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Osteoclast Ontogeny-Experimental Studies in Two Osteopetrotic Mutations in the Rat: A Dissertation

Matthew Joseph Cielinski

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OSTEOCLAST ONTOGENY—EXPERIMENTAL STUDIES IN TWO OSTEOPETROTIC MUTATIONS IN THE RAT

A Dissertation Presented
By
Matthew Joseph Cielinski

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

April 1994

Cell Biology
This dissertation is dedicated to my wife, Jill.

You have shared my dreams,
and
your love and friendship have made them possible.
OSTEOCLAST ONTOGENY-EXPERIMENTAL STUDIES
IN TWO OSTEOPETROTIC MUTATIONS IN THE RAT

A Dissertation Presented

By

Matthew Joseph Cielinski

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ACKNOWLEDGEMENTS

With the greatest pleasure, I would like to acknowledge those who have supported me in the completion of the work presented in this dissertation. No person is an island, and it is through interactions with others, both colleagues and friends, that one grows in life. Accordingly, I would like to extend heartfelt appreciation to my mentor, Dr. Sandy C. Marks, Jr., for accepting this unseasoned and naive graduate student into his lab and providing me the opportunity to pursue a research project with personal and professional interests. His generosity and keen insight have been a constant source of inspiration and have allowed me to experience a breadth of opportunities few graduate students enjoy. Sandy's advice, criticism and encouragement have guided the work embodied in this dissertation.

I wish to extend thanks to Dr. Victoria Shalhoub for her scientific guidance in the isolation and characterization of mRNAs from bone. Her generosity and patience has made the process of Northern hybridization enjoyable.

I wish to acknowledge the diligent work of those "behind-the-scenes", without whom this work would not be complete. I especially thank Michael Jolie, who performed the histomorphometry necessary for enumerating osteoclasts and mononuclear cells around erupting molars in the rat. The many hours he spent on the Gateway 2000 are greatly appreciated. I would also like to extend thanks to April Mason-Savas for the quality thin sections needed for ultrastructural examination of osteoclasts and "down-to-the-wire" photographic printing. Likewise, I wish to thank Cindy Richards for an commendable attention to detail in helping me put all of this work together.

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The yearly influx of visiting postdoctoral fellows was rewarding both scientifically and culturally. I especially wish to thank Dr. Tadashi Lizuka for his
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I would like to express my deepest thanks to close friends Carole MacKay, Dr. Leslie Wisner-Lynch, and Erik Larson. Their professional dedication to and belief in the ongoing work in the lab, together with their willingness to help others has been inspirational. I shall always remember their support and encouragement, which have guided me through the most difficult endeavors.

I would like to express my sincere gratitude to my parents, Martha and Michael Cielinski for believing in me. They never pushed, but instilled in their children the morals and convictions to pursue their dreams, no matter how difficult. I am grateful.

Finally, I would like to share this accomplishment with my wife, Jill. Her love, encouragement, and support have made this endeavor possible. I am fortunate to have in her both wife and best friend.
ABSTRACT:

Osteopetrosis is a metabolic bone disease in mammals characterized by a generalized skeletal sclerosis caused by reduced bone resorption. This reduced bone resorption is manifested in afflicted animals by abnormal bone shape, reduced or absent marrow cavities, extramedullary hemopoiesis, abnormal mineral homeostasis and absent or delayed tooth eruption. The available osteopetrotic animal mutations have been a constant source of fruitful investigations concerning the systemic regulation of osteoclastogenesis and bone metabolism. Tooth eruption, on the other hand, is a localized manifestation of the timely activation of bone resorption and bone formation on opposite sides of an erupting tooth. Its rate-limiting step is the speed of bone resorption to form the eruption pathway. In this dissertation, we used two osteopetrotic rat mutations, toothless (tl) and microphthalmia blanc (mib), to investigate the abnormal development of osteoclasts and tooth eruption in mutant rats with an emphasis on the role of systemic and local factors. The significant contributions to this work are listed below.

1. In the toothless rat, a mutation lacking erupted dentition due to severely reduced bone resorption, colony-stimulating factor-1 (CSF-1) promoted tooth eruption but this was delayed compared to normal rats. Eruption was accompanied by changes in the populations of tartrate-resistant acid phosphatase-positive (TRAP+) mononuclear cells in the dental follicle and TRAP+ osteoclasts on adjacent alveolar bone surfaces. These cell populations were dramatically increased in treated mutants compared to untreated tl rats, but the timing of their appearance was delayed compared to normal littermates. This lag in the appearance of osteoclasts and their precursors corresponded to the delay in eruption of first molars in treated tl rats.

2. CSF-1 also accelerated the eruption of molars in normal rats. CSF-1 increased the number of TRAP+ mononuclear cells in the dental follicle and TRAP+ osteoclasts on
adjacent alveolar bone surfaces, but had no effect on the timing of their appearance in normal rats.

3. Our data revealed a differential effect on tooth eruption of the growth factors CSF-1 and epidermal growth factor (EGF). CSF-1 accelerated eruption of molars in normal rats, but had no effect on incisor eruption. On the other hand, EGF accelerated incisor eruption, but did not affect molar eruption in normal rats.

4. We have described the mechanism for the transient, mild form of osteopetrosis inherited by mib rats. Mutant animals possess a typical sclerosis at birth, which diminished--but was not resolved--during the first postnatal month. These characteristics are caused by early reductions in osteoclast number and function which improve to normal levels by 4 weeks. Osteoclast numbers were severely reduced in mib rats between birth and 2 weeks, but improved to near normal levels by 4 weeks. Neonatal abnormalities in osteoclast function included reduced staining for the functional enzymes TRAP and TrATPase, decreased levels of mRNA for both TrATPase and CALII, and inability to form a well-developed ruffled border. None of these defects were apparent after the first postnatal month.

5. Finally, we have shown that the dental abnormalities caused by the mild, transient form of osteopetrosis in mib rats are limited to incisor defects and delayed eruption of all teeth. Histologic and radiographic examination of mutant incisors revealed that, contrary to the situation in normal rats, the apex of the incisors of mib rats failed to extend past the first molar region to the third molar. The incisor apex of newborn mib rats was misshaped due to ankylosis of incisor matrices with alveolar bone. This ankylosis was temporary, being resolved by the third postnatal day. The delayed eruption of incisors in mib rats and abnormal shape and occlusion of these teeth in older animals is a consequence of the temporary ankylosis in newborn rats.
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COLLABORATORS

It is with great pleasure that I acknowledge the assistance of those with whom I have worked:

CHAPTER I: INTRODUCTION

Part F.1 The manuscript was prepared by Dr. Mark Seifert to summarize topics discussed in detail during a three day symposium on osteopetrosis held at the University of Massachusetts Medical School in July 1991. Coauthors contributed to portions of this manuscript according to individual expertise.

Part F.2 Dr. Sandy C. Marks, Jr. prepared most of this manuscript, and Dr. Kai T. Sundquist contributed key figures concerning alveolar crypt surface morphology in bafilomycin-treated dogs during tooth eruption.

Part F.3 Dr. Sandy C. Marks, Jr. prepared most of this manuscript, and Dr. Kai T. Sundquist contributed key figures concerning alveolar crypt and tooth surface morphology in bafilomycin-treated dogs during tooth eruption.

CHAPTER IV: RESULTS

Part A Carole MacKay prepared radiographs and processed tissues examined in this study. Erik Larson and Carole MacKay treated animals with CSF-1. Dr. Sandy C. Marks, Jr. prepared the manuscript.
Part B
Dr. Tadashi Izuka processed many of the tissues examined in this study, performed photography of prepared slides for Figures 3-8, and assisted in determining age of tooth eruption. Dr. Sandy C. Marks, Jr. photographed the 11 rats for Figure 1. Carole MacKay prepared radiographs of the mandibles used in Figure 2 and treated animals with CSF-1.

Part C
Carol Paradise and Carole MacKay cut sections from prepared methacrylate blocks and performed histochemical staining for TRAP. Michael Jolie completed the histomorphometry.

Part D
Carol Paradise and Carole MacKay cut sections from prepared methacrylate blocks and performed histochemical staining for TRAP. Michael Jolie did the histomorphometry.

Part E
Erik Larson, Carole MacKay, and Beth Jackson assisted during the weighing of animals and tissue harvesting. Carole MacKay prepared radiographs used in Figure 2. Dr. Joe Zerwekh determined circulating levels of vitamin D.

Part F
Carole MacKay and Erik Larson assisted during tissue harvesting. Carole MacKay prepared the radiographs used in Figure 2.

Part G
Erik Larson and Carole MacKay assisted during tissue harvesting.
CHAPTER I
INTRODUCTION

PART A:
The Structure of Osteoclasts

The osteoclast (literally "bone-breaker") is the major cell responsible for bone matrix degradation and was originally described by Kölliker (1873). Under the light microscope, osteoclasts are easily identified and appear as large (50-100μm) cells with several centrally located nuclei, often with prominent nucleoli and a strongly basophilic, frothy cytoplasm. Although they appear infrequently in normal adult bone, osteoclasts are usually found attached to mineralized surfaces, especially endosteal or trabecular surfaces between the calcified matrix and bone marrow. Active osteoclasts reside within a region of excavated bone known as Howship's lacuna, produced by extensive matrix degradation. In addition, osteoclasts take on a number of cell shapes ranging from plump cells facing straight bone surfaces to elongate cells wrapped around the end of a spicule of trabecular bone.

The distinctive ultrastructural features of osteoclasts are highly-developed, perinuclear Golgi complexes, abundant mitochondria, various lysosomes, and free ribosomes. These features are represented in Figure 1. In fact, the ratio of mitochondria to cytoplasmic area is larger than any other cell type in the body (Holtrop 1991). The ultrastructural hallmark of active osteoclasts is the ruffled border, a uniquely polarized apical membrane surface comprised of deep infoldings of the plasma membrane (Scott and Pease 1956). This membrane ruffling produces an immense surface area between the cell and extracellular matrix, thus providing an ideal exchange region for bone degradation. The degradation of bone surfaces opposite the ruffled border
that results from the release of lytic enzymes (Lucht and Norgaard 1976) and acid by the ruffled border (Baron et al. 1985) is evident by the frayed appearance of collagen fibers there and the presence of mineralized particles seen between membrane infoldings (Holtrop 1991). In addition, the cytoplasm of the ruffled border region being highly vesicular is a source of membrane for the rapid expansion of the ruffled border (Väänänen et al. 1989). Ruffled borders are always circumscribed by an area known as the clear zone, an organelle-free region of cytoplasm containing numerous contractile/attachment filaments (also called sealing zone). A microfilament network consisting of a vinculin-talin double circle surrounding F-actin has been shown to be associated with resorption lacunae (Holtrop and King 1977; Lakkakorpi et al. 1989; Lakkakorpi and Väänänen 1991). It is likely that this specialized cytoplasmic region limits the acidic environment necessary for mineral degradation by sealing off the area under the ruffled border from extracellular spaces (Schenk et al. 1967; Lakkakorpi and Väänänen 1991). This microfilament structure corresponds roughly to the edges of resorbing lacunae of active osteoclasts, and is dispersed by inhibitors of osteoclastic bone resorption, such as calcitonin (Lakkakorpi and Väänänen 1990).
Part B:
The Origin of Osteoclasts

Osteoclasts are derived from hemopoietic precursors

The observation that osteoclasts are usually found on mineralized bone surfaces in close association with marrow is both functional and developmental in nature. The former observation, that osteoclasts degrade bone matrix, was realized long ago; however, the origin of the osteoclast eluded researchers until recently. Among the first evidence for a hemopoietic origin of osteoclasts came from the pioneering work of Donald Walker (Walker 1972, 1973). Walker made use of inbred strains of rodents with congenital osteopetrosis, a metabolic bone disease characterized by reduced bone resorption due to interceptions in osteoclast function. When lethally-irradiated mutants were joined to normal littermates by skin flaps to establish a common circulation, they were cured of the disease. Furthermore, their osteoclasts were derived from their normal sibling. In an extension of this work, Walker repeatedly cured mice of osteopetrosis with spleen and bone marrow transplantations and likewise, transmitted osteopetrosis to normal littermates via transplantation of mutant spleen or marrow (Walker 1973, 1975a,b,c). In similar parabiosis experiments, Göthlin and Ericsson (1973) assessed the origin of bone cells in developing fracture calluses. To do so, they joined inbred rats and delivered a dose of radiation sufficient to destroy all hemopoietic tissue in one rat while the other animal was shielded. The cross-circulation was temporarily blocked, and tritiated thymidine was injected into the shielded rat. Electron microscopic and autoradiographic examination of osteoclasts and other bone cells of the fracture calluses in both animals revealed that both osteoblasts and giant cells were labeled in the shielded animal while labeling was confined to osteoclasts in the irradiated sibling. These experiments indicated that osteoclasts could be replaced with cells
derived from the bloodstream and formed the foundation for our current concept of the hemopoietic origin of osteoclasts.

Walker's observations were confirmed in other laboratories through
1. chick/quail transplantation experiments (Kahn and Simmons 1975; Jotereau and Le Douarin 1978), 2. co-cultures of osteoclast-free bone with nonadherent bone marrow cells (Ko and Bernard 1981; Schneider and Relfson 1988b), and 3. cell suspension transplantation studies (Schneider and Byrnes 1983; Schneider and Relfson 1988a):

1. Chick/quail transplantation experiments have provided scientists with a useful tool for constructing chimeras, since the cells from chickens and quails can be easily distinguished by their characteristic interphase nuclear morphology. When bone rudiments of one species were grafted onto the chorioallantoic membrane of the other and allowed to differentiate (endochondral bone formation), the osteoclasts present in the resultant later stage long bones were invariably of recipient origin, while osteoblasts were donor-derived. 2. Osteoclast-free bone formation assays: Osteoclast-free bone can be derived from either preculturing fetal mouse calvaria rudiments or by taking advantage of periosteum and perichondrium-free fetal long bone rudiments; in either case ossification proceeds with the development of osteoblasts in the absence of osteoclasts. When these rudiments are co-cultured with appropriate cell suspensions, for example spleen or marrow, osteoclasts formed. 3. Using density gradients and fluorescent activated-cell-sorting techniques, hemopoietic stem cells were enriched and transplanted to donor rats. These isolated progenitor cells, especially those of CFU-G and CFU-GM, differentiated into osteoclasts in the host or fused with pre-existing host osteoclasts. Taken together, these experiments provide strong evidence that osteoclasts can be replaced and replenished by the blood stream via precursor cells present in the skeleton and extraskeletal hemopoietic tissues.
Characteristics of osteoclast precursors

It is now generally believed that osteoclasts form by the fusion of mononuclear precursors present in blood and marrow. Mononuclear phagocytes have long been considered as candidates for osteoclast progenitors, since they can dissolve both bone mineral and organic matrix under appropriate conditions (Holtrop et al. 1982; Teitelbaum et al. 1979), and respond to many factors either present in bone matrix and/or known to influence bone resorption, including bone mineral itself, osteocalcin, type I collagen, and PTH (Minkin et al. 1977; Malone et al. 1982; Mundy et al. 1978). Histochemical and ultrastructural examinations of these mononuclear phagocytes, osteoclasts, and presumptive osteoclast precursors have revealed both similarities and differences between presumptive preosteoclasts and mononuclear cells of the macrophage lineage (Horton and Helfrich 1992). Both have Fc and C3 receptors, characteristic of macrophages and nonspecific-esterase (NSE), a marker for mononuclear phagocytes (Pierce and Lindskog 1986; Baron et al. 1986). However, preosteoclasts express greater amounts of acid phosphatases--especially tartrate-resistant acid phosphatase--and NADH dehydrogenase, but lower levels of fluoride-inhibited non-specific esterase activity than multinucleated giant cells (Zheng et al. 1991). In addition, phagocytic mononuclear macrophages do not respond to calcitonin nor do they develop ruffled borders (Teitelbaum 1979; Mundy 1983). It has been observed that mononuclear cells with ultrastructural features of preosteoclasts, including abundant mitochondria and lysosomes and extensive Golgi complexes, fuse with avian osteoclasts attached to bone surfaces (Fukushima et al. 1991). These cells also have numerous small vesicles containing alkaline p-NPPase, a marker for the basal ruffled border. Indeed, mononuclear osteoclasts can resorb bone (Domon and Wakita 1991). Recently, a number of laboratories have shown that mouse peripheral blood
monocytes, chicken bone marrow mononuclear phagocytes, and alveolar macrophages fuse with cultured osteoclasts or form syncytia by fusing with themselves in vitro (Zambonin-Zallone and Teti 1985; Zambonin-Zallone et al. 1984; Helfrich et al. 1986; Udagawa et al. 1990). However, these studies have been questioned since it is difficult to obtain pure populations of the cells in question. In addition, other studies have indicated that mature monocytes and macrophages cannot fuse to form osteoclasts (Burger et al. 1982; Takahashi et al. 1991). These data indicate that osteoclasts and macrophages share a common lineage and that osteoclasts arise from mononuclear progenitors before commitment to the macrophage lineage.

**Regulation of osteoclastogenesis**

Recent investigations concerning the development of osteoclasts have relied primarily on the use of long-term marrow or stromal cell cultures. These studies have provided additional evidence for the hemopoietic origin of the osteoclast and shed light on the factors influencing their development and function. Factors which have been shown to stimulate osteoclast formation in stromal or marrow cell cultures include the growth factors macrophage colony-stimulating factor (M-CSF or colony-stimulating factor-1, CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony stimulating factor (G-CSF) (Kodama et al. 1991; Takahashi et al. 1991); cytokines including interleukin 1 (IL-1), interleukin 3 (IL-3), interleukin 6 (IL-6), and tumor necrosis factor (TNF) (Kurihara and Roodman 1990; Hagenaars et al. 1991; Barton and Meyer 1989; Kurihara et al. 1990; Pfeilschifter et al. 1989); and the calcitropic hormones such as 1,25-dihydroxyvitamin D$_3$ and the parathyroid hormone (PTH) family of molecules (Akatsu et al. 1989; Udagawa et al. 1990). Numerous factors which inhibit the formation of osteoclasts in vitro have also been
identified in long-term marrow cell cultures, for example estrogen, TGF-β, PGE2, INF-alpha and gamma, and IL-4 (Jilka et al. 1992; Chenu et al. 1988; Kurihara and Roodman 1990; Shioi et al. 1991). However, it is noteworthy that the specific conditions necessary for generating osteoclasts in vitro remain undefined (Flanagan et al. 1992). Furthermore, these studies are selective in approach, primarily testing the effects of known factors (growth factors, hormones, cytokines, colony-stimulating factors) on the formation of osteoclasts in vitro. In order to identify and understand the factors/signals which are operative during in vivo osteoclastogenesis, non-selective approaches must be utilized. In addition, the possible role of those factors influencing osteoclast development and/or function in vitro must be examined and verified in vivo. In situ hybridization and antibody blockade provide useful tools for such investigations.

