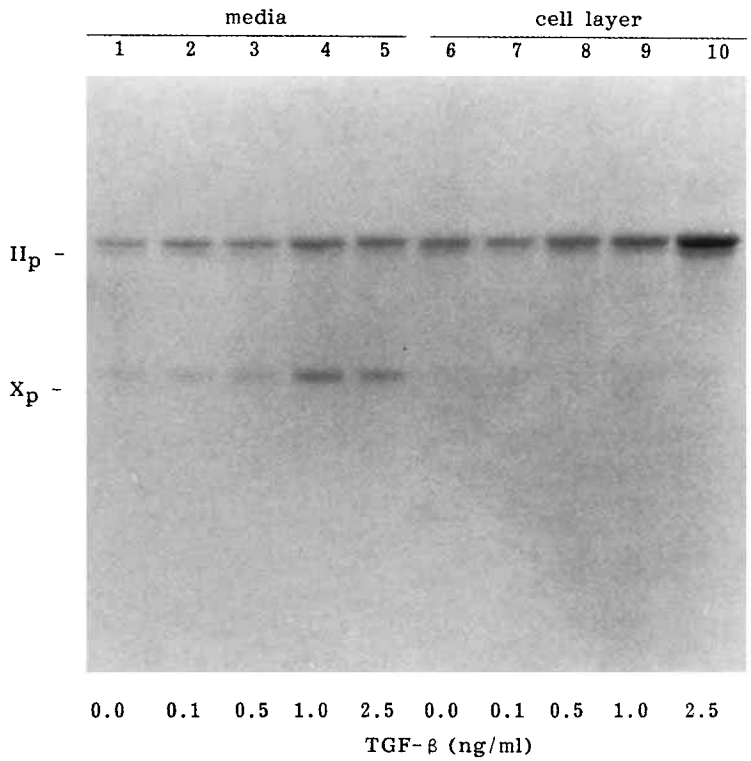


Fig. 5. SDS-PAGE fluorographic analyses of the dose-dependent effect of TGF-β_1 on synthesis of collagens into the media and cell/matrix layer of *post-confluent* growth plate chondrocyte cultures. Cells were cultured in the presence of ascorbate, treated on day 21 for 72 h with the indicated levels of TGF-β_1, and concomitantly labeled with 1 μCi of L-[^{3}H]proline (35 Ci/mmol). The media were harvested, the cell/matrix layer was extracted, precipitated by 30% ammonium sulfate, digested with pepsin, and the newly synthesized collagens analyzed by SDS-PAGE fluorography as described in Materials and Methods. Note the graded response to increasing levels of TGF-β_1 in the secretion of types II (II_p) and X (X_p) collagens into the culture medium (lanes 1–5), and in the synthesis of type II collagen into the cell/matrix layer (lanes 6–10).

dent that TGF-β_1 increased the synthesis of both type II and X collagens. It is also clear that TGF-β_1 stimulated the synthesis of two 22 and 33 kDa proteins in the TCA-precipitable media fraction (lane 2).

TGF-β_1 and bFGF had minimal effect on incorporation of [^{35}S]$SO_4^=$ into proteoglycans secreted into the culture medium by post-confluent chondrocytes. TGF-β_1 at 2.5 ng/ml caused a modest (32%) increase in [^{35}S]$SO_4^=$ incorporation into the cell/matrix layer; bFGF, alone or in combination with higher levels (5 ng/ml) of TGF-β_1, did not increase synthesis of PG by the chondrocytes under these conditions (data not shown).

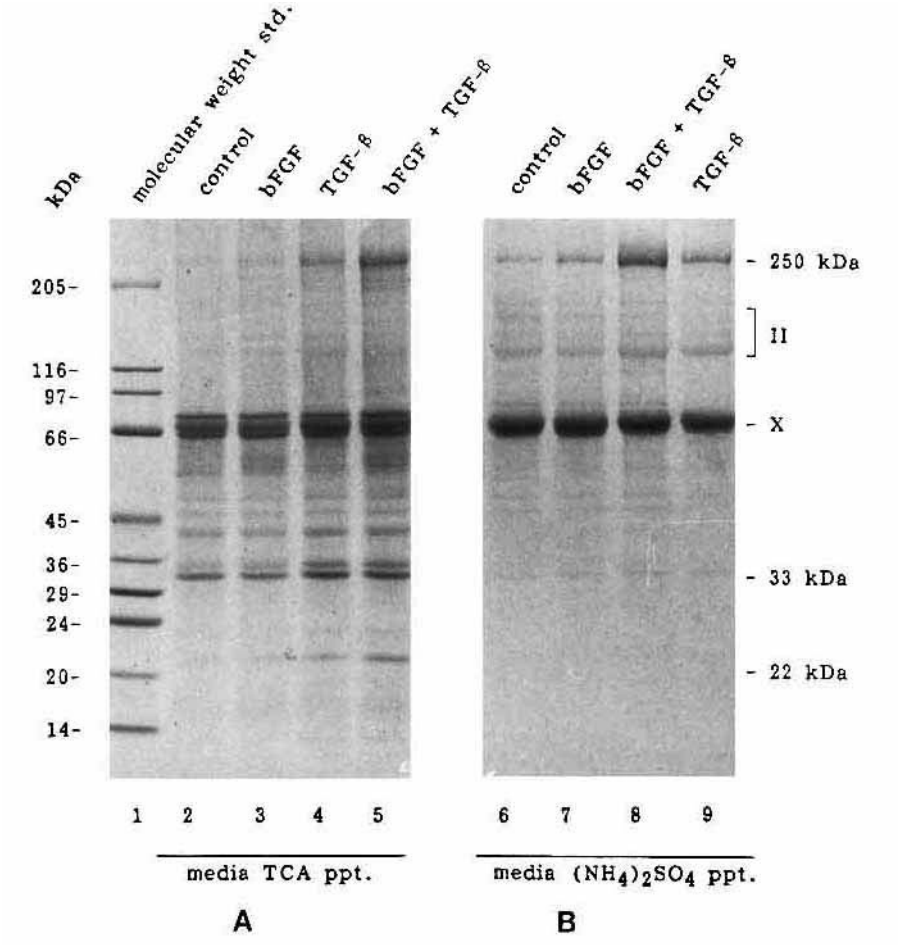
Effect of TGF-β and bFGF on Pre-Confluent Chondrocytes

