Human Fetal and Adult Chondrocytes

Effect of Insulinlike Growth Factors I and II, Insulin, and Growth Hormone on Clonal Growth

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Abstract

Clonal proliferation of freshly isolated human fetal chondrocytes and adult chondrocytes in response to human insulinlike growth factors I and II (IGF I, IGF II), human biosynthetic insulin, and human growth hormone (GH) was assessed. IGF I (25 ng/ml) stimulated clonal growth of fetal chondrocytes (54±12 colonies/1,000 inserted cells, mean±1 SD), but IGF II (25 ng/ml) was significantly more effective (106±12 colonies/1,000 inserted cells, $P < 0.05$, unstimulated control: 14±4 colonies/1,000 inserted cells). In contrast, IGF I (25 ng/ml) was more effective in adult chondrocytes (42±6 colonies/1,000 inserted cells) than IGF II (25 ng/ml) (21±6 colonies/1,000 inserted cells; $P < 0.05$, unstimulated control: 6±3 colonies/1,000 inserted cells). GH and human biosynthetic insulin did not affect clonal growth of fetal or adult chondrocytes.

The clonal growth pattern of IGF-stimulated fetal and adult chondrocytes was not significantly changed when chondrocytes were first grown in monolayer culture, harvested, and then inserted in the clonal culture system. However, the adult chondrocytes showed a time-dependent decrease of stimulation of clonal growth by IGF I and II. This was not true for fetal chondrocytes.

The results are compatible with the concept that IGF II is a more potent stimulant of clonal growth of chondrocytes during fetal life, whereas IGF I is more effective in stimulating clonal growth of chondrocytes during postnatal life.

Introduction

Human skeletal growth is influenced by a variety of hormones and growth factors. Cartilage is the important target tissue for prenatal and postnatal growth (1).

The stimulatory effects of hormones and growth factors on human skeletal growth can be assessed in an in vitro culture system that measures the proliferation of single chondrocytes to cell clones in a semisolid medium (2).

It is widely accepted that throughout postnatal life some of the actions of human growth hormone (GH)[1] are mediated by the somatomedins/insulinlike growth factors (3). The direct growth promoting effect of insulinlike growth factor I (IGF I) has been demonstrated in hypophysectomized rats. Insulinlike growth factor II (IGF II) was clearly less active in these experiments (4, 5).

Whether or not GH acts directly on cartilage growth remains a matter of controversy. Most investigators have been unable to show any effect of GH on DNA or matrix synthesis of cartilage or chondrocytes in vitro (6, 7). Recently, Isaksson (8) reported that GH injected locally into epiphyseal growth plates of hypophysectomized rats leads to a significant increase of growth plate width. Madsen (9, 10) demonstrated GH-stimulated thymidine incorporation and proteoglycan synthesis in high density chondrocyte cultures.

A possible activity of insulin on skeletal growth is suggested by the clinical observation that newborn infants of insulin-dependent diabetic mothers display macrosomia. However, continuous intrauterine infusion of insulin to the pregnant rhesus monkey leads to weight gain only and not to a significant increase in body length (11). In vitro insulin exerts mitogenic action on a variety of cells, including osteoblasts, fibroblasts, and chondrocytes; however, supraphysiologic concentrations are needed (12).

The role of IGF II in fetal and postnatal growth remains to be defined. In postnatal life, levels of IGF II are less growth hormone-dependent than those of IGF I (13). Changes in IGF I and II concentrations before and after birth have been described in the sheep and rat fetus and newborn lambs and rats. In the sheep, IGF I levels rise during gestation to low postnatal levels (14). In the rat fetus IGF I rises during late gestation, falls after birth, and rises again after day 15 of postnatal life (15). IGF II levels are significantly higher in all stages of gestation than during postnatal life in fetal lambs and rats (14, 15). However, this finding was not confirmed in humans. The concentration of IGF II in fetal and cord blood was significantly lower than during postnatal life (16, 17) or similar to the value found in the adult (18).

The present study was designed to determine whether or not insulinlike growth factors I and II, insulin, and growth hormone have a direct effect on the clonal growth of human chondrocytes, and to determine if their effects on clonal growth differ in fetal and postnatal chondrocytes.