The cells which form under culture conditions have many of the features of osteoclasts. These include ultrastructural and histochemical features, responsiveness to calcitonin, and ability to resorb devitalized bone (Skjødt and Russell 1992). Although there is little doubt that some osteoclasts form in long-term marrow/stromal cell cultures, up to 4 weeks is often necessary to produce them. Macrophage polykaryons, which do not resorb bone, often form spontaneously in cell cultures, especially in the presence of factors commonly in these systems, such as 1,25-dihydroxyvitamin D₃ (Schlesinger et al. 1984; Sutton and Weiss 1966). Consequently, caution must be used when interpreting data from in vitro osteoclast-generating cultures, especially when attempting to correlate multinuclear cell number with osteoclast formation or activity. For example, osteoclastic markers such as tartrate-resistant acid phosphatase (TRAP) do not always apply to the in vitro state, since TRAP may be expressed by monocytes and/or macrophages, which resemble osteoclasts in culture (Horton and Helfrich 1992). In addition, TRAP is expressed by hairy leukemia cells and macrophages derived from a variety of tissues (Ketcham et al. 1985; Robinson and Glew 1980; Snipes et al.
Therefore, the only verifiable criterion for assessing in vitro osteoclastogenesis at this time lies in determining that the cell(s) in question are capable of resorbing a mineralized matrix (Hattersley and Chambers 1989).

The role of the hemopoietic growth factor CSF-1 in osteoclastogenesis

One example of discrepancies between in vivo and in vitro data concerning the generation of osteoclasts comes from recent experiments about colony-stimulating factor-1 (CSF-1 or M-CSF). CSF-1 is one of a family of factors known to stimulate growth and differentiation of hemopoietic cells. The primary function of CSF-1 is to stimulate the proliferation, differentiation, function, and survival of cells of the mononuclear phagocyte system (Stanley and Heard 1977; Stanley et al. 1983). CSF-1 is produced by stromal or connective tissue cells including monocytes, keratinocytes, fibroblasts, endothelial cells and osteoblasts (Horowitz and Jilka 1992) and is encoded by a single gene (about 21 kb) composed of at least 10 exons (Ladner et al. 1987). Post-transcriptional modifications of the primary mRNA transcript produce multiple mature mRNAs which encode both a soluble form and at least 3 distinct membrane-bound forms of the factor. The soluble form of CSF-1 is derived from a 554 amino acid precursor that is processed to yield two subunits of approximately 223 amino acids. These smaller subunits form homodimers to yield the soluble form of CSF-1, which is secreted by the cell (Ladner et al 1987). The membrane-bound forms of CSF-1 share a common arrangement which includes a short carboxyterminal intracellular tail, a series of hydrophobic amino acids which serve as the transmembrane segment, a sequence corresponding to the actual soluble factor, and an aminoterminal signal peptide (Kawasaki et al. 1985; Rajavashisth et al. 1987). Consequently, the membrane-bound forms are transported to the plasma membrane where they are expressed as integral
membrane glycoproteins. The proto-oncogene c-fms encodes the receptor for CSF-1 (Rothwell and Rohrschneider 1987).

Numerous studies have provided conflicting evidence for the role of CSF-1 is bone cell function, but recent observations suggest that CSF-1 plays an important role in osteoclastogenesis. CSF-1 stimulates the formation of osteoclast-like cells in organ cultures of embryonic mouse calvaria and weakly adherent bone marrow cells (Burger et al. 1982), suggesting that osteoclasts share a common lineage with phagocytic macrophages. CSF-1 enhances osteoclast formation in long-term marrow cell cultures, presumably by inducing proliferation from precursors and 1,25-dihydroxyvitamin D-mediated fusion of precursors (MacDonald et al. 1986). These data support the concept that CSF-1 influences osteoclast formation from mononuclear precursors. On the contrary, Shinar et al. (1990) reported that CSF-1 had no effect on osteoclast formation in long-term marrow cultures, but actually reduced the number of TRAP+ mononuclear and multinuclear cells. Nevertheless, additional studies using long-term marrow and stromal cell cultures have led some investigators to conclude that CSF-1 in necessary for proliferation and differentiation of osteoclast precursors (Tanaka et al. 1993). These studies illustrate conflicts in the role of CSF-1 in osteoclastogenesis in vitro.

Recent studies in the op mouse and the It rat, osteopetrotic mutations with few osteoclasts, reduced numbers of macrophages and monocytes, and resistance to cure by stem cell replacement (Marks and Lane 1976; Marks et al. 1984; Wiktor-Jedrzejczak et al. 1982, 1990; Marks 1977b; Seifert et al. 1988b) have provided compelling evidence for the importance of CSF-1 in osteoclast development and function. The mutation responsible for the disease in op mice is located in the coding region of the CSF-1 gene (Yoshida et al. 1990) and injections of CSF-1 are reported to cure the
disease in op mice (Felix et al. 1990a; Kodama et al. 1991). Similarly, injections of CSF-1 improve the skeletal manifestations of osteopetrosis in Il rats, although some skeletal sclerosis persists even with prolonged treatment (Marks et al. 1992; lizuka et al. 1992; Marks et al. 1993). In either case, treatment with CSF-1 increases osteoclast numbers and increases bone resorption. Indeed, a single dose of CSF-1 is sufficient to cause a temporary elevation in osteoclast numbers in the skeleton of op mice (Kodama et al. 1993). The residual metaphyseal sclerosis which persists in Il rats treated with CSF-1 is probably due to a failure to restore subepiphyseal osteoclasts (Marks et al. 1993). Despite the reported effects of CSF-1 on op mice by other investigators, Sundquist and others (1993) have shown that, after prolonged CSF-1 treatment, metaphyseal sclerosis persists in these mice. In addition, they report that macrophages are deficient and osteoclasts are maldistributed in the metaphyseal regions of CSF-1 treated op mice. Furthermore, no subepiphyseal osteoclasts were observed. These authors have postulated that the residual metaphyseal sclerosis and failure to restore subepiphyseal osteoclasts in op mice is caused by the failure to restore the cell-bound form of CSF-1 on local cells. This concept is supported by the observation that cells known to produce CSF-1, namely osteoblasts and endothelial cells, are present in the subepiphyseal region and osteoclasts are absent from this region despite the administration of secreted CSF-1. Therefore, CSF-1 treatment of either osteopetrotic mutation improves—but does not cure—the manifestations of the disease. The similar skeletal responses of these two osteopetrotic mutations to exogenous CSF-1 suggest that redundant and related mechanisms for osteoclastogenesis exist in vivo. This concept is supported by the observation that some—albeit few—osteoclasts are present in both mutations prior to treatment.
Part C:
The Function of Osteoclasts

In order to resorb bone, newly formed or quiescent osteoclasts must be activated. It is generally thought that this process is influenced by factors produced by the local environment in response to systemic signals (1,25-dihydroxyvitamin D₃, PTH) or local factors such as matrix components, cytokines, or growth factors produced by cells in the microenvironment. Activating factors stimulate bone resorption by influencing cellular events such as adhesion to bone, formation of a ruffled border, release of acid hydrolases in the ruffled border region, and production of superoxide radicals.

Osteoclast attachment to bone is mediated by the integrin superfamily of adhesion molecules and plays an integral role in the resorbing process (Horton and Davies 1989; Oldberg et al. 1988a,b). Bone contains numerous matrix proteins that contain Arg-Gly-Asp (RGD) sequences, such as type I collagen, osteopontin, bone sialoprotein, thrombospondin, and fibronectin (Bernard et al. 1983; Oldberg et al. 1986; Oldberg et al. 1988; Lawler and Haynes 1986). These proteins facilitate adhesion of both osteoblasts and osteoclasts in culture (Dedhar et al. 1987; Gehron Robey et al. 1989; Helfrich et al. 1992), apparently by interactions with the vitronectin receptor. Indeed, peptides containing the RGD sequence inhibit bone resorption (Lakkakorpi et al. 1991). The vitronectin receptor (alpha-v, beta-3) has been localized to the plasma membrane of osteoclasts (Horton 1988; Davies et al. 1989) and antibodies to this integrin inhibit bone resorption (Chambers et al. 1986; Horton et al. 1991). Although it is tempting to suggest that the vitronectin receptor plays an important role in maintaining the tight attachment (seal zone) of osteoclasts to bone during resorption, recent evidence suggests that the protein mediates osteoclast attachment and migration. The receptor is not found
in association with the clear/sealing zone, but is localized in the ruffled border and the basolateral membrane of osteoclasts (Lakkakorpi et al. 1991).

The process by which osteoclasts resorb bone occurs in two phases and is illustrated in Figure 1. During the first phase of resorption, inorganic matrix is dissolved, a process which takes about 2 hours to reach peak activity in freshly isolated chicken osteoclasts (Blair et al. 1986). During this process, degradation of bone mineral is facilitated by the acidic microenvironment present in the resorption lacuna under the osteoclast. The acidity of this region has been demonstrated by acridine orange staining and by direct micropuncture techniques (Baron et al. 1985; Anderson et al. 1986; Silver et al. 1988). The protons necessary for the generation of this acidic milieu are provided by either intracellular or membrane-associated carbonic anhydrase isoenzyme II and delivered to the extracellular space by vacuolar-type H⁺-ATPase proton pumps in the ruffled border region (Baron et al. 1985; Sundquist et al. 1990; Sundquist 1993). Numerous observations support the role of carbonic anhydrase in bone resorption. First, carbonic anhydrase is influenced by calcitropic hormones. Calcitonin reduces carbonic anhydrase activity and reduces bone resorption in isolated osteoclasts, while PTH increases carbonic anhydrase II (CAII) activity (Anderson et al. 1982). Second, inhibition of carbonic anhydrase II activity by drugs reduces bone resorption in organ culture (Minkin and Jennings 1972) and responsiveness to PTH in vivo (Waite et al. 1970; Waite 1972). Third, congenital defects in carbonic anhydrase are associated with reduced bone resorption (osteopetrosis) (Sly et al. 1983; Gay 1992).

In order to regulate intracellular pH, the osteoclast possesses a chloride/bicarbonate exchanger in the basolateral membrane (Teti et al. 1989). Inhibition of this chloride/bicarbonate exchanger reduces bone resorption, indicating that this exchanger is coupled with bone resorption (Hall and Chambers 1989).
addition, the basolateral membrane of osteoclasts has numerous Na+-K+-ATPases which, like the chloride/bicarbonate exchanger, are coupled to bone resorption (Baron et al. 1986; Krieger and Tashjian 1981).

Degradation of the organic portion of bone matrix is dependent upon exocytosis and endocytosis at the ruffled border (Lucht and Norgaard 1976) and takes 12-24 hours in freshly isolated chicken osteoclasts (Blair et al. 1986). High concentrations of lysosomal enzymes have been observed in the ruffled border and the extracellular space adjacent to the ruffled border (Baron et al. 1988). Among these enzymes are various cysteine proteinases and acid hydrolases, including tartrate-resistant acid phosphatase (TRAP), an enzyme observed in the granules and vesicles of rat osteoclasts and linked to actively resorbing osteoclasts (Hammarström et al. 1971; Chambers et al. 1987). Tartrate-resistant acid ATPase is also present in active osteoclasts (Andersson and Marks 1989). Evidence for the role of these enzymes in the degradation of organic matrix during bone resorption comes from the observation that both TRAP and TrATPase exhibit phosphoprotein phosphatase activity with the bone matrix proteins osteopontin, osteonectin, and bone sialoprotein II as substrates (Ek-Rylander et al. 1991). Osteoclasts also produce and secrete cysteine proteinases capable of solubilizing collagen, including cathepsin B, L, and D (Rifkin et al. 1991; Goto et al. 1992).

Furthermore, it has been proposed that collagenase, produced by the osteoblast and stored in the bony matrix, could play a role in bone resorption (Chambers et al. 1985). Collagenase is present in bone matrix and contributes to the removal of non-mineralized collagen during PTH-stimulated bone resorption in cultured calvaria (Delaisse and Vaes 1992). This activity is prevented by inhibitors of collagenase, but not by calcitonin, indicating that the activity is independent of osteoclasts. Although it is generally agreed that the concerted effects of collagenase and the lysosomal enzymes and cysteine proteases produced by the osteoclast are responsible for the removal of organic
matrix during bone resorption, the source of collagenase remained a mystery until recently. Many investigators speculated that procollagenase is secreted by osteoblasts, incorporated into bone matrix, and activated by the acidic microenvironment of the resorption lacuna (Delaisse and Vaes 1992; Vaes et al. 1992). It was also considered possible that cysteine proteinases activate collagenase from its inactive zymogen, since cathepsin B is known to activate procollagenase (Eechout and Vaes 1977). However, Delaisse and others (1993) have recently demonstrated the presence of procollagenase in the subosteoclastic bone resorbing compartment and in osteoclasts of mice, rats and rabbits. The perinuclear Golgi distribution of this enzyme in both active and quiescent osteoclasts, together with the observation that mRNA encoding for procollagenase is present in odontoclasts (Okamura 1992; Okamura et al. 1993) strongly suggest that procollagenase is produced and secreted by osteoclasts during matrix dissolution.

Recent evidence supports a role for oxygen-derived radicals such as the superoxide anion in bone resorption. Superoxide anions are found in the resorption lacuna and are believed to aid the process of bone resorption by enhancing matrix degradation (Key et al. 1990; Garrett et al. 1990). In addition, superoxide dismutase, the enzyme responsible for converting superoxide anions into hydrogen peroxide ($H_2O_2$), has been identified in the osteoclast membrane (Oursler and Osdoby 1990). Indeed, $H_2O_2$ stimulates bone resorption at nanomolar concentrations (Bax et al. 1992). These authors speculate that the stimulatory effects of $H_2O_2$ are brought about by enhanced osteoclast motility, similar to the effects of PTH (Chambers 1982).

The identification of functional markers is a paramount concern to distinguish osteoclasts and their precursors from cells of other/similar lineages like macrophages. Mononuclear precursors to osteoclasts have been observed at the bone surface and express ultrastructural and histochemical features of osteoclasts, including Golgi complexes, mitochondria, polysomes and cytoplasmic extensions (Baron et al. 1986).
One useful marker is carbonic anhydrase isoenzyme-2 (CA II), an enzyme present in the ruffled border of active osteoclasts and responsible for the delivery of H\(^+\) to the resorption site. Similarly, tartrate-resistant acid phosphatase (TRAP) and acid ATPase (TrATPase) have been implicated in acid production by the osteoclast. As mentioned above for TRAP, these latter markers are not absolute because cells other than osteoclasts can produce them, in vivo and in vitro (Marks and Chambers 1991; Hattersley and Chambers 1989).

The above data concerning osteoclast morphology, development, and function summarize the current understanding of osteoclast biology and illustrate its derivation largely from cultures of isolated osteoclasts and reconstituted marrow/stromal cell systems. How these data relate to in vivo events is, for the most part, unknown. What is the origin and regulation of the specific mononuclear precursors of osteoclasts? What factors determine the tissue and site-specificity of osteoclasts and their subsequent activation? What causes resorption to stop and what is the lifespan of an osteoclast? Many of these issues can be addressed in vivo by the use of specific mutations or analyses of events during tooth eruption. These opportunities are described below.
Figure 1: Schematic representation of an active osteoclast depicting morphological features and details of the resorptive process. Osteoclasts create an acidic environment by secreting protons via acid ATPase in the ruffled border region. This process depends on carbonic anhydrase II. Lysosomal enzymes are delivered to and released at the ruffled border region. This acidic microenvironment activates proteinases and digests bone mineral, which may be released to the circulation via osteoclastic processing. Ionic balance is maintained by Na⁺-K⁺ and Cl⁻ exchangers on the basolateral membrane surface and ruffled border surface, respectively. This schematic representation is based on the work of Delaisse and Vaes (1992).
Part D:

Osteopetrosis--Opportunities

for Studying Systemic Osteoclast Development and Function

Osteopetrosis is a rare metabolic bone disease in mammals characterized by a general skeletal sclerosis caused by reduced bone resorption (Marks 1987a, 1989; Osier and Marks 1992). Because these mutations interrupt the development or function of osteoclasts, they represent reproducible models in which to study the systemic and local factors essential for osteoclast differentiation and function (Marks and Walker 1976; Marks 1992). In addition, because osteoclasts fail to develop in certain skeletal sites in different osteopetrotic mutations, these mutants offer models in which to explore the emerging site-specificities of osteoclast development and function.

While spontaneous mutations have been documented in cattle (Gopal et al. 1980; Zetterholm 1972), dogs (Lees and Sautter 1979), deer (Smits and Bubenik 1990), and humans (Loria-Cortes et al. 1977), the nine distinct mutations found in common laboratory animals (mice, rats, and rabbits) have been most studied. These animal mutations with osteopetrosis have been useful in the study of osteoclast development and function, and are listed with their distinguishing characteristics in Table 1. These mutations are congenital, inherited as an autosomal recessive trait. Consequently, all possess a characteristic skeletal sclerosis at birth, which persists in the severe forms of the disease, but may gradually diminish in milder mutations. The four mouse mutations are on different chromosomes and two of these, grey-lethal (gl) and osteosclerosis (os), are lethal and afflicted individuals die within the first 3-4 weeks of life. The four mutations in the rat are not alleles (Moutier et al. 1989, 1976). One mutation, osteopetrotic (op) is lethal, while both the incisors-absent (ia) and
microphthalmia blanc (mib) are mild mutations capable of normal lifespans (Marks and McGuire 1988).

The general skeletal manifestations of osteopetrosis are remarkably similar. Because the disease is invariably caused by reduced bone resorption, any process dependent on bone resorption is compromised. All mutants have a generalized sclerosis detectable radiographically at birth due to absent or delayed marrow cavity formation. Indeed, individual mutations cannot be distinguished from each other radiographically at that time. The ends of long bones lack the external modeling present in normal animals and exhibit a characteristic blunting commonly referred to as "club-shaped." Delay or failure of tooth eruption is common among the osteopetroses, ranging from complete lack of erupted dentition in the most severe forms of the disease (lizuka et al. 1992) to delays in incisor and/or molar eruption in the less severe ones (Marks 1981). These abnormalities in bone modeling, tooth eruption, and marrow cavity development are the distinct consequences of reductions in bone resorption. Rates of bone resorption have been measured in four mutations and range from undetectable in the toothless (tl) rat (Raisz et al. 1981) to 70% of normal siblings in ja rats (Marks 1973). Other values include <10% normal for microphthalmic (mi) mice (Marks 1977a) and 50-60% normal in qc mice (Reitter et al. 1989).