We also investigated whether TGF-β_1 and bFGF had an influence on cell growth and AP activity during early stages of chondrocytic development. Proliferating chondrocytes were ex-

TABLE I. Effect of TGF-β_1 on Incorporation of [^{3}H]Proline Into Cell/Matrix Layer Collagenous Proteins*

Media levels of TGF-β_1 (*ng/ml*)	[^{3}H]Proline incorporated into 30% $(NH_4)_2SO_4$ ppt. (*cpm/dish*)	[^{3}H]Proline incorporated into the pepsin digest[a] (*cpm/dish*)
0.0	7,567 ± 1,047	5,320 ± 816
0.1	8,680 ± 1,052	5,627 ± 631
0.5	11,470 ± 1,446	8,633 ± 1,137
2.5	11,320 ± 969	6,873 ± 717

*[^{3}H]Proline (1 μCi, 35 Ci/mmol) was incubated on day 21 for 72 h with the post-confluent chondrocytes for measurement of incorporation into matrix collagen. Cell/matrix layer collagen was isolated by 30% ammonium sulfate saturation, and also by pepsin digestion as described in Materials and Methods. Values are the mean ± SD of three cultures.
[a]Pepsin digestion should have removed most noncollagenous proteins.



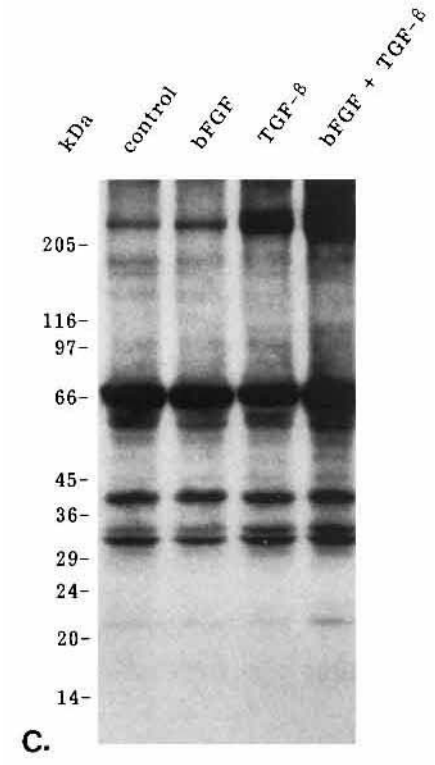


Fig. 6. SDS-PAGE analyses of the comparative effects of bFGF and TGF-β_1 on synthesis of media proteins by *post-confluent* cultured growth plate chondrocytes. Cells grown in the presence of ascorbate (50 μg/ml) in serum-free HL-1 medium were treated for 72 h on day 21 with either bFGF (10 ng/ml) or TGF-β_1 (5 ng/ml), or with bFGF and TGF-β_1 together at the indicated levels. Media were collected and precipitated with either 10% trichloroacetic acid (TCA) (**A**) or 30% $(NH_4)_2SO_4$ (**B**). $(NH_4)_2SO_4$ precipitates were dissolved in 0.5 ml of 0.25 M acetic acid; TCA precipitates were dissolved in 0.5 ml of Laemmli sample buffer, aliquots (25 μl) being applied to the acrylamide gel. Note that the level of the 250 kDa protein, and to a lesser extent of type II (II) and type X (X) collagen, was increased markedly by the *combination* of TGF-β_1 and bFGF. **C**: Fluorographic analyses of the effects of bFGF and TGF-β_1 on synthesis of extracellular media proteins by growth plate chondrocyte cultures. Cells were grown and treated with bFGF or TGF-β_1 alone, or together, as described above. On day 21, the cultures were concomitantly labeled for 72 h with 10 μCi of Tran[^{35}S]methionine/cysteine (>1000 Ci/mmol). The media were harvested and the protein precipitated with 10% TCA; the synthesized proteins were analyzed by SDS-PAGE and fluorography as described in Methods. Note that the combination of both growth factors caused a powerful synergistic increase in the synthesis of the 250 kDa protein.

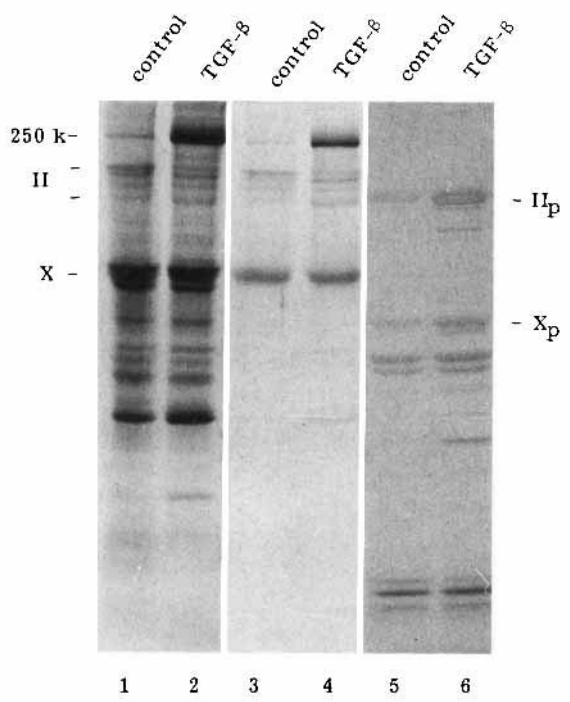


Fig. 7. SDS-PAGE analyses of the effects of longer TGF-β_1 exposure on synthesis of media proteins by *post-confluent* growth plate chondrocyte cultures. Cells were cultured in the presence of ascorbate and treated on day 21 with TGF-β_1 for a longer period (144 h) and the media proteins analyzed as described in Figure 6 as follows: TCA-precipitated media proteins (lanes 1, 2), 30% $(NH_4)_2SO_4$ precipitated proteins (lanes 3, 4), and pepsin digested media proteins (lanes 5, 6). Note that longer exposure of the cells to TGF-β_1 caused greater stimulation of secretion of the cartilage collagens (lanes 5, 6) and the 250 kDa protein (lanes 1–4) into the medium. The doublet seen below X_p in lanes 5 and 6 is residual pepsin from the digestion.

posed to TGF-β_1 or bFGF, alone or in combination, either continuously (days 6 to 22) or discontinuously (days 6 to 10, 6 to 14, 10 to 14, and 14 to 18) of culture, the effects being monitored on days 10, 14, 18, or 22.

