STUDIES ON CONGENITAL OSTEOPETROSIS IN MICROPHTHALMIC MICE USING ORGAN CULTURES: IMPAIRMENT OF BONE RESORPTION IN RESPONSE TO PHYSIOLOGIC STIMULATORS*

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Walker (1–3) has shown that congenital osteopetrosis in microphthalmic (*mi*)[1] mice is associated with a defect in bone resorption which can be reversed by injecting cells from the bone marrow or spleen of normal animals into irradiated mutants (2). Conversely, osteopetrosis could be produced by injecting spleen cells from mutants into normal recipients (3). Abnormalities of osteoclast morphology and histochemistry have been reported in this mutant and in incisor absent (ia) rats with congenital osteopetrosis (4–6). While Walker's studies and earlier work on amphibia by Fischman and Hay (7) suggest that circulating mononuclear cells may be the precursors for osteoclasts, neither the specific cell types which reverse the defect in osteopetrosis nor the mechanism by which they act have been identified. By analogy with some immune responses it is possible that the formation of the osteoclasts requires cooperation among several cell types including both effector cells and helper cells which might secrete humoral mediators. The osteoclast-activating factor (OAF), which is produced by phytohemagglutinin (PHA)-activated human mononuclear cells (8–10), is a potent stimulus for osetoclast-mediated bone resorption in vitro. The cell source for this factor is not certain, but available data suggest that it is derived from activated lymphocytes, but that these require macrophages as helper cells (11). The role of OAF in physiologic skeletal development is unknown. It has been suggested that OAF might be involved in marrow development and that a defect in production or response to this mediator might be important in the pathogenesis of osteopetrosis.

The present studies were undertaken to develop an in vitro model which would enable us to analyze more completely the defects responsible for osteopetrosis in animal models. The *mi* mouse was selected for initial studies because of ease of identification of the different genotypes.

Tissue cultures of long bones and calvaria from *mi* mice were compared with bones from homozygous normal animals and heterozygous carriers. Bones from

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[1] *Abbreviations used in this paper:* AG, β-acetylglucosaminidase; BGJ, a chemically defined medium developed by Biggers et al. (16); β-Gl, β-glucuronidase; Cath D, cathepsin D; CDP, collagenase digestible protein; ia, incisor absent; *mi*, microphthalmic; NCP, noncollagen protein; OAF, osteoclast-activating factor; $1,25(OH)_2D_3$, 1,25 dihydroxy vitamin D_3; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; PTH, parathyroid hormone.

mi mice showed a generalized defect in bone resorption with marked impairment of the resorptive response to both human and mouse OAF as well as other known stimulators of osteoclastic bone resorption including parathyroid hormone (PTH) (12), prostaglandin E_2 (PGE_2) (13), 1,25 dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] (14), and vitamin A (12). Bone formation was not impaired in *mi* mice and could be inhibited by PTH and OAF. OAF production did not appear to be impaired since supernatant fluid of PHA-activated spleen cells from *mi* mice contained as much bone resorbing activity as supernates from normal animals.

Methods

Mice, heterozygous for the *mi* recessive gene, were obtained from Dr. D. W. Walker, The Johns Hopkins University School of Medicine, Baltimore, Md., and from The Jackson Laboratory, Bar Harbor, Maine. Separation of the various genotypes was possible in fetal as well as newborn animals because *mi* mutants have no eye pigment while heterozygotes have a small amount and homozygous normals have a fully pigmented eye. Except for one experiment in which fetal age was estimated from bone size and development, the studies were carried out after a single overnight mating to permit precise dating of pregnancy.

Long Bone Cultures. The technique was similar to that used for measuring resorption in fetal rat long bones (12, 15). The pregnant mice were injected with ^{45}Ca on the day before sacrifice. On the 16th to 19th day the fetuses were removed, the shafts of the radius and ulna dissected free of soft tissue and cartilage, and precultured for 18–24 h in a chemically defined medium, BGJ, at 37°C in an atmosphere of 5% CO_2 in air. Subsequently the bones were transferred to the same medium supplemented with 4 mg/ml bovine serum albumin, to which various stimulators of bone resorption were added, and cultured for 3 days. ^{45}Ca in the medium and bone were measured and resorption estimated from the percent of ^{45}Ca released. Bones killed by three cycles of freezing and thawing were used to correct for any differences in the exchange of ^{45}Ca by bones from the different genotypes.

