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Abstract

Five pairs of immature, non-hemopoietic femur and tibia from 17-day-old gestating female rat fetuses, whose sex was determined by chromosomal analysis of liver cells, were transplanted into subcutaneous tissues of adult male rats. The original bones were about 3 mm in length and they grew to about 17 mm length at 4 wereks after transplantation. Bone deformation was not evident after transplantation and bone marrow hemopoiesis developed. Bone marrow cytohistologic observations were made on smears, and chromosome analyses were performed on bone marrow cells. Active erythro-, myelo- and megakaryopoiesis were conducted by cells of recipient adult rats. Sex chromosome analysis of cartilage cells from the epiphyses of transplanted bones demonstrated that the growing bones were composed of cells from the grafted embryo. The results thus strongly suggest that the transition of hemopoiesis from liver to bone marrow in late embryonic development is conducted by stem cells migrating through circulating blood and settling in the bone marrow and not by in situ cells differentiating in the bone marrow stroma.

KEYWORDS: embryonic bone, subcutaneous transplantation, hemopoiesis induction

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INDUCTION OF HEMOPOIESIS IN RAT EMBRYONIC BONE TRANSPLANTED INTO ADULT SUBCUTANEOUS CONNECTIVE TISSUE

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Abstsact. Five pairs of immature, non-hemopoietic femur and tibia from 17-day-old gestating female rat fetuses, whose sex was determined by chromosomal analysis of liver cells, were transplanted into subcutaneous tissues of adult male rats. The original bones were about 3 mm in length and they grew to about 17 mm length at 4 weeks after transplantation. Bone deformation was not evident after transplantation and bone marrow hemopoiesis developed. Bone marrow cytohistologic observations were made on smears, and chromosome analyses were performed on bone marrow cells. Active erythro-, myelo- and megakaryopoiesis were conducted by cells of recipient adult rats. Sex chromosome analysis of cartilage cells from the epiphyses of transplanted bones demonstrated that the growing bones were composed of cells from the grafted embryo. The results thus strongly suggest that the transition of hemopoiesis from liver to bone marrow in late embryonic development is conducted by stem cells migrating through circulating blood and settling in the bone marrow and not by *in situ* cells differentiating in the bone marrow stroma.

Key words: embryonic bone, subcutaneous transplantation, hemopoiesis induction

Mammalian embryonic hemopoiesis starts with yolk sac erythropoiesis. With the growth of the embryo, the erythropoiesis shifts from the liver to the bone marrow, though the spleen may participate in hemopoiesis in some species. In yolk sac hemopoiesis, erythroid cells are found in primitive blood vessels (1–3), but in fetal liver and bone marrow, distinguishing hemopoietic cells is difficult. It has been generally believed that liver and bone marrow hemopoiesis may be conducted by cells differentiated from the reticuloendothelial system of each organ, but hemopoiesis failed to develop in embryos after removal of the yolk sac (4). This finding indicates that hemopoiesis in the early embryonic stage is conducted by stem cells originating in the yolk sac and migrating to the spleen and liver (5). However, the mechanism of transition of hemopoiesis from the liver to bone marrow in late embryonic stage is not yet entirely clear. To analyse

this transition process, cytomorphologic observations were conducted on rat embryonic bones transplanted into the subcutaneous tissue of adult animals. Embryonic bones with no hemopoiesis were successfully accepted by syngeneic adult animals without immunological rejection (6–8). The transplanted bones increased in size, and the developing bone marrow cells indicated that cells participating in hemopoiesis were from the recipient animal and not from the grafted bone.

In this paper detailed findings are reported on the developing hemopoiesis in bone marrow of grafted immature bones and on chromosome analysis of the bone marrow and cartilage cells. The observations suggest that the transition of hemopoiesis from the liver to bone marrow is by stem cells migrating into the bone marrow through circulating blood, but not by *in situ* cells in bone marrow tissue.