When cell cultures were exposed from days 6 to 14, TGF-β_1 or bFGF, alone, were mitogenic and stimulated protein synthesis (Fig. 8A); bFGF in combination with TGF-β_1 caused an additive increase, except during the period days 14 to 18. TGF-β_1 or bFGF, alone, added from days 6 to 10 or from Days 10 to 14, had little effect on AP activity (Fig. 8B, C). However, in contrast to post-confluent cultures, when added together, TGF-β_1 and bFGF caused marked (eightfold), transient, stimulation of AP activity, especially if present continuously from days 6 to 10 or 6 to 14.

Effects on Cell Morphology

The effects of the growth factors on cell shape was also monitored by phase-contrast microscopy. Cells were grown in the presence or absence of TGF-β_1 and bFGF, alone or the two combined, from day 6 and from day 14 onward (Fig. 9). Addition of TGF-β_1 to pre-confluent (days 6 to 14) cells altered cell shape from flattened polygonal (Control, panel A) to more spherical (panel C), and the cells were more evenly distributed over the surface of the culture dishes; bFGF added under these conditions did not alter cell shape, but increased cell numbers significantly (panel B). When TGF-β_1 and bFGF were added together, cell numbers were highest and cells were rounded up and predominantly spherical/hexagonal in shape (panel D).

On day 18, cell cultures grown in the presence or absence of TGF-β_1 and bFGF were again analyzed microscopically (Fig. 10). Cells treated with TGF-β_1 from days 6 to 14 (panel B) and from days 6 to 18 (panel C) showed multilayer cell growth and spherical/hexagonal cell morphology. Control cells at this stage displayed a more irregular shape and distribution (panel A). Cells treated with TGF-β_1 from days 14 to 18 exhibited some rounding of cell shape (panel D), but the effect was much less obvious than when cells were treated with TGF-β_1 earlier in their development. Cells treated continuously with bFGF from days 6 to 18 had a much higher cell population than the control, but cell shape was not affected (panel E). Cells treated continuously with combined TGF-β_1 and bFGF from days 6 to 18 displayed dense multilayered spherical/hexagonal cell growth, but cell size was somewhat reduced (panel F).

Effect of Growth Factors on [^{3}H]Thymidine Incorporation

To more accurately quantitate the effects of TGF-β_1 and bFGF on cell division, incorporation of [^{3}H]thymidine was assessed under a variety of conditions. Table II shows that when added at early time periods (days 6 to 10, 6 to 14, and 10 to 14) bFGF *alone* was mitogenic, causing on average about 40% increase in [^{3}H]thymidine uptake compared to the control; at later stages of culture (days 14 to 18 and 18 to 22) or when added continuously for an extended time (days 6 to 22), bFGF was not mitogenic. In contrast, TGF-β_1 *alone* was most mitogenic when given between days 6 to 14, 10 to 14, and 18 to 22. The most striking effects on cell division were seen when TGF-β_1 and bFGF were given together, especially between days 6 and 14 and

