

EFFECT OF THE DEGREE OF DIFFERENTIATION ON GROWTH OF ANLAGEN OF THE LONG BONES OF THE LIMB IN MOUSE EMBRYOS IN VITRO

(UDC 612.64-083 : 612.753-019 + 612.753-019 : 612.64-083)

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Translated from *Byulleten' Éksperimental'noi Biologii i Meditsiny*, Vol. 61, No. 3,
pp. 86-89, March, 1966

Original article submitted April 12, 1965

It has been shown that growth of the anlagen of the long bones of the limbs in chick embryos in vitro is largely dependent on the composition of the culture medium [3, 5, 7]. The author has obtained similar results for anlagen of the long bones of the limbs of mouse embryos [1]. Growth of the anlagen of the bones also depends on the degree of differentiation of the anlagen at the time of explantation. Fell [4], for example, found that the relative increase in length of the femur of 5 $\frac{1}{2}$ -day chick embryos during cultivation for 27 days was 226%. However, if the femur of 6-day embryos was explanted, its relative growth was reduced by about half. Another investigation [6] showed that the femur and tibia of mouse embryos on the 20th day of development, at the stage of endochondral ossification, grew very slowly in vitro. During 22 days of cultivation their relative increase in length was only 30%. Meanwhile the relative increase in length of the tibia of 13-day mouse embryos during 8 days of cultivation was 100% [2]. However, no detailed study has yet been made of the influence of the degree of differentiation of anlagen of the limb bones of mammals on their growth and differentiation in culture.

This paper describes the results of a study of the growth and differentiation in vitro of the cartilaginous anlagen of the femur, the tibia, and the fibula of mouse embryos of 13, 14, and 15 days of development,

EXPERIMENTAL METHOD

Anlagen of the femur, tibia, and fibula of line C57Bl/61 mouse embryos of 13, 14, and 15 days of development were grown in culture. The methods of isolation and cultivation were described in the author's previous paper [1]. The composition of the culture medium was as follows: synthetic medium No. 199 (0.5 ml), ox serum (0.45 ml), Earl's solution (0.45 ml), and embryonic extract from 13-day chick embryos (0.1 ml). Glucose and penicillin were added to the culture medium in doses of 18 mg/ml and 200 units/ml respectively. The length of the anlagen of the bones was measured on drawings made by means of a drawing apparatus. The relative increase in length of the anlagen of the femur, tibia, and fibula over 2, 4, and 6 days of cultivation was determined. Measurements were also made of the increase in the volume of anlagen of the long bones of 13-day embryos over 6 days of cultivation by weighing drawings of serial sections. Some of the anlagen of the bones were fixed in Zenker's solution before and on the 2nd, 4th, and 6th days of cultivation. The sections were stained with Delafield's hematoxylin and erythrosin.

EXPERIMENTAL RESULTS

The anlagen of the long bones of the hind limbs of the 13-day mouse embryos continued to grow and differentiate in culture. During the 6 days of cultivation the femur increased in length from 1.09 to 1.38 mm, the tibia from 0.82 to 1.19 mm, and the fibula from 0.66 to 0.94 mm. The relative increase in length of the anlagen of the long bones of the 13-day embryos is shown in the table. Most of the growth of the bones in all cases took place during the first 4 days of cultivation; the differences in the growth between the 4th and 6th days were not significant ($P > 0.05$). During the 6 days of cultivation the volume of the femur increased by $140.1 \pm 19.8\%$, that of the tibia by

Relative Increase in Length of Anlagen of the Femur, Tibia, and Fibula of Mouse Embryos of 13, 14, and 15 Days of Development in Vitro (in %)

Age of embryo (in days)	Duration of cul- tivation (in days)	Number of anlagen	Femur	No. of anlagen	Tibia	No. of anlagen	Fibula
13	2	21	$24 \pm 1,1$	23	$36 \pm 1,6$	18	$33 \pm 1,9$
	4		$28 \pm 1,2$		$44 \pm 1,3$		$40 \pm 1,6$
	6		$29 \pm 1,3$		$46 \pm 1,2$		$42 \pm 1,6$
			$P < 0,05$ $P > 0,05$			$P < 0,001$ $P > 0,05$	$P = 0,01$ $P > 0,05$
14	2	15	$13 \pm 0,6$	17	$17 \pm 0,9$	15	$18 \pm 1,1$
	4		$15 \pm 1,0$		$19 \pm 0,8$		$20 \pm 0,9$
	6		$16 \pm 0,6$		$21 \pm 0,7$		$21 \pm 0,9$
			$P < 0,01$			$P < 0,01$	$P < 0,05$
15	2	19	$10 \pm 0,5$	19	$12 \pm 0,7$	17	$9 \pm 0,5$
	4		$11 \pm 0,6$		$13 \pm 0,6$		$9 \pm 0,5$
	6		$12 \pm 0,7$		$15 \pm 0,7$		$10 \pm 0,6$
			$P < 0,05$			$P < 0,01$	$P > 0,05$

Note: The relative increase in length of the anlagen of the femur, tibia, and fibula of the mouse embryos of 14 and 15 days of development between the 2nd and 4th, and also between the 4th and 6th days of cultivation was not statistically significant ($P > 0,05$). In the case of anlagen of bones of 14-day and 15-day embryos the significance of the difference in growth between the 2nd and 6th days of cultivation is given.