Experiments Combining Bones from Different Genotypes. In some experiments, ^{45}Ca release from long bones of *mi* normal or heterozygous mice were compared when cultured alone or together with long bones from unlabeled animals. Labeled bones were co-cultured with unlabeled bones from the same or different genotype. The labeled and unlabeled bones were placed together on a 2 mm square Millipore filter and the cultures were maintained for 6 days with a change of medium at 3 days.

Calvarial Cultures. Newborn mice were used (21 days post-mating). The bones were labeled with ^{45}Ca by injection of the mother 3 days before birth. The flat, central portions of the frontal and parietal bones were dissected, care being taken to preserve the periosteum. Half calvaria were incubated in BGJ supplemented with 5% heat-inactivated human serum in a manner similar to the long bone cultures. The bones were cultured for one or two 3-day periods. In some of the experiments the bones were transferred to Ehrlenmeyer flasks at the end of the resorption culture period for studies of bone collagen synthesis.

Bone Collagen Synthesis. After 3–6 days culture, bones were transferred to 25 ml Ehrlenmeyer flasks with 2 ml of growth medium containing 1 mM proline and no serum, gassed with 5% CO_2 and air, stoppered, and incubated for 3 h. Labeled proline (2,3-^3H-proline; 5μCi/ml; New England Nuclear, Boston, Mass.) was added for the last 2 h. At the end of incubation the bones were washed with 5% TCA, acetone, and ether; weighed; and homogenized. ^3H-proline in collagen was determined by digestion with repurified bacterial collagenase by the method of Peterkofsky and Diegelmann (17). Labeled collagenase-digestible protein (CDP) and noncollagen protein (NCP) were determined and the percent collagen synthesis calculated using a factor of 5.4 to correct for the relative abundance of proline in collagen and noncollagen protein.

Lysosomal Enzyme Measurements. To measure lysosomal enzyme activity, calvaria were homogenized in 0.1% Triton in water and aliquots of bone homogenate or medium were incubated with appropriate buffers and substrates using standard methods (18). For measurement of β-glucuronidase (β-Gl) the substrate was phenolphthalein-β-glucosiduronate, for n-β-acetylglucosaminidase (AG) the substrate was the nitrophenylglycoside and for cathepsin D (Cath D), ^3H-acetylated hemoglobin was the substrate. Lysosomal data are presented as the percent of total

TABLE I

Effect of Parathyroid Hormone (N-PTH, 100 ng/ml) and Human OAF (1/20 Dilution) on Release of ^{45}Ca from Cultured Long Bone Shafts of Microphthalmic (mi/mi), Heterozygous (mi/+), and Normal (+/+) 16-19-Day Fetal Mice

Treatment	*mi/mi*		*mi/+*		+/+	
	% Total ^{45}Ca released	Treated/ control	% Total ^{45}Ca released	Treated/ control	% Total ^{45}Ca released	Treated/ control
Control	10.2 ± 0.6	–	11.7 ± 0.7		11.1 ± 2.0	
Killed	10.1 ± 0.3	0.99	9.8 ± 0.3*	0.84	9.2 ± 0.5*	0.83
PTH	12.1 ± 0.3*	1.19	20.2 ± 0.8*	1.73	22.9 ± 1.3*	2.07
OAF	11.3 ± 0.3*	1.13	15.7 ± 0.7*	1.35	19.9 ± 2.3*	1.79

Values are means ± standard error for 6–10 bones cultured for 3 days.
* Significantly different from control, $P < 0.05$.

enzyme present in bone and medium that was released into the medium during a 3 day culture period. The recovery of enzyme from bone homogenate appeared to be complete, since the initial values for enzyme in homogenates of the calvaria and in the medium plus bone of calvaria killed by repeated freezing and thawing and then incubated for several days were similar. Moreover, no additional enzyme activity could be obtained by centrifugation and re-extraction of the homogenate.