Despite the marked similarities observed at the tissue level, there exists a remarkable heterogeneity among the osteopetroses with respect to osteoclast morphology and function (Seifert et al. 1993). While the number of osteoclasts is reduced in most mutations, numbers of osteoclasts per unit area of skeleton are elevated in the ja rat and qc mouse (Marks et al. 1984). Ruffled borders, the specialized membrane responsible for delivering protons and enzymes to the resorption compartment during bone resorption, are absent or poorly developed in most mutations (Marks 1984). However, those few osteoclasts in the skeleton of qc mice have extensive ruffled borders (Marks
Osteoclast activity is reduced in all mutations. In addition to the morphological and functional aberrations present in osteoclasts in osteopetrotic animals, osteoblast abnormalities in three rat mutations have recently been reported (Shaloub et al. 1991a,b) as shown in Table 2. Messenger RNA for histone 4 is reduced in op rats from 2-4 weeks after birth and inİL rats at 2 weeks. Elevations in fibronectin, osteopontin, and alkaline phosphatase mRNA are observed in all mutations at some point between the 2nd and 6th postnatal week. Other osteoblast abnormalities include reduced osteoblasts per unit area of bone inİL rats, and structural and functional deficiencies in osteoblasts from os rabbits (Seifert et al. 1988b; Marks et al. 1987; Popoff and Marks 1990). Bone formation--assessed by uptake of 3H-proline--is increased in the four mouse mutations (gl, oc, op, oc), but decreased in both the ia rat and os rabbit (Marks 1989). In addition, biochemical evidence suggests that aberrations in bone matrix may contribute to osteopetrosis, since osteocalcin is reduced in calvarial bone ofİL and op rats (Lian and Marks 1990). The abnormal numbers and/or function of osteoblasts inİL rats and os rabbits, together with the alterations in osteoblastic gene expression among the rat mutations, suggest that derangements in osteoblasts may contribute and/or compound abnormal osteoclast function in osteopetrotic animals. This is particularly true considering that the reported derangements in gene expression are associated with functional properties of the osteoblast, especially those believed to be involved in osteoclast recruitment and/or development (Marks et al. 1989). Indeed, the concept that osteoblasts play a central role in the regulation of osteoclast recruitment and activation has grown in recent years (Marks and Popoff 1988; Rodan and Martin 1981).

Additional morphological and physiologic evidence for alterations in the skeletal microenvironment has recently been reported. An amorphous material between the calcified cartilage and newly-formed bone is present in growing longbones of oc mice (Seifert and Marks 1985). This material cannot be resorbed by either mutant or
normal osteoclasts (Van Slyke and Marks 1987), suggesting that the matrix may be
deficient in factors necessary for the recruitment or activation of osteoclasts. Impaired
production of colony-stimulating factor-1 (CSF-1 or M-CSF) by osteopetrotic bone,
bone cells, and fibroblasts of the op mouse has been demonstrated (Wiktor-Jedrzejczak

In addition to the skeletal manifestations of osteopetrosis, numerous
extraskeletal distinctions exist. Osteopetrosis in animals is often accompanied by
aberrations in mineral homeostasis, and many mutants are hypocalcemic and
hypophosphatemic (see Table 1). Three of the four mouse mutations (mi, oc, gl) and the
os rabbit have reduced serum levels of phosphate and calcium. Serum levels of 1,25-
dihydroxyvitamin D3 (1,25(OH)2D) are elevated at some point in life for all of the
seven mutations examined to date, including mi, op, oc mice, ja, il, op rats, and the os
rabbit (Zerwekh et al. 1987; Seifert et al. 1993). Serum levels of 1,25(OH)2D are
elevated in il rats at 1 week after birth, peak to almost 4 times the value for normal
siblings at 3 weeks of age, and decline to normal levels by the 5th postnatal week.
Serum levels of 1,25(OH)2D in op rats remain up to 4 times those seen for normal
littermates 4-6 weeks after birth. Furthermore, infusions of high doses of
1,25(OH)2D do not induce a calcemic response in os rabbits. These data suggest that
osteopetrotic individuals possess a skeletal resistance to 1,25(OH)2D (Marx and
Barsony 1988). Additional evidence for skeletal resistance to 1,25(OH)2D has come
from renal studies. Nesbitt and others (1988) have reported elevated levels of renal
25-hydroxyvitamin D-1-alpha-hydroxylase in oc mice. This enzyme, which produces
the active metabolite of vitamin D, is suppressed with a high calcium diet, suggesting
that increased synthesis is the major cause of high levels of circulating 1,25(OH)2D in
these mutants (Nesbitt et al. 1988). These data, together with elevated serum levels of
1,25(OH)2D in osteopetrotic animals suggest that skeletal resistance to 1,25(OH)2D is
characteristic of congenital osteopetrosis in mammals (Seifert et al. 1993). Clearly, the relationship between the skeleton and endocrine systems is complex, and deserves further study.

Immunological complications of osteopetrosis have been reported. Early studies in the op rat indicated that the skeletal manifestations of the disease were caused by thymus dysfunction. These reports described premature thymus atrophy, reduced mitogenic responses of splenic and thymic lymphocytes, and cure of the disease by transplantation with normal thymic tissues (Milhaud and Labat 1978, 1979; Milhaud et al. 1975, 1977). Similarly, decreased mitogen-induced lymphocyte proliferation in isolated spleen cells from mi and op mice has been reported (Minkin et al. 1982; Olsen et al. 1978). These data have led some investigators to conclude that osteopetrosis is a thymic disorder with skeletal complications (Labat 1991). However, this hypothesis is challenged by numerous findings. First, athymic mice are not osteopetrotic (McCauley et al. 1989). Second, thymic transplants cure neither mi and op mice nor op rats (Schneider and Marks 1983; Wiktor-Jedrzejczak et al. 1981; Evans et al. 1985). Third, qualitative and quantitative studies of the cellular composition of thymus and the T-cell zones of both spleen and lymph nodes demonstrate no differences between the mi or ja mutants and their normal littermates (Schneider 1978; Schneider and Marks 1983). Fourth, Popoff et al. (1990) have shown increased proliferation of op thymocytes compared to normal thymocytes, when cultured in the presence of interleukin 1. Nevertheless, immune dysfunction secondary to the skeletal abnormalities is likely.

Some osteopetrotic mutations are cured by stem cell replacement (see Table 1), and include the op and ja rats and the mi and gl mice (Walker 1973, 1975a,b; Milhaud et al. 1975; Marks 1976). As mentioned above, these findings were the result of the pioneering work of Walker, who first cured osteopetrosis in a mouse by parabiosis with
a normal littermate. However, stem cell therapy does not cure osteopetrosis in op or occ mice, fl rats, or os rabbits (Marks et al. 1984; Seifert and Marks 1987; Marks 1977b; Popoff and Marks 1991). This indicates that the defect in these mutations is not in the stem cells themselves but in other factors including the skeletal microenvironment.

Experimental investigations of osteopetrotic mutations have contributed to our understanding of bone cell biology, especially osteoclast development and function (Marks 1989). These contributions include advances in understanding calcium and phosphate homeostasis, the regulation of bone resorption, and the origin and function of osteoclasts. In addition, the transplantation experiments of Walker and colleagues provided the first successful clinical treatment of human malignant osteopetrosis (Coccia et al. 1980; Sorell et al. 1981). These animal models are likely to provide additional insights concerning bone cell biology, including the role of CSF-1 and other factors in the regulation of generalized and site-specific osteoclastogenesis, end organ resistance to vitamin D, control of bone modeling and remodeling by local factors and cells, and the clinical management of the osteopetroses.

The human osteopetroses exhibit both autosomal recessive and dominant forms of inheritance. The autosomal recessive forms of osteopetrosis are usually malignant and follow a progressively deteriorating course within the first decade of life (Nussey 1938). Clinical complications of reduced bone resorption include obstruction of cranial foramina resulting in increased cranial pressure, mental retardation, optic atrophy, blindness, and deafness (Cournot et al. 1992). Infantile osteopetrosis is often accompanied with low levels of serum calcium and phosphorous (Reeves et al. 1981), increased parathyroid gland activity (Glorieux et al. 1981), and extramedullary hemopoiesis (Freedman and Saunders 1981; Cook and Moore 1992). The autosomal dominant forms of the disease are relatively benign and follow a progressive course with
aging (Bollerslev et al. 1993). Clinical attempts to manage infants with osteopetrosis remain experimental and have resulted in mixed success (Sorell et al. 1981; Key et al. 1984; van Lie Peters et al. 1993; Coccia et al. 1980; Coccia 1993), thus illustrating the heterogeneity among human osteopetroses.

Recently, a transient form of infantile osteopetrosis was described (Monaghan et al. 1991) in which an infant presented clinical signs of severe malignant osteopetrosis at birth that diminished during the first year of life. This mild form of osteopetrosis demonstrates the need to distinguish at birth, those infants in whom the disease will become progressively lethal, from those who will gradually improve.
## Characteristics of Available Osteopetrotic Mutations

### Mouse

<table>
<thead>
<tr>
<th>Bone Resorption</th>
<th>Bone</th>
<th>Serum</th>
<th>BMT</th>
<th>Cure</th>
<th>Defect</th>
<th>Lethal?</th>
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<tbody>
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<td>MOUSE</td>
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<td>RB</td>
<td>QUANT</td>
<td>FORMn</td>
<td>Ca</td>
<td>Pi</td>
</tr>
<tr>
<td>op/op</td>
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<td>I/D</td>
<td>N</td>
<td>I</td>
</tr>
<tr>
<td>mi/mi</td>
<td>N</td>
<td>No</td>
<td>1/20th</td>
<td>I</td>
<td>D</td>
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</tr>
<tr>
<td>oc/oc</td>
<td>I</td>
<td>No</td>
<td>D</td>
<td>I</td>
<td>D</td>
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</tr>
<tr>
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<td>FORMn</td>
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<tr>
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### Rat

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<td>?</td>
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<tr>
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<th>Rabbit</th>
<th>Others</th>
<th>Others</th>
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<tr>
<td>op/op = osteopetrotic</td>
<td>mi/mi = microphthalmic</td>
<td>os/os (osteosclerotic)</td>
<td>RB = ruffled border</td>
<td>BMT = bone marrow transplant</td>
</tr>
<tr>
<td>mi/mi = microphthalmic</td>
<td>oc/oc = osteosclerotic</td>
<td>op/op = osteopetrotic</td>
<td>Ocl = osteoclast</td>
<td>Ocl = osteoclast</td>
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<td>tl/tl = toothless</td>
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<td></td>
<td>PI = phosphate</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CSF-1 = colony-stimulating factor</td>
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### Expression of Bone Matrix-Related Genes in Calvarial Bone of Osteopetrotic Rat Mutations

#### Table: Differentiation, Matrix Formation, Growth, and Bone Occlusion

<table>
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<th>RAT MUTATION</th>
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<th>AP</th>
<th>OP</th>
<th>OC</th>
<th>C1</th>
<th>FN</th>
<th>H4</th>
<th>GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ia/ia incisor absent</td>
<td>2 wk</td>
<td>I</td>
<td>N</td>
<td>N</td>
<td>D</td>
<td>D</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>I</td>
<td>I</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>hard to extract</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>I</td>
<td>N</td>
<td>D</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>op/op osteopetrotic</td>
<td>2 wk</td>
<td>I</td>
<td>I</td>
<td>D</td>
<td>N</td>
<td>I</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>I</td>
<td>I</td>
<td>D</td>
<td>N</td>
<td>I</td>
<td>D</td>
<td>(50%)</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>N</td>
<td>I</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>tl/tl toothless</td>
<td>2 wk</td>
<td>I</td>
<td>N</td>
<td>D</td>
<td>N</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>I</td>
<td>N</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>N</td>
<td>(73%)</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>I</td>
<td>N</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
</tbody>
</table>

**Key:**
- **AP** = Alkaline Phosphatase
- **OP** = Osteopontin
- **OC** = Osteocalcin
- **C1** = Type-I Collagen
- **F** = Fibronectin
- **H4** = Histone 4
- **I** = Increase
- **D** = Decrease
- **N** = Normal
- **I** = Increase Trend
- **D** = Decrease Trend
Part E:

Tooth Eruption--An Opportunity to Study Localized Osteoclast Development and Function

Tooth eruption is defined as the dynamic process whereby a tooth is translocated from its developmental site in alveolar bone to its functional position in the oral cavity (Massler and Schour 1941). For this process to occur, bone above the erupting tooth must be removed while new bone forms below it. These events represent a localized, bilaterally symmetrical, and precisely timed biological growth process (Cahill et al. 1988; Marks et al. 1988). The precise timing of the cellular, biochemical and molecular events (discussed below) which cause tooth eruption, and the necessity of bone resorption for eruption, provide a unique opportunity for the study of local osteoclast development and function.

Theories of Eruption.

Many attempts to explain the process of tooth eruption have centered on the belief that mechanical forces, rather than biological processes account for the movement of teeth during eruption (Cahill et al. 1988). Despite recent advances in understanding the biology of tooth eruption, mechanical force explanations remain popular, perhaps a consequence of orthodontic success in causing erupted teeth to move by influencing bone resorption and formation at opposite sides of the tooth via applied orthodontic apparati. Mechanically-based theories are based on forces generated by pulp, root elongation, alveolar bone growth, periodontal ligament, and vasculature.

Pulp-based theories state that the propulsive forces necessary to move teeth originate in the dental pulp. Presumably, forces are produced by the generation of higher pressure below the tooth and lower pressure above by extrusion of pulpal tissues during dentin growth, interstitial pulp growth, or hydraulic pressure provided by the pulp vasculature (Sicher 1942; Bryer 1957; Eccles 1961). The vascular theory of
eruption was proposed by Constant (1900). The concept is similar to the pulp theory in that hydraulic pressure within or beneath the tooth provides the force necessary for eruption. These theories were supported by the observation that the average tissue pressure was higher in the pulp of erupting dog teeth than in tissues above those teeth (Van Hassel and McMinn 1972). Despite their careful measurements, Van Hassel and McMinn failed to compare these findings with pressure measured in and around nonerupting teeth, which may also possess higher pulp pressure. In addition, teeth erupt when pulp is surgically removed (Herzberg and Schour 1941). Furthermore, metal (pulpless) tooth replicas erupt in beagle dogs (Marks and Cahill 1984).

Alveolar bone growth-based theories stem from the work of Brash (1928), who determined that the mandible grows in height during prefunctional eruption and concluded that appositional bone growth beneath the erupting teeth raises the teeth to their functional site. Others have shown that significant amounts of bone form below erupting teeth (Cahill 1970; Cahill and Marks 1980). While bone apposition below erupting teeth may partially explain the process of eruption, these authors are cautious and point out that other factors are likely to be involved (see below).

Support for the theory that the periodontal ligament (PDL) produces the mechanism for tooth eruption comes from studies on the continuously erupting rat incisor (Berkovitz and Thomas 1969). Forces of between 5 and 7 grams were found necessary to impede incisor eruption in the rat. Additional evidence stems from root resection experiments in which removal of the proliferative growth zone of the incisor impeded eruption (Ness 1957). Moxham and Berkovitz (1974) continued these studies in transection experiments--known as root resection methods--in which the distal incisor segment continued to erupt after surgical separation from its proximal or growing end. Since the distal segment was connected to alveolar bone by the PDL alone, these experiments clearly indicated a role for the PDL in tooth eruption. These clever
experiments eliminated theories based on forces generated by the pulp, root growth, and alveolar bone growth. Some investigators have suggested that contractile forces generated by fibroblasts and/or collagen fibrils within the PDL account for these observations (Ten Cate 1985; Berkovitz et al. 1982).

Clinical observations have discounted the mechanical theories of eruption. For example, root growth theories are compromised by the observations that teeth rendered rootless by irradiation, congenital renal disease, or surgical interventions erupt in children (Carl and Wood 1980; Brin et al. 1985; Barfoed et al. 1984).

The development of the tooth offers some insights concerning the biological mechanism(s) of eruption. Teeth develop within bony crypts and are derived from the collaborative interaction of epithelial and mesenchymal cells of the first brachial arch (Marks et al. 1994; Slavkin 1990; Thesleff et al. 1991). As teeth develop, the surrounding mesenchyme condenses to form bone of the mandible and maxilla. This formation of alveolar bone depends upon the timely development of the dentition, since failure of tooth formation impedes formation of alveolar bone (Landsberger 1924; Brash 1928). The bony crypts of developing teeth are connected to the gingiva by an epithelial strand known as the gubernaculum, the remnant of the original epithelial invagination from which the teeth developed (Mallassez 1885). In many mammals, the gubernaculum narrows to form a stalk-like structure which passes through a gubernacular foramen in the jaw. In other mammals the gubernaculum remains broad, and the bony crypts of developing teeth are connected to the oral epithelium by a trough or groove (Park 1973). In any case, the developing tooth is surrounded by alveolar bone. Therefore, in order to erupt, the bone between the tooth and the oral cavity must be removed.
A new concept of tooth eruption

These observations led to another theory of tooth eruption, which asserts that localized bone resorption above the tooth produces an eruption pathway from which the tooth emerges (Cahill and Marks 1980). Several lines of evidence support this concept. First, the radiographic and histologic hallmark of bone resorption during tooth eruption is the timely formation of an eruption pathway (Marks et al. 1993). Second, teeth do not erupt when bone resorption is absent or reduced. This is perhaps best illustrated in the spectrum of aberrant tooth eruption observed among the mammalian osteopetroses, a metabolic bone disease caused by compromised osteoclastic bone resorption (see above). Teeth do not erupt in the most severe forms of the disease (e.g., tl, toothless rat), while absence of erupted incisors and delays and/or absence of erupted molars is observed in mutations with less severe reductions in bone resorption (mib, microphthalmia blanc, ja, incisors-absent rats) (Seifert et al. 1993). Earlier erupting teeth are most affected in these mild forms. Third, tooth eruption is restored in osteopetrotic animals with treatments that cure or improve the disease, such as stem cell replacement and administration of CSF-1, respectively (Iizuka et al. 1992). Daily injections of this hemopoietic growth factor cause an increase in osteoclast number and dramatically improve the skeletal manifestations of the disease in tl rats. However, timing is critical in these cases since delays in treatment compromise eruption of particular teeth. Both incisors and molars erupt in individuals treated from birth with CSF-1, but delaying treatment by only one day inhibits incisor eruption (Iizuka et al. 1992). Fourth, tooth eruption is blocked by surgical removal of the dental follicle, which prevents bone resorption and formation of an eruption pathway (Marks and Cahill 1987; Cahill and Marks 1980). Fifth, local delivery of bafilomycin A₁, an antagonist of osteoclastic proton pumps, results in a temporary inhibition of tooth eruption by blocking bone
resorption (Sundquist and Marks 1994). These data also support the recent hypothesis that the dental follicle is needed for the bone resorption of tooth eruption.

Scanning electron microscopy of mineralized surfaces of the bony crypts surrounding erupting teeth demonstrates that the crypt consists of three different topographical regions: an upper area characteristic of bone resorption, a lower region characteristic of forming bone, and an intermediate or transitional zone typical of resting bone undergoing neither resorption nor formation (Marks et al. 1994). Light and electron microscopy of these surfaces reveals that they are covered with the appropriate cellular correlates which include numerous active osteoclasts and osteoblasts on upper and lower crypt surfaces, respectively (Marks et al. 1983; Marks and Cahill 1986).

