Autoradiographic Studies of Cell Proliferation in the Periosteum of Intact and Fractured Femora of Mice Utilizing DNA Labeling with H^3-Thymidine.*† (26733)

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Opinions on the origin of periosteal cells differ(1). The periosteum consists of 2 layers, an outer layer of fibroblasts and an inner layer of pre-osteoblasts and osteoblasts. The origin of the preceding cell types is of fundamental interest. Apparent morphologic transitions between various cell types exist (Fig. 1). Therefore, it is difficult to tell which cells produce others without a marker that is permanent and is carried through the transitions. Such a marker is tritiated thymidine which labels DNA during replication prior to mitosis and is visible by autoradiographic methods for 5 to 6 cell generative cycles(2, 3,4). The availability of such a marker should make it possible to determine the sequential relations between pre-osteoblasts, osteoblasts, osteoclasts, osteocytes, the chondrocytic series, and fibroblasts.

The aim of these experiments is: 1. To determine the original cell types participating in fracture repair, and 2. To find out whether a common progenitor of these cells exists.

The normal cellular complement of the skeletal system has been recently studied autoradiographically with tritiated thymidine during growth and aging(5).

Methods and materials. A total of 45 female mice of the Brookhaven National Laboratory strain of Swiss Albino were divided into 5 age groups, namely 1, 5, 8, 26 and 52 weeks of age. The right femur was fractured by digital pressure at the mid-diaphyseal aspect, under ether anaesthesia. The left leg was used as a control. It has been established from normal and fracture studies previously reported(5,6) that no qualitative or quantitative changes are observed in tritiated thymidine autoradiographs of non-fractured femora when the opposite limbs have been fractured. Animals were killed 24 hours, 1 week and 2 weeks post-fracturing. One hour prior to killing, the mice received a subcutaneous injection of 0.5 μc of tritiated thymidine per gram of body weight. Tissues were fixed in acetic-alcohol for 3 hours, followed by an additional 24 hours of fixation in formol-saline and washed for 24 hours with running tap water. Decalcification was carried out in a 10% solution of Versene. Paraffin sections were cut at 5 μ and subsequently covered with a liquid emulsion of Kodak NTB_3 and exposed for 20 days in a cold, dry atmosphere. Preparations were stained with Harris hematoxylin after developing. Additional histological sections were stained with hematoxylin and eosin.

Measurements of the thickness of the periosteum at each age were made with an ocular micrometer.

Results and discussion. "Flash labeling" by injecting H^3TDR and then killing the animals before sufficient time has elapsed to permit labeled cells to divide, gives one an estimate of the proliferative rate at that instance by marking the fraction of cells that were synthesizing DNA preparatory to the next mitosis.

In the periosteum of normal intact mice, pre-osteoblasts are "flash" labeled more frequently than osteoblasts or fibroblasts. Examples are seen in Fig. 2. Osteocytes do not "flash" label. With increasing age the fraction of cells "flash" labeled diminishes. The fractions labeled in non-fractured femora were relatively constant 1 day, 1 and 2 weeks after fracture of the opposite femur.

Following fracture of the femur the fraction of cells labeled in the periosteum 24 hours after fracture was greater throughout the entire shaft (Fig. 3). One week after fracture, labeling was most prevalent at the fracture site but was less than after 24 hours.

* Research supported by U. S. Atomic Energy Commission.

† Work was presented in part at Seventh International Congress of Anatomists, New York City, April 11, 1960.

Two weeks after fracture the periosteal labeling was similar to the control leg except at the fracture site. After fractures there is more abundant labeling of pre-osteoblasts than osteoblasts and only a slight increase in labeling of fibroblasts.

Striking changes are observed in the periosteum with increasing age. The total thickness decreases from about 136 μ to 48 μ and the osteogenic layer from 77 to 14 μ with increasing age (Table I). The per cent reduction in thickness is 42% in the fibrous layer and 81% in the osteogenic layer between 1 and 52 weeks of age. The diminution in thickness is due in part to the presence of fewer and smaller cells. However, following trauma, the periosteum can respond at all ages by increased cell production with resultant thicker periosteum. The decreased rate of callus formation in fractures of older animals corresponds to the increased time needed to produce sufficient cells to repair the fracture.