Methods

Materials. Pure IGF I and II were generously supplied by Dr. Humbel, Institute of Biochemistry, University of Zürich, Switzerland (19, 20). Biosynthetic human insulin was purchased from Eli Lilly & Co., Indianapolis, IN. Human growth hormone was kindly provided by Dr. Schleyer of the Proteohormon Laboratory of the University of Ulm, Federal Republic of Germany. Its specific activity was 2.5 U/mg protein.

Cartilage. Human fetal cartilage was obtained from the proximal and distal femoral epiphyses of four fetuses (18th–24th wk of gestation)

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1. *Abbreviations used in this paper:* GH, human growth hormone; IGF I and II, insulinlike growth factors I and II.
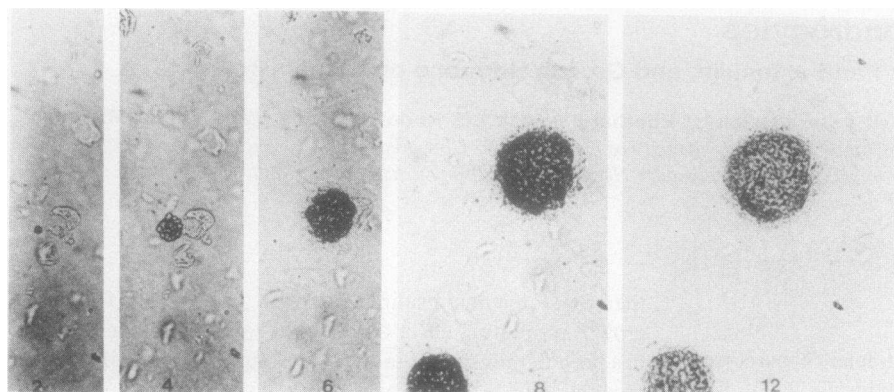

Figure 1. Colony growth of human fetal chondrocytes (day 2 through 12). At day 2 a four-cell stage is reached and at day 4 an eight-cell stage is reached; at day 12 a chondrocyte colony contains ~50–60 cells, and displays metachromatic staining and collagen type II by immunohistochemical staining (courtesy of Dr. K. von der Mark MPI, Munich) (magnification, × 125) (34).

after legal abortion. Adult articular cartilage from hip joints was obtained from three individuals aged 27–35 yr during surgery after accidental injury. None of the above fetuses or individuals had known endocrine or genetic disorders, degenerative or inflammatory disease, or generalized growth disturbances.

Isolation of chondrocytes. The cartilage was minced with a razor blade. The minced cartilage was transferred to 20 ml Ham's F10 Medium containing 0.25% trypsin and 2 mg/ml of collagenase (CLS II; Worthington Diagnostics Div., Millipore Corp., Freehold, NJ) and incubated at 37°C for 30 min to remove adherent perichondrium and connective tissue. The incubation was stopped by the addition of 2 ml fetal calf serum (FCS) and the incubates were centrifuged at 300 *g* for 10 min. The sediment was resuspended in 20 ml F10 Medium supplemented with 10% FCS, 200 mM L-glutamin, Eagle's minimal essential medium (MEM)-multivitamins, and 2 mg/ml of collagenase, and was incubated for 16 h at 37°C.

After digestion, a single cell sediment was obtained by centrifugation at 300 *g* for 10 min. After resuspension of the cells in Ham's F10 Medium, the cell number and viability of the cells was determined by trypan blue exclusion. The viability of fetal and adult chondrocytes after isolation averaged 90–95%. Microscopic evaluation proved that the chondrocytes were suspended as single cells and did not show aggregates.

Clonal proliferation assay of chondrocytes. The assay system used to measure the proliferative capacity of human chondrocytes was adapted from Bradley and Metcalf (2), who determined clonal proliferation of hematopoietic stem cells. The culture technique is based on the observation that the use of semisolid culture media prevents the sedimentation of cells and therefore enables proliferating chondrocytes to form colonies (21).