Surgical experiments and clinical observations provided the first evidence that formation of an eruption pathway requires only the dental follicle, the loose connective tissue investment of the tooth. Cahill (1969, 1974) demonstrated that an eruption pathway is produced by osteoclastic bone resorption even when tooth movement--and its resultant pressure on bone--is blocked by transmandibular wires. Indeed, eruption pathway formation is independent of the tooth, since metal replicas of dog premolars erupt on schedule, provided they are surgically placed within the corresponding dental follicle (Marks and Cahill 1984). Surgical ablation of the follicle impedes eruption in dogs and clinical procedures which result in damage to the follicle block eruption in children (Cahill and Marks 1980; Barfoed et al. 1984). Additional surgical interventions by Marks and Cahill (1987) demonstrated that the coronal portion of the follicle is required for bone resorption and formation of an eruption pathway above the tooth, while the basal follicle is needed for bone formation below the tooth. These observations imply that the dental follicle coordinates events at opposite ends of the
tooth, namely osteoclastic bone resorption on adjacent coronal crypt surfaces and osteoblastic bone formation on adjacent basal crypt surfaces.

The mechanisms by which the follicle regulates physiologic processes on opposite ends of the tooth are currently being examined. Tooth eruption is preceded by a cellular infiltration of mononuclear cells into the dental follicle in rats and dogs (Wise and Fan 1989; Marks and Grolman 1987). It is generally believed that these cells are progenitors to the osteoclasts responsible for the bone resorption of tooth eruption, since they have ultrastructural and histochemical features of preosteoclasts and their arrival in the follicle precedes or coincides with the appearance of osteoclasts on adjacent alveolar bone surfaces (Marks et al. 1983; Marks and Grolman 1987; Wise and Fan 1989).

Biochemical analyses of the dental follicle of canine permanent premolars have provided additional evidence for the role of the dental follicle in tooth eruption. The dog premolar dental follicle is a highly hydrated structure that reaches its greatest mass at the onset of prefunctional eruption. During eruption, collagen and proteoglycan content increase 250% and 45%, respectively (Gorski et al. 1988a, b). These authors identified more than 20 proteins in the follicle prior to eruption, including a sialoprotein of 95,000 relative molecular weight (DF-95). It is interesting to note that DF-95 content is reduced at the onset of eruption, when three new, small molecular weight sialoproteins appear. Since the relative abundance of the three new sialoproteins present at the onset of eruption coincides with the total content of DF-95 prior to eruption, the authors concluded that fragmentation of DF-95 is a marker for the onset of eruption. Additional studies have shown that the dental follicle contains collagenolytic activity (Woessner and Cahill 1974) and the amount of metalloproteinases (collagenase and stromelysin) is reduced during eruption (Gorski and Marks 1992), suggesting a role for these enzymes in tooth eruption. Recently, Gorski and others (1994)
demonstrated that DF-9S is localized to the reduced enamel epithelium, providing biochemical evidence that this derivative of the oral epithelium is involved in eruption. Numerous proteases have been identified in the enamel organ during tooth development (Nanci et al. 1989, Smith et al. 1989; Den Besten et al. 1989), and their activation at the completion of crown formation may contribute to the eruption process by fragmenting DF-95 and initiating the release of metalloproteinases from the follicle.

The molecular regulation of tooth eruption remains a mystery. Cohen (1962) demonstrated that injections of epidermal growth factor (EGF) cause precocious eruption of incisors in rodents. Recently, EGF and EGF-receptor were localized to cells of the dental follicle and enamel organ early in erupting rat molars (Wise et al. 1992). In addition, cells of the dental follicle produce mRNA for CSF-1 and secrete the protein in culture (Wise et al. 1994). These data, the observation that EGF upregulates the production of CSF-1 message and protein (Abboud 1992), and the aforementioned data concerning the effects of CSF-1 on osteoclastogenesis suggest that both of these growth factors are involved in the molecular cascade which produces the bone resorption of tooth eruption (Figure 3). The precise timing of these cellular and molecular events, together with the necessity of bone resorption in tooth eruption, provide a unique opportunity for studying osteoclast ontogeny, tooth eruption, and bone resorption in general.
Part F:
Future Directions

As in most fields of biological research, bone cell biologists are confronted with the problem of how to study the complex local regulation of skeletal metabolism. Skeletal metabolism consists of the production and mineralization of a complex matrix by one group of cells and removal of the mineralized matrix by another. These processes are all timed and coordinated so that, in skeletal development, matrix formation and mineralization exceed resorption to produce a functional skeleton; later these processes are coordinated and balanced to maintain skeletal mass. These successive periods require a precise coordination of the development and function of several different cell populations, and may involve different mechanisms for each. In a sincere scientific effort to limit the known and unknown variables operating in vivo, bone cell biologists have used an ever increasing variety of cultured bone cell and tissue preparations to study skeletal development and regulation (Marks and Popoff 1988). While in vitro approaches have provided reliable observations in some instances, they have been almost universally deficient in demonstrating the mechanisms for precise, local regulation of skeletal metabolism. This is in part due to the redundancy of such controls, lessons which are emerging from the initially disappointing but surprising results from specific gene knockouts (Erickson 1993). On another level in vitro observations suffer from the varying inability of culture conditions to duplicate those in vivo.

Specifically, in the recent past, studies of macrophages as osteoclast surrogates (Kahn et al. 1978) and cloned osteosarcoma cells as osteoblast surrogates (Majeska and Rodan 1982) have been disappointing at best and erroneous at worst (Hattersley and Chambers 1989; Grigoriadis et al. 1985). Similarly, studies of the effects of the growth factor, colony-stimulating factor-1 (CSF-1), on cultured osteoclasts have been
contradictory (Tanaka et al. 1993; Shinar et al. 1990). In addition, the use of bone organ cultures in the initial report of the skeletal effects of the prostaglandins produced limited, erroneous information about their true effect in vivo (Marks and Miller 1993). Because organ cultures of bone invariably resorb, the initial report described resorption as the major effect (Klein and Raisz 1970), and it has taken more than two decades to reverse this view, and establish enhanced bone formation (Ueda et al. 1980; Mori et al. 1990; Miller and Marks 1993) as the true effect in vivo. Finally, no current methods for the isolation and culture of specific bone cells can produce pure populations, and contaminants are seldom characterized with precision (Chatterjee et al. 1992). At best, these methods permit evaluation of either bone formation or resorption in presumed isolation, a phenomenon rarely encountered in vivo.

The relative simplicity of all in vitro methods to study bone cell biology will at best result in oversimplification of the developmental and functional capacity of the skeleton in vivo. We have shown this to be the case for selected aspects of bone cell biology, but numerous other examples are available.

One alternative is to undertake skeletal research in vivo. It is important that those in bone research be willing to move increasingly in this direction not only to understand the true complexities of skeletal versatility, but also to avoid repetition and perpetuation of erroneous or irrelevant conclusions which waste resources.

Toward this end, we have described two situations, osteopetrosis and tooth eruption, in which reproducible aberrations or local activations of bone resorption can be examined in vivo. This dissertation used these examples to explore aspects of osteoclast development and function.
Part G:

Manuscripts

Some of these topics have been described in the three publications below which I have co-authored.

1. Experimental studies of osteopetrosis in laboratory animals
2. The role of bone resorption in tooth eruption
3. Bone surface morphology reflects local skeletal metabolism
Experimental Studies of Osteopetrosis in Laboratory Animals

MARK F. SEIFERT, PH.D.,* STEVEN N. POPOFF, PH.D.,** MARY E. JACKSON, M.S.,† CAROLE A. MACKAY, B.S.,† MATTHEW CIELINSKI, B.S.,† AND SANDY C. MARKS, JR., D.D.S., PH.D.†

Osteopetrosis is a metabolic bone disease characterized by a systemic increase in skeletal mass. It results from a defect in the production or function of osteoclasts and is inherited in nine genetically distinct osteopetrotic animal mutations and man. Studies of these mutations have revealed that osteopetrosis is a complex, heterogeneous disorder in its expression, etiology, and response to treatment by bone marrow transplantation or by hormone/growth factor therapy. These animal mutations have been valuable tools for probing the pathogenesis and treatment of osteopetrosis, and information obtained from these studies has been used clinically for the treatment of humans with osteopetrosis. In addition, studies of these mutations have contributed significantly to understanding normal bone cell biology, including the origin of the osteoclast and the significance of colony-stimulating factor-1 in osteoclast development. The resistance of some of these mutations to cure by stem cell transplantation and hormone therapy, coupled with similar observations and experiences in the human condition, indicates that these animal mutations will continue to serve important roles in the development of alternative therapies to treat resistant forms of the disease. These studies are bound to improve the understanding of normal bone biology by providing additional insights into the regulation of osteoclasts by osteoblasts and their products or by other elements of the skeletal microenvironment.

Osteopetrosis in mammals is an inherited disorder of bone metabolism resulting from reduced osteoclast function and is characterized by a generalized, net accumulation of skeletal mass. Recent reviews of osteopetrosis have emphasized the skeletal parameters of the disease, and its heterogeneous expression has been explained on the basis of interrupted osteoclast function sometime within their complex life history. This review summarizes these parameters and concentrates on the implications of new information about cytokines and matrix factors in the pathogenesis and therapy of osteopetrosis. This information reinforces the remarkable heterogeneity among the mammalian osteopetroses with respect to pathogenetic mechanisms of expression of this disease. Recent studies show that deficiencies in osteoclast number and/or function can be caused by genetic defects in other cells in bone, including, but not necessarily limited to, osteoblasts. Thus, successful treatment of some of these mutations may require methodology more complex than presently imagined and, as a result, will provide new insights into bone biology.

The most commonly studied animal mutations and their distinguishing characteris-
SUMMARY OF OBSERVATIONS ON OSTEOCLASTS AND THE CURATIVE ABILITY OF BONE MARROW TRANSPLANTS IN THE INHERITED OSTEOPETROSSES

<table>
<thead>
<tr>
<th>Osteoclasts:</th>
<th>MOUSE</th>
<th>RAY</th>
<th>RABBIT</th>
<th>HUMAN JUVENILE</th>
<th>HUMAN ADULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruffled Border</td>
<td>+ - - + - + +</td>
<td>±</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Cured by Bone Marrow Transplants</td>
<td>+ + - - + + -</td>
<td>±</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Genetic Transplant</td>
<td>R R E R R R R R</td>
<td>R E R or D R or D</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIG. 1. Comparison of data regarding osteoclast biology and response to cure by bone marrow transplantation of animal and human osteopetroses. Increases or decreases relative to unaffected animals indicated by arrows, presence or absence by + or −, and recessive or dominant inheritance by R or D, unknown. Opposite parameters reported together indicate both have been observed.

SKELETAL MANIFESTATIONS

Tissue Similarities Produced by Diverse Aberrations in the Skeletal Microenvironment

At the tissue level, the skeletal manifestations of the osteopetroses are remarkably similar. Bone shape is abnormal. The ends of long bones are typically broad and cylindrical and lack the external modeling characteristic of normal bones. Internally, marrow cavity formation is either delayed or, as more often the case, absent. This produces the pathognomonic radiographic appearance of homogeneously dense bone. At birth, individual mutations cannot be distinguished from each other radiographically. Later, however, mutations whose skeletons become progressively less dense with age (i.e., ia, mib) can be distinguished from those whose skeletons change little in density with age (i.e., mi, tl). External modeling of long bones and the formation of marrow spaces require bone resorption, and thus, the tissue manifestations of osteopetrosis are the direct result of reduced bone resorption. Bone resorption has been measured in four mutations, and the results are shown in Figure 2. Resorption rates range from undetectable in tl rats7 to 70% of that in normal littermates of ia stock.18 Values for mi22 and oc mice5 lie in between. Reduced bone resorption is also responsible for a delay or fail-
ure of tooth eruption that occurs in all osteopetrotic mutants.\textsuperscript{20,23,53}

In contrast to the tissue level similarities noted above, there are differences in skeletal cells and matrix among the mutations. Osteoclast numbers per unit area of the skeleton are reduced in most mutations but increased in \textit{ia} rats and in tibial bone from \textit{oc} mice.\textsuperscript{17} In addition, the active site for bone resorption in osteoclasts, the ruffled border,\textsuperscript{31} is absent or poorly developed in most mutants.\textsuperscript{26} An exception is the \textit{op} mouse where extensive ruffled borders are seen on the few osteoclasts present in the skeleton.\textsuperscript{24} Regardless of the numbers and ultrastructure of osteoclasts, net osteoclast function is reduced in all mutations. Abnormalities in osteoblasts have been reported but have not been studied extensively. Reductions in osteoblasts per unit area of the skeleton in \textit{tl} rats\textsuperscript{74} and ultrastructural and functional evidence of degradative changes in osteoblasts from \textit{os} rabbits\textsuperscript{20,53} have been reported. Bone formation, evaluated by \textsuperscript{3}H-proline uptake is increased in the four osteopetrotic mutations in the mouse,\textsuperscript{35,36} but decreased in all other mutations in which it has been examined.\textsuperscript{28} Thus, it appears that in the mouse mutations, elevated bone formation complements reduced resorption to accentuate skeletal sclerosis. The reductions in osteoblast numbers and/or functional capacity in \textit{tl} rats and \textit{os} rabbits may be related to the reduction in bone resorption. The role of osteoblasts in regulating osteoclast function is increasingly appreciated.\textsuperscript{31,59} Thus, the failure of osteoblasts to produce local mediators of resorption could produce the reduced resorption seen in some osteopetrotic mutants.

Morphologic, physiologic, biochemical, and molecular evidence of aberrations in the skeletal microenvironment in osteopetrosis has appeared recently, providing additional support for a primary osteoblast defect in some osteopetrotic mutations. The persistence of an amorphous material between calcified cartilage and bone in metaphyseal trabeculae from \textit{oc} mice\textsuperscript{72} provides morphologic evidence of a matrix abnormality in this mutation. This amorphous material overlies the lamina limitans of calcified cartilage, and neither the material nor the lamina limitans can be resorbed by either mutant or normal osteoclasts.\textsuperscript{72,80}

Physiologic evidence for an abnormality in the skeletal microenvironment in osteopetrosis is the impaired production of colony-stimulating factor-1 (CSF-1, or macrophage colony-stimulating factor, M-CSF) by mutant bone,\textsuperscript{89} bone cells,\textsuperscript{7} and fibroblasts in \textit{op} mice.\textsuperscript{7,89,91} Further characterization of the CSF-1 defect\textsuperscript{91} has shown that a base pair insertion in the coding region for the gene results in synthesis of a truncated molecule without biologic activity. This, the first molecular breakthrough in osteopetrosis, suggests that the \textit{op} mouse phenotype, having few macrophages\textsuperscript{88} and osteoclasts, with the latter possessing well-developed ruffled borders,\textsuperscript{24} is caused by the absence of CSF-1. This condition is not lethal, and the postnatal skeletal sclerosis in \textit{op} mice improves with age.\textsuperscript{79} These data suggest that CSF-1 is needed for macrophage and osteoclast development, but not function. Treatment of young \textit{op} mice with CSF-1 (see below) restores bone resorption and cures the skeletal sclerosis.\textsuperscript{6} Recently, it has been shown that osteopetrosis and tooth eruption in \textit{tl} rats is improved but not cured by treatment with CSF-1.\textsuperscript{10,37} These different effects of CSF-1 treatment in \textit{op} mice and \textit{tl} rats suggest that use of this growth factor might stimulate bone resorption in other mutations.

Biochemical evidence of matrix aberrations in osteopetrosis consists of the observation that osteocalcin is reduced in calvarial bone from \textit{tl} and \textit{op} rats.\textsuperscript{15} Gene expression in \textit{ia}, \textit{tl}, and \textit{op} rats during development shows distinct differences among these mutations with respect to bone cell proliferation (histone H4) and extracellular matrix (collagen Type I, alkaline phosphatase, osteopontin, and osteocalcin) mRNA levels compared with normal littermates.\textsuperscript{75} These data raise questions about osteoblast competence in
some osteopetrotic mutations (i.e., \textit{op} and \textit{t}/
rats) and suggest that aberrations in matrix
synthesis or composition may contribute to
the reduced bone resorption in these muta-
tions.

The reviews above show that the common
tissue phenotype in osteopetrosis, skeletal
sclerosis with deformities in bone shape, is
accompanied by a host of differences in bone
cell function and matrix components.

CURE OF THE SKELETAL PHENOTYPE

THE VARIABLE SUCCESS OF STEM CELL
TRANSPLANTS AND HORMONE/GROWTH
FACTOR THERAPY

The first cellular breakthrough in osteope-
trosis was the demonstration by Walker\textsuperscript{82}
that parabiotic union of a \textit{mi} mouse and its
normal littermate cured the skeletal sclerosis
in the mutant. Further work by this imagina-
tive pioneer\textsuperscript{84} showed that temporary para-
biotic union for two weeks resulted in a cure
two months later and that the same effect
could be produced by a single transplant of
normal hemopoietic tissue (bone marrow
or spleen in the mouse) into \textit{mi} or \textit{gl}
mutants.\textsuperscript{85-87} This was soon confirmed by
others\textsuperscript{16} and also was shown to cure skeletal
sclerosis in the \textit{op}\textsuperscript{62} and \textit{ia}\textsuperscript{19} mutations in the
rat. The cellular bases of these results have
been pursued by fluorescence-activated cell
sorting to show that the cure is effected by
hemopoietic stem cells\textsuperscript{62} of the granulocyte-
monocyte lineage.\textsuperscript{65} This pioneering work
was directly responsible for the first success-
ful clinical treatments of children with osteo-
petrosis.\textsuperscript{1,4,11,76}

However, not all mutations are cured by
stem cell transplantation. This treatment
does not produce a cure in \textit{op}\textsuperscript{34} or \textit{oc}\textsuperscript{73} mice,
\textit{t}/
rats,\textsuperscript{21} or \textit{os} rabbits,\textsuperscript{54} and has not been suc-
cessful in several children.\textsuperscript{77} This implies that
in these mutations, the defect lies in cells
other than transplantable stem cells or in the
local microenvironment. One example is the
CSF-1 deficiency in the \textit{op} mouse.\textsuperscript{89,91}

The fact that bone resorption is reduced in
osteopetrosis prompted the use of pharaco-
logic doses of known promoters of resorption
in an attempt to overcome a suspected resis-
tance. This treatment is not new. Barnicot\textsuperscript{2}
noted that \textit{gl} mice thrived on amounts of
parathyroid extract that otherwise killed their
normal littermates by prolonged hypercalce-
mia. Resistance to the hypercalcemic effects
of parathyroid hormone (PTH) has also been
noted in \textit{mi}, \textit{oc},\textsuperscript{35} and \textit{op}\textsuperscript{29} mice, and \textit{ia}\textsuperscript{18} and
\textit{t/p}\textsuperscript{1} rats. Prolonged treatment with pharaco-
logic doses of PTH fails to cure osteopetrosis
in \textit{gl} mice,\textsuperscript{2} \textit{ia} rats,\textsuperscript{18} and \textit{os} rabbits.\textsuperscript{83}
The use of pharmacologic doses of 1,25-
dihydroxyvitamin D [1,25(OH)\textsubscript{2}D] to cure
osteopetrotic mutants has been equally frus-
trating.\textsuperscript{55,56} Serum levels of 1,25(OH)\textsubscript{2}D are
elevated in all mutations examined,\textsuperscript{92} and has
led to the suggestion that osteoclasts are resis-
tant to the effects of this hormone in these
animals.\textsuperscript{92} Administration of high doses of
1,25(OH)\textsubscript{2}D in the \textit{os} rabbit failed to increase
bone resorption\textsuperscript{56} despite increases in osteo-
clast number and acid hydrolase content.\textsuperscript{8}
Similarly, such treatment has met with vari-
able clinical success in children with osteope-
trosis.\textsuperscript{12-14}

The recent cure of osteopetrosis in the \textit{op}
mouse by injections of CSF-1 represents the
specific delivery of a substance known to be
deficient in this mutation.\textsuperscript{7,89,91} Whether the
use of this or other cytokines will be success-
ful in children will depend in large measure
on the cellular and biochemical parameters
of their disease. Children or other mutations
with low numbers of monocytes, macro-
phages, and osteoclasts will be good candi-
dates. The rationale for the use of CSF-1 will
be significantly improved if fibroblasts or
other cells from these individuals produce
low or undetectable levels of this growth fac-
tor. Therapeutic options for the treatment of
osteopetrosis have been enhanced by this
demonstration of the effectiveness of CSF-1.
However, the challenges of developing crite-
ria for specific treatments remain.