These autoradiographic studies may be considered as "flash labeled" since the one hour interval between injection of the DNA label and killing of the animals does not permit cell division to occur. Thus the proliferative potentials of all cells at that instance are recorded. Pre-osteobasts, osteoblasts and fibroblasts possess proliferative potentials of decreasing order. Osteocytes and osteoclasts are presumably non-proliferative "sterile cells" produced only by transformation or fusion of precursor cells(7).

From these studies it is highly unlikely that fibroblasts produce osteogenic cells or that osteogenic cells produce fibroblasts. It appears that fibroblasts either have an autonomous origin in part or are in part produced by migration of mononuclear cells from the blood(8).

Tentatively it is believed that pre-osteoblasts produce chondrogenic cells and osteoblasts in fracture repair. The fibrous layer of the periosteum apparently contributes little if any to the osteogenic layer, whereas, during fracture repair the thickening osteogenic layer is produced by production of new osteoblasts (Fig. 1, No. 3).

Actual cellular flow rates and pathways

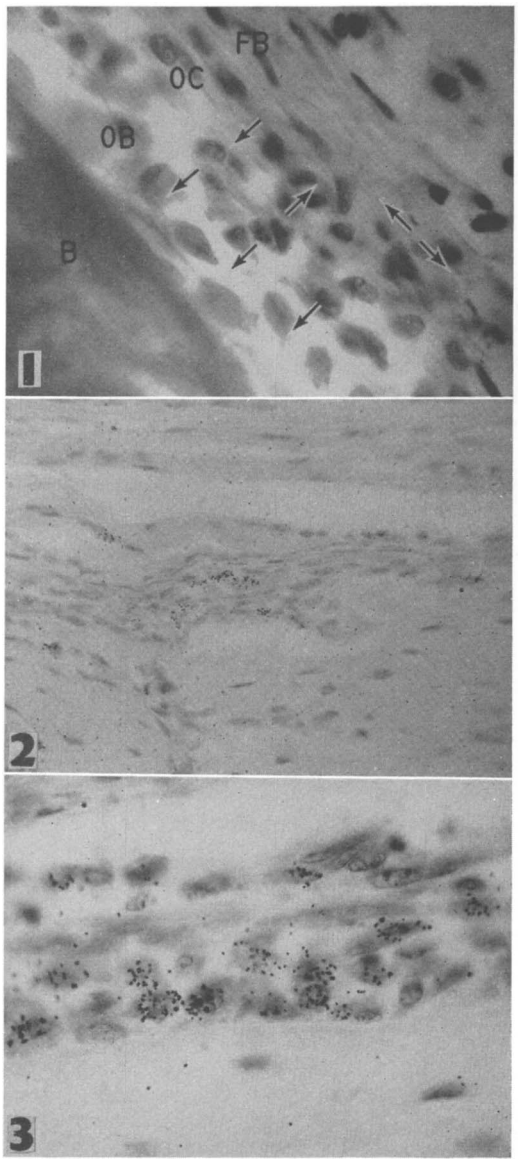

FIG. 1. The femoral periosteum of a new-born mouse is shown depicting the possible origin and pathways of various cell types. (FB) represents the fibrous layer of periosteum, (OC) preosteoblasts, (OB) osteoblasts, and (B) cortical bone. (1) shows formation of pre-osteoblasts from fibroblasts and osteoblasts from pre-osteoblasts. (2) indicates formation of osteoblasts and fibroblasts from pre-osteoblasts. (3) shows that each cell type can contribute to each compartment and that the pre-osteoblasts serve as the progenitor pool for the supply of osteoblasts not fibroblasts.