116.2 \pm 9.9%, and that of the fibula by 79.3 \pm 14%. In other words, the relative increase in the volume of the anlagen was much greater than the increase in their length. However, greater variability was observed in the volume than in the length of the individual anlagen of the bones, so that no measurements of the volume of the anlagen to the bones were made in the case of the 14-day and 15-day embryos.

At the time of beginning of cultivation the cartilaginous anlagen of the long bones of the hind limbs were only very slightly differentiated. The cells of the diaphyses differed from the cells of the future epiphyses only in the regular arrangement and the large amount of ground substance between the cells. The zone of hypertrophied chondrocytes in the femoral diaphysis was larger than in the tibia. The fibula showed least differentiation. After only 2 days of cultivation changes were found in the shape of the anlagen of all the long bones. The outlines of the anlagen were clearer and their shape resembled that of the definitive bone more closely than at the beginning of cultivation. Growth of the bones took place on account of lengthening of the diaphysis; at the same time the epiphysis became wider. The zone of hypertrophied cells became much larger during cultivation in the femur and tibia, and such a zone appeared also in the diaphysis of the fibula.

In all the anlagen the cells of the epiphyses remained small, and only the amount of ground substance between the cells grew. The zone of flattened cells did not form in the anlagen of the femur, tibia, and fibula during cultivation. By the second day of cultivation mitoses were found extremely rarely in the anlagen of the bones, and by the 6th day degenerative changes had appeared in the cells. Hence, growth of the anlagen of the bones of the 13-day embryos took place mainly on account of hypertrophy of the cells of the diaphysis and of an increase in the amount of ground substance between the cells. The degree of differentiation of the anlagen of the femur, tibia, and fibula of the 13-day embryos after 4 days of cultivation corresponded approximately to 1 day of development in situ.

The anlagen of the femur, tibia, and fibula of the 14-day embryos were more highly developed at the moment of cultivation than the anlagen of the same bones of the 13-day embryos. They had a zone of flattened cells and a zone of hypertrophied cartilage, and these zones were more clearly defined in the tibia than in the tibia and fibula. Over 6 days of cultivation the length of the femur increased from 1.65 to 1.9 mm, that of the tibia from 1.49 to 1.8 mm, and that of the fibula from 1.32 to 1.6 mm.

As the table shows, the relative increase in length of all the anlagen of the bones of the 14-day embryos was much smaller than in the case of the 13-day embryos. The increase in length in situ of the femur, tibia, and fibula

of the 14-day embryos likewise was smaller than that of the anlagen of the corresponding bones in the 13-day embryos. In culture, as in situ, significant differences remained between the growth of the femur and the fibula, and the femur and the tibia. The zone of flattened cells remained in the femur after cultivation, and a thin cuff of periosteal bone appeared in the center of the diaphysis. The zone of hypertrophied cartilage in the diaphysis of the tibia and fibula was considerably widened.

In the femur and tibia of the 15-day mouse embryos the diaphysis was surrounded by a bony cuff, while in the fibula the zone of hypertrophied cartilage occupied about half the diaphysis, and there was still no periosteal ossification. After 6 days of cultivation the length of the femur increased from 2.28 to 2.55 mm, that of the tibia from 2.22 to 2.55 mm, and that of the fibula from 2.07 to 2.30 mm. As the table shows, growth of all the cultivated bones of the 15-day embryos was much less than growth of the anlagen of these bones of the 14-day embryos. The tibia grew the most. The relative increase in the length of the femur and fibula during the period of cultivation was significantly less than the increase in length of the tibia. As it grew, the anlagen continued to differentiate in culture. The bony cuff in the femur and tibia became much thicker. In the femoral diaphysis the hypertrophied cartilage was completely destroyed and the process of endochondral ossification began. In the diaphysis of the fibula a bony cuff appeared.

Hence, the results of the study of the increase in length of the anlagen of the femur, tibia, and fibula of 13-day, 14-day, and 15-day mouse embryos of line C57B1/61 showed that growth in vitro took place mainly in the first 2 days of cultivation and on account of hypertrophy of the chondrocytes in the diaphysis and an increase in the amount of ground substance between the cells. On the subsequent days of cultivation the relative increase in the length of the anlagen of the bones was greatly reduced, and after the 4th day they began to show degenerative changes. Both in culture and in situ the most highly differentiated anlagen of the bones showed the smallest increase in length. The difference in the relative increase in the length of the various anlagen persisted. This shows that the method of organ culture may be used to study the pathogenesis of inherited anomalies of the mammalian limbs.

LITERATURE CITED

1. E. K. Ginter, In book: Proceedings of a Conference of Junior Scientists of the Institute of Experimental Biology of the AMN SSSR [in Russian], Moscow (1965), p. 12.
2. J. D. Biggers, R. B. Gwatkin, and S. Heyner, *Exp. Cell. Res.*, 25 (1961), p. 41.
3. J. M. Chen, *Ibid.*, 7 (1954), p. 518.
4. H. B. Fell, *Arch. exp. Zellforsch.*, Bd. 11, S. 225 (1931).
5. M. Kieny, *Arch. Anat. micr. Morph. exp.*, 47 (1958), p. 85.
6. J. S. Niven, *J. Path. Bact.*, 34 (1931), p. 307.
7. Et. Wolff, K. Haffen, M. Kieny, et al., *J. Embriol. exp. Morph.*, 1 (1953), p. 55.