Cultures of Mouse Spleen for OAF. Homozygous normal and *mi* newborn mice spleens were used. The remaining heterozygous animals were returned to the mother and ultimately added to the breeding colony. The spleens were minced and the cells dispersed by repeated pipetting, the cell suspension pelleted by brief centrifugation and resuspended in BGJ medium at 10^6 mononuclear cells/ml. 10 ml of cell suspension was incubated in a 150 mm Petri dish with 1% PHA (PHA-M; Grand Island Biological Corp., Grand Island, N. Y.). After 24 h the supernate was removed, centrifuged, and the clear supernate frozen. This crude supernate which contained bone-resorbing activity is referred to as mouse OAF. In addition, a human OAF preparation partially purified by Sephadex G100 chromatography, as previously described (9), was used in some cultures.

Materials. In addition to the OAF preparations, bovine parathyroid hormone 1-84 (N-PTH; kindly provided by the National Institutes of Health, Bethesda, Md.), synthetic bovine PTH 1-34 (B-PTH, Beckman Instruments, Inc., Fullerton, Calif.), PGE_2 (kindly provided by the Upjohn Co., Kalamazoo, Mich.), $1,25(OH)_2D_3$ (kindly provided by Hoffman-La Roche, Inc., Nutley, N. J.), and vitamin A (retinol; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) were used.

Results

Long Bone Cultures. The radius and ulna from 16- to 19-day fetal mice provided satisfactory material for measurement of bone resorptive response to stimulators. Bones from homozygous normal or heterozygous animals showed up to twofold increase in ^{45}Ca release in response to PTH and a significant response to partially purified human OAF (Table I). Control resorption rates were low, but the difference from killed bones was significant for homozygous normals and for heterozygotes. In contrast, bones from *mi/mi* mice showed minimal response to PTH or OAF and no control resorption. The response to bones from heterozygotes appeared less than that for homozygous normals, and in another experiment (Table II) the response to a submaximal dose of PTH was significantly less in bones from heterozygous animals ($P < 0.05$). Histologic studies showed that both heterozygous and normal bone treated with PTH contained numerous osteoclasts on the bone surface, with loss of matrix. Stable calcium content of the bone was reduced by treatment with PTH by a percentage

TABLE II

Effect of Parathyroid Hormone (N-PTH) on Release of ^{45}Ca from Cultured Long Bone Shafts of Microphthalmic (mi/mi), Heterozygous (mi/+), and Normal (+/+) 17-Day Fetal Mice

Treatment	*mi/mi*		*mi/+*		+/+	
	% Total ^{45}Ca released	Treated/control	% Total ^{45}Ca released	Treated/control	% Total ^{45}Ca released	Treated/control
Control	14.2 ± 0.7	–	16.0 ± 0.5	–	16.9 ± 0.3	–
Killed	13.5 ± 1.0	0.95	14.4 ± 0.4	0.90	14.7 ± 0.4*	0.87
PTH (10 ng/ml)	13.8 ± 1.9	0.97	17.8 ± 1.0	1.11	25.7 ± 2.4*	1.52
PTH (100 ng/ml)	16.5 ± 2.3	1.16	35.5 ± 3.7*	2.21	47.4 ± 7.5*	2.80

Values are means ± standard error for 9–17 bones cultured for 3 days.
* Significantly different from control, $P < 0.05$.

TABLE III

Effect of Combined Culture with Unlabeled Bones and Treatment with Parathyroid Hormone (N-PTH, 40 ng/ml) on Release of ^{45}Ca from Cultured Long Bone Shafts of Microphthalmic (mi/mi) vs. Heterozygous (mi/+) or Normal (+/+) 17-Day Fetal Mice

Treatment	% Total ^{45}Ca released	
	Labeled *mi/mi* bone	Labeled *mi/+* or +/+ bone
PTH alone	31.5 ± 1.7	65.8 ± 11.3
PTH plus unlabeled *mi/+* or +/+ bone	32.9 ± 1.7	68.6 ± 6.9
PTH plus unlabeled *mi/mi* bone	31.2 ± 0.1	61.6 ± 1.8

Values are means ± standard error for three to seven bones cultured for 6 days.

similar to the amount of ^{45}Ca lost. In contrast, *mi/mi* bones showed no identifiable osteoclasts on bone surfaces and did not lose stable calcium with PTH.