1×10^3 chondrocytes were incubated in 100 μl BM Whissler Medium (Boehringer Mannheim, Federal Republic of Germany) per individual incubate supplemented with 5% heat-inactivated FCS (FCS incubated for 60 min at 70°C), 10^{-4} M 2-mercaptoethanol, MEM-multivitamins, 40 μg/ml of gentamicin, 0.8% methylcellulose, and different concentrations of hormones and growth factors. The cultures were incubated without medium change for 14 d at 37°C, 5% CO_2, and 5% O_2 and 95% relative humidity. At the end of the incubation period the number of colonies per well was counted, 6–8 wells could be analyzed at different dose levels of hormones and growth factors (Fig. 1).

Monolayer culture of human fetal and adult chondrocytes. Freshly isolated chondrocytes were suspended in Ham's F10 Medium supplemented with 10% FCS, 10^{-4} mol 2-mercaptoethanol, 200 mM L-Glutamin, MEM-multivitamins, and 40 μg/ml of gentamicin. Volumes of 5 ml (5×10^5 chondrocytes/ml) were placed in Greiner culture flasks

Table I. Colony Formation of Human Fetal and Adult Chondrocytes

	ng/ml	n	Fetal chondrocytes		n	Adult chondrocytes	
			colonies/1,000 inserted chondrocytes			colonies/1,000 inserted chondrocytes	
Control		24	14.4±3.7		20	6.5±3.0	
10% FCS		24	99.2±28.7		19	52.0±17.8	
IGF I	6	26	19.8±4.8 NS		18	10.2±4.2 NS	
	12	25	32.6±5.6 <0.02		18	25.0±5.9 <0.01	
	25	23	53.9±11.4 <0.02	<0.05	17	42.0±15.9 <0.01	<0.05
IGF II	6	27	32.0±6.1 <0.05		18	7.2±4.3 NS	
	12	22	58.1±9.3 <0.02		18	13.3±3.9 NS	
	25	23	106.2±12.3 <0.001		18	20.8±6.2 <0.01	
Insulin	1	16	16.4±5.6 NS		14	5.6±2.6 NS	
	10	16	18.6±4.7 NS		17	8.7±4.2 NS	
	100	27	20.4±6.0 NS		17	12.6±9.5 NS	
Human GH	50	25	15.8±4.8 NS		16	6.4±3.1 NS	
	100	25	16.0±5.9 NS		16	6.2±2.9 NS	

Colony formation of human fetal and adult chondrocytes stimulated by 10% FCS IGF I and II, insulin, and growth hormone (GH) (mean±1 SD). *n* represents the total number of dishes incubated in four independent experiments of isolated chondrocytes from four different fetuses, and three independent experiments of isolated chondrocytes from three adult donors.

(50 ml/25 cm^2) and incubated at 37°C, 5% O_2 and 5% CO_2 and 95% relative humidity. Every third day a complete change of the medium was performed. At days 8, 15, and 22 the monolayer was scraped off the bottom of the flask and incubated for 2 h at 37°C in Ham's F10 Medium supplemented with 10% FCS, 200 mMol L-Glutamin, MEM-multivitamins, and 2 mg/ml of collagenase (CLS II; Worthington Diagnostics Div., Millipore Corp.). At the end of the incubations the cell suspension was centrifuged at 300 g and the cell sediments were washed twice with 10 ml Ham's F10 Medium. After resuspension of the cells in Ham's F10 Medium, the cell number and viability of the cells were determined as above.

The chondrocytes were then incubated in the clonal assay system as described above.

Statistics. Statistical analysis was performed using unpaired Student's *t* test.

Results

Colony growth of freshly isolated fetal and adult chondrocytes. The results of clonal growth promotion by growth hormone, insulin, and insulinlike growth factors I and II of human fetal and adult chondrocytes are summarized in Table I. Growth hormone (50 and 100 ng/ml) did not stimulate clonal growth of either fetal or adult human chondrocytes. Insulin (1, 10, and 100 ng/ml) also failed to enhance clonal growth of human fetal and adult chondrocytes. Insulinlike growth factor I at a concentration of 12 ng/ml and 25 ng/ml and insulinlike growth factor

II in all tested concentrations led to a significant stimulation of the clonal proliferation of fetal chondrocytes. Insulinlike growth factor II at a concentration of 25 ng/ml was significantly more effective than insulinlike growth factor I (25 ng/ml) in stimulating the clonal growth of fetal chondrocytes ($P < 0.05$).

