EFFECT OF INSULIN ON CARTILAGE DIFFERENTIATION IN VITRO

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Opinions differ on the action of insulin on cartilage tissue. High doses of insulin, when injected into the yolk sac of chick embryos, caused deformation of the limbs of the chondromalacia type [13, 14], whereas small doses had an anabolic action on cartilage cells [3, 4]. Meanwhile Merker and Günter [12] showed that insulin in a concentration of 80-160 μg/ml of medium in which limb buds of chick embryos were cultured, delays cartilage tissue formation. The aim of the present investigation was to study the effect of insulin in serum-free medium on DNA biosynthesis and on differentiation of cartilage tissue in high density culture. Serum-free medium was used because serum added to a nutrient medium contains hormones which had an anabolic action on cartilage (STH, parathormone, calcitonin, insulin, and so on), as well as insulin-like growth factors (IGF I, IGF II), somatomedins A and S, NSiLH, cartilage growth factor, and so on, which simulate the action of insulin [1, 11, 16, 17].

EXPERIMENTAL METHOD

A high-density culture was obtained by the method described previously [7] from limb buds of chick embryos (Eurybrid) at the 23rd-24th stage of development according to Hamburger and Hamilton. A drop of 0.01 ml of medium with $2 \cdot 10^5$ cells in an area of 0.25 cm^2 was applied to the surface of a slide. During the first day the cultures were incubated in F-12 nutrient medium (Flow Laboratories, UK) with the addition of 50 μg/ml of ascrobic acid and 10% embryonic calf serum (Human, Hungary). On the second day all the material of the cultures was divided into three series: I) incubation in medium containing serum; II) in medium not containing serum; III) in medium without serum, but in the presence of various doses of insulin: 0.025, 0.25, 2.5, 5, 10, 20, 40 and 80 μg/ml (crystalline insulin, from Serva, USA). The cultures were incubated at 37°C in 95% humidity and in an atmosphere with 5% CO_2. The nutrient medium was changed every second day. Cultures for histological analysis were stained with toluidine blue. DNA [6] and uronic acid [5] were investigated by quantitative biochemical methods, and proteoglycan synthesis was studied with the aid of radioactive sulfate ($Na_2{}^{35}SO_4$, Izocommerz, East Germany) [15]. The radioactivity of the culture and nutrient medium was measured in an Isocap 300 liquid scintillation counter (Nuclear Chicago, USA).

EXPERIMENTAL RESULTS

On the 2nd day of culture, solitary nodules of cartilage tissue were observed to be appearing among the mesenchymal cells. At subsequent stages of development of the cultures after the use of nutrient media of different composition, different types of cartilage formation were observed. For instance, in series I on the 4th day, small discrete nodules of cartilage tissue could be identified, and on the 6th day they formed large agglomerations (Fig. 1). In series II at these same times the culture was still able to form cartilage, evidence that this process can take place even under suboptimal conditions, although at a low level of intensity (Fig. 1b). In series III insulin had a stimulating action on chondrogenesis compared with the cultures of series II only in doses of between 0.25 and 10 μg/ml medium, manifested on the 6th day by an increase in the number of foci and widening of the area of cartilage formation, and also an increase in the intensity of staining of glycosaminoglycans in the matrix of the newly formed cartilage tissue (Fig. 1c, d). An increase in the insulin concentration (from 40 μg and over) led to delay of chondrogenesis (Fig. 1d).

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TABLE 1. DNA (in μg/culture) and Uronic Acid (in μg/μg DNA) Concentrations on the Sixth Day of Culture in Nutrient Media of Varied Composition

Parameter studied	Series I	Series II	Series III							
			insulin concentration in nutrient medium without serum, μg/ml							
			0.025	0.25	2.5	5	10	20	40	80
DNA absolute	4,53±0,40	1,39±0,23	1,57±0,36	1,58±0,44	1,98±0,48**	2,48±0,39**	2,96±0,41**	3,15±0,43	3,36±0,45	3,37±0,64
%		100	112,7	113,5	142,1	177,6	212,0	225,6	239,3	241,7
Uronic acids	2,82±0,47	0,60±0,12	0,57±0,1	0,90±0,19*	1,14±0,32*	1,30±0,28*	—	—	—	1,17±0,14
%	100	21,3	20,2	31,9	40,4	46,1	—	—	—	41,5

Legend. *p < 0.001, **p < 0.005, ***p < 0.002 (compared with previous value). DNA content determined from 12 experiments, one of these using ten cultures, uronic acids were determined in 15 experiments, 12 cultures being used for one determination.