MATERIALS AND METHODS

Twenty-six adult male Wistar rats and about 30 embryos of 17 ± 1 gestation days of age were used. The adult animals served as recipients and the embryos as donors and controls. The embryos were obtained from four pregnant Wistar rats by total hysterectomy under ether anesthesia. One femur and one tibia from one embryo were removed and transplanted into the inguinal subcutaneous tissues of a recipient animal that was a litter mate of the bearing mother. The bones were transplanted under anesthesia by intravenous injection of Nembutal (1.0g/kg). The other pair of femur and tibia were fixed with 10% formal for control observations. The sex of the embryos was determined by chromosome analysis of liver cells in about 10 chromosome plates of metaphase cells. Five male recipients were successfully transplanted female embryonic bones for observations of bone marrow cells in grafted bones. Four weeks after transplantation, the recipient animals were injected with colchicine (1.0 mg/kg) subcutaneously, and 2 h later the animals were decapitated. After sacrifice, the transplanted bones were removed, and the surrounding granulation tissues carefully removed. Then, the epiphyses were cut off, and the bone marrow tissues were pumped out into a watch glass using a syringe containing saline solution. The bone marrow cells were freed from the tissue using a small glass homogenizer. After removal of the connective tissue masses, the cell suspension was centrifuged at 1,200 rpm for 5 min. For cytological examinations, one drop of sedimented cells was added with a small amount of rat serum, smeared and stained with Giemsa. For chromosome analyses, the cells were spread by the Omura method (9). Karyotypic analyses were made on about 100 chromosome plates.

Eighteen recipient male animals transplanted with male embryonic bones were used for control histologic observations of bone marrow and granulation tissues surrounding the transplanted bones. The bones with the surrounding tissues were fixed with 10% formal, embedded in paraffin, sectioned and stained with hematoxylin-eosin.

For analysis of bone tissue, the cartilage of the epiphysis was resected by aseptic knives and cultured in 6 ml of TC 199 (GIBCO) with 15% fetal calf serum in tissue culture flasks (Falcon 3012). The cells were cultured at 37°C for 3 weeks with the medium being changed twice a week. At the termination of culture, 2 drops of colcemid (10 mcg/ml) were added to the medium in each flask, and incubated for a further 12 h. After incubation the cells in suspension culture were sedimented by centrifugation at 1,200 rpm for 5 min. Some sedimented cells were smeared, fixed with methanol and stained with Giemsa or Alcian blue. The remaining cells were washed 3 times with 0.6% sodium citrate by repeated centrifugation and used for chromosome analysis. The cells were separated from large clusters by repeated pumping with a small pipette, 1-2 times per second, for one

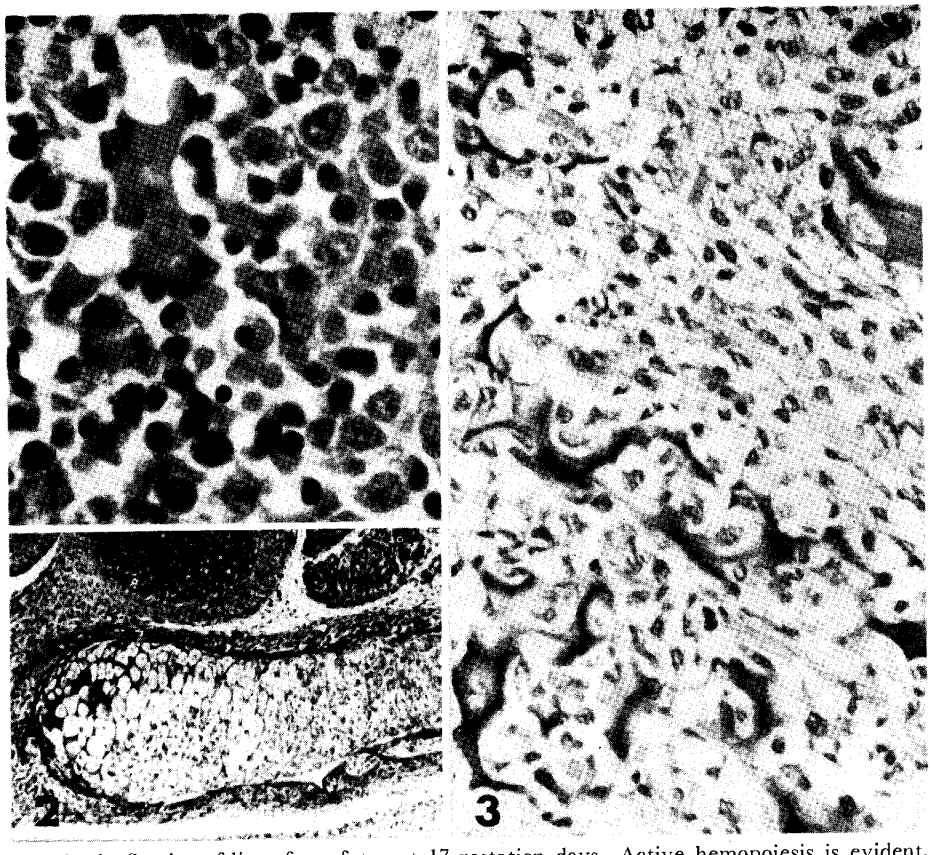


Fig. 1. Section of liver from fetus at 17 gestation days. Active hemopoiesis is evident. H. E. stain. ×400.