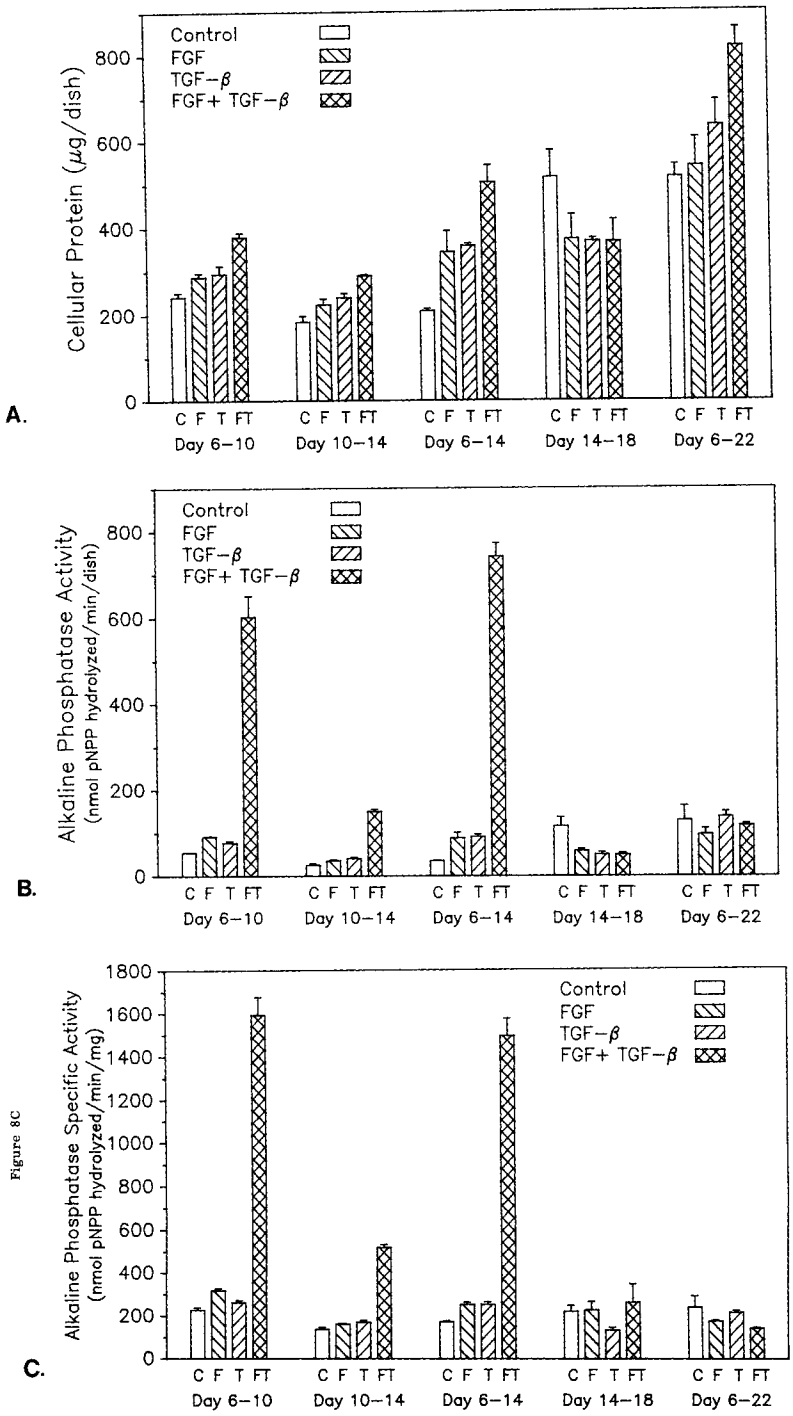


Figure 8C

Fig. 8. Effect of continuous exposure of rapidly growing *preconfluent* chondrocytes to TGF-β_1 and bFGF, alone or in combination, on levels of protein and cellular AP activity. Primary cultures of growth plate chondrocytes were grown in HL-1 medium and ascorbate as described in Methods. Cells were treated with TGF-β_1 (1 ng/ml) and bFGF (10 ng/ml) alone or in combination every 4 days at each change of medium from days 6 to 14 and 6 to 22. Also, transient exposure of the cells to growth factors from days 6 to 10, 10 to 14, and 14 to 18 were also studied. On days 6, 10, 14, 18, and 22 of exposure to the growth factors, total culture protein (**A**), cellular AP activity (**B**), and specific activity (**C**) were measured as described in Methods. Values are the mean ± SE of three samples each. When both bFGF and TGF-β_1 were present together, the initial response was to greatly stimulate cellular AP activity and protein levels (days 6 to 14).

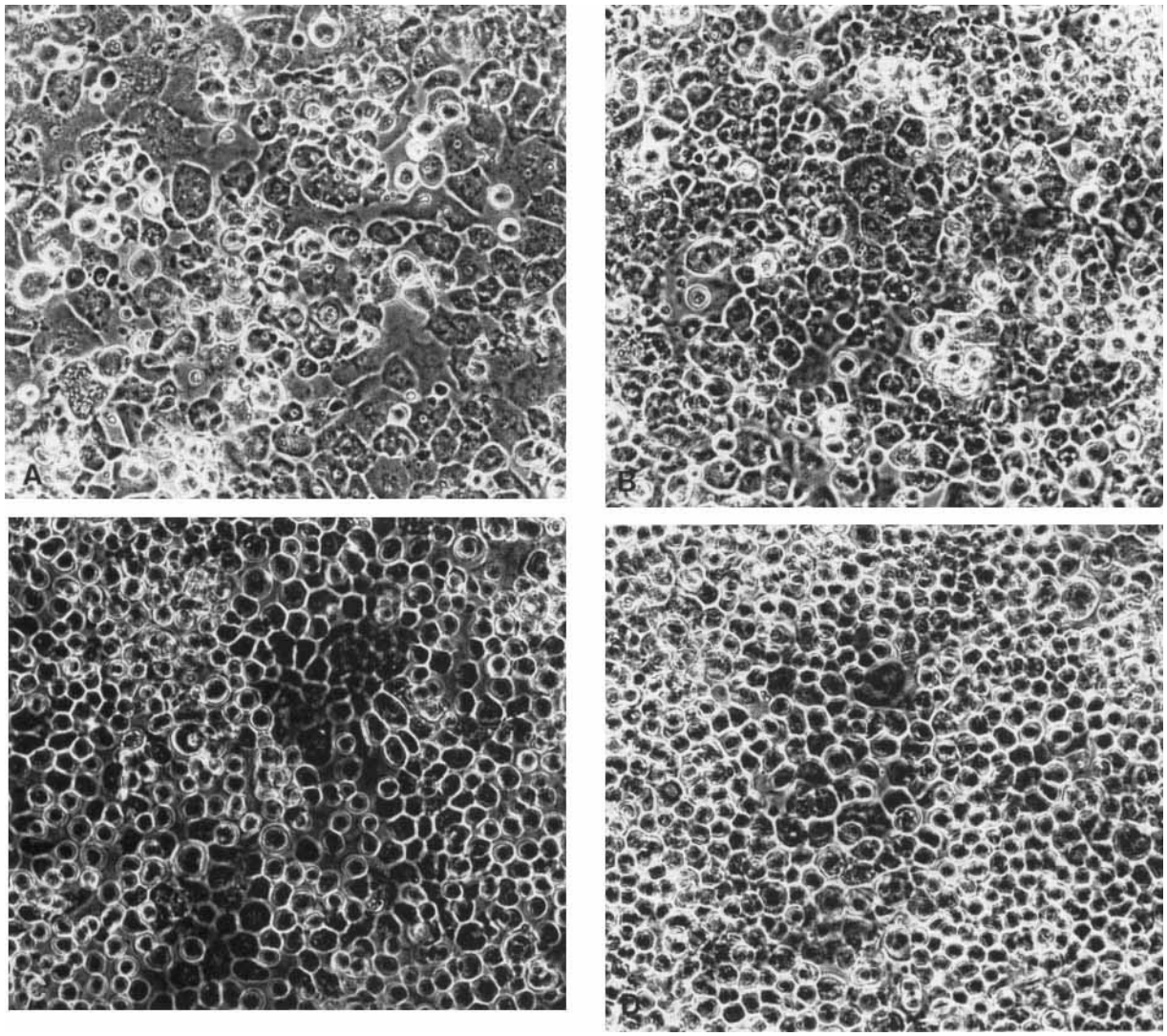


Fig. 9. Phase-contrast microscopic analysis of cells grown in HL-1 medium in the presence of TGF-β_1, or bFGF, or both. After exposure to growth factors from days 6 to 14 as described in Figure 8, cell morphology was examined on day 14. **(A)** Control, **(B)** bFGF, **(C)** TGF-β_1, and **(D)** bFGF + TGF-β_1. Note the marked increase in cell numbers and rounding of cells observed with TGF-β_1 (C), especially when combined with bFGF (D).

10 and 14, when they caused more than doubling of the rate of [^{3}H]thymidine incorporation.

Effects of Growth Factors on Media Matrix Proteins of Pre-Confluent Cells

To ascertain whether secretion of collagen or other proteins was increased when TGF-β_1 or bFGF were added to the growing cultures (days 6 to 18), SDS-PAGE analysis was made of 30% ammonium sulfate precipitates of the culture medium. Figure 11 shows that after exposure from days 6 to 14, secretion of type II and X collagens and a 250 kDa protein into the media was stimulated by TGF-β_1 (lane 3), and that longer treatment with TGF-β_1 (days 6 to 18) further increased this effect (lane 8). bFGF also increased type X collagen secretion (lane 4). Exposure to combined TGF-β_1 + bFGF from days 6 to 14 stimulated collagen production (both type II and X) to a greater extent (lane 5). Longer exposure to combined TGF-β_1 + bFGF (days 6 to 18, lane 11), delayed exposure to TGF-β_1 (days 14 to 18, lane 9), or discontinuous exposure to TGF-β_1 (days 6 to 14, lane 7) caused a lesser response.

Identification of the 250 kDa Protein as Fibronectin

While many studies have indicated the TGF-β_1 stimulates fibronectin synthesis, it was impor-

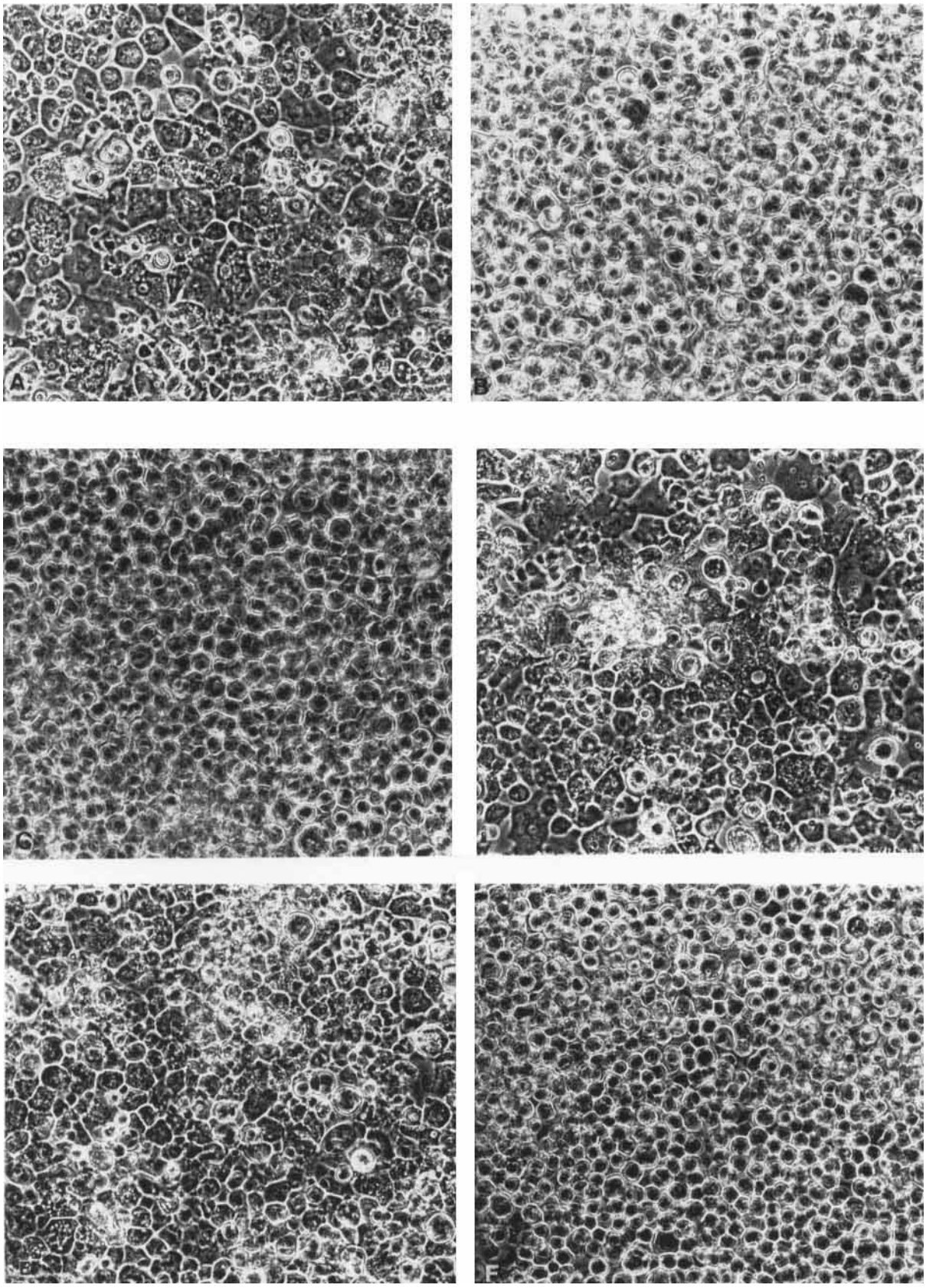


Figure 10.

TABLE II. Effect of TGF-β_1 and bFGF on [^{3}H]Thymidine Incorporation Into Cultured Growth Plate Chondrocytes*

Treatment period (days)	Treatment	[^{3}H]Thymidine uptake (cpm/samples)	Experimental control
6–10	Control	304 ± 26	1.00
	bFGF	430 ± 14	1.41
	TGF-β_1	342 ± 28	1.13
	bFGF + TGF-β_1	524 ± 44	1.72
6–14	Control	462 ± 37	1.00
	bFGF	692 ± 31	1.49
	TGF-β_1	782 ± 28	1.69
	bFGF + TGF-β_1	1,188 ± 110	2.57
10–14	Control	416 ± 39	1.00
	bFGF	562 ± 81	1.35
	TGF-β_1	697 ± 23	1.68
	bFGF + TGF-β_1	886 ± 84	2.13
14–18	Control	1,041 ± 141	1.00
	bFGF	1,118 ± 194	1.07
	TGF-β_1	969 ± 47	0.92
	bFGF + TGF-β_1	995 ± 179	0.96
18–22	Control	966 ± 62	1.00
	bFGF	1,040 ± 138	1.08
	TGF-β_1	1,400 ± 178	1.45
	bFGF + TGF-β_1	1,328 ± 170	1.37
6–22	Control	1,415 ± 79	1.00
	bFGF	1,172 ± 62	0.83
	TGF-β_1	1,142 ± 75	0.81
	bFGF + TGF-β_1	1,760 ± 69	1.24

*Methyl[^{3}H]thymidine (2 μCi, ~20 Ci/mmol) was incubated on the final day of each test period for 16 h with the chondrocytes for measurement of relative rates of cell division. After rinsing twice with TMS buffer to remove unincorporated labels, the cell layer harvested and then extracted with sonication into 1.0 ml of TMT buffer as described in Methods. For counting, 0.1 ml samples were used; values are the mean ± SD of three samples each. Note that when bFGF on TGF-β_1 were added to the pre-confluent chondrocytes between days 6 and 14, and uptake of radiolabel measured on day 14, [^{3}H]thymidine incorporation was greatly stimulated, especially when both growth factors were present together.

tant to establish the identity of the 250 kDa protein. SDS-PAGE immunoblot analyses of the 30% ammonium sulfate fraction from the control and treated cell media showed strong reactivity of the 250 kDa bands with antibodies to fibronectin in the TGF-β_1 and TGF-β_1 + bFGF lanes, but none in the control or bFGF lane (Fig. 12). This indicates that the 250 kDa protein, which was effectively increased by TGF-β_1, is fibronectin.

Fig. 10. Phase-contrast microscopic analysis of cells grown in HL-1 medium in the presence of TGF-β_1, or bFGF, or both. Experimental conditions were as described in Figures 8 and 9 except that all microscopic examinations were made on day 18. (**A**) Control, (**B**) TGF-β_1, days 6 to 14, (**C**) TGF-β_1, days 6 to 18, (**D**) TGF-β_1, days 14 to 18, (**E**) bFGF, days 6 to 18, and (**F**) TGF-β_1 + bFGF, days 6 to 18. Note that early exposure of the cells to TGF-β_1 from days 6 to 14 (B) or 6 to 18 (C) caused marked proliferation and rounding of the cells; however if exposure to TGF-β_1 was delayed to days 14 to 18 (D), there was a marked reduction in the stimulation of cell proliferation. Note also that while exposure to bFGF from days 6 to 14 (E) stimulated cell proliferation, morphology was not spherical, but remained more polygonal, like the control. When both TGF-β_1 and bFGF were administered together from days 6 to 14 (F), there was a marked increase in cell proliferation, but cell size was slightly reduced.

Effect on Ca^{2+} and Pi Accumulation

The effect of these growth factors on the accumulation of calcium and phosphate by both pre- and post-confluent growth plate chondrocytes cultured was also investigated. With *post-confluent* cells, TGF-β_1 was mildly (10–20%) inhibitory to Ca^{2+} and Pi deposition by the mineralizing cultures; whereas, bFGF was modestly (11–15%) stimulatory. However, with *pre-confluent* cells exposed continuously to either growth factor from days 6 to 14, there was an approximate doubling of Ca^{2+} levels from 1,270 ± 270 (control) to 2,080 ± 450 (bFGF) or 2,100 ± 200 (TGF-β_1) cpm/dish. When both TGF-β_1 and bFGF were present together from

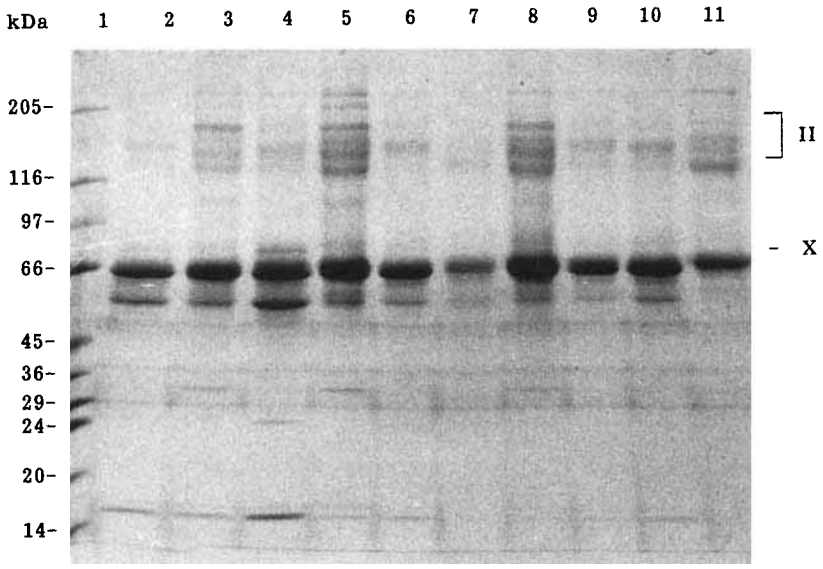


Fig. 11. SDS-PAGE analyses of the effects of bFGF and TGF-β_1 on synthesis of media proteins by pre-confluent chondrocyte cultures. Cells were treated with TGF-β_1 and bFGF, alone or in combination, from day 6 (lanes 3–5, 7–8, 10–11) or day 14 (lane 9), and harvested either on day 14 (lanes 2–5) or day 18 (lanes 6–11), as indicated in Figure 8. Lane 1, molecular weight standards; lane 2, control, harvested day 14; lane 3, TGF-β_1 treated from days 6 to 14 harvested day 14; lane 4, bFGF, as in lane 3; lane 5, TGF-β_1 + bFGF, as in lane 3; lane 6, control, harvested day 18; lane 7, TGF-β_1 treated from days 6 to 14, harvested on day 18; lane 8, TGF-β_1 treated from days 6 to 18, harvested on day 18; lane 9, TGF-β_1 treated from days 14 to 18, harvested on day 18; lane 10, bFGF treated from days 6 to 18, harvested on day 18; lane 11, TGF-β_1 + bFGF treated from days 6 to 18, harvested on day 18.

days 6 to 14, there was a marked 36-fold increase in Ca^{2+} levels to 46,240 ± 15,600 cpm/dish. These findings again illustrate the marked differences in response between pre- and post-confluent chondrocytes.

DISCUSSION

Expression of certain enzymes and connective tissue proteins change significantly during differentiation of growth plate chondrocytes. For example, levels of AP and certain hydrolases increase markedly going from the zone of proliferation to the zone of hypertrophy (Follis, 1949; Granda and Posner, 1971; Wuthier, 1973); similarly the expression of type X collagen is notably enhanced in the zone of hypertrophy (Schmid and Linsenmayer, 1983). The cause for the marked increase in expression of these proteins in growth plate chondrocytes is not well understood. However, since the growth plate is relatively avascular, and induction appears to coincide with vascular penetration from the marrow space, it is probable that factors present in blood plasma or its perfusate are in part responsible for the enhanced expression of these proteins.

Previously, we showed that nutritional factors are necessary for the expression of AP by cultured avian growth plate chondrocytes. If the levels of amino acids in the culture medium are increased to match those found in growth plate cartilage extracellular fluid (Ishikawa et al., 1985), marked enhancement in cellular and matrix vesicle AP activity is observed (Ishikawa et al., 1986b). Recent studies have also shown that ascorbate enhances AP activity in cultured growth plate chondrocytes and stimulates matrix vesicle formation (Ishikawa et al., 1986a; Leboy et al., 1989; Wu et al., 1989). Type II and X collagen secretion into the culture medium was also stimulated, and the amount of collagen in the cell/matrix layer was also markedly increased by ascorbate (Leboy et al., 1989; Wu et al., 1989).

On the other hand, we have found that various humoral factors also affect AP expression by these cells. Parathyroid hormone markedly inhibits the expression of AP by cultured growth plate chondrocytes (Chin et al., 1986); whereas the vitamin D metabolite (24R,25-dihydroxy vitamin D_3) (Hale et al., 1986), and prostaglandins E_1, E_2, and D_2 significantly enhanced AP expression when added at low levels in serum-free media (Kemick et al., 1989). AP-IF, a fetuin/α_2HS-glycoprotein present in the sera of several species, has been shown to enhance expression

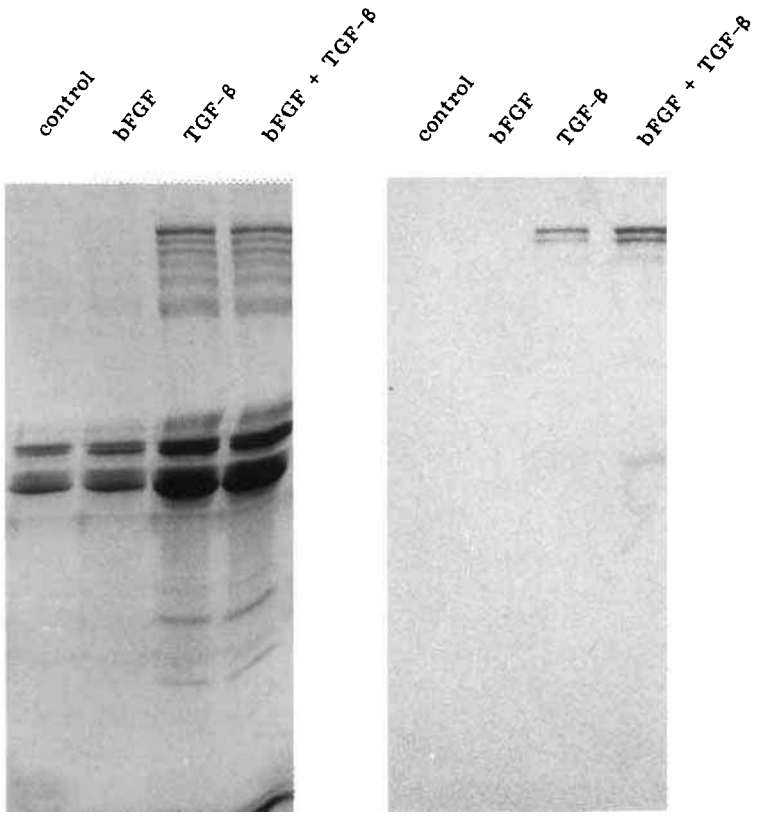


Fig. 12. Identification of the 250 kDa protein as fibronectin. Cultures were treated with TGF-β_1 and/or bFGF from days 6 to 14 and media were harvested and precipitated with 30% ammonium sulfate, analyzed by SDS-PAGE, and then transferred to a nitrocellulose sheet. The left panel shows the Coomassie blue stained gels; the right panel shows the western blot probed with anti-fibronectin antibody.

of AP in serum-free (HL-1) cultures of avian growth plate chondrocytes (Ishikawa et al., 1987, 1989, 1991).

The current studies were aimed at further elucidating the regulation of AP and secreted matrix proteins by TGF-β_1 in the growth plate, and attention was also directed toward elucidating the interplay that occurs between TGF-β_1 and bFGF. Our findings reveal that when given alone, TGF-β_1 exhibited a clear, dosage-dependent inhibition of AP activity in mature, differentiated chondrocytes grown in serum-free HL-1 medium supplemented with ascorbate (Fig. 1). On the contrary, when TGF-β_1 alone was added to growing pre-confluent chondrocytes, it did not affect, or slightly stimulated, AP activity (Fig. 8B, C). And when given together with bFGF during this stage, TGF-β_1 caused a transient, but marked (eightfold) increase in AP activity.

In mature, post-confluent chondrocytes, other phenotypic expressions were not inhibited by TGF-β_1; in fact, [^{35}S]sulfate incorporation into matrix layer PG was mildly stimulated, and collagen and fibronectin (250 kDa protein) synthesis was clearly stimulated. Especially in proliferating chondrocytes, TGF-β_1 stimulated fibronectin and both type II and type X collagen synthesis (Figs. 11, 12) when supplied continuously to the cultured cells between day 6 (preconfluent) and day 18 (post-confluent) (Fig. 11). When applied at confluency (day 14, Fig. 11) or well after confluency (day 21, Figs. 4, 5), TGF-β_1 alone caused only modest stimulation of collagen synthesis. However, even when applied after confluency, TGF-β_1 clearly increased fibronectin secretion into the media (Figs. 4, 6).

One of the most dramatic effects of TGF-β_1, especially when given in combination with bFGF, was to stimulate proliferation of growth plate chondrocytes. Together these growth factors markedly increased cell growth (as measured by cell protein levels, Fig. 8, and DNA synthesis, Table II) and matrix synthesis (collagen type II and X, and fibronectin, Fig. 11). Phase-microscopic examination of these cultures (Figs. 9, 10) showed remarkable cell proliferation; cellular multilayers and nodules were abundant through-

out the cultures. Administered together with bFGF to confluent, mature, differentiated chondrocyte cultures, TGF-β_1 caused much less stimulation of cell growth (Figs. 3, Table II), but markedly enhanced synthesis of fibronectin (Figs. 4–7). The fact that both bFGF and TGF-β_1 were required to obtain maximal synthesis of fibronectin indicates that these factors were acting cooperatively.

TGF-β is expressed in significant levels in growth plate cartilage (Sandberg et al., 1988), and has been shown to accumulate in latent form within the mineralized matrix during the conversion from cartilage calcification to bone formation in vivo (Carrington et al., 1988). Activation of latent TGF-β during osteoclastic resorption is thought to prime osteoblasts for bone reformation (Roberts et al., 1988). Studies by Jingushi et al. (1990) have revealed that bFGF and TGF-β_1 are both localized to the same nuclear and matrix areas within the growth plate. In addition, recent studies indicate that bFGF and TGF-β_1 together cause synergistic stimulation of cell division ([^{3}H]thymidine incorporation) in growth plate chondrocytes (Crabb et al., 1990), in agreement with current findings. TGF-β_1 also has been shown to be an autocrine factor for growth plate chondrocytes (Rosier et al., 1989). Maximal stimulation of cell division was seen in cells isolated from the maturation zone between proliferating and early hypertrophic chondrocytes. Insight into the mechanism by which bFGF and TGF-β_1 act synergistically has come from studies which showed that bFGF markedly stimulates TGF-β_1 formation by growth plate chondrocytes (Gelb et al., 1990). Taken together, it is clear that the cooperative interactions between these growth factors have physiological significance since both are expressed in native tissue.

TGF-β_1 has been shown to induce chondrogenesis when implanted into muscle (Seyedin et al., 1985, 1986), and when injected into the periosteal layer of normal bone (Joyce et al., 1990). In the periosteal injection studies, as long as the TGF-β_1 injections were continued, stimulation of chondrocytic phenotype was maintained; however, upon cessation of the injections, the induced cartilaginous callous rapidly underwent calcification and transformation to bone. In both tissue sites, TGF-β_1 appeared to cause the cells to revert back to a mesenchymal precursor form prior to proliferation and induction of chondrocytic phenotype.

Other studies suggest that part of the growth-regulatory effects of TGF-β_1 may be mediated via enhancement of fibronectin synthesis. While TGF-β_1 has been shown to rapidly stimulate the expression of fibronectin in several fibroblastic, epithelial, and chondroblast cell lines (Ignotz and Massagué, 1986; Rosen et al., 1988), our findings also demonstrate enhanced fibronectin synthesis by growth plate chondrocytes. Others have shown that fibronectin can mimic the effects of TGF-β in induction of anchorage-independent growth of normal fibroblasts, and that this can be specifically blocked by arginine-glycine-aspartate (RGD) containing peptides that inhibit fibronectin binding to cells (Pierschbacker and Ruoslahti, 1984). Thus, fibronectin appears to mediate at least some of the effects of TGF-β. The recent demonstration of TGF-β binding to immobilized fibronectin (Mooradian et al., 1989) suggests a mechanism for localizing TGF-β_1 to sites of tissue injury or inflammation, and for mediating its known auto-upregulatory effects (Obberghen-Schilling et al., 1988).

While we have shown that the 250 kDa protein bands react immunologically with monospecific antibodies to fibronectin, it is also possible that some of this protein may be tenascin (Erickson and Bourdon, 1989). Tenascin also has a MW of 250 kDa and is known to be associated with developing embryonic cartilage (Vaughan et al., 1987) and the periosteal layer of developing bone (Mackie et al., 1987). However, given the previously known association between fibronectin and TGF-β_1 in other tissues, it seems plausible that the 250 kDa protein induced by bFGF + TGF-β_1 is fibronectin. To our knowledge, this is the first demonstration that bFGF and TGF-β_1 act synergistically to stimulate synthesis of this protein.

Thus, multiple factors (nutritional, hormonal, and growth factors) affect the phenotypic expression of chondrocytes. Their actions depend on concentration, presence of other humoral factors, and developmental state of the responsive cells. The effects may be either stimulatory or inhibitory depending on the timing of exposure, dosage, length of treatment, presence of other factors, and cell type. TGF-β_1 in particular regulates expression of AP activity, and of collagen and fibronectin in cartilage, suggesting that it is a key modulator of cartilage repair. The effects observed at any given time are highly dependent on interaction with the various tissue factors. This complex interplay needs to be kept in mind

in interpreting the numerous studies now being reported on the growing repertoir of agents known to affect the skeletal system.

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