In vitro proliferation of achondroplastic and normal mouse chondrocytes, before and after basic fibroblast growth factor stimulation

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Abstract. Achondroplasia in mice is a recessive genetic disorder, characterized by disproportionate dwarfism with reduced bone growth. The cause of this chondrodystrophy is unknown.

In this study normal and achondroplastic mouse chondrocytes were cultured in monolayer primary culture, their differentiation was verified by immunofluorescence and their growth was compared.

The results showed that achondroplastic cells exhibited a higher proliferative activity than control cells of the same age, confirmed also by a thymidine incorporation assay. Furthermore, basic fibroblast growth factor treatment was found to induce a strong increase in growth of normal mouse chondrocytes, while it did not stimulate statistically significant proliferation of achondroplastic mouse cells.

We suppose that this different growth rate could play a role in achondroplastic phenotype development.

INTRODUCTION

Achondroplasia is a common form of disproportionate dwarfism, reported in many vertebrates, including humans and mice (Maroteaux & Lamy 1964; Rimoin 1975). It is characterized by the alteration of the growth process of the long bones, particularly of the growth cartilage that determines the skeleton's longitudinal growth capacity (Rimoin *et al.* 1970). This defect is hereditary and in mice manifests itself in homozygous recessive conditions due to the *cn* allele, located on chromosome 4 (Lane & Dickie 1968; Lane 1973). Also in humans achondroplasia was mapped on chromosome 4 (Francomano *et al.* 1994; Velinov *et al.* 1994), however, its genetic base differs from mice, since it is an autosomal dominant trait. Furthermore human achondroplasia seems to be due to amino acid alterations in the transmembrane domain of fibroblast growth factor receptor 3 (Rousseau *et al.* 1994; Shiang *et al.* 1994), while in mice the cause of this chondrodystrophy remains unexplained.

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Previous histological and histochemical investigations on skeleton growth showed that in achondroplastic mice endochondral ossification occurs in much the same way as in control, notwithstanding the reduction of length of long bones (Silberberger & Lesker 1975; Bonucci *et al.* 1976). Electron microscope studies of *cn/cn* mouse cartilage confirmed that the structure of cartilage and bone cells differs very little from that found in control mice and indicated that the main difference between achondroplastic mice and their normal offsprings is represented by early ageing-like changes in cartilage matrix and abnormal maturation of chondrocytes, with progressive reduction in cell number (Bonucci *et al.* 1977). Further studies on development of the growth plate showed that the process of chondrocyte differentiation, occurring during their hypertrophy, is disturbed. In particular, the hypertrophic zone is reduced in mice *cn/cn*, probably because of a loss of cells (De Marco 1981). No data on biochemical lesions in achondroplastic mice are known. Recent studies on the cartilage matrix protein gene (*Crtm*), mapped to the distal part of chromosome 4, to the same region of *cn*, excluded that mutations in *Crtm* can cause achondroplasia (Aszodi *et al.* 1998).

During the process of endochondral ossification, the chondrocytes undergo a series of tightly regulated maturational changes and many physiological factors are required for normal proliferation and differentiation of chondrocytes. Among these the basic fibroblast growth factor (bFGF) seems to play a role based on its ability to modulate chondrocytes phenotype. Studies indicated that bFGF is a potent mitogen for cultured chondrocytes (Kato & Gospodarowicz 1985; Kato, Iwamoto & Koike 1987) and recently, it was found that it is useful for improving cartilage repair *in vivo* (Fujimoto *et al.* 1999).

The aim of this study was to evaluate the growth of costal chondrocytes *in vitro*, comparing the cell proliferation of chondrocytes from achondroplastic and normal mice and also to determine the effects of bFGF on the cell growth in the two types of cultures. The results indicate that *in vitro* chondrocytes from achondroplastic mice proliferate more than normal controls and demonstrate that the growth factor, mitogen for 'normal' cells, does not stimulate significantly the growth of 'achondroplastic' chondrocytes.