Fig. 2.. Bone marrow section of a femur from the same embryo as in Fig. 1. Bone marrow is composed of cartilage cells and immature reticular cells, but no hemopoietic cells are seen. H. E. stain ×40.

Fig. 3. An enlargement of the bone section in Fig. 2, showing the details of the cells. ×200.

hour. Then, cells were sedimented again by centrifugation at 1,200 rpm for 5 min and resuspended in 0.6% sodium citrate solution. Chromosome spreading was conducted on these cells by the Omura method. Karyotypic analyses were made on 6–10 chromosome plates from microphotographs.

RESULTS

Rat fetuses of about 17 gestation days of age showed active hemopoiesis in the liver (Fig. 1). The fetus femurs and tibias were about 3 mm in length. They were composed of cartilage tissues and some undifferentiated reticulum cells but hemopoiesis was not present in the bone marrow (Figs. 2, 3). In transplantation into the subcutaneous tissue of adults, the fetal femur and tibia grew to about 17 mm in length, or 300 times in volume 4 weeks after transplantation

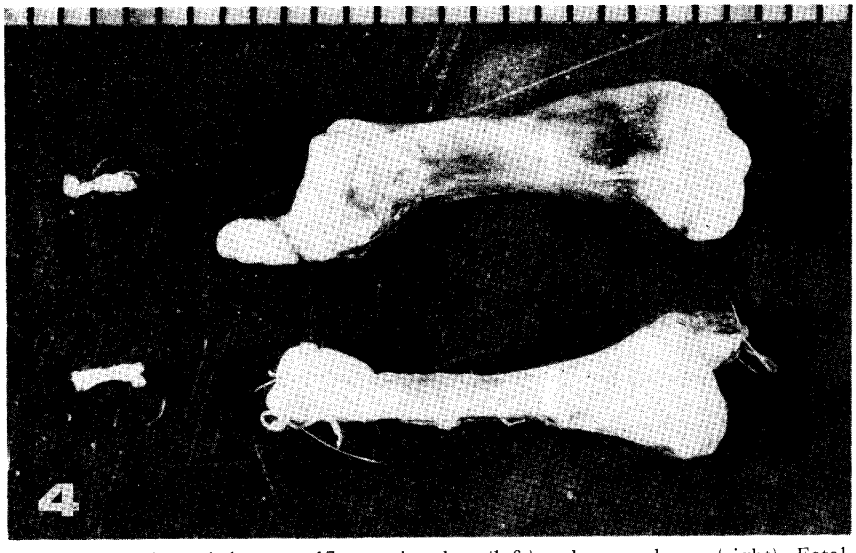
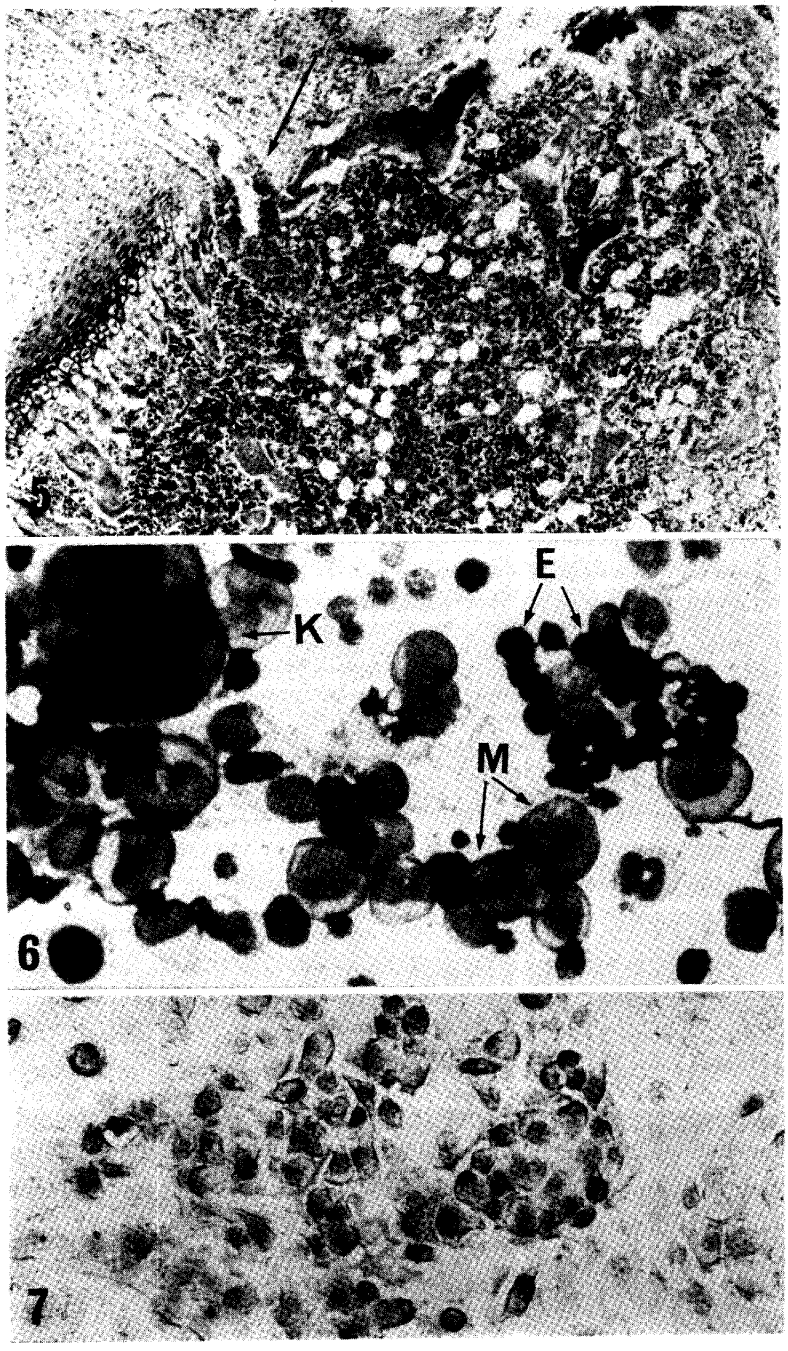


