

# Investigations on Congenital and Induced Osteopetrosis

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Osteopetrosis, or marble bone disease, is a disturbance of skeletal development in which the rate of bone resorption fails to keep pace with the rate of bone formation. Bone matrix accumulates excessively throughout the skeletal system, causing damage to neighboring tissues—particularly the dental, hematopoietic and nervous tissues.

The line of investigation on osteopetrosis which has led to the current point of view that the thyroid gland represents the primary site of the disturbance was initiated soon after an experimental animal became available. In 1935 Hans Grüneberg described a mutant of the house mouse, named the grey-lethal, whose skeletal system showed manifestations of osteopetrosis which closely resembled those in man originally described by Albers-Schönberg in 1904. Barnicot (1948), working at University College, was the first to show that osteopetrosis is an endocrine disorder. His most interesting work involved the reciprocal transplantation of parathyroid tissue and bone. In one experiment he took from a grey-lethal mouse a piece of calvarial bone and a parathyroid body, attached the gland to the surface of the bone, and implanted the graft intracerebrally in a normal sibling. A few days later, when the graft was removed for examination, the calvarial bone was found to have been perforated in the region underlying the parathyroid gland. When the grey-lethal mouse was used as host for grafting a parathy-

roid body and bone obtained from a normal littermate, little or no evidence of bone erosion was demonstrable at the end of the culture period.

In another experiment (Barnicot, 1941), ribs from a grey-lethal mouse were transplanted into a normal littermate. When examined histologically two weeks later, the rib grafts appeared normal. In the reciprocal transplantation, in which a rib from a normal mouse was cultured subcutaneously in a grey-lethal littermate, the graft underwent osteopetrotic changes. From these results it was evident that the failure of bone remodelling in the osteopetrotic mouse was not explicable on the basis of incompetence on the part of either osteoclasts or the chief cells of the parathyroid gland. In an effort to promote the resorption of the excess bone, Barnicot (1945) discovered that the grey-lethal mouse suffered no ill effects from repeated injections of parathyroid extract (PTE) at a dose level that was toxic enough to kill the normal siblings after only one exposure. Thus, Barnicot terminated his study of the grey-lethal mouse by suggesting that osteopetrosis is a form of hypoparathyroidism which cannot be cured by parathormone therapy alone.

There are two reasons why the "hypoparathyroid hypothesis" is not tenable. It has long been known that normal mice which have been thyroparathyroidectomized at birth do not develop osteopetrosis. Fur-

thermore, the fact that bone erosion failed to occur, even when the parathyroid body was in direct contact with the calvarial bone, rules out the possibility that a parathormone-destroying tissue intervenes between the gland and its target.

In seeking additional information as a basis for an alternative to Barnicot's explanation, I first devoted my efforts to measuring the plasma calcium concentration in untreated animals of both sexes at various ages. All of the grey-lethals examined, including the younger well-nourished group, were found to be hypocalcemic, with a total plasma calcium averaging 20% below the mean value of plasma calcium obtained from the normal littermates. In response to a single injection of PTE at a dosage of 5 units/gm of body weight, the plasma calcium level of normal mice one to four weeks of age increased by 100% in 18 hours and 200% in 36 hours. Grey-lethal mice given the same dosage of PTE showed a maximum elevation of plasma calcium concentration of only 25%, reached within 18 hours. By 36 hours the plasma calcium fell to a tetanic threshold level. In response to injections of PTE administered at intervals of 12 hours for as long as eight days, the grey-lethal mouse never became hypercalcemic, whereas the normal littermates invariably died by the second or third day with extreme hypercalcemia and nephrocalcinosis.