To test the possibilities that normal bone cells were producing a humoral factor essential for osteoclast activation or that osteopetrotic bones contain an inhibitor of osteoclasts, paired cultures of mutant and normal or heterozygous bones were maintained in culture with the addition of PTH. There was no difference in ^{45}Ca release when *mi/mi* bones were cultured alone, with paired bones from normal or heterozygous animals or with paired bones from *mi* animals (Table III). Similarly, the data for bones from normals and heterozygotes, which are pooled, showed no difference in response whether cultured with unlabeled bones from the same genotypes or from *mi* animals. Since these small bones (less than 1 mg wet weight) were cultured in 0.5 ml of medium, the data do not rule out the existence of a humoral factor which was ineffective because of dilution.

Culvaria Cultures. Further studies were carried out on half calvaria from *mi/mi*, heterozygous, and normal animals, because (*a*) they had been used in previous studies of osteopetrosis, (*b*) they show relatively greater rates of control resorption, and (*c*) they provide larger amounts of bone for studies of

TABLE IV

Effects of Parathyroid Hormone (β-PTH, 400 ng/ml), PGE_2 (10^{-6} M) $1,25(OH)_2D_3$ (10^{-8} M) Vitamin A (Vit A, 10^{-5} M), and OAF from Normal or Mutant Mice (OAF mi/mi and OAF +/+, 1/8 Dilution) on Release of ^{45}Ca from Cultured Calvaria of Microphthalmic (mi/mi), Heterozygous (mi/+), and Normal (+/+) Newborn Mice

	% Total ^{45}Ca released		
	mi/mi	*mi/+*	*+/+*
Control	13.2 ± 0.9	23.1 ± 1.0	23.3 ± 2.0
Killed	11.3 ± 0.8	9.8 ± 0.3*	11.3 ± 0.7*
PTH	13.9 ± 1.9	32.1 ± 4.6*	39.2 ± 2.8*
PGE_2	13.4 ± 0.4	38.4 ± 1.9*	38.2 ± 5.7*
$1,25(OH)_2D_3$	11.6 ± 0.2	36.4 ± 2.3*	36.7 ± 5.9*
Vit A	12.1 ± 1.2	30.1 ± 1.1*	
OAF *mi/mi* 1/8	14.8 ± 1.1	36.2 ± 4.4*	
OAF +/+ 1/8	12.2 ± 3.0	37.8 ± 1.6*	

Values are means ± standard error for 5–26 bones cultured for 3 days.
* Significantly different from control, $P < 0.05$.

lysosomal enzyme release and collagen synthesis. Both heterozygotes and homozygous normals showed substantial control resorption and responded to PTH, PGE_2, and $1,25(OH)_2D_3$ (Table IV). PTH at supramaximal initial concentration had a greater effect on normal bone than on bone from heterozygotes. However, the response to PGE_2 and $1,25(OH)_2D_3$ at supramaximal concentrations was not different for bones from normals or heterozygotes. Bones from *mi/mi* mice showed no significant control resorption and no response to any of the stimulators.

Because the number of bones was limited, only *mi/mi* and heterozygote bone was used to test the ^{45}Ca response to vitamin A and mouse OAF. Vitamin A was found to stimulate resorption of bone from heterozygotes but not from *mi/mi* animals. When tested on bones from heterozygotes, the bone-resorbing activity in supernates of PHA-stimulated spleen cell cultures from *mi/mi* mice was as great as for spleen cells from homozygous normals. However, mouse OAF did not stimulate resorption of *mi/mi* bone. In other studies (data not shown) mouse OAF from normal and mutant animals was assayed at several dilutions and no difference in potency or dose-response curve was observed.