Fig. 4. Embryonic bones at 17 gestation days (left) and grown bones (right). Fetal femur (top left) and tibia (bottom left) transplanted in subcutaneous tissue of adult animal for 4 weeks. Grown femur (top right) and tibia (bottom right). Note that the fetal bone growth had morphologic expressions typical for the specific bone.

Fig. 5. Section of embryonic femur appearing in the top right of Fig. 4. An artery is seen (arrow) entering the bone marrow from the surrounding tissue. H. E. stain. ×40.

Fig. 6. Cell smear of bone marrow cells from the femur Fig. 5, showing development of myeloid cell (M), erythroid cells (E) and a megakaryocyte (K). Giemsa stain. ×400.

Fig. 7. Cartilage cells separated from the epiphyses of the embryonic bone 4 weeks after transplantation. The sample was cultured for 21 days in TC 199 medium and used for chromosome analysis. For methods, see the text. Alcian blue-PAS stain. ×200.


5
K
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(Fig. 4). The bones developed with shapes characteristic of the specific adult bones. Active hemopoiesis developed with the appearance of all types of hemopoietic cells (Figs. 5, 6). Cell classification on smears revealed active erythropoiesis with moderate myelopoiesis (Table 1).

TABLE 1. CLASSIFICATION OF BONE MARROW CELLS (%) FROM EMBRYONIC RAT FEMUR TRANSPLANTED IN ADULT RAT SUBCUTANEOUS TISSUE FOR 4 WEEKS

Cells	Animal no.				
	1	2	3	4	5
Myeloid cells					
Immature	6.4	7.2	4.6	5.0	5.6
Mature					
Neutrophils	8.8	4.0	0.8	3.8	4.8
Eosinophils	4.6	13.2	5.2	7.2	5.0
Basophils	0	0	0	0	0
Erythroblasts	42.4	41.2	13.8	31.4	36.6
Lymphoid cells	31.8	30.8	74.6	44.0	45.2
Plasma cells	2.0	1.8	0.2	5.4	1.6
Others including megakaryocytes	0.5	1.8	0.8	4.2	1.2

The bones were surrounded by granulation tissues with abundant regenerating capillaries, some penetrating into bones (Fig. 5). No lymphocytic reaction suggesting immunological rejection was observed in the surrounding tissues. The transplant appeared readily accepted by the host. Chromosome analyses of the developing bone marrow cells in the transplanted embryonic bone revealed that almost all cells had XY chromosomes and were thus of the recipient animal (Table 2).

TABLE 2. SEX CHROMOSOME ANALYSIS OF MITOTIC BONE MARROW CELLS OF RAT EMBRYO TIBIA AND FEMUR TRANSPLANTED IN ADULT RAT SUBCUTANEOUS TISSUE FOR 4 WEEKS

Animal no.	Sex of* donor (Embryo)	Sex of hosts (Adult)	Chromosome* plates analysed	Karyotypes	
				XY	XX
1	Female	Male	106	106	0
2	Female	Male	107	106	1
3	Female	Male	97	97	0
4	Female	Male	104	103	1
5	Female	Male	88	88	0

*For methods, see the text.