Pathogenetic and therapeutic characteris-
Studies of Osteopetrosis in Animals

Studies of various osteopetrotic mutations are summarized in Figure 3. Among the animal mutations, there are great differences in numbers of osteoclasts, presence of a ruffled border, and response to cure by transplants of stem cells. Many of these parameters are unknown for the human osteopetroses, and when known, they are variable. It is likely that the various interceptions of osteoclast function present in the animal mutations are also seen in the human conditions. However, the absence of an animal mutation with carbonic anhydrase II deficiency should preclude undue reliance on parallels between the two groups. Furthermore, criteria to distinguish between mutations cured and those not cured by stem cell transplantation and development of rational options for those not responding to such transplants, such as a combination of multiple treatments, remain important challenges to basic scientists and clinicians.

EXTRASKELETAL MANIFESTATIONS

A major extraskeletal manifestation in osteopetrotic animals is significantly elevated serum 1,25-dihydroxyvitamin D. Figure 3 shows that serum 1,25(OH)₂D is markedly elevated in the seven mutations examined to date compared with their normal littermates. Also note the variation in serum levels of normal animals among the various stocks, particularly the op rat. Age-related determinations of serum 1,25(OH)₂D levels have only been done in a few mutations. Serum levels in tl rats are already elevated one week after birth, peak almost four times the level in normal littermates at three weeks of age, and return toward normal levels by five weeks. Serum levels in op rats remain four times those in normal littermates four and six weeks after birth, whereas those in ia rats peak about three weeks after birth. These data in the presence of reduced bone resorption suggest a skeletal resistance to 1,25(OH)₂D. Whether other tissues are resistant to 1,25(OH)₂D remains to be shown. However, studies of vitamin D-dependent intestinal calcium binding protein (calbindin-D₂₅) in oc mice and tl rats show precocious development parallel to the elevations in serum 1,25(OH)₂D. There are few studies of renal function in osteopetrotic animals, but data of Nesbitt and colleagues show that renal 25-hydroxyvitamin D-1α-hydroxylase is elevated in oc mice and that this enzyme is suppressible with a high calcium diet, suggesting increased synthesis as a major cause of high-circulating 1,25(OH)₂D. These data together suggest that skeletal resistance to 1,25(OH)₂D and abnormal production of this vitamin D metabolite are characteristics of congenital osteopetrosis in animals. The relationship of the skeletal and endocrine manifestations of the disease clearly deserve more study.

Studies of immune function in osteopetrosis have produced conflicting results. Early studies in the op rat described premature thymic atrophy, reduced mitogenic responses of
splenic and thymic lymphocytes, normalization of these parameters after bone marrow transplantation from normal littermates, and cure of skeletal sclerosis after transplantation of normal thymic tissue.\textsuperscript{40,41,43} Reduced mitogen-induced lymphocyte proliferation has also been reported in spleen cell isolates from \textit{mi} and \textit{op} mice\textsuperscript{45,51} and \textit{op} rats.\textsuperscript{9} These data have led to the hypothesis that osteopetrosis is the skeletal result of a thymic disorder.\textsuperscript{40,41} However, thymic transplants fail to cure osteopetrosis in \textit{mi} mice\textsuperscript{16} and \textit{op} rats,\textsuperscript{50} and when the compensatory splenic hemopoiesis of osteoprotic mutants is taken into account by comparing the mitogenic responses of mononuclear cell isolates (lymphocytes) rather than whole spleen cell suspensions, no differences in mitogenesis are seen in \textit{op} and \textit{mi} mice\textsuperscript{64,90} and \textit{op} rats.\textsuperscript{5} Additional evidence for normal thymic function in osteopetrosis is the demonstration of a normal delayed-hypersensitivity response in \textit{ia} rats,\textsuperscript{60} the absence of qualitative and quantitative differences between mutants and normal littermates of \textit{mi} and \textit{ia} stock in the cellular composition of the thymus and thymus-dependent (T-cell) zones in lymph nodes and spleen,\textsuperscript{60,64} the absence of cytopenia in T-cell subsets from thymus and peripheral lymphoid organs of \textit{op} rats, and the increased proliferation of \textit{op} compared to normal thymocytes cultured in the presence of interleukin-1 and a co-mitogen.\textsuperscript{52} These data together with the observation that athymic rats are not osteoprotic,\textsuperscript{39} have been interpreted to mean that the thymus is not directly involved in bone resorption and that any thymic abnormalities are likely to be secondary to the skeletal or nutritional condition.\textsuperscript{5,50,64}

Aberrations in T-cell independent immune function in osteopetrosis are supported by the following: reduced macrophage oxidative metabolism and iodination in \textit{ia} rats;\textsuperscript{61} decreased fusion ability of \textit{mi} macrophages;\textsuperscript{79} impaired macrophage migration and chemotaxis in \textit{ia} rats\textsuperscript{69} and \textit{mi} mice;\textsuperscript{44} reduced natural killer (NK) cell activity coexisting with decreased (\textit{mi} mouse), normal (\textit{il} and \textit{op} rats), or increased (\textit{ia} rat) numbers of NK cells;\textsuperscript{52,67,68,78} and an interleukin-2 (IL-2) deficiency in \textit{ia} rats.\textsuperscript{66} In \textit{ia} rats and \textit{mi} mice, there are parallels between NK/macrophage and osteoclast numbers and function perhaps reflecting a common progenitor or common developmental influences. This relationship is supported by the ability of IL-2 infusions to restore NK cell function\textsuperscript{66} and elevate bone resorption\textsuperscript{63} in \textit{ia} rats. Although skeletal and immune dysfunctions coexist in osteoprotic animals, their relationships remain unclear. Whether they are causative (\textit{i.e.}, CSF-1 in \textit{op} mice or perhaps IL-2 in \textit{ia} rats) or coincidental will require additional work.

**THE CARE AND NURTURE OF OSTEOPETROTIC MUTANTS**

Any investigator using osteoprotic animals must maintain a breeding colony for each mutation to be studied and must consider the fragile condition of most mutants if the results of any study are to be reliable. Maintaining a breeding colony for osteoprotic mutations is greatly facilitated by providing an optimal environment. Periodic changes of clean cages, bedding, and water, circulation of clean, warm air, and protection of individual cages or groups of animals by air filtration or laminar flow rack systems are good investments for maintaining a healthy colony and for producing healthy offspring.\textsuperscript{27,48} Meticulous record keeping is required for monitoring breeding performance, output of mutant offspring, and dates of birth. Additionally, a test breeding program is necessary for most mutations (rats, rabbit) to identify heterozygote carriers that will serve to replace retired breeders. Reduced bone resorption interferes with tooth eruption in mutants,\textsuperscript{23,27} making the daily provision of a soft diet to these animals after weaning a requirement for maintaining their health. In addition, compromises in immune function of osteoprotic mutants can be easily produced by the stress of malnutrition or the failure to limit exposure of mutants to
Studies of Osteopetrosis in Animals

other strains or species of rodents. Body weights of all mutants will eventually lag behind those of normal littermates, and this may be seen in some mutations before weaning. After weaning, mutants should be provided with a wet, ground mash to ensure adequate nutrition. In addition, before mutants are included in the study, body weights should be checked for several days, and animals with decreasing body weight should be excluded. Mutants in poor health typically lose weight for two or more days before they die. These precautions safeguard against including malnourished or dying mutants in experimental studies.

THE CONTRIBUTIONS OF CONGENITAL OSTEOPETROSIS TO BONE CELL BIOLOGY

Studies of osteopetrotic mutations have made significant contributions to an understanding of the regulatory biology of bone cells, particularly the osteoclast. These include the origin of the osteoclast, the role of carbonic anhydrase II in bone resorption, and the significance of CSF-1 in osteoclast development. In addition, the resistance of several mutations to cure by stem cell transplantation and hormone therapy indicates that studies of these mutations are likely to provide additional insights into bone cell biology in the areas of regulation of osteoclasts by osteoblasts and their products, selective skeletal resistance to vitamin D, and control of bone modeling. These opportunities, recently reviewed, await exploration using osteopetrotic mutations.

PATHOGENETIC PATHWAYS AND THE CLINICAL SIGNIFICANCE OF CONGENITAL OSTEOPETROSIS IN ANIMALS

A summary of pathogenetic possibilities for osteopetrosis in animals is shown in Figure 4. This figure incorporates the data discussed above but is likely incomplete because the mechanisms of osteoclast inactivation are not known in several mutations. Osteoclasts (Fig. 4) are derived from mononuclear precursors that are themselves the products of proliferation of stem cells in hemopoietic tissues. These precursors migrate to the skeleton, where they develop the ability to make acid hydrolases and fuse with each other or with preexisting osteoclasts. Some of these steps in osteoclast ontogeny are regulated by CSF-1 and 1,25(OH)2D. Osteoclasts, in turn, need to be activated to resorb bone, a process that includes the production and localized release of acid hydrolases, the acidification of the extracellular space at the ruffled border, and cell membrane turnover (exocytosis and endocytosis). Bone resorption is inhibited by calcitonin and activated by PTH, 1,25(OH)2D, and other hormones and factors through osteoblasts and other cells in the local microenvironment. Some mutations (Fig. 3) have few osteoclasts, suggesting abnormalities in osteoclast development. In other mutations, the absence of ruffled borders indicates aberrations in osteoclast activation. In mutations cured by bone marrow transplantation (Fig. 3), stem cell incompetence is believed to be involved, whereas in those not cured by this procedure, the primary defect may involve an abnormal skeletal microen-
vironment that neither supports endogenous osteoclast function nor the development of transplanted normal osteoclast precursors (Figs. 3 and 4).

The clinical significance and rationale for studying osteopetrosis in laboratory animals include their relevance to understanding normal bone cell biology and human osteopetrosis. The former is summarized in the preceding section and will have wide clinical application in preventing bone loss and in the therapeutic management of metabolic bone diseases. The latter includes establishing criteria for mutations amenable to treatment by bone marrow transplantation and developing rational therapies for mutations not curable by transplantation. Fibrosis of marrow spaces is a feature common to animals and children not curable by bone marrow transplantation. These stromal differences may reflect changes in cells or growth factors in the local environment, and if measurable, a bone biopsy could provide sufficient material to indicate if transplantation will be successful. The rapid postnatal reduction in skeletal sclerosis and the normal lifespan of mib rats (Cielinski, unpublished data) implies that not all infants with osteopetrosis will need treatment. Further characterization of this rat mutation should reveal the pathogenetic mechanisms of this transient type of osteopetrosis.

Animal mutations not curable by stem cell transplantation (Fig. 3) could be used to develop alternative therapies. These might include transplantation and 1,25(OH)2D and/or cytokines/growth factors, with different sequences or timing for each mutation. The comparison of various features of these mutations, including their responses to multi-procedure therapy, could lead to a host of alternative therapies.

These data show the complicated pathogenetic pathways of osteopetrosis in laboratory animals and how this diversity can be exploited for the benefit of those who inherit osteopetrosis, those who endure a variety of other skeletal disorders, and those who seek to unlock the mysteries of normal skeletal biology.

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2. The Role of Bone Resorption in Tooth Eruption

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SUMMARY

Teeth develop within crypts in alveolar bone and this bone is resorbed by osteoclasts during tooth eruption. This activity is dependent upon the adjacent part of the dental follicle which accumulates early in eruption a population of mononuclear cells which have ultrastructural and cytochemical features of osteoclast precursors. Evidence that bone resorption plays a key role in tooth eruption includes a bone surface topography and cellular composition reflecting resorption, a delayed or absent eruption in conditions of reduced resorption and the local production of matrix metalloproteinases during eruption. Furthermore, eruption can be accelerated and retarded by substances which respectively increase or inhibit osteoclastic activity. Understanding the spatial and temporal contributions of alveolar bone resorption to tooth eruption will have clinical application in the pharmacological regulation of tooth eruption, tooth movement and the stabilization of retained deciduous teeth and of dental implants.

Key words - tooth eruption, bone resorption, osteoclast, monocyte, dental follicle, colony-stimulating factor-1, bafilomycin, osteopetrosis.

Teeth Develop Within Bone

The embryology of the mammalian dentition demonstrates that teeth develop from the collaborative interaction of epithelial and mesenchymal cells in the first branchial arch (Ruch, 1990; Slavkin, 1990; Thesleff et al., 1991). During tooth morphogenesis the surrounding mesenchyme condenses to form the bone of alveolar processes of the mandible and maxilla. It has been appreciated for almost a century that development of alveolar bone in a jaw segment depends on tooth formation in that same area.
Failure of tooth development precludes formation of adjacent alveolar bone. The dependence of teeth on alveolar bone support illustrates the interdependence of the dentition and its osseous support.

The bony crypts of developing teeth are connected to the oral epithelium by an epithelial strand, the gubernaculum, which is the persistent connection of the enamel organ to the oral ectoderm from which it arose (Mallassez, 1885). In some mammals this connection narrows to a cord-like structure and passes through a gubernacular foramen in the jaw (Fig. 1) while in others this connection remains broad and a trough or groove, rather than a foramen, connects the bony crypt of the developing tooth and the oral cavity (Park, 1973).

Thus, the contemporaneous development of a tooth and its adjacent alveolar bone results in the tooth's being surrounded by a bony crypt. For a tooth to erupt, the bone between the tooth and the oral cavity must be removed. This is the indispensable role of bone resorption during tooth eruption.

**Evidence that Tooth Eruption Requires Bone Resorption**

1. In the direction of tooth movement the topography of bone surfaces and the cellular investment of these surfaces show resorption and its cellular correlate, osteoclasts. By scanning electron microscopy the anorganic crypt surfaces around erupting teeth have three distinct phenotypes (Fig. 2); a scalloped surface in the direction of movement, an intervening smooth surface and a trabeculated area with a granulated surface on the opposite surface of the crypt (Marks, 1987). These are, respectively, the pathognomonic hallmarks of resorbing, resting and forming bone surfaces (Boyde and Hobdell, 1969). Furthermore, the scalloped surfaces of such a crypt are essentially a carpet of osteoclasts which have ultrastructural and cytochemical features of active cells (Marks, Cahill and Wise, 1983; Marks and Cahill, 1986; Marks and Grolman, 1987). The radiographic and histological hallmark of bone resorption during
tooth eruption is formation of a distinct eruption pathway (Cahill, 1969; Cahill and Marks, 1980; 1982).

2. Bone resorption and tooth eruption depend upon the dental follicle. Surgical removal of the dental follicle, a distinct connective tissue investment of the tooth, prevents tooth eruption (Cahill and Marks, 1980) and removal of that part of the follicle in the direction of eruption prevents tooth eruption by intercepting bone resorption and precluding the timely development of an eruption pathway (Marks and Cahill, 1987).

3. Teeth do not erupt when bone resorption is reduced or absent. The best illustration of this principle is the status of tooth eruption in congenital mammalian osteopetrosis, an inherited metabolic bone disease caused by variable reductions in bone resorption (Seifert et al., 1993). In this disease tooth eruption is either absent or delayed depending upon the extent to which bone resorption is reduced. In mutations with little or no resorption, such as toothless (tl) in the rat, no teeth erupt. In other mutations with less severe reductions in bone resorption such as incisors-absent (ja) or microphthalmia blanc (mib) in the rat incisors don't erupt or are delayed but some first molars and all second or third molars erupt (Cielinski and Marks, 1992; Schour et al., 1949). In all cases the status of bone resorption and the probability of eruption of a tooth can be ascertained by analysis of the surfaces of its crypt during the period of normal eruption (Fig. 3).

Regulation of the Bone Resorption of Tooth Eruption

The dental follicle (see above) is required for tooth eruption and appears to coordinate the resorption and formation of alveolar bone that occur on opposite sides of a tooth during its eruption (Cahill and Marks, 1980; Marks and Cahill, 1987). These activities could be produced by either the dental follicle or the reduced enamel epithelium because both of these structures were removed in these surgical experiments. However, the recent demonstration that teeth with only the enamel
epithelium fail to erupt suggests that regulation of tooth eruption is a property of the dental follicle (Larson et al., 1993).

The infiltration of mononuclear cells into the dental follicle coincidental to alveolar bone resorption and formation of the eruption pathway has been noted above. Recent biochemical analyses of the dental follicle have identified numerous proteins (Gorski et al., 1988). Some of these proteins change during eruption suggesting that they may play a role in the process. Prominent among them are the matrix metalloproteinases collagenase and stromelysin which can be found in the follicle prior to eruption but whose concentrations decrease dramatically as eruption proceeds (Gorski and Marks, 1992). Analysis of follicular extracts has also provided evidence for developmentally regulated proteolytic activity during tooth eruption (Gorski and Marks, 1992). The potential roles of these enzymes in cell migration, cell communication, matrix degradation and/or growth factor activation are currently being examined.

The bone resorption of tooth eruption can be accelerated by substances which promote the differentiation and/or activation of osteoclasts. A prime example of this is the premature eruption of incisors and molars in rats treated with colony-stimulating factor-1 (CSF-1) (Cielinski, Jolie and Marks, 1993 and in the Proceedings of this conference). This growth factor produces its effect by increasing the number and accelerating the appearance of monocytes in the dental follicle and of osteoclasts on crypt surfaces of the mandibular first molar. Wise and Lin (1994) have recently demonstrated that follicles from these teeth transcribe and translate CSF-1 mRNA and that interleukin 1_ and CSF-1 itself increase transcription of CSF-1 mRNA. These data taken together indicate that the dental follicle can be the source of CSF-1 and that activation of CSF-1 gene expression may be a marker of tooth eruption. The local bilaterally symmetrical regulation of CSF-1 gene expression in alveolar bone during
tooth eruption and how systemic administration of this growth factor causes its spatial and temporal effects in the jaws deserve further study.