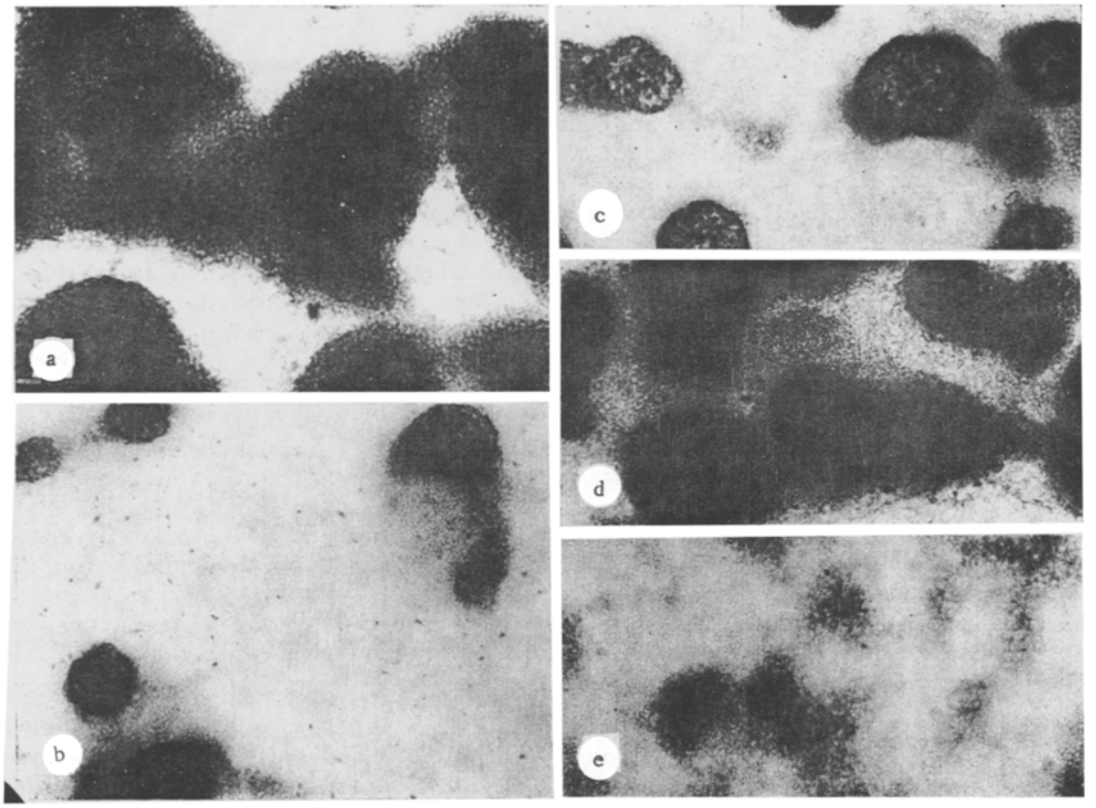

Fig. 1. High-density cultures obtained from limb buds of chick embryos on 23rd-24th stage of development, cultured in media of different composition for six days. Toluidine blue. 90 ×. a) Medium with embryonic calf serum. Extensive areas of chondrogenesis; b) medium without serum. Solitary areas of chondrogenesis; c) insulin 0.25 μg/ml. Increase in number and area of cartilage nodules compared with Fig. 1b; d) insulin 10 μg/ml. Further intensification of chondrogenesis; e) insulin 80 μg/ml. Inhibition of chondrogenesis.

Comparison of the DNA content in the cells on the 4th and 6th days of culture showed that the increase was greatest in the experiments of series I (2.81 ± 0.43 and 4.53 ± 0.40 μg) per culture depending on the time of investigation), and minimal values were obtained in series II (1.30 ± 0.20 and 1.33 ± 0.23 μg). Insulin in a concentration of 5 μg/ml medium increase the DNA concentration (2.76 ± 0.24 and 2.48 ± 0.39 μg/compared with series II, but compared with series I a decrease by 17 and 55%, respectively, was found). Neither in series II nor in series III was any increase in DNA synthesis observed between the 4th and 6th days. More complete information on the character of DNA synthesis in the cultures was obtained by the use of insulin in different concentrations on the 6th day of the investigation (Table 1). Within the dose range of 2.5-80 μg/ml stimulation of DNA synthesis was observed compared with series II, but the increase in the DNA concentration was more marked at concentrations of

TABLE 2. Change in Activity of Incorporation of Radioactive Sulfate (in cpm•10^3/μg DNA) in Cultures Growing in Nutrient Media with Serum (Series I), in the Absence of Serum (Series II), and without Serum But with the Addition of Insulin, 5 μg/ml of Medium (Series III) and in the Nutrient Media

Time of exposure to 2.2 MBq per 2 ml of medium, h	Culture (5 experiments, 5 tests in each)			Nutrient medium***		
	series I	series II	series III	series I	series II	series III
2 absolute	37,2±4,1*	0,73±0,14*	22,01±2,1	3,7±0,3	0,25±0,09*	3,78±0,40
%	100	100	100	100	100	100
4 absolute	47,88±5,82	1,24±0,24*	42,03±5,30	4,26±1,3*	9,5±0,26*	7,89±0,01
%	146	170	191	115	200	209
8 absolute	89,8±5,34**	1,59±0,47	62,25±8,45	14,9±0,97*	1,15±0,2*	21,8±1,58
%	275	218	315	403	460	577

Legend. *$p < 0.001$, **$p < 0.005$ for comparison of values between series of experiments (relative to series III); ***) after incubation for 20 h in medium with radioactive sulfate the cultures were washed and transferred into nutrient media without the isotope, and these were investigated at the specified times.