In an effort to locate the basis

for the great resistance to parathyroid hormone observed in the grey-lethal, a thorough cytological and histochemical assessment of osteoclasts was conducted. Bone samples obtained from grey-lethals between 12 and 24 hours after the administration of parathormone showed widespread or intense signs of osteoclastic activity, including an exceptionally high level of collagenase (Walker, 1966a). However, in the untreated mice and at all time points later than 24 hours in the parathormone-treated animals, osteolytic activity was subnormal, whereas the signs of increased osteoblastic activity were most impressive. The endosteum was hyperplastic, containing several layers of osteoprogenitor cells as well as a prominent layer of large osteoblasts; adjacent to the endosteum was a thick seam of osteoid. The hyperplastic endosteum was distributed over most of the trabecular surface in the grey-lethal bones. In order to place the osteogenic evaluation on a quantitative basis, I employed tritiated proline incorporation studies. Tritiated proline was administered intraperitoneally at a dosage of  $4\mu\text{c}$  to  $8\mu\text{c}$  per gm of body weight, and the animal was sacrificed six hours later. Samples of calvarial and tibial bone were pulverized, extracted in ether, suspended in scintillation fluid and counted by means of a well-type automated scintillation counter. The counts obtained from the untreated animals indicated that the grey-lethal incorporated 50% more of the  $^3\text{H}$ -proline into bone matrix than the normal control. Parathyroid hormone was found to depress incorporation in the normal by about 50% but in the grey-lethal by only about 15% (Walker, 1966b).

According to our interpretation of the results of the evaluation of osteolytic and osteogenic activities in the osteopetrotic mouse, the imbalance leading to the excessive accumulation of bone is more likely to be due to increased osteoblastic

activity than to decreased osteoclastic activity. In attempting to locate an abnormally enlarged source of an osteoblastic-stimulating factor, the pituitary and thyroid glands were surveyed thoroughly by means of light and electron microscopy. No evidence of increased somatotroph or follicle cell activities was disclosed; however, a parafollicular cell hyperplasia was discovered in the parathormone-treated grey-lethals. In electron micrographs the parafollicular cell differs from the follicle cell in several respects. It is of a larger size, has a shape that is oval instead of polygonal, and occupies a more peripheral (parafollicular) position, resting against the basement membrane of the follicle but never bordering directly on the colloid. Unlike all other epithelial cells, the parafollicular cells are devoid of intercellular attachment devices, such as desmosomes, light junctions, and interlocking processes. The most distinctive feature of the parafollicular cells is the abundance of cytoplasmic secretory vesicles. These vesicles are about  $0.2\mu$  in diameter and appear to be empty but may contain thyrocalcitonin, a substance of homogeneous appearance and low electron opacity.

Since the parafollicular cell has been shown to be the source of thyrocalcitonin, the presence of a parafollicular cell hyperplasia may represent an excessive capacity to produce thyrocalcitonin. Chronic hypercalcitoninemia would be associated with sustained hypocalcemia, hypophosphatemia, and a retardation of bone resorption. All of these signs are characteristic of osteopetrosis as manifested in the grey-lethal mouse. The increased rate of bone formation observed in the grey-lethal might indicate that thyrocalcitonin has a dual action on bone—inhibiting osteolytic activity, yet stimulating osteogenic activity—a combination of effects diametrically opposed to the effects of parathormone.

In addition to the grey-lethal,

three other mutants with osteopetrosis have recently been studied. The implication of the findings obtained in microphthalmic and osteosclerotic mice is essentially the same as the implication of the findings obtained from the grey-lethal (S. C. Marks and D. G. Walker, unpublished data).

Rabbits with hereditary osteopetrosis as well as the mice with congenital osteopetrosis are hypocalcemic throughout life. They fail to develop hypercalcemia even when placed on an intensive parathormone regimen. However, the abnormal tolerance to parathormone in the rabbit does not seem to be related to parafollicular cell activity, as it was in osteopetrotic mice. The parafollicular cell population is not elevated in the rabbits, and, even after thyroidectomy or thyroparathyroidectomy, the osteopetrotic rabbits are refractory to the influence of parathormone. Furthermore, the rabbit mutants never show increased osteogenic activity; in fact, according to tritiated proline incorporating studies carried out at various ages from birth to one month of age, their capacity to make bone matrix is only about half that of the normal littermates. Osteopetrotic rabbit bones show reduced numbers of osteoblasts, osteoclasts of abnormal appearance and an abundance of mesenchyme throughout the intertrabecular spaces (D. G. Walker and R. R. Fox, unpublished data).