MATERIALS AND METHODS

Cell culture

Mice of the '*cn*' strain, obtained from Jackson laboratory, were kept by mating heterozygous pairs yielding *cn/cn* and normal offspring.

Chondrocytes were obtained from rib cartilage of 7–10-day-old normal (*cn⁺/cn⁺* or *cn⁺/cn*) and achondroplastic (*cn/cn*) mice and cultured in monolayer culture, as described by Argentin, Cicchetti & Nicoletti (1993). Briefly, sterna were separated from the soft tissue, washed with phosphate-buffered saline (PBS) and cut into small pieces; then the fragments were incubated in 10 ml of PBS containing 5 mg/ml of collagenase and 10% of fetal calf serum (FCS). After 7 h the resulting cell suspension was centrifuged at 1000 rpm for 10 mins and the pellet resuspended in Ham's-F12 supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin. Cultures were incubated at 37°C in 5% CO₂ atmosphere and the medium was changed every 48 h until confluence.

Growth experiments

For growth experiments, chondrocytes were seeded at a density of 1×10^5 cells per 35 mm dish in 2 ml of complete medium. After an adherent step of 48 h, to allow the cells to attach to the culture plates, triplicate plates were incubated every 12 h for 10 mins in PBS

containing 0.02% trypsin and cells were harvested and cell number was determined with a haemocytometer.

In order to test the growth factor, chondrocytes were seeded (1×10^4 cells/ml) and maintained on plastic tissue culture dishes in supplemented medium either in the absence or presence of 1.0 ng/ml bFGF. This concentration was known to have an effect on chondrocyte proliferation. bFGF was added every other day via 10 μ l aliquots of medium. Control cultures were given the same amount of medium without bFGF. Within a period of 2–8 day in culture, triplicate plates were harvested, as above described, and growth was assayed every 24 h by counting the number of cells.

DNA synthesis determination

Chondrocytes were initially plated at a density of 1×10^3 cells/ml in 2 ml of Ham's-F12 with 10% FCS. Forty-eight hours before confluence, cells were synchronized in phase G1 by exposure to serum-free medium. After 24 h medium was replaced with 2 ml of Ham's-F12 with 20% FCS. When cultures reached confluence, cells were pulse labelled with [3 H] thymidine at 2 h intervals during 24 h. At each time, medium was removed and replaced with 2 ml Ham's-F12 without thymidine with 20% FCS and 185 kBq/ml (5 μ Ci/ml) [3 H] thymidine 247.9 GBq/mM (6.7 Ci/mM)). After the indicated intervals, cells were fixed in methanol and radioactivity was determined by scintillation counting.

Data were obtained from three cultures at each time point.

Immunofluorescence analysis

To confirm chondrocyte differentiation, cells were seeded at a density of 1×10^5 cells/ml. Subconfluent and confluent cultures were rinsed three times in PBS, fixed in methanol : acetone for 5 mins and dried at room temperature. The slides were then rinsed in PBS and incubated for 40 mins in a moist chamber in each of the following antibodies: to type II collagen (1 : 100) and to chondroitin sulphate (1 : 200). Then, they were incubated with fluorescein conjugated goat antirabbit IgG (1 : 200) for 30 mins, under the same conditions. Afterwards, the slides were mounted in glycerol-PBS, sealed and examined under fluorescent light.

Statistical procedure

All statistical analyses were performed using Student's *t*-test

RESULTS

Figure 1 shows the comparison between the growth curve of chondrocytes from normal and achondroplastic mice. The points of the curve represent the mean values of three separate cultures for each time interval; the linear bars refer to S.D. We shall refer to chondrocyte cultures as NOR for those from normal mice and ACH for those from achondroplastic mice. Both types of chondrocytes were seeded at a density of 1×10^5 cells per 35 mm dish and cultured in Ham's-F12 containing 10% FCS. After an adherent step of 48 h, the cells were harvested and counted (see M.M.). The proliferation rate of ACH chondrocytes was statistically different from that of NOR cells and the difference in behaviour of the two cultures was evident from the first counting, when ACH cells doubled their number, while NOR cells increased only by 60%. On the 6th day, the growth of ACH chondrocytes slowed down, probably because cultures reached confluence, while on the same day no slowing was

Figure 1. Comparison of growth curve of normal and achondroplastic cultured chondrocytes. Cells were seeded at 1×10^5 per 35-mm dish in complete medium. Cell number was determined as described under Materials and Methods. Values presented are averages $\pm$ SD for triplicate samples.

observed in normal cultures. This significant difference in cell proliferation of the two culture types remained until the last counting.

