ACTION OF TRIIODOTHYRONINE ON IN VITRO GROWTH OF ANLAGEN OF MOUSE LIMB BONES IN A MUTANT OF LINES BRACHYPODISM-H AND C57BL/61

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The processes of growth and differentiation of tissues are stimulated to a considerable degree by thyroid hormones, in association with their action on metabolism and, in particular, on the processes of oxidative phosphorylation [3]. It has recently been shown that thyroxine also stimulates the incorporation of amino acids into the tissue proteins of mammals in vivo and in vitro [8, 14]. The direct action of thyroxine and of triiodothyronine on growth of the bone anlagen of 4-8-day chick embryos has been demonstrated by organ culture. The action was dependent on the presence of the bone anlage and on the degree of its differentiation. These hormones inhibited growth of bones which in vivo and in vitro possess a high rate of growth, the femur and the tibia and,conversely, they stimulated growth of bones with a slower rate of growth, the radius and ulna [6, 7, 11, 12]. The degree of stimulation of growth of the bone anlagen was largely dependent on the concentration of triiodothyronine in the culture medium [13].

Several hereditary anomalies of the skeleton in man and animals are due to inhibition of the growth of cartilage and bone tissue at different stages of ontogenesis [9]. The object of the present investigation was to study the effect of triiodothyronine on growth of genetically injured bone anlagen in vitro.

The investigation of the action of the gene brachypodism-H (gene symbol bp^H) on growth of the anlagen of the long bones of the hind limbs of mouse embryos at the 13th-18th day of development showed that the main effect of the bp^H gene consisted of inhibition of the growth and differentiation of the fibula. The effect of this gene was also shown in vitro, and the strongest inhibition of growth was observed during cultivation of the fibula of 13-day embryos [2].

EXPERIMENTAL METHOD

Experiments were carried out on mice of the mutant line brachypodism-H, discovered in Harwell (England) as a repeated mutation of the brachypodism gene. The effects of the bp^H gene were described by Landauer [10].

The anlagen of the femur, tibia, and fibula of bp^H bp^H embryos of the 13th day of development were grown in culture. Cultivation was by Fell's method [5], as modified by Chen [4] in a wet chamber on a liquid medium in watch glasses at 37.2°. The composition of the culture medium was described previously by the authors [1]. The length of the bone anlagen was measured on drawings made by means of a drawing apparatus, and their relative growth after 2 and 4 days of cultivation was determined. Subculture, with complete replacement of the medium in one series of experiments was carried out after 48 h and in another series after 12 h. The 3,5,3'-triiodo-1-thyronine (T_3) was dissolved in 0.1% Na_2CO_3 solution and the concentration was adjusted to its final level by addition of medium No. 199. The following concentrations of T_3 were used: 0.02, 0.2, 0.4, 0.8, 2.0, and 7.0 μg/ml of medium. For the histological investigation the bone anlagen before cultivation and on the 4th day of cultivation were fixed in Zenker's solution and sections were stained with Delafield's hematoxylin.

TABLE 1. Effect of T_3 on Growth in Vitro of Anlagen of Limb Bones of $bp^H bp^H$ Embryos at the 13th Day of Development

Conc. of T_3 (in μg/ml medium)	Duration of cultivation (in days)	Relative increase in length (in percent)					
		femur		tibia		fibula	
		n	$M \pm m$	*n*	$M \pm m$	*n*	$M \pm m$
0,02	2	18	15±1,1	17	28±2,1	16	4±1,5
	4	18	16±1,0	17	36±1,4	16	5±1,6
0,2	2	24	14±0,9	21	31±1,5	24	17±1,0
	4	24	18±0,8	21	34±1,3	24	18±1,2
0,4	2	22	15±1,2	23	30±1,5	16	19±2,1
	4	22	18±1,0	23	33±1,5	16	21±1,6
0,8	2	14	16±1,2	13	38±1,7	14	19±1,0
	4	14	18±1,2	13	40±1,4	14	20±1,0
2,0	2	22	15±0,9	23	29±1,2	19	11±0,9
	4	22	16±0,8	23	34±0,8	19	12±1,0
7,0	2	20	13±0,9	19	21±1,5	19	0
	4	20	16±0,7	19	27±1,5	19	0
Control (without T_3)	2	23	14±0,8	18	30±1,7	23	0
	4	23	17±1,1	18	36±2,0	23	0
Length of bone anlagen before cultivation (in mm)		143	1,00±0,006	134	0,92±0,010	131	0,55±0,005

TABLE 2. Effect of T_3 on Growth in Vitro of Anlagen of Limb Bones of + + Embryos (C57BL/61) at the 13th Day of Development