Cartilage cells were separated from the epiphyses of femur and tibia and cultured to examine the origin of the bony tissue. The cells grew well forming clusters (Fig. 7). Chromosome analysis of the epiphyseal cells cultured for 3 weeks had XX chromosome, and were thus from the transplanted bones (Table 3). The cells had the characteristics of chondrocytes, being stained blue with Alcian blue (Fig. 7).

TABLE 3. SEX CHROMOSOME IDENTIFICATION OF CHONDROCYTES OF RAT EMBRYONIC FEMUR TRANSPLANTED IN ADULT SUBCUTANEOUS TISSUE FOR 3 WEEKS FOLLOWED BY CULTURE IN TC 199 MEDIUM

Animal no.	Chromosome plates analysed	Karyotypes		
		XX	XY	Unidentified
1	7	6	0	1
2	6	6	0	0
3	7	6	0	1

For methods, see the text.

DISCUSSION

In rat embryogenesis, hemopoiesis is evident first in the yolk sac (1–3), and then later in the liver and spleen at about 11 gestation days of age. Bone marrow hemopoiesis usually first appears after birth. Embryos with the yolk sac removed failed to develop blood cells even though the vessels and heart developed (4). These results strongly suggest that the hemopoietic precursor cells or stem cells are produced in the yolk sac and migrate into the liver and spleen forming hemopoietic tissues there. The transition of hemopoietic tissues from the liver to bone marrow is probably conducted in a similar way, but no definitive evidence is yet presented that the initiation of bone marrow hemopoiesis is conducted by stem cells migrating from the liver or spleen. Another possibility is that hemopoietic cells may arise from bone marrow reticular cells. "Myeloid metaplasia" means differentiation of hemopoietic cells from local tissue (10–12).

In the present investigation, an attempt was made to obtain reliable data on bone marrow development in immature bones transplanted into the subcutaneous tissue of adults. Preliminary experiments performed on embryonic bones at various developmental stages indicated that the 17-day-old embryo was most suitable for this purpose. Bones transplanted after 17 gestation days indicated poor bone marrow development in the host, probably due to insufficient vascularization. Bones transplanted from younger fetuses were not vigorous, small and difficult to handle in transplantation.

Morphological observations of bone tissue sections of 17 day embryos at 4 weeks after transplantation revealed neither inflammatory reaction nor changes

suggesting immunological rejection. Blood capillaries in subcutaneous tissue penetrated the transplanted bones and bone marrows developed.

Other investigators (8) reported that about 70% of hemopoietic cells in implanted bones of adult mice were of host origin; however, if the implanted bones were obtained from x-ray irradiated donors, all cells mitosing in the implant were of host origin. The present findings may suggest that in embryonic development the cartilageous bony tissue at 17 days of age has no hemopoietic stem cells and that there is no hemopoietic precursor migration from peripheral blood. In this experiment the sex chromosome of almost all hemopoietic cells in transplanted bones were male, and female chromosomes were rarely encountered, whereas cartilage cells separated from transplanted bones had female chromosomes. The results clearly indicate that hemopoietic cells in transplanted bones came from the recipient adult animal through circulating blood. This is consistent with stem cells or CFUs being found in circulating blood (13-17). Amsel and Dell (6) indicated that osseous tissue is probably both donor and host origin. In the present experiment all cultured cartilage cells were of donor origin (Table 3), and the growing embryonic bones at 4 weeks after transplantation had morphologic expressions typical for the specific bones (Fig. 4).

One problem in this experimental system is that the graft versus host (GVH) reaction may inhibit the growth and differentiation of *in situ* hemopoietic cells of the transplanted bone. All animals used in this experiment were inbred Wistar rats raised in this laboratory, with both the male recipient and pregnant female being litter mates. The GVH reaction thus would be effectively minimized. No abnormal lymphocytic proliferations nor plasma cell reactions were observed in the bone marrow, or in the tissues of the transplanted bones. Four weeks after transplantation, the embryonic bones showed growth with a structural expression consistent with adult rat bones.

The results of present experiment indicate the induction mechanism of bone marrow hemopoiesis is triggered and conducted by migrating stem cells in peripheral blood. Therefore the transition of hemopoietic function during embryogenesis is probably in the sequence: stem cells production in the yolk sac in early embryonic stage (5), followed by movement of stem cells through peripheral blood to the spleen and liver, and finally to the bone marrow.

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