On the basis of the fragmentary data available at present, the primary defect in hereditary osteopetrosis of the rabbit appears to be located in osseous tissue rather than in the endocrine system.

Recently, osteopetrosis has been induced in normal mice (S. C. Marks, unpublished data), rabbits and rats (D. G. Walker and M. A. Shepp, unpublished data) by means of daily injections of PTE at the moderately low dose level of 0.5 units/gm of body weight. Throughout the two-week or longer PTE regimen initiated at birth, the med-

ullary cavities of the long bones failed to enlarge, and the cancellous bone of the epiphyses and metaphyses became increasingly dense, gradually approaching compact bone in appearance. The experimentally osteopetrotic animals, like the congenitally osteopetrotic mice, showed increased extent of osteoblastic activity in autoradiographic studies and elevated levels of tritiated proline incorporation into bone matrix as determined by counting of radioactivity. In general, the counts indicated that the experimental animals incorporated two to three times as much of the administered label as did their normal littermate controls. In addition, parafollicular cell hyperplasia was shown to exist in all of the animals with induced osteopetrosis. The parafollicular cell population in the experimental animals was increased about eightfold over that of the controls.

The explanation for the mechanism which induced osteopetrosis in normal mice was as follows. The daily injection of PTE caused a transient hypercalcemia. This, in turn, stimulated the secretory and proliferative activities of the parafollicular cells, leading to an overproduction of thyrocalcitonin. The explanation received additional support from the finding that osteopetrosis could not be induced in mice that had been completely thyroidectomized. In those instances where the thyroidectomy was not complete, the degree of osteopetrosis induced by the parathormone regimen correlated directly with the number of parafollicular cells counted in the remnant of thyroid gland (S. C. Marks and D. G. Walker, unpublished data).

From these investigations on hereditary and induced osteopetrosis has emerged the theory that the parafollicular cell is the source of a potent osteoblast-stimulating factor which may be an entity distinct from thyrocalcitonin. According to general consensus, thyrocalcitonin has but one action, namely,

inhibiting bone resorption. Therefore, at the present time we are investigating other possible sources for the osteoblast-stimulating influence. In order to determine whether or not the growth-promoting effect was mediated by the pituitary gland, rats hypophysectomized at two weeks of age were injected with either growth hormone or PTE or a combination of the two hormones for a period of three weeks. As compared with the untreated hypophysectomized controls, hormone-injected hypophysectomized rats showed approximately a tenfold increase in the number of parafollicular cells of the thyroid gland and a threefold to fivefold increase in the bone label-incorporating capacity. The excessive accumulation of bone matrix was observed only in those animals receiving PTE, but the osteopetrotic trend developed more rapidly in animals receiving growth hormone in addition to PTE. The disturbance of calcium homeostasis that leads to the excessive accumulation of bone matrix occurs not only when the endogenous source of parathormone is inhibited, but also when thyrocalcitonin production is increased. This dual effect is created by the administration of PTE at a low dose level. However, administration of growth hormone indirectly stimulates the activities of the chief cell of the parathyroid gland as well as the parafollicular cell of the thyroid and, thus, calcium homeostasis is not disturbed. The fact that growth hormone acts synergistically with PTE in producing osteopetrosis can be explained simply by the fact that growth hormone stimulates chondrogenesis, and the more rapid the rate of cartilage production by the epiphyseal plate, the more rapid the bone accumulation under conditions where bone resorption has been inhibited.

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