To confirm these data, DNA synthesis was quantified by pulsing cells with [3 H] thymidine at 2 h intervals during 24 h. Figure 2 gives the results of the isotope incorporation for cells measured at 2, 6, 12, 18 and 24 h. The level of [3 H] thymidine incorporation in cultures of ACH chondrocytes was considerably higher than that of normal chondrocyte cultures, reflecting a more active DNA synthesis i.e. a more rapid cell proliferation. In particular, in ACH cultures the doubling time for the cpm was 6 h and maximum stimulation of DNA synthesis was observed at 18 h; in contrast, in normal cultures the doubling time and maximum incorporation of [3 H] thymidine was 12 h and 24 h, respectively.

To ascertain whether this rapid proliferation of achondroplastic chondrocytes was not due to the loss of differentiated phenotype, their ability to synthesize cartilage-characteristic matrix was evaluated by an immunofluorescence assay with type II collagen and chondroitin sulphate (Fig. 3). The staining showed clearly that both substances were present in intercellular matrix and in the cell cytoplasm, confirming a good differentiation of the achondroplastic chondrocytes.

Furthermore, chondrocyte cultures were treated with basic fibroblast growth factor (bFGF) to evaluate the effect of this mitogen on cell proliferation. The results are illustrated in Fig. 4. Comparison between the growth curves of cultures maintained with or without bFGF showed the mitogenic effect of the growth factor for normal mouse chondrocytes but not for achondroplastic mouse cells. In fact, although a statistically significant difference was found between untreated and treated cells at second ($P \leq 0.001$) and third ($P \leq 0.001$) counting points, from fourth counting point onwards no significant difference was observed between ACH untreated and treated chondrocytes ($0.30 > P > 0.05$), whereas a difference remained between NOR untreated and treated cells ($0.008 > P > 0.0001$). Surprisingly no

Figure 2. Incorporation of [³H] thymidine in normal and achondroplastic chondrocyte cultures. Cells were seeded at a density of 1×10^3 /ml and confluent cultures were pulsed with 185 kBq/ml of [³H] thymidine for 2 h intervals during 24 h. The figure shows more representative experimental points. Triplicate dishes were determined for every point. The standard deviation in the different determinations did not exceed 5% of the mean.

significant difference was observed between NOR treated and ACH untreated chondrocytes ($0.53 > P > 0.12$).

DISCUSSION

The initial aim of the experiments was to evaluate and compare the growth of chondrocytes from normal and achondroplastic mice. The data clearly indicate that ACH chondrocytes displayed a higher proliferative activity in respect of those from normal mice of the same age and under the same culture conditions. These results, confirmed also by a thymidine incorporation assay, were unexpected since previous studies reported that the main difference between achondroplastic mice and normal controls seems to be represented by anomalous evolution of ACH cell cartilage that leads to reduction of the hypertrophic zone (De Marco 1981). It is well known that the endochondral ossification occurs during a series of successive steps; i.e. chondrocytes develop from mesenchymal cells, proliferate, undergo hypertrophy and finally are replaced by osteoblasts. Since hypertrophic chondrocytes are terminally differentiated cells that are derived from the maturation of proliferating chondrocytes, the reduction of the hypertrophic zone could be due to the decreased proliferation of chondrocytes: it was therefore expected that ACH cells in culture would show a decreased growth rate in comparison with NOR cells. In contrast, our data indicated that ACH cells proliferated more actively than NOR cells. Considering the good differentiation shown by our cultured cells, one plausible explanation for these results could be that they depended on the heterogeneity of isolated chondrocytes. In fact this study was performed using cells isolated