In similar fashion, temporary inhibition of bone resorption can delay tooth eruption. Bone resorption by osteoclasts requires the participation of a unique proton pump on the ruffled border of these cells (Blair et al., 1989; Väänänen et al., 1990; Sundquist et al., 1990). Bafilomycin A$_1$ is a potent, specific inhibitor of vacuolar H$^+$-ATPases (Bowman, Siebers and Altendorf, 1988) and blocks resorption by irreversibly binding to one of its subunits (Rautiala, Koskinen and Väänänen, 1993). The action of bafilomycin is reversible upon its withdrawal because osteoclasts synthesize new, functional proton pumps (Lakkakorpi and Väänänen, personal communication). Local delivery of bafilomycin A to the dental follicle of erupting mandibular premolars in dogs suspends resorption during the period of infusion and temporarily prevents the formation of an eruption pathway (Sundquist and Marks, 1994 and Fig. 4).

Other proteins are known to influence tooth eruption but their mechanisms are unknown. Injections of epidermal growth factor (EGF) cause premature eruption of incisors and eyelid opening in mice (Cohen, 1962) but has no effect on molar eruption (Lin, Fan and Wise, 1992; Cielinski and Marks, 1992). Proteins of 167kDa and 200kDa isolated from the rat dental follicle retard eruption of incisors and molars in rats when injected subcutaneously in the perinatal period (Lin et al., 1992). Whether the action of these and other compounds capable of modulating tooth eruption work by influencing bone resorption or other mechanisms remains to be established.
Clinical Applications of the Biology of Tooth Eruption

Tooth eruption is a complicated process involving not only alveolar bone resorption but also alveolar bone formation and formation and renewal of the periodontal ligament and the epithelial attachment between tooth and gingiva. While knowledge of each component and its regulation is rudimentary at present, the clinical applications of current and future information will extend to most oral health disciplines. Knowledge of the local initiation and directionality of tooth eruption will lead to rational, predictable control of ectopic eruption and tooth movement. Permanent local inhibition of bone resorption will insure the retention of deciduous teeth without a permanent successor, will limit the growth of jaw cysts and may stabilize the progressive loss of bone around teeth and their replacements in the periodontal diseases. More significantly, the predictable formation of alveolar bone that occurs during tooth eruption (Fig. 2f,g) or in response to the local delivery of growth factors such as prostaglandin E₁ (Marks and Miller, 1993) can produce accelerated repair of facial fractures and integration of transplants or implants and provide predictable periodontal regeneration. Finally, the molecular mechanisms determining position and renewal of the junctional epithelium will provide ways to position and stabilize a healthy esthetic epithelial attachment on teeth and dental implants regardless of the position of the alveolar crest.

These examples illustrate the variety of clinical applications of current and future studies of the biology of tooth eruption.
FIGURE 1: Palatal view of the skull of a 5-year-old child showing the gubernacular foramina (arrows) of the permanent incisors. The foramina of the maxillary central incisors are larger than those of the lateral incisors because they are being enlarged as part of the eruption pathway of the permanent central incisors, which can be seen in their crypts. Ruler at bottom indicates mm.
FIGURE 2: Scanning electron micrographs of an anorganic preparation of crypt surfaces during eruption of the mandibular fourth permanent premolar in a 19-week-old dog. The medial surface of the crypt (Fig. 2A) lies above the mandibular canal (MC) and can be divided into three different regions indicated by number. The superior part of the crypt (labelled 1 in Fig. 2A,B,C and D) is scalloped and is the result of resorption by osteoclasts. The middle area (labelled 2 in Fig. 2A,D and E) is smooth, reflecting an inactive bone surface. The inferior part of the crypt (labelled 3 in Fig. 2A,E) is trabeculated and consists of newly formed bone with surface depressions of osteocytes (arrowheads in Fig. 2F and O in Fig. 2G). Bars = 200 µm in A thru F and 10 µm in G.
FIGURE 3: Scanning electron micrographs of first molar crypt surfaces in a 14-day-old normal rat (a) and its osteopetrotic (toothless, II) littermate (b). In normal rats at this age crypt surfaces are scalloped (arrows in a) reflecting the formation of an eruption pathway by osteoclasts as this tooth erupts. In osteopetrotic rats (b) first molars do not erupt and bone formation, not bone resorption, is seen on crypt surfaces. Bar = 200 μm.
FIGURE 4: Radiographs of the mandibular third (3) and fourth (4) permanent premolars and first molar (M) in two 20-week-old dogs. The one on the left (Fig. 4a) shows the usual progression of eruption of the premolars with formation of an eruption pathway (arrowhead) and root growth. The dog on the right received bafilomycin A₁, a potent inhibitor of bone resorption, delivered to the crypt of the fourth premolar for the previous two weeks by a cannulated osmotic minipump whose metal tip and retaining screws are clearly visible. Notice that in this dog the eruption pathway (arrowhead) still contains bone, tooth movement has not progressed and root growth on this tooth has invaded the mandibular canal (asterisk). Bar = 10 mm.
3. Bone Surface Morphology Reflects Local Skeletal Metabolism

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ABSTRACT

The metabolic activity of bone cells is faithfully reflected in the surface topography of mineralized bone surfaces and this can be easily detected by scanning electron microscopy (SEM). Forming bone surfaces exhibit knobby projections which represent foci of mineralization, resorbing surfaces are scalloped and resting surfaces undergoing neither activity are smooth as shown by Boyde and Hobdell 25 years ago. These phenomena are illustrated in vivo by tooth eruption, a local activity in alveolar bone where resorption and formation are polarized around an erupting tooth, and osteopetrosis, a metabolic bone disease characterized by a congenital reduction or absence of bone resorption. The ability to analyze bone metabolism over large areas of the skeleton by SEM offers a convenient and powerful microscopic technique to assess regional and global bone cell activity in an era where the investigative focus is increasingly molecular.
INTRODUCTION

Twenty-five years ago Boyde and Hobdell (1969) reported that mineralized bone surfaces exhibited three distinct topographies by scanning electron microscopy (SEM) and that these corresponded to different overlying cellular activities. Bone formation by osteoblasts consists of the production and mineralization of a complex extracellular matrix and forming bone surfaces seen by SEM exhibit knobby projections which represent foci of mineralization. Bone resorption by osteoclasts results in a confined, localized removal of mineral and matrix and resorbing bone surfaces seen by SEM are scalloped. In a growing skeleton most bone surfaces are undergoing formation or resorption and they will appear either knobby or scalloped by SEM. As the skeleton matures the extent of these activities decrease and a third, smooth bone surface phenotype becomes most prominent. Thus, a smooth bone surface indicates a region undergoing neither formation nor resorption and is covered by squamous bone living cells or flattened osteoblasts.

These seminal observations by Boyde and Hobdell (1969) have been confirmed and used by others to evaluate bone metabolism in a variety of situations in vivo and in vitro. The fidelity of bone surface topography to overlying metabolism and the ease of tissue preparation and analysis by SEM make this a convenient microscopic method for analysis of regional or systemic activities in the skeleton. The applications of this method will undoubtedly expand with the increasing molecularization of biology and the need to analyze the skeletal effects in transgenic animals and after gene deletions or knockouts. We review tissue preparation procedures for bone and present examples of the application of SEM to study local skeletal metabolism during tooth eruption and the generalized skeletal metabolic derangements in the congenital bone disease osteopetrosis.
Tissue Preparation for Analysis of Mineralized Surfaces by SEM

While much can be gained by analyzing the cellular investment of bone by SEM, analysis of metabolic activity as described by Boyde and Hobdell (1969) necessitates removal of all organic material because organic material covers all forming and quiescent bone surfaces making them indistinguishable without this step (Chow and Chambers, 1992). Bone specimens are fixed in 10% neutral-buffered formalin or 3% gluteraldehyde in 0.1M cacodylate buffer. Fixation protocols are not as rigorous as those for cell preservation because there is little inherent deterioration of mineralized surfaces. Caution should be used with phosphate-based buffers, however, because they tend to promote precipitation of crystals on mineralized surfaces. Fixed specimens are rendered anorganic by treatment with commercial bleach (5% NaOCl) for 2 to 12 hours, depending on the density of the organic coating. Specimens are subsequently washed extensively in distilled water to remove cell and organic matrix debris, dehydrated in a graded series of alcohols, air-dried, mounted and sputter-coated prior to examination in a scanning electron microscope.

Tooth Eruption - an example of discretely localized changes in bone metabolism

Tooth eruption is the process by which teeth that develop within alveolar bony crypts in each jaw move into their functional positions (see Gorski and Marks, 1992; Marks, 1987 for reviews). This process is illustrated radiographically in Figure 1 which shows the eruption of the third and fourth permanent mandibular premolars in dogs. Notice that as these teeth move upward, the overlying bone and roots of the deciduous precursors are resorbed and that below the erupting teeth roots elongate and bone is formed, most prominently between the roots of each tooth. Tooth eruption is accompanied, indeed accomplished, by bone resorption and bone formation on opposite
sides of the erupting tooth. This is illustrated in Figures 2 and 3 which are of anorganic preparations of mineralized crypt surfaces from a dog premolar midway through eruption. At low magnification (Fig. 2) one can see three different topographies (numbered in Fig. 2) on the surface of each crypt during eruption. The superior surface of the crypt (1 in Fig. 2 and 3A,B) is scalloped, indicative of resorption. A thin strip in the middle (2 in Fig. 2 and 3C,D) is smooth and is quiescent, undergoing neither resorption nor formation. The inferior part of the crypt is trabeculated and has a knobby surface (3 in Fig. 2 and 3E,F) indicative of bone formation. The cells in these regions of the crypt have been shown to have the appropriate ultrastructural and histochemical features for bone resorption, maturation and formation, respectively (Marks et al., 1983; Marks and Cahill, 1986; Marks and Grolman, 1987; Wise et al., 1985). Thus, bone surfaces during tooth eruption are elegant local examples of the principles demonstrated by Boyde and Hobdell (1969). The demonstration that eruption itself is dependent upon the regulation of cell migration and differentiation by the dental follicle (Cahill and Marks, 1980; Marks and Cahill, 1984; 1987) has led to recent attempts to characterize the molecular regulation of eruption (Marks et al., 1994; Wise et al., 1994).

Tooth eruption has been shown to be accelerated by systemic administration of the growth factor, colony-stimulating factor-1 (CSF-1), to neonatal rats (Cielinski et al., 1994) and to be retarded by local delivery of bafilomycin A, to crypts of dog premolars (Sundquist and Marks, 1994). Bafilomycin A1 is a specific inhibitor of the vacuolar H\textsuperscript{+} ATPase in osteoclasts (Bowman et al., 1988) and blocks resorption by binding to one of its subunits (Rantiala et al., 1993). The retardation of eruption by bafilomycin is accomplished by a temporary inhibition of bone resorption. This can be seen radiographically in Figure 4 where, on the experimental side, an eruption pathway has not formed by resorption and tooth root growth has invaded the mandibular canal in
the absence of eruption. The suspension of bone resorption is also evident on deciduous tooth root (Fig. 5A,B) and bone (Fig. 5C,D) surfaces in the direction of eruption. In control crypts both tooth root (Fig. 5A) and bone (Fig. 5C) surfaces show clearly the scalloped surfaces of resorption. In the bafilomycin-treated crypt (Fig. 5B and D) the scalloped surfaces of previous resorption can be distinguished but they have been made much less distinctive by two weeks of arrested resorption.

Osteopetrosis, an example of a generalized reduction in bone resorption

Congenital osteopetrosis in mammals is an inherited metabolic bone disease characterized by reduced bone resorption due to interceptions of osteoclast development or function (Marks 1984; 1987; Osier and Marks, 1992; Seifert et al., 1993). The disease is diagnosed radiographically and, as expected, skeletal effects are easily detected by SEM. Figure 6 is such an example. It demonstrates that the sites where bone resorption occurs in the normal skeleton (Fig. 6A,D) are replaced in mutants with resting or forming bone surfaces (Fig. 6B,E), distorting bone contours both externally and internally. Furthermore, if resorption is restored (Marks et al., 1992), as with CSF-1 in the toothless mutation, resorption pits appear in these sites as scalloped surfaces (Fig. 6C,F).

As shown above, tooth eruption is a bone resorption-dependent process and tooth eruption is delayed or absent in all osteopetrotic mutations. This can be detected by SEM of crypt surfaces as illustrated in Figure 7. Osteopetrotic mutants have crypt surfaces with features of bone formation (Fig. 7A) only. In affected animals of the toothless (II) mutation, CSF-1 injections restore bone resorption (Marks et al., 1992) and if begun at birth result in eruption of incisors and molars (Iizuka et al., 1992). Treatment of these mutants from birth with CSF-1 results in scalloped molar crypt surfaces in the direction of eruption (Fig. 7B) which are indistinguishable from those seen in normal littermates (not shown).
Conclusions

Scanning electron microscopy of mineralized surfaces can reveal the state of bone metabolism at that site as shown initially by Boyde and Hobdell (1969) 25 years ago. The use of this method of sample preparation and analysis since that time has provided significant insights on a variety of biological processes. Two illustrated here, tooth eruption and osteopetrosis, show that both local and systemic skeletal conditions and their regulation can be accurately analyzed at least qualitatively by SEM of mineralized surfaces.

This morphologic method can have wide application as the increasing molecularization of biology produces a plethora of transgenic and gene deletion strains in which the skeletal consequences can be rapidly and accurately assessed.
FIG. 1: Radiographs of erupting third (3) and fourth (4) permanent premolars in a young dog at 16, 18 and 23 weeks (Figs. 1A, B and C, respectively). Eruption of these teeth begins at 15 weeks when the first basal and interradicular bone (arrows) is evident radiographically between the crown and the mandibular canal (mc - Fig. 1A). As eruption proceeds (Fig. 1B) roots lengthen and alveolar bone resorption removes the roots of the overlying deciduous tooth (open arrow) and creates a radiolucent eruption pathway (arrows) most visible above the main cusp of these teeth. Immediately after eruption is complete (Fig. 1C) basal and supporting bone is consolidated around the roots (arrows). X 2.5
FIG. 2: Scanning electron micrograph of an anorganic preparation (see text) of the medial crypt surface of an erupting mandibular fourth permanent premolar in a 19-week-old dog. Three distinct regions are identified by surface topology and reveal the metabolic state of the bone at the time of preparation: a scalloped superior surface (1) indicative of bone resorption, a smooth middle area (2) reflecting a resting/inactive bone surface, and a trabeculated inferior portion consisting of newly formed bone. MC = mandibular canal, arrow = direction of tooth eruption. X 20
FIG. 3: Detailed SEM analysis of the crypt surfaces identified in Figure 2.

A,B: The superior surface (1) is scalloped (3A) due to resorption of bone by osteoclasts, which form an eruption pathway for the mandibular permanent fourth premolar. Individual resorption lacunae (R) are evident at higher magnification (3B).

C,D: The superior resorbing region (1) gives way to a smooth middle surface (2) indicative of resting/inactive bone (3C). The inferior surface (3) lies below the resting bone (3D) and consists of trabeculated, newly formed bone.

E,F: Higher magnification of the inferior bone-forming region reveals numerous osteocyte lacunae (arrows in 3E, O in 3F) on the trabeculated surface which is covered with knobby projections (open arrows, 3F) caused by new matrix mineralization. V = vascular canal

A,C and D = X 82; B = X 245; E = X 490; F = X 1630
Radiographs of erupting third (3) and fourth (4) mandibular permanent premolars in an untreated dog at 20 weeks (Fig. 4A) and an age-matched dog in which the fourth premolar was treated with $10^{-7}$ bafilomycin $A_1$ for the last two weeks (Fig. 4B) by a cannulated osmotic minipump. In the control dog (Fig. 4A) alveolar bone superior to the cusp of the fourth premolar (4) has been removed by osteoclastic bone resorption (arrowhead) and new bone has formed between the roots of the permanent premolar (arrowhead). Local delivery of bafilomycin $A_1$ (Fig. 4B) delays tooth eruption by inhibiting resorption of alveolar bone superior to the permanent premolar (arrowhead). Arrow in both figures shows new bone formation below the erupting fourth premolar. Root growth in impeded premolar (Fig. 4B) has invaded the mandibular canal (asterisk). M=first molar, X 2.2
Scanning electron micrographs of resorbing deciduous premolar (Fig. 5A,B) and adjacent bone (Fig. 5C,D) surfaces during tooth eruption in an untreated dog (Fig. 5A,C) and an age-matched dog in which the fourth permanent premolar was treated for two weeks with $10^{-7}$ bafilomycin A₁. In the control dog numerous resorption lacunae (R) are present on the surfaces of both tooth (Fig. 5A) and bone (Fig. 5C). Resorption lacunae (R) are also present on these surfaces in the bafilomycin-treated dog (Fig. 5B,D) but their depth and number are blunted by inhibition of osteoclastic bone resorption. Arrows = dentin tubules. X 490
Scanning electron micrographs of anterior tibial surfaces from 10 day old rats of \( \text{II} \) (osteopetrotic) stock. The characteristic shape of the tibia in the normal rat (Fig. 6A) is caused by resorption of bone by osteoclasts on the lateral surface (arrowhead) resulting in a flaring of the anterior end of the bone and a central tibial crest (double-ended arrow). High magnification (Fig. 6D) of the resorbing surface of the same bone shows scalloped surfaces (open arrows) and resorption lacunae (arrowheads) characteristic of active bone resorption. The tibia of the untreated mutant littermate (Fig. 6B) is club-shaped due to reduced bone resorption on the lateral surface (open arrowhead). High magnification (Fig. 6E) reveals that this surface lacks the scalloping characteristic of resorption, and consists of areas of inactive bone (asterisks) and forming regions with osteocyte lacunae (arrows). The tibia of a mutant rat treated daily from birth with \( 10^6 \) U CSF-1 (Fig. 6C) has a shape intermediate to those of the normal littermate (Fig. 6A) and its untreated littermate (Fig. 6B) due to resorption of the lateral surface (arrowhead). High magnification of the lateral surface of the same bone (Fig. 6F) shows scalloped surfaces (open arrows) and osteoclast lacunae (arrowheads) similar to those seen in normal rats (Fig. 6D). NLM = normal littermate; II utx = II untreated; II + CSF-1 = II treated with CSF-1. A,B,C = X 15; D,E,F = X 100
Scanning electron micrographs of mandibular first molar crypts from 14-day-old toothless (osteopetrotic) rats untreated (Fig. 7A) and treated systemically with $10^6$ units colony-stimulating factor-1 (CSF-1) (Fig. 7B). Bone forming surfaces (F) are prominent in the untreated mutant (Fig. 7A) and have characteristic knobby projections (arrows). Treatment of toothless rats with CSF-1 from birth (Fig. 7B) promotes bone resorption by osteoclasts as evidenced by scalloped surfaces and osteoclast lacunae (large arrows). $R =$ resting bone. $X$ 150
CHAPTER II

SPECIFIC AIMS

SPECIFIC AIM 1: TO EXAMINE THE SKELETAL AND DENTAL EFFECTS OF THE OSTEOCLAST GROWTH FACTOR, COLONY-STIMULATING FACTOR-1 (CSF-1 OR M-CSF) ON THE COMPROMISED OSTEOCLAST DEVELOPMENT AND FUNCTION IN TOOTHLESS (OSTEOPETROTIC) RATS.