Conc. of T_3 (in μg/ml medium)	Duration of cultivation (in days)	Relative increase in length (in percent)					
		femur		tibia		fibula	
		n	$M \pm m$	*n*	$M \pm m$	*n*	$M \pm m$
0,02	2	15	22±1,0	19	30±1,2	13	31±1,3
	4	15	24±1,2	19	36±0,8	13	35±0,9
0,4	2	20	25±0,8	20	35±2,1	18	24±1,9
	4	20	28±1,0	20	40±1,4	18	28±1,9
2,0	2	20	22±1,3	22	31±1,4	20	20±1,6
	4	20	25±1,2	22	37±1,5	20	22±1,4
Control (without T_3)	2	13	22±1,1	12	32±1,2	11	31±1,9
	4	13	25±1,2	12	36±1,2	11	36±1,5
Length of bone anlagen before cultivation (in mm)		68	1,08±0,01	73	0,96± 0,02	62	0,69±0,02

Hypertrophy of the chondrocytes of the fibula of the bp^H bp^H embryos was determined after cultivation in a medium with T_3 in a concentration of 0.2 μg/ml. For this purpose, 100 cells for each bone embryo were drawn by means of a drawing apparatus in 10 cases. The outlines of the cells drawn on graph paper were cut out and weighed. Knowing the weight of 1 cm^2 of paper and the linear enlargement of the section of the cell, the mean area was calculated. The effect of T_3 on growth of the anlagen of the long bones from the hind limbs of mouse embryos of inbred line C57BL/61 (gonotype + +) at the 13th day of development was also studied.

EXPERIMENTAL RESULTS

The results of the experiments of series I in which anlagen of the limb bones of bp^H bp^H embryos were cultivated with complete replacement of the medium after 48 h are given in Table 1.

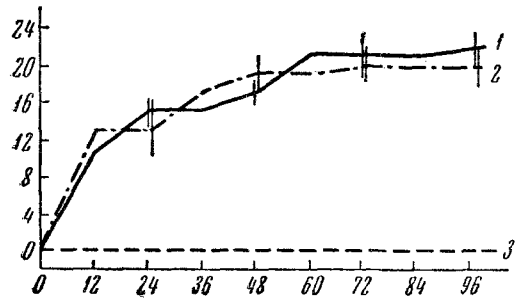

Fig. 1. Growth in vitro of anlage of the fibula of $bp^H bp^H$ mouse embryos at the 13th day of development. Along the axis of abscissas—duration of cultivation (in hours); along the axis of ordinates—relative increase in length of bone (in %). Replacement of medium with T_3 in a concentration of 0.8 μg/ml medium after 12 h (1) and 48 h (2), and of medium without T_3 after 12 h (3).

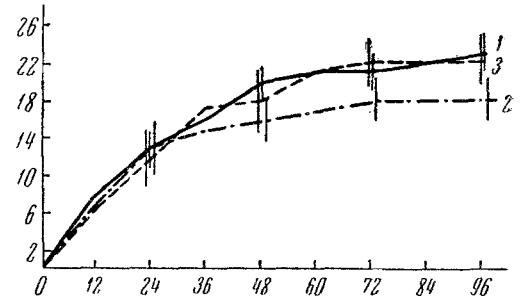

Fig. 2. Growth in vitro of anlage of the femur of $bp^H bp^H$ mouse embryos at the 13th day of development. Legend as in Fig. 1.

The femur and the tibia continued to grow and differentiate throughout the four days of cultivation in a medium without T_3. Meanwhile the fibula neither grew nor differentiated. The relative increase in length of the tibia of the bp^H bp^H embryos was the same as the increase in length of this bone in ++ embryos. The increase in length of the femur of the bp^H bp^H embryos was less than that of ++ embryos (Table 1 and 2). In a T_3 concentration of 0.02 μg/ml the relative increase in length of the fibula of the bp^H bp^H embryos increased by 5% during the four days of cultivation ($P < 0.01$). Maximal stimulation of growth of the fibula was observed in T_3 concentrations of 0.2, 0.4, and 0.8 μg/ml. With these concentrations of T_3, the relative increase in length of the bone during four days of cultivation was 18, 21, and 20% respectively (the differences in the increase in length between these three series of experiments were not statistically significant, $P > 0.05$). In a medium with a T_3 concentration of 2.0 μg/ml, growth of the fibula was slightly less ($P < 0.001$), while with a T_3 concentration of 7.0 μg/ml, the fibula, as also in the medium without T_3, did not grow. The relative increase in length of the femur and tibia of the bp^H bp^H embryos in a medium with T_3 concentrations of .02, 0.2, 0.4, 0.8, and 2.0 μg/ml was the same as in the medium without T_3. Only when the T_3 concentration was 7.0 μg/ml was growth of the tibia inhibited by 25% ($P = 0.001$).