FIG. 2. Autoradiograph of femoral periosteum of a 1-wk-old mouse labeled with tritiated thymidine. Hematoxylin stained. ×100.

FIG. 3. Autoradiograph of femoral periosteum of a 1-wk-old mouse, 32 hr after fracture, heavily labeled with tritiated thymidine. Hematoxylin stained. ×320 oil immersion.

TABLE I. Average Thickness of Mouse Periosteum (in $\mu \pm$ AD) Taken from the Anterior Aspect of the Mid-Shaft of the Femur.*

Age (wk)	1	5	8	26	52
Osteogenic layer	76.9 ± 17.5	53.9 ± 11.3	32.0 ± 6.3	18.5 ± 7.7	14.1 ± 10.5
Periosteum (fibrous + osteogenic layers)	136.3 ± 20.9	90.3 ± 15.3	87.6 ± 14.3	60.8 ± 10.2	48.1 ± 13.2

* 15 areas were measured in each case.

can only be proved by serial killing after a single injection of label.

Summary. An autoradiographic study of cell proliferation in intact and fractured femoral periosteum of mice using H^3-thymidine showed that osteogenic cells are a relatively quiescent cell population awaiting a signal for proliferation and transformation as in fracture repair. Osteogenic cells constitute a self-sustaining cell population, which becomes diminished in size with increasing age. Osteoblasts are in part self-reproducing and in part are produced by transformation of pre-osteoblasts.

The authors wish to thank Miss M. Pavelec for her technical assistance and Mr. Robert F. Smith for the photomicrography.

1. Ham, A. W., Harris, W. R., in *The Biochemistry and Physiology of Bone,* ed. G. H. Bourne, Academic Press, 1956, p477.

2. Hughes, W. L., Bond, V. P., Brecher, G., Cronkite, E. P., *Proc. Nat. Acad. Sci.,* 1958, v44, 476.

3. Cronkite, E. P., Fliedner, T. M., Bond, V. P., Rubini, J., Quastler, H., *Ann. N. Y. Acad. Sci.,* 1959, v77, 803.

4. Quastler, H., Sherman, F. G., *Exp. Cell Res.,* 1959, v17, 420.

5. Tonna, E. A., *J. Biophys. & Biochem. Cytol.,* 1961, v9, 813.

6. Tonna, E. A., Cronkite, E. P., *J. Bone and Joint Surg.,* 1961, v43A, 352.

7. ———, *Nature,* 1961, v190, 459.

8. Cronkite, E. P., Bond, V. P., Fliedner, T. M., Killmann, S. A., *Ciba Foundation Symp. on Haemopoiesis,* ed. G. E. W. Wolstenholme, and M. O'Connor, J. & A. Churchill Ltd., London, 1960, 70.

Received March 27, 1961. P.S.E.B.M., 1961, v107.

Epinephrine Metabolism in Phenylketonuria.* (26734)

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A possible disturbance in the metabolism of epinephrine in phenylketonuria has been suggested. Cawte(1,2) has demonstrated an increased rise of blood pressure following an intravenous dose of adrenalin among phenylketonurics and attributed this to an impairment of epinephrine production. Weil-Malherbe(3) has shown a decrease of plasma epinephrine and norepinephrine in various forms of mental defect, with phenylketonurics being among the lowest.

The present paper will describe the decrease of dopamine, norepinephrine, and epinephrine in phenylketonuria and discuss evidence for the *in vivo* inhibition of dopa decarboxylase by phenylalanine derivatives.

Materials and methods. Two groups of phenylketonurics were studied. Eight patients† (ages 9-15) had received no specific treatment and 6 patients (ages 3-7) had been treated with low-phenylalanine diet from 6 months to 4 years. Identical studies were carried out in 8 normal and 8 mentally-retarded controls of comparable age and sex to the untreated phenylketonurics.

* Aided by grants from Illinois Mental Fund and U. S. Public Health Service.

† The kind cooperation of Dr. Tillman and staff members of Dixon State School is gratefully acknowledged.