SPECIFIC AIM 2: TO CHARACTERIZE THE SKELETAL AND DENTAL EFFECTS OF OSTEOPETROSIS IN A NEW OSTEOPETROTIC MUTATION IN THE RAT, MICROPHTHALMIA BLANC (MIB), IN RELATION TO OSTEOCLAST DEVELOPMENT AND FUNCTION.
CHAPTER II

The following procedures and techniques were used to obtain the data for this dissertation. They are described below in alphabetical order.

Animals: care and breeding:

The \textit{mib} animals used in this study were the offspring of \textit{mib/mib} males and \textit{+/mib} females derived from breeding stock donated by Dr. René Moutier, Centre de Sélection et d'Elevage d'Animaux de Laboratoire, CNRS, Orléans, France. In June 1990, Dr. Moutier sent 6 \textit{+/?} animals (3 of each sex) from which the colony at the University of Massachusetts Medical School was derived. The animals were housed, maintained, and used in accordance with the \textit{NIH Guide for the Care and Use of Laboratory Animals}, 1985 and the guidelines of the University of Massachusetts Animal Advisory Committee. The colony was managed by Matthew Cielinski, who was responsible for breeding to perpetuate the colony, determining sex and phenotype of newborn animals, and other related duties. Breeding cages were arranged with one male \textit{mib/mib} and 2-3 females (\textit{+/mib}). Offspring of mated \textit{mib/mib} males and \textit{+/mib} females were either of normal phenotype (\textit{+/mib}) or mutant homozygotes (\textit{mib/mib}); therefore the normal animals used in this dissertation were heterozygous for the wildtype and \textit{mib} characteristics. This differs from studies concerning other animal mutations, in which tested breeders (\textit{+/op}, for example) typically have progeny consisting of three genotypes (\textit{+/op, +/- normals, and op/op mutants}). In these cases, normal animals are of either genotype, are indistinguishable from one another except by breeding, and are denoted \textit{+/?}. Normal females were typically bred up to 1 year, but occasionally produced viable litters up to 1.5 years, and \textit{mib} females were determined to be sterile. Mutant males were sexually viable up to 1 year, but typically useful up to 6 months. Therefore, spent males were
killed when litter production ceased and fresh mutant males provided to established breeding cages when necessary. Mutant rats were identified at birth by lack of retina pigment. All animals were weighed prior to use and their body weight recorded at regular intervals.

The 11 rats used to derive data for this thesis were offspring of tested breeders (+/11) perpetuated from stock originally donated by Dr. Carl Hansen of the Veterinary Resources Branch, Division of Research Services, N.I.H., Bethesda, MD, USA. All rats were bred, maintained, and used according to recommendations in the Guide for Care and Use of Laboratory Animals, prepared by the ILAR, NRC (DHEW Publication No. NIH 86-23-1985) and guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School. Carole MacKay was responsible for maintaining the colony, determining phenotype of offspring, and related tasks, and Matthew Cielinski assisted when necessary. Primary breeding cages contained 2-4 young untested females (+/?/) and test-proven males (+/11) were rotated between these cages to prove or test-out females, as normal animals are indistinguishable except by breeding. Secondary breeding cages contained 2 test-proven females (+/11), which were either bred to test-proven males (+/11) to produce progeny for experimental use, or used to test males of unknown genotype to perpetuate the colony. Homozygous toothless (11/11) rats were identified in litters at birth under hypothermic anesthesia (Schneider et al. 1979). As heterozygous (+/11) and homozygous (+/+ ) normal rats are indistinguishable except by breeding, the normal rats used in this dissertation were of either genotype.

Rats born to timed-pregnant CD rats (Charles River) were used for studies concerning the effects of EGF, CSF-1, and combinations of both growth factors on the eruption of incisors and molars.
Animals: treatment

Purified human recombinant CSF-1 was generously provided by Chiron Corporation (Ladner et al. 1987; Halenbeck et al. 1989). Unless otherwise noted, one million units of CSF-1 were injected subcutaneously daily into 11 rats, beginning at birth or varying times thereafter. For dose-response experiments, CSF-1 doses ranging from $10^2$ to $10^7$ units were given as above. Untreated or sham (phosphate-buffered saline)-injected normal and mutant littermates served as controls.

To monitor the effects of EGF on tooth eruption, EGF (Upstate Biotechnology Inc., receptor grade, purified from submaxillary gland of male mice) was injected daily, beginning at birth, into the dorsal neck of normal rats (0.1 μg/1.0g body weight, subcutaneously). At least 6 control/sham-treated rats (phosphate-buffered saline) and 6 EGF-treated rats were killed at each age examined.

Bone formation

To study the formation rate of alveolar bone and dentin, at least 4 animals of each genotype (+/mib, mib/mib) at 1, 7, 14, 21, and 28 days were given 5.0 μCi $^3$H-proline (New England Nuclear) per gram body weight and killed 6 hours later (Walker 1966, Marks 1969). Serial sections were cut from paraffin-embedded mandibles, dipped in Ilford-L4 emulsion, and alternate slides were placed in dry chambers for 1 or 2 week exposures, respectively. Exposed slides were developed in D-19 (Kodak) for 4 minutes, rinsed in tap water for 1 minute, fixed in 30% sodium thiolsulfate for 5 minutes, and rinsed in tap water. Some sections were then counterstained with hematoxylin and eosin.

To quantitate bone formation at least 5 mutant and 5 normal littermates of mib stock were injected intraparietally with 5.0 μCi $^3$H-proline (New England Nuclear) per gram body weight at birth and weekly intervals during the first postnatal month.
(Walker 1966, Marks 1969). Rats were killed 6 hrs later, and the calvariae were removed, cleaned of excess soft tissue, air dried, weighed, minced into a fine powder with scissors, and subjected to digestion with 1M HCl at 60°C overnight. Samples were then neutralized with an equivalent volume of 1M NaOH, mixed with 10mL scintillation fluid, and placed in a scintillation counter and values normalized to counts per minute per gram calvarial bone. Rate of bone formation at each age was expressed as values of mutants divided by values of normal littermates.

**Bone histochemistry**

Tibiae and femora were rapidly removed, bisected longitudinally, and fixed for 2 hr in 2.5% glutaraldehyde in 0.1M cacodylate buffer with 7% sucrose, demineralized in 10% EDTA, dehydrated in a graded series of ethanol, and embedded in resin for histochemical analyses of tartrate-resistant acid phosphatase (TRAP) and tartrate-resistant acid ATPase (TrATPase) as previously reported (Cole and Walters 1987, Lindunger et al. 1990). Controls included incubation without substrate or with 10 mM sodium orthovanadate, both of which were negative in all cases. In addition, sections of bone from normal rats of another stock known to be positive for TRAP and TrATPase were included in reactions as positive controls.

Mandibles were rapidly removed, cleaned of soft tissue, and processed described above.

**Histomorphometry**

For histomorphometric analyses, anaesthetized rats were killed at 1-2, 3, 5, 7, 10, 14, and 18 days after birth. Mandibles were then rapidly removed, transected anterior and posterior to the gubernacular trough of the first molar and immersion-fixed in 2.5% gluteraldehyde in 0.1M cacodylate buffer with 7% sucrose for 2.5 hours
at 4°C and demineralized in 10% EDTA. These blocks were then dehydrated in a graded series of ethanol, transected through the center of the first molar and embedded in resin for histochemical analyses of tartrate-resistant acid phosphatase (TRAP) as reported previously (Cole and Walters 1987).

The number of TRAP+ (TRAP+) mononuclear cells in the dental follicle and TRAP+ osteoclasts on adjacent alveolar bone surfaces were enumerated at 100X in transverse sections of the first molar using the software package Osteomeasure (Osteometrics, Inc., Atlanta, GA). A representative section and corresponding Osteomeasure field are shown in CHAPTER IV, Part C. Cell density was expressed as number per unit length of dental follicle (mononuclear cells) or alveolar bone surface (osteoclasts). The statistical significance of differences between groups at various ages was determined using a two-tailed student-test.

Northern analyses of osteoclast-specific messages

Total cellular RNA was isolated from long bones (tibiae and femora) by a modification of the Chirgwin procedure (Chirgwin et al. 1979) as described previously (Shalhoub et al. 1991). Briefly, long bones were isolated from at least 4 normal and mlb littermates, soft tissue removed, snap-frozen in liquid N2, and stored at -70°C until use. Bone samples were crushed into powder using a pre-cooled (liquid N2) Bessman tissue pulverizer (Fisher). Total RNA was extracted (5M guanidine thiocyanate with 2% sarcosyl and 72 mM β-mercaptoethanol) and recovered as a translucent pellet after centrifugation through a cesium chloride (5.7 M). This RNA pellet was resuspended (10mM Tris, pH 7.4 and 5 mM EDTA), precipitated with 2.5 volumes ethanol, partially dried under vacuum, and resuspended and precipitated a second time in TE buffer and 2.5 volumes ethanol, respectively. RNA was resuspended in ultrapure water (Sigma), quantitated by absorbence at 260 nm, and stored at -70°C. The quality of RNA was monitored with respect to representation of ribosomal RNA (28S...
and 18S) as an internal standard, and the intactness monitored by electrophoretic fractionation (1% agarose gels, 6.6% formaldehyde, ethidium bromide staining).

Between 10 and 15 μg of each RNA sample was then transferred by capillary action to Genescreen Plus hybridization transfer membranes (Dupont, NEN Research Products). Transferred RNA was cross-linked to the membrane by UV light exposure for 1.5-2.0 min and stored at 4 °C in sealed plastic bags.

Probes were labeled with [α-32P]dCTP by the random primer method (Freiberg and Vogelstein 1983) to a specific activity of at least 1 x 10⁹ dpm/μg DNA. Prehybridization and hybridization of membranes were performed in 50% formamide, 5X SSC, 10X Denhardt's solution (100X Denhardt's solution is 2% Ficoll, 2% polyvinylpyrolidone), 50 mM sodium phosphate (pH 6.5), 1% SDS, 50 μg/ml single stranded salmon sperm DNA (Sigma) for 24 hours at 42°C. Blots were washed 3 x 20 mins with 2X SSC, 0.1% SDS at 50°C, 2 x 20 mins with 1X SSC, 0.1% SDS at 50°C, and once with 0.1X SSC, 0.1% SDS at 65°C. The blots were then exposed to X-OMAT AR x-ray film (Eastman Kodak) using a Cronex Lightning Plus intensifying screen at -70°C. The resultant autoradiograms were quantitated by scanning laser densitometry (LKB 2400 GelScan XL) within the linear range of signals. All data obtained in this way was normalized with respect to 18S ribosomal RNA and expressed in densitometric units. Statistical significance was defined as differences with a probability less than 0.5% (two-tailed student-test).

**Radiography**

High-resolution radiographs were used for the following: 1. to identify newborn litters and their normal littersmates at birth under hypothermic anesthesia (Schneider et al. 1979); 2. to evaluate treatment of litters in anesthetized animals (6:1 Ketamine-Zylagene at 4.0 ml/kg intramuscularly); 3. to document and compare changes in the
amount of skeletal sclerosis and marrow cavity size in the hindlimbs and axial skeleton of mib rats and their normal littermates.

**Scanning electron microscopy**

Scanning electron microscopy of anorganic bone surfaces was used to evaluate local skeletal metabolism as previously described (Boyde and Hobdell 1969, Marks et al. 1994). To examine the bony crypt of first molars during eruption, the lingual cortical plate and underlying first molar were removed, fixed in 10% neutral-buffered formalin (NBF), and the exposed crypt was subjected to organic digestion by immersion in 2% sodium hypochloride. For evaluation of local bone metabolism in long bones in normal and mutant rats of mib stock, tibiae were rapidly removed, cleaned of excess soft tissue, and fixed overnight in 10% NBF. Fixed specimens were rendered anorganic by immersion in 2% sodium hypochloride. Anorganic crypt surfaces or tibiae were then washed in ddH₂O, dehydrated in a graded ethanol series, blotted onto paper towels, and mounted on aluminum stubs using silver paint as adhesive for sputtercoating and observation in an ETEC scanning electron microscope.

**Tooth eruption**

Tooth eruption was defined as the age at which the given tooth first pierced through the oral epithelium. For incisors, eruption was monitored by daily inspection beginning seven days after birth. Molar eruption was determined by palpation of anesthetized animals on alternate days (7:1 Ketamine-Zylagene at 0.4 ml/Kg, intramuscularly) beginning fourteen days after birth. Because EGF injections promote precocious incisor eruption (Cohen 1962), daily inspection for eruption began at 5 days in treated animals.

**Ultrastructural observations**

At birth and 7 weeks a minimum of 4 normal and 4 mib littermates were killed by decapitation and their tibiae and femora rapidly removed, cleaned of soft tissue,
transected longitudinally to facilitate penetration of fixative, and fixed in 4% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1.5 hrs at 4°C. Fixed specimens were rinsed in 0.1 M sodium cacodylate buffer, demineralized in 10% EDTA in 10 mM Tris buffer, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in epon. Semi-thin sections (1 μm) were stained with toluidine blue to study cytological details before cutting thin sections for transmission electron microscopy. These were collected on 200-mesh grids and stained with 8% uranyl acetate and 2% lead citrate.

Statistical Analyses:

All quantitative data were expressed as means and standard deviations of observations from at least 3 determinations. The statistical significance of differences between groups (mutant versus normal, treated versus untreated, etc.) was determined using a two-tailed student's t-test.
CHAPTER IV

RESULTS

SPECIFIC AIM 1

Part A:
The Effects of CSF-1 on Osteoclast Development and Function in Rats of Toothless (II, osteopetrotic) Stock

Rationale:
Toothless (II) is an osteopetrotic mutation in the rat characterized by few osteoclasts, undetectable bone resorption, and resistance to cure by stem cell replacement. Treatment of mutant II rats with daily systemic doses of 1×10⁶ units of CSF-1 from birth results in a dramatic skeletal improvement, as evidenced by the formation of marrow spaces and increased numbers of osteoclasts; however, metaphyseal sclerosis persists. The purpose of this study was to determine the range of doses of CSF-1 which are beneficial, and ascertain the cause of the persistent metaphyseal sclerosis following such treatments.

Summary of Results and Conclusions:
Within a range of 10³ to 2 × 10⁶ units, CSF-1 caused marrow cavities to form in the long bones of toothless rats and osteoclast numbers increase. However, osteoclast numbers remained below those seen in normal siblings and their function, as determined by histochemical staining for TRAP and TrATPase, was reduced compared to normal littermates. Despite increases in cell number and function, osteoclasts were absent from the subepiphyseal areas of long bones of treated mutant rats. Withdrawal of CSF-1 caused the return of skeletal sclerosis. These results indicate that regular treatments of mutants with exogenous CSF-1 are needed to maintain the beneficial effects of this
growth factor. The persistent sclerosis which is present after CSF-1 treatments is related to the failure to restore osteoclasts to the subepiphyseal regions of long bones, indicating that the cause of osteopetrosis in II rats is not merely a lack of circulating CSF-1.

**Publication:**

This study has been published and a reprint follows. Please refer to it for specific details.
The Skeletal Effects of Colony-Stimulating Factor-1 in Toothless (Osteopetrotic) Rats: persistent metaphyseal sclerosis and the failure to restore subepiphyseal osteoclasts

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ABSTRACT

Toothless, (I) one of four osteopetrotic mutations in the rat, is characterized by few osteoclasts, undetectable bone resorption and failure to be corrected by bone marrow transplantation. We recently reported that CSF-1 treatment improves these skeletal problems but that metaphyseal sclerosis persists. In the present study we demonstrate that optimal reduction of the skeletal sclerosis in I rats following CSF-1 treatment has lower and upper dosage thresholds and that skeletal sclerosis returns after CSF-1 withdrawal. Osteoclasts increase significantly in I rats after CSF-1 treatment but compared to untreated normal littermates histochemical staining for characteristic enzymes and osteoclast number are reduced and no osteoclasts appear in the subepiphyseal areas of long bones. These data are interpreted to mean that there are dosage limits to the beneficial skeletal effects of CSF-1, that persistent sclerosis is related to the failure to restore subepiphyseal osteoclasts, that osteoclast or progenitor populations may be deficient or differ in their responses to CSF-1, and that the defect in I rats is not merely lack of a circulating, biologically active form of CSF-1.

KEY WORDS: Colony-Stimulating Factor-1 (CSF-1), Macrophage Colony-Stimulating Factor (M-CSF), Osteopetrosis, Osteoclast, Tartrate-Resistant Acid Phosphatase (TRAP), Rat
INTRODUCTION

Osteopetrosis in mammals is a heterogeneous group of inherited metabolic bone disorders characterized by a generalized skeletal sclerosis, the result of reduced bone resorption (Osier and Marks 1992). The toothless (Il/Il) mutation in the rat exhibits the usual skeletal and dental manifestations, failure of both marrow cavity development and tooth eruption, and is characterized by severe reductions in osteoclasts, monocytes and macrophages (Cotton and Gaines 1974; Marks 1977; Seifert et al. 1988; Marks et al. 1992). Osteoclasts in Il/Il rats stain weakly for the characteristic enzymes tartrate-resistant acid phosphatase and tartrate-resistant acid ATPase (Ek-Rylander et al. 1989) and bone resorption is undetectable in vitro (Raisz et al. 1981).

The osteopetrosis (op/op) mutation in the mouse has numerous similarities with the toothless mutation in the rat including reductions in osteoclasts, macrophages and monocytes and failure to be cured by bone marrow transplantation (Marks and Lane 1976; Marks 1982; Wiktor-Jedrzejczak et al. 1982). The op/op mouse mutation has been shown to be associated with a total absence of colony-stimulating factor-1 (CSF-1, also known as M-CSF), to be located within the coding region of the CSF-1 gene (Yoshida et al. 1990; Wiktor-Jedrzejczak et al. 1990; Felix et al. 1990a) and to be cured by treatment with CSF-1 (Felix et al. 1990b; Kodama et al. 1991a; Wiktor-Jedrzejczak et al. 1991).

Recently we have shown that CSF-1 treatment of Il/Il rats has beneficial effects on the skeletal, dental and macrophage defects in this mutation but that significant skeletal sclerosis persists (Marks et al. 1992; Izuka et al. 1992). In the present study we describe the dose response to CSF-1, the effects of withdrawal of treatment and the effects of CSF-1 treatment on osteoclast number, location and enzyme content in Il/Il rats. These data suggest a differential sensitivity of osteoclast populations or...
progenitors to CSF-1 and a broader base than mere CSF-1 deficiency for the skeletal derangements in II/II rats.