The action of T_3 on growth of the fibula on the ++ embryos was opposite to its effect on growth of the analogous bone in the bp^H bp^H embryos. Triiodothyronine in a concentration of 0.4 μg/ml caused a decrease in the growth of the fibula of the ++ embryos ($P < 0.01$). Still more marked inhibition on growth was observed with a T_3 concentration of 2.0 μg/ml (the difference in the size of the increase in length with T_3 concentrations of 0.4 and 2.0 μg/ml was statistically significant, $P = 0.01$). Growth of the femur and tibia of the ++ embryos, like growth of the corresponding bone anlagen of the bp^H bp^H embryos was unchanged with T_3 concentrations of 0.02, 0.4, and 2.0 μg/ml (Table 2).

The question arose whether a greater increase in length of the fibula of the bp^H bp^H embryos could be obtained by a more frequent replacement of the medium with the T_3 concentration yielding maximal increase in growth of this bone in the series of experiments described above. For this purpose, complete replacement of the culture medium with a T_3 concentration of 0.8 μg/ml was carried out every 12 h. The fibula of the bp^H bp^H embryos neither grew nor differentiated following frequent replacement of the medium to which T_3 was not added. The relative increase in size of this bone following replacement of the medium with 0.8 μg/ml every 12 h was the same as the increase in size when the medium with 0.8 μg/ml of T_3 was replaced every 48 h, $P > 0.05$ (Fig. 1). More frequent replacement of the medium had a slight stimulant effect on growth of the femur of the bp^H bp^H embryos. The relative increase in size of the femur after cultivation for four days with change of medium every 12 and 48 h was 22 and 18% respectively ($P < 0.05$). Meanwhile, as in the preceding series of experiments, no action of T_3 was observed on the growth of this bone (Fig. 2). The relative increase in the size of the tibia when the medium was changed every 12 h with hormone and without hormone was the same as the increase in the size of this bone when the medium was changed every 48 h (Fig. 3).

Triiodothyronine influenced not only the growth of the anlage of the fibula of the bp^H bp^H embryos, but also its differentiation. Hypertrophy of the chondrocytes in the middle part of the anlage of this bone was on the average 80% when the T_3 concentration was 0.2 μg/ml. Meanwhile, no hypertrophy of the chondrocytes took place when the fibula of the bp^H bp^H embryos was cultivated in a medium without T_3. It is interesting to note that T_3, in concentrations having no stimulant action on growth of the femur and tibia, caused some increase in the rate of

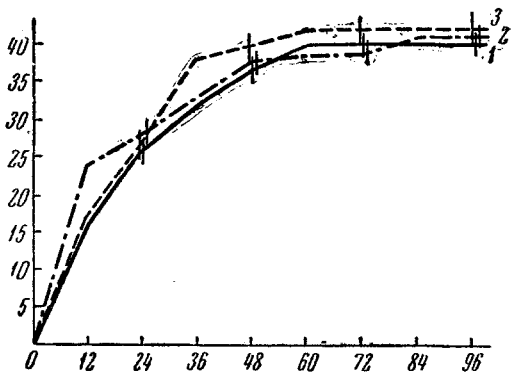

Fig. 3. Growth in vitro of anlage of the tibia of bp^H bp^H mouse embryos at the 13th day of development. Legend as in Figs. 1 and 2.

differentiation of the anlagen of these bones. In particular, this was shown by the appearance of signs of periosteal ossification, absent when cultivation took place in a medium without T_3. In all cases growth of the anlagen of the bones in a medium with and without T_3 was observed mainly during the first two days of cultivation. On the following days of cultivation the rate of growth of the bones was appreciably slowed.

Hence, the results of these investigations show that T_3, in concentrations of 0.2-0.8 μg /ml of medium has a marked stimulant effect on growth of the fibula of bp^H bp^H embryos. Triiodothyronine in a concentration of 0.02 μg/ml stimulated growth very slightly, while large concentrations of the hormone (2.0 and 7.0 μg/ml), on the contrary, slowed the growth of the fibula of bp^H bp^H embryos. The relative increase in size of the femur and tibia of the bp^H bp^H embryos did not increase in the presence of T_3. The stimulation of growth of the fibula of the bp^H bp^H embryos cannot be attributed to a lower degree of differentiation of this anlage by comparison with that of the femur and tibia. Triiodothyronine not only did not stimulate, but actually inhibited growth of the fibula of the ++ embryos, which was also less well differentiated than the femur and tibia of these embryos.

The difference in the action of T_3 on growth of the fibula, on the one hand, and of the tibia and femur on the other hand, was evidently due to the different origin of the mesenchyme in the postaxial and preaxial parts of the limb bud from which the anlagen of these bones are formed. This hypothesis is supported by the fact that in several mutant lines of animals, anomalies affecting mainly either the tibia or the fibula are observed [9].

As already mentioned, triiodothyronine accelerates growth and differentiation of the cartilaginous anlagen of the slowly growing bones of chick embryos [6, 7, 11, 12]. These results are in agreement with those obtained in present investigation in which the stimulant effect of T_3 was demonstrated on growth of the fibula of bp^H bp^H embryos. Addition of T_3 to the culture medium leads to a partial correction of the genetic defect of the fibula, which may be explained by a change in the primary action of the bp^H gene.

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