Insulinlike growth factor I at a concentration of 12 ng/ml and 25 ng/ml and insulinlike growth factor II at a concentration of 25 ng/ml significantly stimulated clonal growth of adult chondrocytes. Insulinlike growth factor I (25 ng/ml), however, was more effective than insulinlike growth factor II in adult chondrocytes ($P < 0.05$). Both in human fetal and adult chondrocytes, insulinlike growth factors I and II showed a dose dependent pattern of clonal growth promotion.

Colony growth of fetal and adult chondrocytes after preincubation in monolayer culture. To investigate the influence of in vitro preincubation on the growth response pattern to insulin, GH, and IGF I and II, we grew human fetal and adult chondrocytes in monolayer culture for 22 d. The colony formation of freshly isolated chondrocytes (day 0) was compared with the colony formation of chondrocytes that were harvested for at different times (days 8, 15, 22) during the 22-d monolayer culture. Fig. 2 shows the morphology of chondrocytes. By day 8 of culture, chondrocytes expressed a typical polygonal shape and were partially growing in clones in the monolayer. At day 22 the chondrocytes had lost their phenotype and became fibroblastic in morphology.
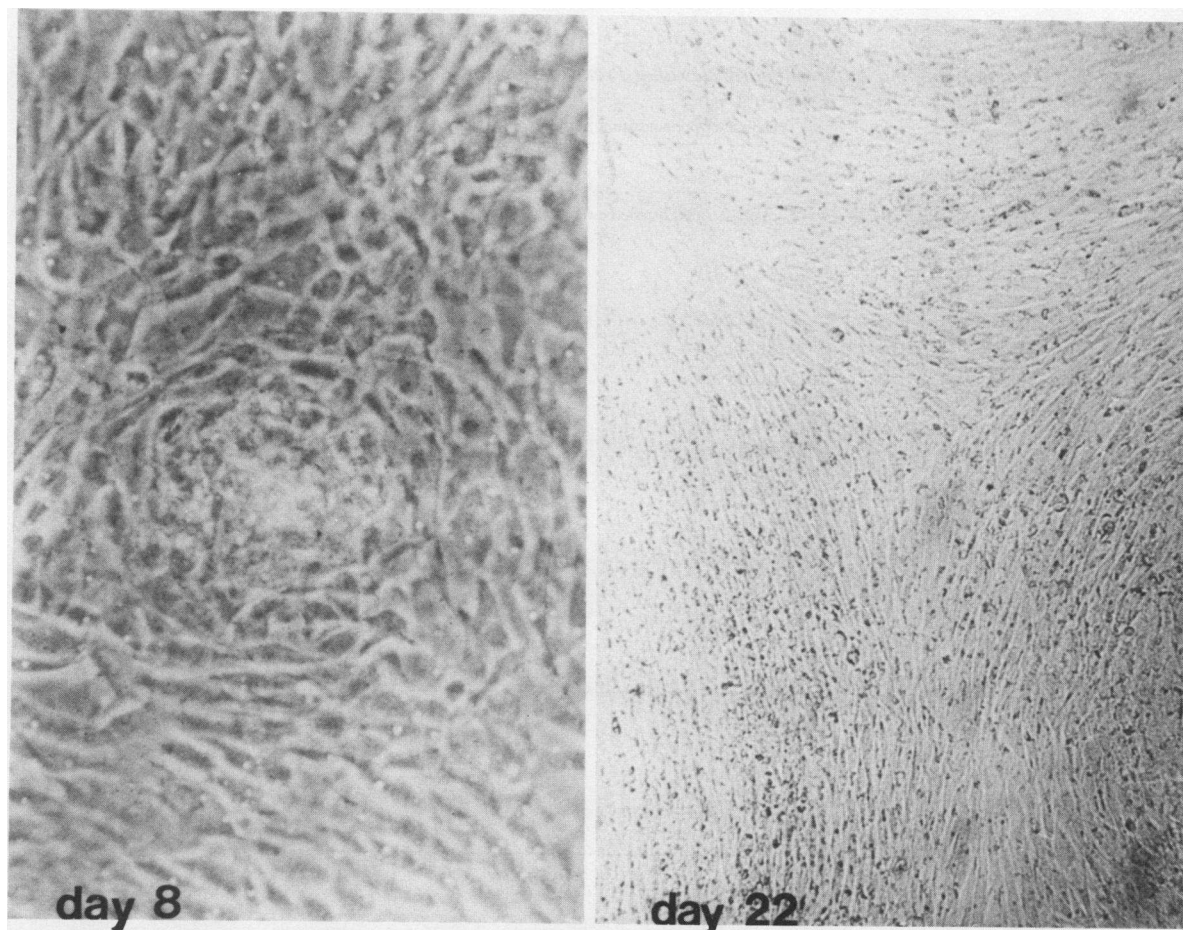