An increase in the lysosomal enzyme release into the medium was associated with stimulation of resorption by PTH or vitamin A in cultures of normal bone and was similar for all three enzymes tested (Table V). In contrast, bone from *mi/mi* animals showed no increase in enzyme release with stimulation. Control release of enzymes was similar for mutant and normal bone, and there was no difference in total enzyme content of normal and mutant bone. In other experiments (data not shown) the medium content of β-Gl was increased with stimulation of bone resorption by PGE_2 in normal but not in mutant bones.

PTH and human OAF are inhibitors of bone collagen synthesis in vitro at high concentrations (10). Mouse calvaria were cultured for 3–6 days and transferred to a growth medium to measure proline incorporation into CDP (Table VI). Bones from *mi/mi* mutants and normal or heterozygous mice incorporated

TABLE V

Effects of Parathyroid Hormone (β-PTH 400 ng/ml) and Vitamin A (10^{-5} M) on Release of β-Gl, n-β-Acetyl Glucosaminidase (AG) and Cath D from Cultured Newborn Mouse Calvaria

	mi/mi			+/+		
	% release			% release		
	β-Gl	AG	Cath D	β-Gl	AG	Cath D
Control	11.2 ± 1.2	11.2 ± 1.1	8.8 ± 0.9	11.3 ± 1.8	10.4 ± 0.9	9.4 ± 1.0
PTH	9.9 ± 0.6	9.3 ± 1.2	9.2 ± 1.1	28.3 ± 2.0*	25.1 ± 1.3*	23.8 ± 1.1*
Vit A	14.0 ± 0.9	12.2 ± 2.1	12.2 ± 2.0	21.6 ± 1.9*	19.9 ± 1.7*	19.8 ± 1.4*

Values are means ± standard error for six bones cultured for percent of total enzyme released into medium in 48 h.

* Significantly greater than control, $P < 0.01$.

TABLE VI

Effect of Parathyroid Hormone (β-PTH, 400 ng/ml) and OAF from Normal Mice on Incorporation of Labeled Proline into CDP and NCP of Cultured Calvaria from Microphthalmic (mi/mi) and Heterozygous or Normal (mi/+ or +/+) Mice

	mi/mi		% Collagen synthesized	*mi/+* or +/+		% Collagen synthesized
	dpm/μg dry wt			dpm/μg dry wt		
	CDP	NCP		CDP	NCP	
Control	25 ± 6	36 ± 5	10.5 ± 1.0	17 ± 1	31 ± 1	8.9 ± 0.7
PTH	12 ± 1*	27 ± 3	6.1 ± 0.6*	9 ± 2*	29 = 5	5.4 ± 0.7*
Mouse OAF	11 ± 1*	29 ± 6	7.0 ± 1.0*	8 ± 1*	29 ± 2	5.0 ± 0.3*

Values are means ± standard error of 3 to 12 bone cultures treated for 3–6 days and pulsed with ^{3}H-proline for the last 2 h of culture.

* Significantly less than control, $P < 0.05$.

substantial amounts of proline into CDP, and the value was somewhat higher for the mutants. Both PTH and OAF caused similar inhibition of the incorporation of labeled proline into collagen in *mi/mi* and normal or heterozygous mouse calvaria. NCP synthesis was not affected.

Discussion

The present studies have used techniques for the study of the pathogenesis of osteopetrosis in vitro which should make it possible to identify the specific defects in the various animal models of this disorder. The *mi* mouse was used initially because the defect is severe and it is easy to identify the genotypes in fetal and newborn animals. Cultured long bones and calvaria from *mi* mice showed decreased spontaneous or control resorption and failed to respond to a number of potent stimulators of resorption including PTH, PGE_2, 1,25 $(OH)_2D_3$, vitamin A, and OAF from both human peripheral leukocyte and mouse spleen cultures. These results indicate that there is a generalized bone resorption defect in *mi* mice which is responsible for the development of osteopetrosis. The most likely explanation for this is an abnormality in osteoclast formation or function. We have looked for a local humoral factor which might be deficient in the microphthalmic mouse or which might inhibit bone resorption. Co-culture

of mutant and normal bones provided no evidence for such a humoral factor, however, dilution or inactivation could have been responsible for the negative results. The failure to respond to widely differing stimulators of bone resorption makes it extremely unlikely that there was any defect in receptor sites on the osteoclasts or their precursors.

