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 35S-sulfate incorporation into newly synthesized proteoglycans 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$_{II}$ and α$_{I}$ 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.

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,
on ajoute soit IGF₁ (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 protéoglycans 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 α₁ et α₂. 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'IGF₁ 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 protéoglycans 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. IGF₁ ou de fortes concentrations d'insuline peuvent prévenir l'effet dédifférenciant de bFGF sans inhiber son effet stimulant sur la synthèse d'ADN.

protéoglycans / 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, IGF₁ is originally considered to act as a systemic or autocrine regulator of cartilage growth and differentiation. Recent observations have suggested that bFGF and IGF₁ 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 IGF₁ 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

Chondrocyte 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 x 10⁴ cells/well for DNA measurement and ³⁵S-sulfate incorporation either into 75 cm² culture flasks at a density of 8 x 10⁵ cells/flask for RNA determination. The culture medium used was Ham F₁₂ 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% CO₂ 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 IGF₁ (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 Kapsucinski and Skoczylas (1977). Results were expressed as the mean ± SEM μg DNA per well of 4 similarly treated wells. Results were compared to the amount of DNA or cell number observed in cultures completely grown in the presence of 10% FCS.

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 triacarb 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 cm² flasks were incubated, as described above, in the presence or absence of bFGF for 3 d with or without IGF₁, 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 α₁II procollagen (BC7) and human α₁I procollagen (HF 677), kindly provided by Dr Ramirez (USA) (Chu et al, 1982; Sangiorgi et al, 1985), were labelled by nick translation using [α²³P]-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 IGF1 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, IGF1 and Insulin on 35S-sulfate incorporation into chondrocytes

The 35S-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 35S-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 IGF1 (1–40 ng/ml) was added to these cultures during the last 24 h incubation period, there was an increase in 35S-sulfate incorporation (figure 2A). The maximum effect of IGF1 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 35S-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, IGF1 and Insulin on mRNA level encoding the procollagen α1, II and α1, I 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 α1, II mRNA is significantly lower in cultures developed in the pres-
ence of bFGF. Figure 3 represents 1 out of 3 experiments which show a significant 60 ± 10% lower level of α1Ii 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 α1I mRNA is not modified, as measured by dot blot hybridization with a Hf 677 cDNA probe.

The combined effect of bFGF with IGF₁ (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. IGF₁ added at 2 ng/ml, has no effect on α1Ii 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.
**EFFECT OF FGF ON CHONDROCYTE PHENOTYPE**

**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 IGF₁, as described in Methods, and hybridized with either α₁(II) (Bc7) or α₁(II) (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 proαI 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 IGF₁ is added to the cells at 2.20 or 80 ng/ml, the α₁I 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 IGF₁ (40 ng/ml) on α₁I, as well as on α₁II 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, IGF₁ 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 α₁I mRNA, followed later by synthesis of α₂I mRNA (Duchene et al, 1982). For this reason, cDNA encoding for α₁I 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 epiphysial 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 epiphysial 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
### bFGF, IGF₁ and chondrocyte phenotype

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**EFFECT OF IGF₁ OR INSULIN ON CHONDROCYTE PHENOTYPE IN THE PRESENCE OF FGF**

![Graph showing effects of FGF, IGF₁, and Insulin on procollagen mRNA levels.](image)

**Fig 5.** Northern Blot analysis of the mRNA levels of cultured chondrocytes treated with bFGF alone or combined with either 40 ng/ml IGF₁, 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 (α1I)3 and to collagen type I (α1I)2 (α2I)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). IGF1 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 IGF1 on sulfated proteoglycan synthesis is confirmed even in the presence of bFGF. IGF1 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 Piet er et al (1982).

The stimulating effect of IGF1 on chondrocyte phenotype is even better characterized when looking at collagen gene expression. IGF1 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 IGF1 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 IGF1 receptors. There is also the possibility of interplay of both autocrine and exogenous action of IGF1 on cartilage cells since chondrocytes have been shown to locally produce IGF1 (Froger-Gaillard et al, 1989). It has been proposed by Zezulak and Green (1986) that IGF1 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 IGF1, as shown for adipocytes by Doglio et al (1987). In the present study, the stimulating effect of IGF1 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, action on chondrocyte phenotype, as was shown on adipocytes, remains to be seen.

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