Figure 2. Monolayer culture of human fetal chondrocytes. Day 8, polygonal shape of chondrocytes that partly grow in colonies. Immunohistochemical analysis mainly shows collagen type II. Day 22, fibroblast-like appearance of dedifferentiated human fetal chondrocytes. Immunohistochemical analysis mainly shows collagen type I (magnification, day 8, × 125; day 22, × 50).

GH and insulin did not stimulate the clonal growth of fetal and adult chondrocytes at any time (results not shown). IGF II (25 ng/ml) was significantly more effective in stimulating clonal growth of fetal chondrocytes than IGF I (25 ng/ml) ($P < 0.05$) given. IGF I (25 ng/ml) was significantly more effective in stimulating clonal growth of adult chondrocytes than IGF II (25 ng/ml) ($P < 0.05$) (Table II).

The stimulating effect of IGF I and II on fetal chondrocytes did not decrease relative to time in the preincubation period. In contrast, the stimulation of adult chondrocytes by IGF I and II diminished significantly ($P < 0.01$) between day 0 and 22.

Discussion

There has been a longstanding interest in clarification of the stimulatory effects of insulin, GH, and IGF I and II on human fetal and postnatal growth. To investigate the direct mitogenetic action of these hormones and growth factors on skeletal growth, we studied the ability of human fetal and postnatal chondrocytes to proliferate in a clonal manner. Clonal growth is characteristic for chondrocytes in vivo and in vitro (22, 23). The assay system used enables the chondrocytes to proliferate in cell clones over a relatively long period of time and direct evidence of cell multiplication is gained in contrast to incorporation of labeled thymidin. This method merely defines cell populations that incorporate thymidin into the cell nucleus during S-phase of the cell cycle.

The direct action of IGF I and II has been demonstrated by systemic application to hypophysectomized rats (4, 5). IGF I was much more active in stimulating [^{3}H]thymidine incorporation into tibial epiphyseal growth plate than IGF II. In our in vitro assay system, IGF I was twice as active as IGF II in promoting the clonal growth of human adult chondrocytes.

Special interest has been directed to the possible role of IGF II in fetal skeletal growth. Several studies in rats (15, 24) and sheep (14) have shown that a serum growth factor displaying IGF II-like properties is elevated during fetal life when compared to postnatal life. Rat fetal fibroblasts in culture released predominantly rat IGF II and only small amounts of rat IGF I (25). However, postnatal cultured rat fibroblasts released increasing amounts of rat IGF I and decreasing amounts of rat IGF II in an age-dependent manner (25). Our studies using chondrocytes isolated from midgestational fetuses showed that IGF II was twice as active in stimulating clonal growth as IGF I. These findings are suggestive of a role of IGF II in human fetal skeletal growth, although IGF II concentrations in cord blood are low compared with adult values (18). Whether these discrepancies in the effects of IGF can be explained by special physiochemical properties of the IGF binding proteins during fetal life remains to be clarified, since the IGFs are almost exclusively bound to the 50,000 mol wt subunit and not to the 150,000 mol wt subunit of the binding protein as in adult life (26). When human fetal and postnatal chondrocytes were grown in monolayer culture for up to 22 d and subsequently cultured at different times, in