Spleen cells from *mi* mice produced as much bone-resorbing activity in PHA-stimulated cultures as did spleen cells from normal animals. These results suggest that defective OAF production is not important in the pathogenesis of osteopetrosis in this animal model. The physiologic role of OAF is not known and it is possible that its spontaneous release may be important in initiating endosteal bone resorption. Failure of such release could be responsible for osteopetrosis in other mutants.

Our results differ from those reported by Marks, who found that ^{45}Ca loss was increased in cultured bones from ia rats, despite evidence that inhibition of bone resportion was present in vivo (19). This discrepancy could be due to the difference in species, particularly since the abnormality in resorption is transient in the ia rat. Reynolds and his associates found that control resorption was impaired in cultures of calvaria from mice with the gray-lethal mutation which produces severe osteopetrosis (20). Reynolds et al. (20) also demonstrated that administration of a potent inhibitor of bone resorption, dichloro-methylene diphosphonate, to newborn normal mice could mimic the osteopetrotic lesion of the gray-lethal mutants.

The studies with calvaria confirmed and extended the results with long bones, and provided a model in which biochemical studies were more easily carried out. Measurements of several lysosomal enzymes showed that the increased enzyme release occurred from normal bones when resorption was stimulated but did not occur in mutant bone. Failure of enzyme release has been suggested on the basis of morphologic observations of acid phosphatase accumulation in osteoclasts in other animal models of osteopetrosis (4–6). We found no difference in enzyme release between normal and mutant bone in the absence of stimulators, although ^{45}Ca release was different. Many of the cells in the calvaria are not resorbing cells. These may release substantial amounts of lysosomal enzymes due to cell damage or normal turnover and lead to high control values which could obscure differences in the behavior of bone-resorbing cells.

Measurements of bone collagen synthesis in this study have indicated that *mi* mutant bone shows inhibition of labeled proline incorporation into collagenase digestible protein in response to both PTH and OAF. This is considered to be due to inhibition of collagen synthesis rather than changes in amino acid transport or precursor pool size because an excess of cold proline was added to the culture medium. The results indicate that the bone-forming cells in mutant animals do have receptors for PTH and OAF and do not appear to have any functional defect in collagen synthesis or its regulation. The rate of proline incorporation was actually somewhat increased in mutant bone cultures. Walker has shown that proline incorporation is higher in gray-lethal mice in vivo, and that this incorporation is inhibited by injections of parathyroid extract, although to a less extent than in controls (21). A marked increase in bone formation has not been observed in most congenital osteopetrotic models, however, and changes in

osteoblastic activity do not appear to be sufficient to produce the defects in remodeling and growth observed. The fact that osteoblastic activity was inhibited by PTH and OAF in *mi* mice while osteoclastic activity was not stimulated is consistent with the hypothesis that these cells are derived from different precursors, and that the defect is in the osteoclast or its progenitor cell.

Summary

The mechanism of congenital osteopetrosis in microphthalmic (*mi*) mice has been examined in bone organ cultures. Resorption was measured by the release of previously incorporated ^{45}Ca in fetal long bones and newborn calvaria from *mi* mice and heterozygous or homozygous normal litter mates. Bones from *mi* mice showed a generalized resorption defect with decreased spontaneous or control resorption and failure to respond to parathyroid hormone (PTH), prostaglandin E_2, 1,25 dihydroxy vitamin D_3, vitamin A, or osteoclast activating factor (OAF) from human peripheral leukocytes or mouse spleen cells. Bones from heterozygotes showed a smaller response to PTH than bones from homozygous normals. Mutant bones failed to show an increase in lysosomal enzyme release in response to PTH or vitamin A, agents which increased release from bones of homozygous normals. Proline incorporation into collagenase-digestible protein was similar in cultures of normal and mutant bone and was inhibited by PTH and OAF. These results indicate that congenital osteopetrosis in *mi* mice is due to a generalized defect in the function and hormonal response of osteoclasts and suggests that this cell line is separate from the osteoblast cell line which shows no impairment of hormonal response.

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