Figure 3. Immunofluorescence micrographs of achondroplastic chondrocyte cultures, stained with appropriate antibodies to (a) type II collagen and to (b) chondroitin sulphate. Micrographs were taken at confluence (magnification 250 $\times$).

from whole sterna, which represented a heterogeneous population of resting, proliferating and hypertrophic chondrocytes. Since a previous study showed a slower cellular evolution in ACH chondrocytes compared to controls (Bonucci *et al.* 1977), it is possible that sterna from achondroplastic mice, consisting of different cell populations heterogeneous in terms of development and differentiation, could arrest more proliferating cells than controls of the same age. Therefore the unusual behaviour in the growth rate could be due to the amount of proliferating cells and not to the different rapidity of growth.

To evaluate this hypothesis, we examined the effect of the basic fibroblast growth factor (bFGF) on the proliferation of ACH and NOR chondrocyte cultures, as it is well

Figure 4. Effect of bFGF on the proliferation of NOR (black symbols) and ACH (white symbols) chondrocytes. Cells were seeded at 1×10^4 per 35-mm dish and treated (squares) or not treated (triangles) by bFGF (1.0 ng/ml). Cell counts were determined as described under Materials and Methods. Values presented are averages $\pm$ SD for triplicate samples.

known that bFGF is the most potent mitogen for cultured chondrocytes (Kato & Gospodarowicz 1985; Kato *et al.* 1987). If our assumption was correct, bFGF treatment should have led a stimulation of cell growth more evident in ACH cells. The results of these experiments demonstrate that the behaviour of NOR and ACH chondrocytes differs with respect to the response to the mitogen factor: NOR cells show a strong statistically significant increase in their proliferation rate, while ACH cell cultures did not, except in the initial counting points. In this context, it is interesting to observe that the growth curves of untreated and treated ACH cultures show the same growth trend, with only a slight non-statistically significant increase in treated cultures. These data suggest two main implications: first, that chondrocytes from achondroplastic mice seem less sensitive towards bFGF than cells from normal mice and second that, in spite of this, their proliferation rate is similar to that of NOR chondrocytes cultured in the presence of bFGF.

With regards to the behaviour of ACH chondrocytes to bFGF, no studies are reported on a possible connection between murine achondroplasia and alterations in the receptors involved in the signal transduction of various fibroblast growth factors, as seen in man between mutations in a domain of FGFR-3 and achondroplasia (Rousseau *et al.* 1994; Shiang *et al.* 1994). Previous work showed that FGFR-3 deficient mice revealed a recessive phenotype with an enhanced bone growth, contrary to that observed in achondroplastic mice, suggesting that FGFR-3 is a negative regulator of bone growth, limiting rather than promoting osteogenesis (Deng *et al.* 1996). Moreover a recent study described the generation of achondroplastic mice by introduction of human achondroplasia point mutation into murine FGFR-3, resulting in a dwarf phenotype similar to that of *cn/cn* mice, yet dominant (Wang *et al.* 1999). On the other hand, it is very unlikely that this skeletal disorder is due to

the same alteration in both species, since in man it is the result of dominant mutation, while in mice it is recessive.

Since the results of the effect of bFGF do not support our previous hypothesis, i.e. the higher proliferation rate of ACH cells is due to the heterogeneity of examined cultures, we assume that the defect in *cn/cn* mice is due to both a disturbance of the cell cycle and a decreased period of proliferation. Indeed, chondrocytes from achondroplastic mice should have a more rapid cell division rate during a shorter period of time in respect of NOR cells, with a consequent premature degeneration and calcification of the matrix, that gives the cartilage an 'old' appearance. The pattern of cartilage maturation of young achondroplastic mice should therefore be similar to that of elderly normal mice, as reported by Bonucci *et al.* (1976, 1977).

This more rapid proliferative capacity of ACH chondrocytes could play an important role in the failed effect of the growth factor on these cells, because bFGF could be not able to enhance mitotic activity of cells, which already have a peculiar acceleration in growth rate.

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