Table II. Colony Formation of Human Fetal and Adult Chondrocytes after Preincubation in Monolayer Culture

Chondrocytes	ng/ml	Freshly isolated	Days after preincubation in monolayer culture		
			8	14	22
		colonies/1,000 inserted cells	colonies/1,000 inserted cells	colonies/1,000 inserted cells	colonies/1,000 inserted cells
Fetal					
Control		11.6±2.6	16.7±4.1	13.6±2.4	15.0±4.5
IGF I	6	20.0±4.5	19.2±4.5	15.8±3.8	15.0±4.1
	12	33.3±6.0	36.4±5.4	31.6±2.6	32.5±4.2
	25	57.7±5.2	60.0±5.4	55.8±8.3	70.0±5.0
IGF II	6	32.5±4.2	30.7±8.9	21.7±2.6	30.0±3.2
	12	59.2±11.1	56.6±6.0	49.2±8.0	67.0±5.7
	25	108.3±8.2	97.5±14.0	95.0±10.0	99.2±5.8
Adult					
Control		17.5±4.2	5.0±3.2	8.3±4.0	4.2±2.0
IGF I	6	28.0±5.7	22.1±7.5	13.3±2.6	9.0±2.2
	12	56.0±4.1	45.0±4.5	27.5±4.2	20.0±4.5
	25	88.0±7.6	73.0±5.7	50.8±8.0	31.7±6.0

$P < 0.01$

IGF II	6	21.6±2.6	8.3±2.6	14.2±3.8	3.0±2.7
	12	43.6±4.8	28.3±3.3	23.3±5.1	11.7±6.0
	25	51.7±5.2	38.3±7.5	36.6±4.1	18.3±2.6

$P < 0.01$

Colony formation of human fetal and adult chondrocytes freshly isolated from cartilage and isolated from monolayer culture at days 8, 14, and 22. Stimulatory effects of IGF I and II (mean±1 SD). n represents the number of dishes. The chondrocytes were obtained from one fetus (20th week of pregnancy) and one adult donor (age, 28 yr).

our assay system the characteristic pattern of insulinlike growth factor I/II actions was not changed. The responsiveness of fetal chondrocytes to IGF I and II was not altered with increasing time of monolayer culture, whereas adult chondrocytes showed a time dependent decrease in responsiveness to both, IGF I and II, which might be due to a specific biochemical event such as a time-dependent loss of receptors during the preincubation of adult cultured chondrocytes.

The fetal and adult type responsiveness to IGF I and II obviously reflects a very stable gene expression which cannot apparently be changed under in vitro conditions such as those leading to a switch in the synthesis of type II to type I collagen (27).

In our in vitro assay system, growth hormone did not stimulate isolated human fetal and postnatal chondrocytes to proliferate in a clonal manner. This finding confirms the results of other investigators (6, 7), who were unable to demonstrate direct effects of growth hormone in different in vitro systems and clearly shows that growth hormone has no direct mitogenic effect on isolated chondrocytes. The observed increase of tibial width after local injection of growth hormone in hypophysectomized rats and the increased DNA and matrix synthesis in high density chondrocyte culture indicate that a possible direct action of growth hormone needs a special microenvironment which in itself might provide mediators for its action (8–10).

The growth stimulatory effect of insulin on cartilage and chondrocytes in in vitro systems has only been demonstrable using pharmacological doses (28). However, in a rat chondrosarcoma cell line (29, 30), physiological concentrations of insulin have been reported to stimulate matrix synthesis. Physiological insulin concentrations also stimulate collagen synthesis in fetal rat bone in vitro, which indicates that bone growth by matrix synthesis is enhanced by insulin. However, collagen synthesis in the subperiosteal cartilagenous cell layer representing appositional growth is not influenced (31). Administration of long-acting insulin to hypophysectomized rats leads to increase in body weight and length (32). Intrauterine application of insulin, however, has failed to provoke an increase of fetal body length in fetal rhesus monkeys and pigs (11, 33). In our study, insulin, even at supraphysiological concentrations, did not significantly promote clonal growth of either human fetal or adult chondrocytes.

Acknowledgments

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