2.5, 5, and 10 μg/ml. When the intensity of chondrogenesis is compared with DNA content in the cultures, it must be noted that correlation between these parameters was found only within the range of concentrations mentioned above, and higher doses of insulin, while they increased DNA synthesis, inhibited chondrogenesis. Thus to judge from data in the literature [2, 10] and our observations, insulin stimulates DNA synthesis in high density culture, but under these circumstances high doses, in all probability, stimulate proliferation on non-skeletogenic cells in it, whereas lower doses stimulate proliferation of cartilage cells. Previous investigations [8] showed that in high density cultures obtained from limb buds of chick embryos, parallel with the appearance and widening of a zone of cartilage formation, the number of cells in the G_0 phase increased considerably. It could thus be concluded that differentiation begins in cells in this phase. The fact must be borne in mind that under the influence of definite insulin concentrations the DNA content and number of cartilage cells both increase, and the possibility cannot be ruled out that the generation time may be lengthened during culture without serum, and under these circumstances, it can be postulated that besides cell proliferation, the number of cells in the G_0 phase also increases. However, the question of the actual phase of the cell cycle at which insulin acts on the cells requires further investigation.

The results of determination of uronic acid levels, reflecting the appearance of phenotypic features of cartilage cells, are given in Table 1. They show that in the experiments of series II the uronic acid concentration fell by 79% compared with series I. Addition of insulin to the medium in a dose of 0.025 μg/ml caused no increase in this parameter, but a further increase in the insulin concentration (0.25, 2.5, 5.0, and 10.0 μg/ml) correlated positively with the uronic acid levels and the intensity of chondrogenesis. These results were evidence that insulin enhances cartilage differentiation, but this process is closely dose-dependent. These results were compared with those obtained by Merker and Günter [12], showing that insulin delays chondrogenesis, and it was concluded that the dose of insulin used by those workers must be considered to be toxic.

Another important indicator of differentiation of cartilage cells is their ability to undertake proteoglycan biosynthesis. The dynamics of formation of proteoglycans and their Secretion into the nutrient medium is shown in Table 2. These results bear evidence that insulin, in a dose of 5 μg/ml, increased the intensity of incorporation of radioactive sulfate into cartilage tissue compared with cultures grown without serum, and also stimulated secretion of proteoglycans into the culture medium by comparison with cultures growing in medium with serum, indicating that levels of differentiation of the cartilage cells and of their metabolism were raised.

The study of the action of insulin on chondrogenesis in high density culture obtained from limb buds of chick embryos thus revealed dose-dependent stimulation of proliferation and differentiation of cartilage cells.

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SEASONAL DYNAMICS OF ADRENAL MINERALOCORTICOID FUNCTION IN RATS

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Ever-increasing attention is currently being paid to the study of seasonal changes in function of the endocrine glands as an important factor in adaptation of the living organism to changing external environmental conditions. An important role in the chain of these adaptive mechanisms is played by the adrenals. The problem of circadian and circannual fluctuations of glucocorticoid secretion both in man and in various species of animals has been discussed sufficiently fully in the literature; as regards rhythms of mineralocorticoid activity of the adrenal cortex, however, only fragmentary information is available, pointing to considerable seasonal fluctuations [4, 10]. Accordingly it was decided to investigate seasonal rhythms of aldosterone metabolism in albino rats and to compare them with the time course of the concentration of pineal melatonin, a central regulator of adaptation of the organism to changing conditions of illumination.

EXPERIMENTAL METHOD

Male Wistar rats (10 to 15 individuals) weighing 150-180 g were used in the experiments, which were carried out in January, March, July, and October. Values of the plasma aldosterone level and its daily excretion with the urine were determined by radioimmunoassay, using standard test kits from CEA-IRE-Sörin (France), and the plasma metabolic clearance was determined by calculation by Van Slyke's formula. The melatonin content in the pineal gland was determined fluorometrically [3]. The animals were kept on an ordinary diet and water intake, under natural conditions of illumination. To collect the 24-hour urine and subsequently to determine its aldosterone concentration, the rats were kept under conditions of relative hypokinesia, by being confined to metabolic cages of small size. The diuresis and level of excretion of the hormone were determined separately on the 1st and 2nd days of the experiment.

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