MATERIALS AND METHODS

Source and Treatment of Animals

Rats were obtained from our breeding colonies at the University of Massachusetts Medical School. Mutants (II/II) were identified radiographically at birth (Schneider et al. 1979) by the failure of development of marrow cavities in long bones. Normal homozygotes (+/+) and heterozygotes (II/+) are distinguishable only by breeding, and normal littermates included both these genotypes. Animals were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985). Rats were treated with highly purified recombinant human CSF-1 (M-CSF; Ladner et al. 1987; Halenbeck et al. 1989) by subcutaneous injection at daily doses ranging from $10^2$ to $10^7$ units (Ralph et al. 1986), beginning at birth for up to eight weeks. Untreated or sham (phosphate buffered saline) injected normal and mutant littermates served as controls.

Evaluation of Treatment

Phosphate buffered saline (PBS)-injected (sham) and CSF-1 injected rats were observed daily for weight gain. Subsequently, anesthetized (6:1 Ketamine-Zylagone at 4.0 ml/Kg intramuscularly) animals were killed and evaluated using radiologic and histopathologic methods. High resolution radiographs were made of the hind limbs and axial skeleton (Schneider et al. 1979). Tibiae and femora were rapidly removed, bisected longitudinally and fixed for 2 hr in 2.5% glutaraldehyde in 0.1 M cacodylate
buffer with 7% sucrose, demineralized in 10% EDTA, dehydrated in a graded series of ethanol, and embedded in resin for histochemical analyses of tartrate-resistant acid phosphatase (TRAP) and tartrate-resistant acid ATPase (TrATPase) as previously reported (Cole and Walters, 1987; Lindunger et al., 1990). Controls included incubation without substrate or with 10 mM sodium orthovanadate, both of which were negative. Sections were evaluated by microscopic morphometry as previously described (Marks et al. 1984). Osteoclast profiles, identified as TRAP or TrATPase positive cells in sections without a counterstain, were counted in at least eight non-adjacent sections of tibial and femoral metaphysis from 26 animals using the software package Osteomeasure (Osteometrics, Inc., Atlanta, GA). The statistical significance of quantitative differences between groups of animals was determined using a two-tailed Student's t-test.

**RESULTS**

The skeletal effects of CSF-1 treatment of neonatal toothless (I/I) rats could be detected radiographically within four days and a prominent marrow cavity was present in one week (Fig. 1). However, as has been reported earlier, sclerotic metaphyseal bone persisted in long bones of treated mutants (Fig. 1B). This metaphyseal sclerosis remained in animals treated for six weeks (Fig. 2A,B) and was especially prominent at costochondral junctions (Fig. 3A,B). CSF-1 treatment for up to eight weeks did not reduce the persistent skeletal sclerosis in mutants.

The beneficial skeletal effects of CSF-1 treatment required repeated injections of the growth factor. With cessation of treatment metaphyseal sclerosis slowly returned (compare Fig. 2B and C). Again, this can be most easily appreciated at the costochondral junctions (Fig. 3B and C).
Treatment of neonatal mutants with various doses of CSF-1 showed that beneficial skeletal effects have upper and lower limits (Fig. 4). Daily doses of $10^2$ and $10^7$ units did not produce radiographic evidence of marrow cavity formation within one week (Fig. 4d, h). Between these doses (Fig. 4b, c, e, f, g) marrow cavity development could be detected radiographically.

To determine the cause of persistent metaphyseal sclerosis in CSF-1-treated $tl$ rats we compared the metaphyseal distribution of osteoclasts in these animals and in untreated normal and mutant littermates. We took advantage of the selective histochemical staining of osteoclasts by TRAP and TrATPase to examine their distribution in the proximal tibia and distal femur in sections without counterstains (Fig. 5). In untreated normal rats osteoclasts are seen in three regions of the metaphysis (Fig. 5A); immediately below the epiphyseal plate (the capillary invasion front), on the marrow cavity ends of metaphyseal trabeculae and in the periosteum between these two locations. In untreated mutants (Fig. 5B) osteoclasts were routinely absent in all of these locations. In CSF-1-treated $tl$ rats (Fig. 5C) osteoclasts were present on marrow cavity ends of trabeculae and in the adjacent periosteum. Osteoclasts could be detected in CSF-1-treated mutants within three days after treatment was begun. Examination of TRAP-stained osteoclasts at higher magnification showed more intense staining of osteoclasts in normal rats (Fig. 6A) than in treated mutant littermates (Fig. 6B). The distribution and staining intensity of osteoclasts for TRAP and TrATPase in treated mutants were similar at all doses. However, osteoclasts were absent in subepiphyseal regions of all CSF-1-treated mutants regardless of dose, and periosteal osteoclasts were located more toward the center of the tibia than in normal littermates. Because the number of osteoclasts varied, we counted osteoclast profiles per unit area of tibial metaphysis at several doses. These data are shown in Table 1.
CSF-1 treatment of newborn mutants for one week produced a significant increase in osteoclast numbers in the proximal tibial metaphysis in a dose-related manner but even at the highest dose osteoclast density was not restored to normal levels (Table 1). Because osteoclasts in treated mutants do not have the same distribution as in untreated normal littermates (Fig. 5A,C), we counted osteoclasts in areas where they were found in each group. By not including the osteoclast-free subepiphyseal region in the area analyzed in treated mutants our counts actually overestimated the osteoclastogenic response/unit area of the mutant tibia to different doses of CSF-1.

DISCUSSION

These data show that daily CSF-1 treatment of neonatal toothless rats is followed by the appearance of osteoclasts and bone resorption in the center of long bones within a week, that treatment must be continued to maintain these beneficial skeletal effects and that daily doses between $10^3$ and $2 \times 10^6$ units can produce these effects. However, in treated mutants osteoclasts were reduced in number and staining for TRAP and TrATPase, osteoclasts were absent in subepiphyseal and adjacent periosteal sites and metaphyseal length and sclerosis were greater than in normal littermates at all doses even after treatment for up to eight weeks.

The failure to restore subepiphyseal osteoclasts in 11 rats after CSF-1 treatment could result from: 1. a sub-optimal local CSF-1 production reflecting different regional sensitivities of osteoclast or progenitor populations to this cytokine via local regulation, or 2. aberrations in vascular patterns or endothelial cell signaling for mononuclear precursors of osteoclasts which are known to exit vascular pathways and enter the skeleton to form osteoclasts at this site, the capillary invasion front (Kimmel and Jee 1980), or 3. the failure of rh CSF-1 to exhibit the full spectrum of activities in the rat as the native molecule in this species. In the CSF-1 deficient osteopetrotic (op/op) mouse, there is substantial evidence that macrophage subpopulations are affected...
differently by the deficiency of this growth factor (Cecchini et al. 1992; Naito et al. 1991; Pollard et al. 1992; Wiktor-Jedrzejczak et al. 1992). Macrophages in the bone marrow and uterus of op/op mice are greatly reduced whereas those in the epidermis are unaffected by CSF-1 deficiency. Macrophages in liver and spleen are present in reduced numbers. Furthermore, changes in these populations with age in op/op mice suggest a differential dependency of local macrophage populations on CSF-1 for both differentiation and maintenance. The failure of CSF-1 to restore subepiphyseal osteoclasts in l/l rats suggests that there may be similar regional differences among osteoclasts or precursors in different skeletal sites. Osteoclasts in subepiphyseal sites have been shown to arise from fusion of thymidine-labeled mononuclear precursors which exit venous sinuses in this region (Kimmel and Jee 1980). Nothing is known about the local control for these events. However, CSF-1 is known to play a role in osteoclastogenesis (Hattersley et al. 1991; Kodama et al. 1991b; Corboz et al. 1992), osteoblasts are present at this site, are known to produce CSF-1 (Horowitz et al. 1989) and osteoblasts are reduced in number and function in l/l rats (Seifert and Pedigo 1992). Therefore, either aberrations in venous sinusoidal patterns or inadequate signals from local cells (endothelial or osteoblast) or species differences could prevent the appearance of osteoclasts at this site in l/l rats treated with rh CSF-1. These possibilities deserve further study.

It is interesting that osteopetrotic mutations cured by bone marrow transplantation show the first radiographic signs of cure in subepiphyseal sites and marrow cavity formation proceeds centripetally (Walker 1975). In CSF-1 treated l/l rats the first signs are seen centrally and marrow cavity formation proceeds centrifugally. The key to both these differences and the failure of CSF-1 treatment to
produce a normal skeleton may be the absence of subepiphyseal osteoclasts in treated jj/jj rats.

Both the op/op mutation in the mouse and the jj/jj mutation in the rat exhibit severely reduced numbers of monocytes, macrophages and osteoclasts, are resistant to cure by bone marrow transplantation and show improvement in the cellular and skeletal aberrations following injections of CSF-1. However, the following observations indicate that these mutations are produced by different mechanisms: 1. untreated op mice but not jj rats exhibit a gradual resolution of skeletal, hematopoietic and some macrophage deficiencies beginning at six weeks after birth (Marks and Lane, 1976; Marks 1977; Begg et al 1992; Wiktor-Jedrzejczak et al 1992) 2. CSF-1 treatment results in greater improvement in the cellular and skeletal defects in op/op mice than in jj/jj rats (Felix et al 1990 ab; Kodama et al 1991 a; Marks et al 1992; Wiktor -Jedrzejczak et al 1991; data in present study). This suggests that analyses of skeletal and hematopoietic cells in each mutation will continue to reveal different aspects of the biological effects of CSF-1.

Taken together with previous demonstrations of aberrations in osteoblast gene expression (Shalhoub et al. 1991) in untreated jj/jj rats, reduced TRAP and TrATPase staining in osteoclasts of CSF-1 treated jj rats (Iizuka et al. 1992), and the existence of CSF-1 in both secreted and cell- surface-bound forms (Baccarini and Stanley, 1990 Ralph and Sampson-Johannes, 1990) data presented in the present study indicate that the defect in this mutation is not merely lack of the secreted form of CSF-1 but may be another local cell population or its product.
Table 1. Number of osteoclast profiles in eight-day-old rats of toothless stock treated from birth with CSF-1

<table>
<thead>
<tr>
<th>Daily dose of CSF-1 (units)</th>
<th># Animals</th>
<th>Osteoclasts/mm² (± SEM)</th>
<th>Osteoclasts (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLM 0</td>
<td>6</td>
<td>78.0 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>tl 0</td>
<td>6</td>
<td>0.6 ± 0.3*</td>
<td>1</td>
</tr>
<tr>
<td>tl 10³</td>
<td>2</td>
<td>11.0 ± 3*</td>
<td>14</td>
</tr>
<tr>
<td>tl 5 x 10⁴</td>
<td>4</td>
<td>13.0 ± 3*</td>
<td>17</td>
</tr>
<tr>
<td>tl 10⁶</td>
<td>8</td>
<td>50.0 ± 4*</td>
<td>64</td>
</tr>
</tbody>
</table>

*At least eight nonadjacent sections of the proximal tibia, stained histochemically for TrATPase, examined per animal. NLM = normal littermate, tl = toothless mutant. *Significantly different from untreated mutants and normal littermates, p < 0.001.
Fig. 1. Radiographs of the hind limbs of one-week-old littermate rats: a normal rat (A), a mutant treated with 1 million units CSF-1 daily from birth (B) and an untreated mutant (C). Note the differences in density and extent of metaphyseal bone (arrows) in the untreated normal (A) and mutant (C). Marrow spaces have developed in the treated mutant (B), but metaphyseal sclerosis persists, most easily seen in the proximal tibia (arrow), distal femur and distal forelimb (asterisk). X 1.0
Fig. 2, 3. CSF-1 injections are required to maintain the skeletal improvement seen in [1] rats. Hindlimb (Fig. 2) and chest (Fig. 3) radiographs of six-week-old littermate rats, untreated normal (A), mutant continuously treated from birth with $10^6$ units/day (B), and mutant treated for the first three weeks and untreated for the last three weeks (C). Skeletal sclerosis returns in the metaphyseal areas of long bones and costochondral junctions of mutants after cessation of treatment (arrows, C). $X$ 0.9
Fig. 4. Radiographic evidence that the skeletal response to CSF-1 has upper and lower dosage limits. Radiographs of a 1-week-old normal (a), an untreated mutant (i), and mutants treated daily from birth for 1 week at the doses indicated (x10^6). Skeletal improvement was obvious only within the range of 10^3 to 2x10^6 units daily. X 1.4
Fig. 5, 6. Following CSF-1 treatment of 11 rats, subepiphyseal osteoclasts are not restored (fig. 5) and the intensity of staining of osteoclasts for tartrate-resistant acid phosphatase (TRAP) is less than that in normal littermates (fig. 6). Figure 5 shows the proximal tibial metaphysis from 1-week-old littermates stained for TRAP without counterstaining; a normal rat (A), an untreated mutant (B) and a mutant treated daily from birth with CSF-1 (10⁶ units/day). The epiphyseal plate (EP) is at the top of each field. In normal rats (fig. 5A) osteoclasts (arrows) stain heavily for TRAP and are located primarily in three regions; immediately below the epiphyseal plate, at the marrow cavity (M) ends of the metaphyseal trabeculae and in the periosteum (right margin of figure). In untreated mutants (fig. 5B) there are no osteoclasts in these areas and in CSF-1 treated mutants (fig. 5C) osteoclasts are seen only on the marrow cavity (M) ends of trabeculae (arrows) and periosteum (not shown). At higher magnification (fig. 6) osteoclasts in normal rats (arrows, 6A) stain more intensely for TRAP than those in CSF-1-treated mutants (arrows, 6B).

Fig. 5 X 80 : Fig. 6 X 350.
Part B:
The Effects of CSF-1 on Tooth Eruption in Toothless Rats

Rationale:
The toothless (tt) rat is a congenital osteopetrotic mutation characterized by a
generalized skeletal sclerosis from birth, few osteoclasts, and complete lack of erupted
teeth due to a severe reduction in osteoclastic bone resorption. Treatment of mutants
from birth with systemic administration of CSF-1 improves the skeletal manifestations
of the disease and promotes eruption of some teeth. The purpose of this study was to
investigate the effects of treating mutants with CSF-1 on the following: 1. the eruption
of all teeth; 2. the timing of treatments; 3. the morphology of developing teeth; and
4. the cytochemistry of osteoclasts around erupting molars.

Summary of results and conclusions:
Untreated tt rats had no erupted teeth and the roots of incisors and molars were
severely distorted by compression against bone. The incisor apex did not extend past the
first molar and continued growth of its apical end produced odontoma-like abnormalities
due to fusion of dentin and enamel matrices with bony matrix. Treatment of mutants
with CSF-1 from birth caused the eruption of all teeth, but this was delayed compared to
normal littermates. Delays in treatment compromised eruption in a time-dependent
manner, with earlier erupting teeth more severely affected than later ones.
Morphological analysis of erupting molars and incisors in treated tt rats revealed
normal root and crown development. Histological staining for TRAP and TrATPase was
reduced or negligible in osteoclasts of untreated tt rats, heavy in normal siblings, and
intermediate in treated tt rats. These data support the concept that bone resorption is a
necessary and rate-limiting component of tooth eruption and that complete failure of
eruption in untreated mutant rats is a consequence of reduced resorption caused by the
disease. Increased bone resorption--provided by CSF-1 injections--restores tooth eruption by increasing bone resorption, provided that treatment begins early after birth.

Publication:

This study has been published and a reprint follows. Please refer to it for specific details.
THE EFFECTS OF COLONY-STIMULATING FACTOR-1 ON TOOTH ERUPTION IN THE TOOTHLESS (OSTEOPETROTIC) RAT ILLUSTRATE THE CRITICAL PERIODS FOR BONE RESORPTION DURING TOOTH ERUPTION


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Short title: Tooth eruption in tl rats

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The toothless (tl) rat is an osteopetrotic mutation characterized by a generalized skeletal sclerosis, reduced bone resorption, few osteoclasts and a total absence of erupted teeth. This mutation is not cured by bone marrow transplants from normal littermates. We have previously shown that the skeletal defects in tl rats are greatly improved following treatment with colony-stimulating factor-1 (CSF-1). In this study, we investigated the effects of CSF-1 treatment on the development and eruption of the dentition of tl rats using radiography, histology and histochemistry. Untreated tl rats had no erupted teeth by 56 days after birth and the roots of incisors and molars were severely distorted by compression against bone. The apex of the mandibular incisor did not extend past the first molar and continued growth of its apical end produced odontoma-like masses consisting of distorted dentin and enamel matrices. In addition, few osteoclasts were seen on alveolar bone surfaces surrounding the teeth. Mutants treated with CSF-1 were characterized by delayed eruption of all molars and sometimes incisors. The incidence of incisor eruption was related inversely to the age at which CSF-1 treatment began. Molars of treated tl rats had well developed roots similar to those in normal rats. Treated mutants had numerous osteoclasts in alveolar bone and well developed hemopoietic marrow spaces in the mandible. Histochemical staining for both tartrate-resistant acid phosphatase and tartrate-resistant acid ATPase was reduced or negligible in osteoclasts of untreated tl rats, heavy in normal osteoclasts and of intermediate intensity in CSF-1-treated mutants. These data demonstrate that the complete failure of tooth eruption in tl rats is directly related to the reduced bone resorption characteristic of the disease and that an early (neonatal) increase in bone resorption, provided by injections of CSF-1, restores tooth eruption in tl rats.

KEY WORDS: colony-stimulation factor-1, osteopetrosis, tooth eruption
INTRODUCTION

Osteopetrosis is a metabolic bone disease in mammals characterized by a generalized skeletal sclerosis caused by reduced bone resorption. The toothless \( \text{\textit{t}l} \) rat, originally described by Cotton and Gaines (1974) as an osteopetrotic mutation completely lacking erupted teeth, possesses few osteoclasts that, when present, are small cells with little cytoplasmic vacuoles that stain weakly for tartrate-resistant acid phosphatases (Marks 1977; Seifert et al. 1988). The \( \text{\textit{t}l} \) rat is unable to elevate serum calcium levels in response to exogenous parathyroid extract (Marks 1977) and cultured \( \text{\textit{t}l} \) bone does not release previously incorporated calcium-45 in the presence of potent stimulators of bone resorption (Raisz et al. 1981). Thus, bone resorption in this mutation is extremely low or negligible. The pathogenesis of the disease is believed to be due to reduced bone resorption resulting from the severe reduction in the number of osteoclasts (Marks 1977; Seifert et al. 1988). Despite reduced medullary hemopoiesis, mutants are not anemic and typically survive one year if maintained on a powdered diet (Cotton and Gaines 1974; Marks 1977).

Toothless rats are also among the osteopetrotic mutations not cured by transplantation of bone marrow or spleen cells from normal littermates (Marks 1977; