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Research article

Modulation of sulfated proteoglycan synthesis and collagen gene expression by chondrocytes grown in the presence of bFGF alone or combined with IGF_1

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Summary — Prepubertal rabbit epiphyseal chondrocytes were grown in high density primary culture for 3 d. They were then incubated for 3 additional d in serum-free culture medium to which bFGF (1–50 ng/ml) was added. During the last 24 h incubation period, either IGF_1 (1–80 ng/ml) or Insulin (1–5 μg/ml) was added to the culture medium. Chondrocyte DNA was significantly augmented with the increasing concentration of bFGF used, thus confirming its mitogenic effect on chondrocytes. On the other hand, bFGF was also shown to modulate the phenotypic expression of the chondrocytes. The ^{35}S-sulfate incorporation into newly synthesized proteglycans by the cultured cells decreased in a dose-dependent manner with bFGF concentration used. In addition, chondrocyte collagen gene expression was also shown to be modulated by bFGF. Total RNA extracted from the cultured cells was analyzed by dot blot and Northern blot with cDNA probes encoding for α_1II and α_1I procollagen chains.

A significant lower level of type II collagen mRNA, the marker of chondrocytic phenotype, was observed when cells were grown in the presence of bFGF while the level of type I mRNA remained unchanged. When IGF_1 or a high concentration of insulin was added to the cells during the last 24 h of incubation with bFGF, sulfated proteoglycan synthesis, as well as collagen type II mRNA level, were significantly stimulated when compared with chondrocytes incubated with bFGF alone.

In conclusion, in the present experimental conditions, bFGF appears to be a growth promoting agent for chondrocytes *in vitro* with dedifferentiating action on chondrocyte phenotype. IGF_1 or insulin used at a high concentration can prevent the dedifferentiating effect of bFGF without inhibiting its stimulating effect on chondrocyte DNA synthesis.

proteoglycan / collagen / chondrocyte phenotype / bFGF, IGF_1 / growth

Résumé — Modulation de la synthèse des protéoglycanes sulfates et de l'expression des gènes de collagène par des chondrocytes cultivés en présence de bFGF seul ou associé à IGF. *Les chondrocytes épiphysaires de lapin prépubères sont mis en culture à haute densité cellulaire pendant 3 j. Les cellules sont ensuite incubées pendant 3 j supplémentaires dans un milieu de culture sans sérum en présence de bFGF (1 à 50 ng/ml). Pendant les dernières 24 h d'incubation,*

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on ajoute soit IGF_1 (1 à 50 ng/ml), soit Insuline (1 ou 5 µg/ml), soit rien. Le taux d'ADN cellulaire augmente en fonction de la concentration de bFGF utilisé, confirmant l'effet mitogène de ce facteur sur les chondrocytes. D'autre part, l'addition de bFGF au milieu de culture pendant 3 j modifie l'expression phénotypique des chondrocytes. En effet, la synthèse des protéoglycanes mesurée par l'incorporation de soufre 35 dans les cellules est diminuée de façon significative et cette diminution dépend de la concentration de bFGF présent dans le milieu. De plus, l'expression des gènes de collagène est modulée par l'addition de bFGF. Les ARN totaux des chondrocytes sont analysées par dot Blot et Northern Blot avec les sondes ADNc des procollagènes α_1II et α_1I. Quand les cellules sont incubées avec bFGF, on note une diminution significative des taux d'ARNm de collagène de type II, marqueur du phénotype chondrocytaire, alors que les taux d'ARNm de collagène de type I ne sont pas modifiés. L'addition d'IGF_1 ou de fortes concentrations d'insuline pendant les 24 dernières h d'incubation en présence de bFGF inhibe les effets observés avec bFGF seul : on observe en effet une augmentation de la synthèse des protéoglycanes sulfatés ainsi que des taux d'ARNm de collagène de type II. En conclusion, dans nos conditions expérimentales, le bFGF stimule la multiplication cellulaire des chondrocytes in vitro *et a une action dédifférenciante sur le phénotype des chondrocytes. IGF_1 ou de fortes concentrations d'insuline peuvent prévenir l'effet dédifférenciant de bFGF sans inhiber son effet stimulant sur la synthèse d'ADN.*

protéoglycanes / collagène / phénotype du chondrocyte / bFGF, IGF1 / facteurs de croissance

INTRODUCTION

It has been previously shown that bFGF is a potent mitotic agent for mesoderm-derived cells and particularly for chondrocytes *in vitro* (Corvol *et al,* 1972; Jones and Addison, 1975; Sachs *et al,* 1982). It has also been suggested that bFGF stabilizes the phenotypic expression of other cell types in culture, but its effect on chondrocyte differentiation remains unclear (Kato and Gospodarowicz, 1985). In addition, IGF_1 is originally considered to act as a systemic or autocrine regulator of cartilage growth and differentiation. Recent observations have suggested that bFGF and IGF_1 could have a synergistic effect on growth and differentiation of muscle cells (Allen *et al,* 1985). This could also be the case for cartilage.

In the present study, the effects of bFGF-alone or combined with IGF_1 on chondrocyte phenotype *in vitro*, were studied using a short term, high-density culture system of differentiated rabbit epiphyseal chondrocytes. Chondrocyte differentiation was followed at the protein level by the synthesis of sulfated proteoglycans and at the mRNA level by the expression of α1 II. Chondrocyte dedifferentiation was studied by measurement of an increasing level of α1 I collagen mRNA.

MATERIALS AND METHODS

Chrondrocyte culture

The animals used were Fauve de Bourgogne prepubertal rabbits, 250-300 g body weight.

Primary cultures of chondrocytes were developed using rabbit epiphyseal cartilage from the hip, knee and shoulder according to Green (1971). After sequential enzymatic digestion of the cartilage tissue, the chondrocyte suspension was distributed either into 24 microwell tissue culture plates at a density of 8×10^4 cells/well for DNA measurement and ^{35}S-sulfate incorporation either into 75 cm^2 culture flasks at a density of 8×10^5 cells/flask for RNA determination. The culture medium used was Ham F_{12} supplemented with antibiotics (100 UI/ml penicillin, 100 µg/ml streptomycin) and containing 10% fetal calf serum (FCS). The cells were incubated at

37 °C in 5% CO_2 atmosphere. Three d later the chondrocytes formed multilayered non-confluent colonies. The culture medium was replaced by serum-free Dulbecco's medium to which basic fibroblast growth factor (bFGF) was added at a concentration ranging between 1–50 ng/ml, and the cells were incubated for a further 3 days. In some experiments, 24 h prior to the end of incubation, the medium was replaced by fresh medium containing a similar concentration of bFGF and either IGF_1 (1–80 g/ml), insulin (1 or 5 μg/ml) or nothing else. Cells incubated in serum-free Dulbecco's alone were used as controls.

DNA measurement

Chondrocytes grown in 24 well tissue culture plates were used for DNA measurement. After the 3 d incubation period in the presence or absence of bFGF, the medium was discarded, the cells were washed twice with Gey's balanced salt solution and trypsinized in EDTA – 0.25% – trypsin for 15 min at 37 °C. DNA was measured by the fluorometric technique described by Kapuscinski and Skoczylas (1977). Results were expressed as the mean ± SEM μg DNA per well of 4 similarly treated wells. In some experiments, an aliquot of the trypsin extract was also used for cell count in a hemocytometer and results were expressed as cell number per well. Results were compared to the amount of DNA or cell number observed in cultures completely grown in the presence of 10% FCS.

^{35}S-sulfate-incorporation into chondrocyte proteoglycans

Microwell tissue culture plates similar to those used for DNA measurement were prepared. After 2 d incubation in the presence or absence of bFGF, the cells were incubated in fresh serum and sulfate-free Dulbecco's medium containing : 1.5 μCi/ml of ^{35}S-sulfate to which 10 ng/ml bFGF was added alone or in combination with IGF_1 or insulin at the same concentrations as above. The incubation was performed at 37 °C for 20 h. Medium and cell layer were then treated separately with 3 mol/l guanidinium hydrochloride in 0.05 mol/l tris HCl buffer pH 7.4. Newly synthesized radio-labelled proteoglycans secreted by the cells were then purified onto a DEAE cellulose column with more than 75% recovery, and measured as previously described (Corvol *et al*, 1987). Aliquots of cell pellet and culture medium extracts were added separately to 5 ml scintillation liquid and counted in a β scintillation tricarb with an efficiency of 80%. The counts were expressed as the means ± SEM dpm/well of 4 similarly treated wells. Total amount of sulfated proteoglycans synthesized and secreted (cell + medium) by cultured chondrocytes in each well was then expressed as dpm/μg DNA measured in similarly treated wells.

Statistics

Data reported as mean ± SEM were compared using the Student's *t* test. *P* values < 0.05 were considered to be statistically significant.

RNA extraction and analysis

Chondrocytes cultured in 75 cm^2 flasks were incubated, as described above, in the presence or absence of bFGF for 3 d with or without IGF_1 or insulin added during the last 24 h incubation period. Groups of 4 similarly treated flasks were prepared. The guanidinium isothiocyanate/CsCl method of Chirgwin *et al* (1979) was used to extract total cellular RNA from the chondrocytes. Fibroblasts total RNA was extracted from rabbit skin fibroblasts in culture.

Total RNA was denatured with a mixture containing formamide, formaldehyde and morpholine-propanesulfonic acid (Mops), according to the procedure described by Maniatis *et al* (1985). For dot blot analysis aliquots, (0.5, 1, 2 and 4 μg), were deposited directly on hybond N under vacuum. For Northern blot analysis, aliquots of 2 and 4 μg of denatured RNA were run on 1% denaturing agarose gel and transferred to hybond N.

Recombinant cDNA for bovine α_1II procollagen (BC7) and human α_1I procollagen (Hf 677), kindly provided by Dr Ramirez (USA) (Chu *et al*, 1982; Sangiorgi *et al*, 1985), were labelled by nick translation using [α^{32}P]–dCTP (800 Ci/mmol). The mean specific activity observed was

2–4 x 10^8 cpm/µg. The rat β actin cDNA was used as an internal control.

The hybond membranes were prehybridized and hybridized at 42 °C for 4 h and 24 h, respectively, in a solution containing 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.5% SDS, 5% Dextran sulfate and 100 µg/ml salmon sperm DNA. The hybridization solution was supplemented with 4–8 x 10^6 cmp/ml of the appropriate cDNA probe.

After hybridization, the membranes were washed at 65 °C several times with 2X SSC/ 0.1% SDS for 15 min and exposed to Cronex films for various time periods. Quantitative results were performed using scanning densitometry.

RESULTS

Effect of bFGF on chondrocyte proliferation

When primary cultured chondrocytes are incubated for 3 d in serum-free medium supplemented with bFGF at different concentrations, there is a dose-dependent increase in DNA content and cell number of the cultures, as shown in figure 1. The addition of IGF_1 or a high concentration of Insulin during the last 24 h incubation period did not modify the chondrocyte DNA content (data not shown).

Effect of bFGF, IGF_1 and Insulin on ^{35}S-sulfate incorporation into chondrocytes

The ^{35}S-sulfate incorporation into chondrocytes grown in serum-free medium supplemented with bFGF (5, 10 or 50 ng/ml) for 3 d decreases in a dose-dependent manner with the concentration of bFGF used. The total ^{35}S-sulfate incorporation (medium + cell) is observed, respectively, at 1.6 ± 0.08 x 10^4, 1.1 ± 0.16 x 10^4 and 0.95 ± 0.17 x 10^4 dpm per µg DNA.

When IGF_1 (1–40 ng/ml) was added to these cultures during the last 24 h incubation period, there was an increase in ^{35}S-sulfate incorporation (figure 2A). The maximum effect of IGF_1 is observed at 40 ng/ml in the presence of 10 ng/ml bFGF. Insulin added at a high concentration (0.5, 1 or 5 µg/ml) also increases ^{35}S-sulfate incorporation with a maximum effect at 5 µg/ml, but no significant variation occurs with the bFGF concentrations used (figure 2B).

Effect of bFGF, IGF_1 and Insulin on mRNA level encoding the procollagen α_1II and α_1I chains

The level of chondrocyte mRNA was studied after a 3 d incubation period in the presence of 10 ng/ml bFGF and compared to chondrocytes grown in 10% FCS culture medium, and incubated with either 1% or 0% FCS during the last 20 h incubation period. Dot blot analysis of total RNA shows that the level of α_1II mRNA is significantly lower in cultures developed in the pres-

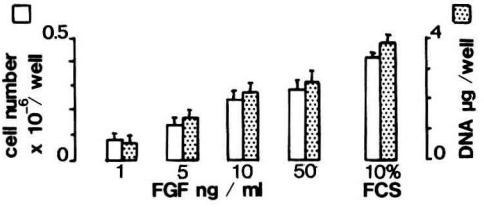


Fig 1. Effect of increasing concentration of bFGF on DNA and cell number. Chondrocytes were grown in primary culture for 3 d in the presence of FCS. They were then incubated for 3 additional d in serum-free medium containing bFGF at different concentrations. DNA and cell number were measured and compared with results observed in cultures developed in the presence of 10% FCS. Each point represents the mean ± SEM of 4 similarly treated culture wells.

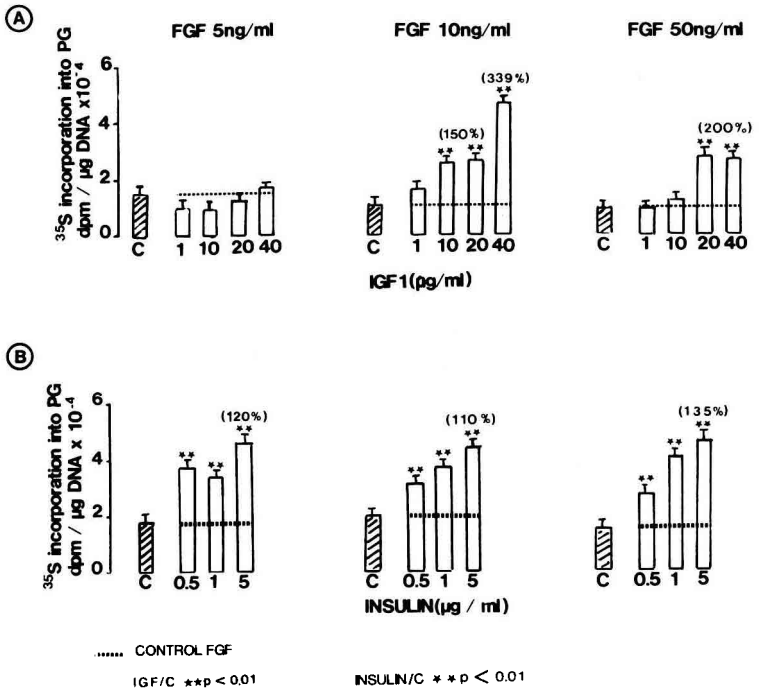


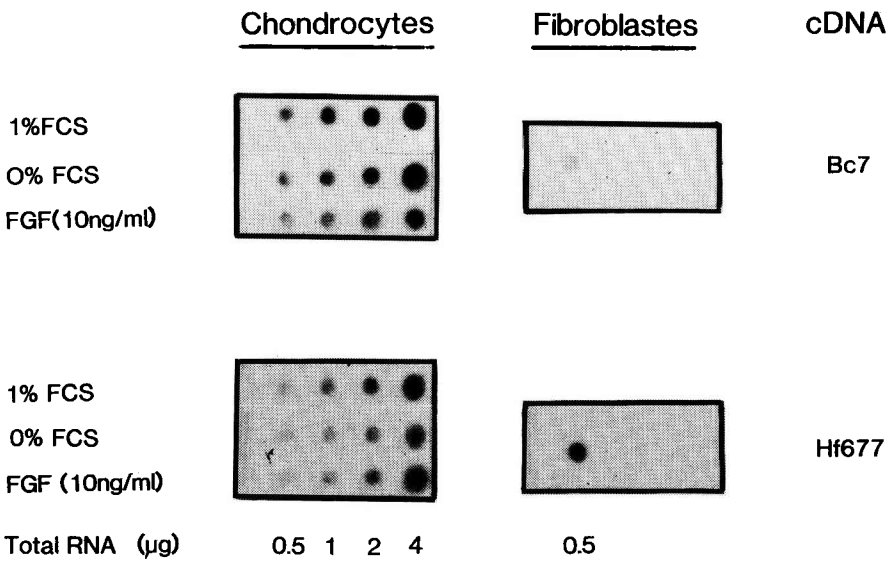
Fig 2. Effect of bFGF alone or combined with IGF_1 or Insulin on ^{35}S-Sulfate incorporation by cultured chondrocytes. Chondrocytes were grown in primary culture for 3 d with FCS. They were then incubated for 3 additional d in serum-free medium to which bFGF was added at different concentrations. During the last 24 h incubation period, either IGF_1 or Insulin were added. ^{35}S-Sulfate incorporation into newly synthesized proteoglycans was measured as in *Methods*. Results observed in chondrocytes treated with bFGF + IGF_1 or bFGF + Insulin were compared to chondrocytes incubated with bFGF alone. Each point is the mean ± sem of 4 similarly treated wells. ** $P < 0.01$.

ence of bFGF. Figure 3 represents 1 out of 3 experiments which show a significant 60 ± 10% lower level of α_1II mRNA in the presence of bFGF than with 1% or 0% FCS. Control is performed using BC7 hybridization with total mRNA from cultured fibroblasts.

The level of α_1I mRNA is not modified, as measured by dot blot hybridization with a Hf 677 cDNA probe.

The combined effect of bFGF with IGF_1 (2–20–80 ng/ml) added to the cultures during the last 20 h incubation period was studied by Northern blot analysis of chondrocyte total RNA and is presented in figure 4.

The cDNA BC7 probe demonstrates the presence of a single transcript of 5 kb in cultured chondrocytes with only very slight cross-hybridization with fibroblast collagen transcripts. IGF_1 added at 2 ng/ml, has no effect on α_1II mRNA level, but at higher concentrations, it significantly increases Type II collagen mRNA in a dose-dependent manner when compared to cells incubated with bFGF alone.



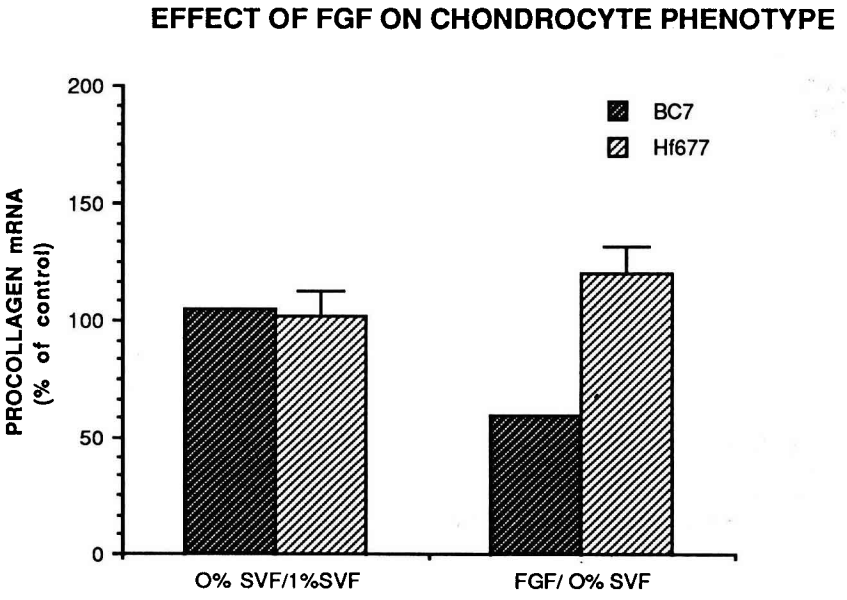


Fig 3. Dot blot analysis of total RNA extracted from chondrocytes cultured in the presence of bFGF (10 ng/ml) for 3 d and compared to cells incubated without bFGF, or with 1% FCS. Quantitative results were measured by densitometry and are represented on the diagram.

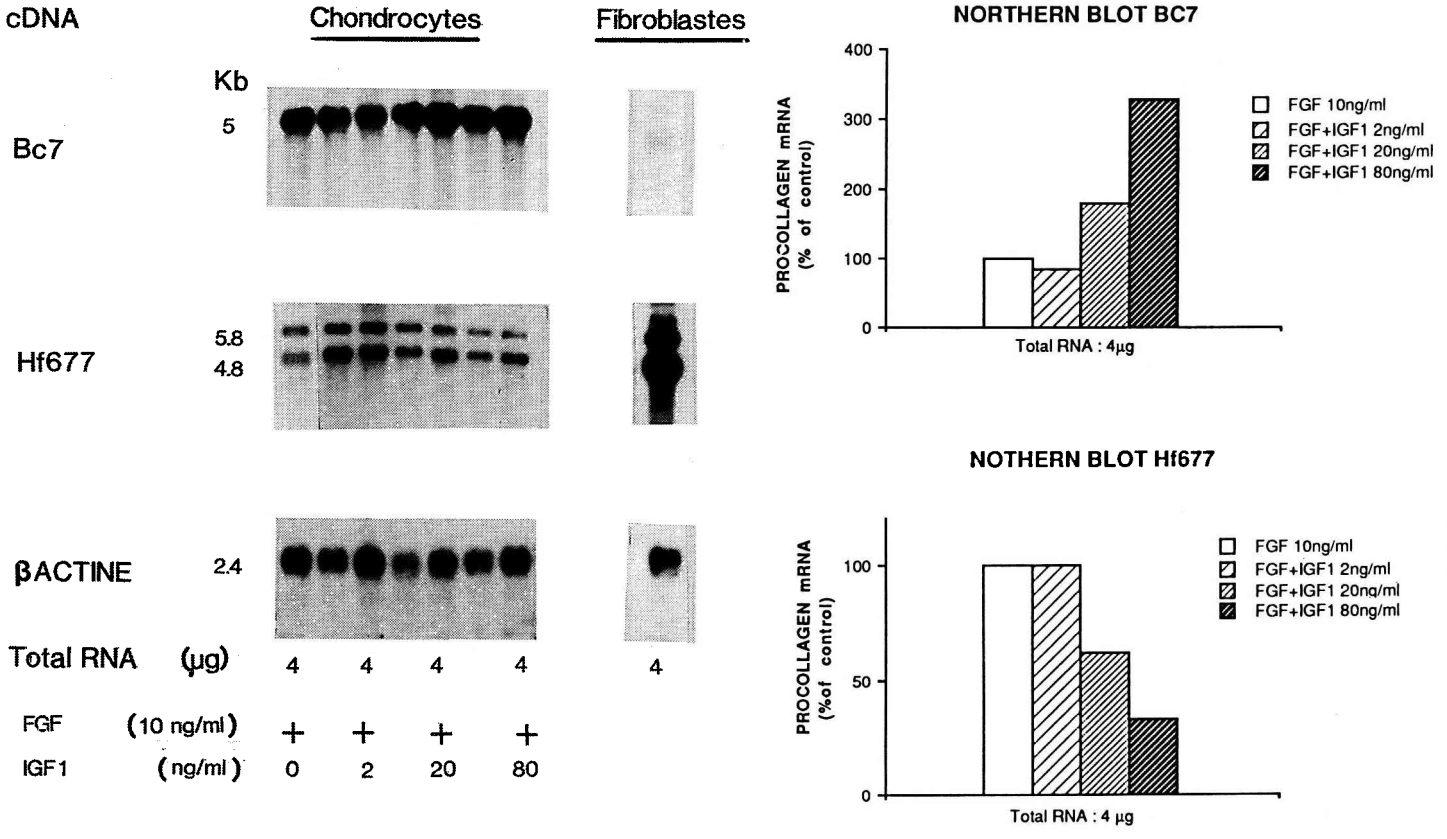


Fig 4. Northern Blot analysis of the mRNA levels of cultured chondrocytes treated with bFGF alone or combined with IGF$_1$, as described in *Methods*, and hybridized with either α_1II (Bc7) or α_1I (Hf 677) cDNA probes. RNA extracted from cultured rabbit fibroblasts was used as negative control. Filters were exposed for an identical length of time (24 h). Quantitative results, as measured by densitometry, are shown on the diagram.

Hybridization with Hf 677 cDNA probe shows the 2 specific proα_1I transcripts of 5.8 and 4.8 kb expressed in cultured fibroblasts. These transcripts are also detected in chondrocytes but at a lower level than in fibroblasts.

When IGF_1 is added to the cells at 2.20 or 80 ng/ml, the α_1I mRNA level represents 95%, 60%, and 35%, respectively, of that extracted from chondrocytes grown in the presence of 10 ng/ml bFGF alone.

Insulin (5 µg/ml) has a similar effect as IGF_1 (40 ng/ml) on α_1II, as well as on α_1II mRNA levels (fig 5).

DISCUSSION

The data presented in this paper elucidate some correlation between a dedifferentiating action of bFGF on chondrocytes and its growth promoting effect on these cells *in vitro*. In addition, IGF_1 appears to be able to reverse the dedifferentiating effect of bFGF on chondrocyte phenotype.

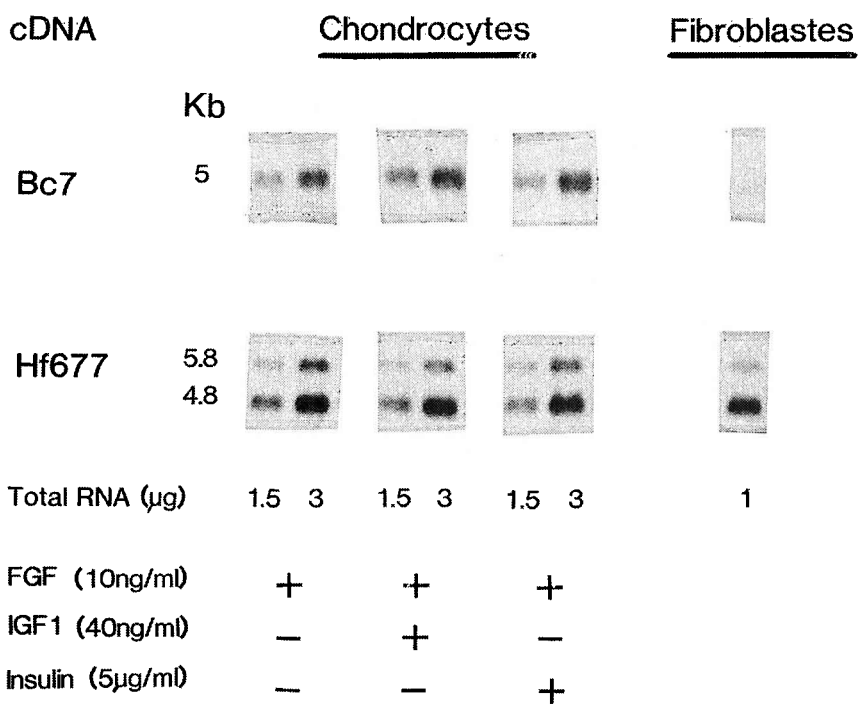
Differentiation and phenotypic expression of chondrocyte can be studied by expression of the main specific proteins synthesized by these cells, represented by proteoglycans of cartilage type and collagen type II. The recent availability to use specific cDNA probes for analysing mRNA procollagen chains allows us to study the co-ordinate regulation of different collagen genes in the same cells.

It is now well established that chondrocyte is phenotypically unstable in culture. Collagen type II mRNA is the major parameter of chondrocyte differentiation. When dedifferentiating, either spontaneously or in response to different agents, chondrocytes switch from type II to type I collagen synthesis (von der Mark *et al*, 1977) and first take up synthesis of α_1I mRNA, followed later by synthesis of α_2I mRNA (Duchene *et al*, 1982). For this reason, cDNA encoding for α_1I procollagen mRNA was used in this study to follow the dedifferentiating process of cultured chondrocytes treated by the growth factors.

Cartilage has been suggested to be a target tissue for bFGF, which strongly stimulates DNA synthesis in chondrocytes *in vitro* (Pieter *et al*, 1982; Sachs *et al*, 1982). In addition, structural studies and recent cloning of bFGF gene (Esch *et al*, 1985) have shown its homology with many other growth factors, among which, the "cartilage growth factor" first purified from cartilage tissue by Klagsbrun *et al* (1986). It is now likely that bFGF may be produced by cartilage cells or by endothelial cells from the microvessels heading chondrocytes in the resting zone of growth plate cartilage, or even by bone cells surrounding the mineralized matrix of epiphyseal plate (Hauschka *et al*, 1986). bFGF could thus act either as an autocrine or paracrine growth factor in growth plate cartilage. However, the mechanism of action of this factor remains unclear. The widespread distribution of bFGF, as well as the wide range of cell types on which it acts, suggest that its growth promoting effect is not tissue specific (Gospodarowicz *et al*, 1987).

Indeed, in the present study, bFGF induces DNA synthesis and cell multiplication of cultured epiphyseal chondrocytes, but is also modulates chondrocyte metabolism in a dedifferentiating pass way. When incubated for 3 d in the presence of bFGF, the chondrocytes become elongated (data not shown), produce smaller amounts of sulfated proteoglycans and contain a lower level of type II collagen mRNA.

Such a dedifferentiating effect of bFGF on chondrocytes is in contradiction with the data presented by Kato and Gospodarowicz on cultured costal chondrocytes (Kato and Gospodarowicz, 1985). In fact, one can suggest that the positive effect of



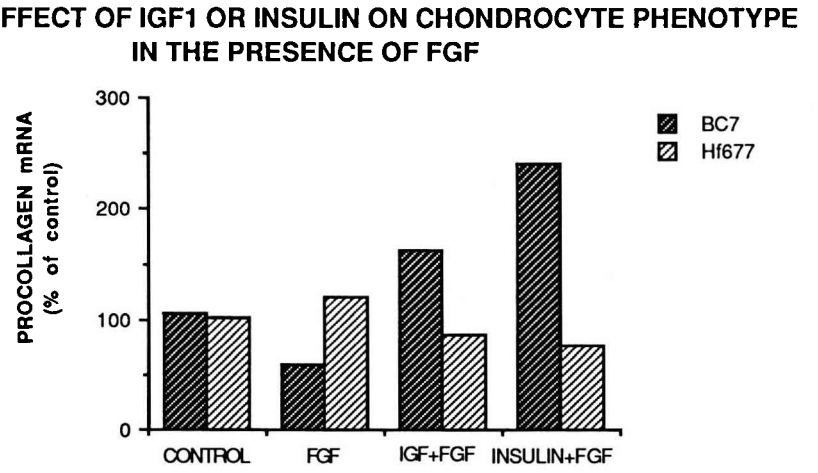


Fig 5. Northern Blot analysis of the mRNA levels of cultured chondrocytes treated with bFGF alone or combined with either 40 ng/ml IGF_1 or 5 µg/ml insulin. Hybridization with specific cDNA probes was performed as in fig 4. Quantitative results, as measured by densitometry, are shown on the diagram.

FGF, observed at that time, was due to impurities contained in the bovine pituitary extract used. Other publications presented by the same group are in favour of this hypothesis. They first observed a stimulating effect of differentiation on myoblasts when using a crude FGF preparation (Esch *et al,* 1985), while an inhibition of differentiation was shown with a more purified bFGF preparation.

A dedifferentiating effect on cultured chondrocytes has also been described when using several other growth promoting agents, including the thymidine analog 5-BrDU, embryo extracts (Mayne *et al,* 1976), or serum (Gauss *et al,* 1981). In these studies, chondrocyte proliferation is enhanced and is followed by a progressive loss of type II collagen gene expression and a shift to the expression of a type I trimer $(\alpha_1 I)_3$ and to collagen type I $(\alpha_1 I)_2$ $(\alpha_2 I)_1$. This dedifferentiating effect was suggested to be the consequence of the effect of growth factors on inducing the cells to enter the S phase and to synthesize DNA, thus blocking the expression of cellular protein genes. It seems likely that in our experimental conditions, such a mechanism of action of bFGF could occur.

More recently, Spizz *et al* (1986) suggested that bFGF may also have a direct specific inhibitory effect on the phenotypic expression of muscle cells independently of its stimulating effect on cell proliferation. Our experimental conditions do not allow us to determine whether or not this direct effect occurs in chondrocytes. Further investigations, using non-dividing confluent chondrocyte colonies, will be useful answering this question.

In fact, bFGF has been proposed to act as a competence factor, as well as other factors such as PDGF, EGF (Jiminez de Asua *et al,* 1977), while another group of factors is responsible for the progression of normal diploid cells committed to divide by brief exposure to competence factor (Stiles *et al,* 1979). IGF_1 was proposed to be one major progression factor for cartilage growth (Adashi *et al,* 1984), with a specific stimulating effect on cartilage sulfation.

In the present study, the stimulating effect of IGF_1 on sulfated proteoglycan synthesis is confirmed even in the presence of bFGF. IGF_1 thus appears to be able to either prevent or reverse the inhibitory effect of bFGF on proteoglycan synthesis, as was previously shown with insulin by Pieter *et al* (1982).

The stimulating effect of IGF_1 on chondrocyte phenotype is even better characterized when looking at collagen gene expression. IGF_1 is not only capable of enhancing type II collagen mRNA in bFGF treated cells, but also of reducing the expression of α1I procollagen chains. These data strongly suggest that IGF_1 may be a major regulating agent of collagen gene expression in chondrocytes.

Similar effects on chondrocyte proteoglycan and collagen are observed with a high concentration of insulin, suggesting involvement of IGF_1 receptors. There is also the possibility of interplay of both autocrine and exogenous action of IGF_1 on cartilage cells since chondrocytes have been shown to locally produce IGF_1 (Froger-Gaillard *et al,* 1989). It has been proposed by Zezulak and Green (1986) that IGF_1 could be one of the major factors stimulating the differentiation of mesenchym-derived cells such as chondrocytes or adipocytes. In this hypothesis, progenitor cells would be induced into the first step of differentiation by growth hormone and stimulated into the final differentiating process by IGF_1, as shown for adipocytes by Doglio et al (1987). In the present study, the stimulating effect of IGF_1 on chondrocyte differentiation, is observed even when these cells have previously

been slightly dedifferentiated by bFGF. Whether of not there is a dissociation of GH and IGF_1 action on chondrocyte phenotype, as was shown on adipocytes, remains to be seen.

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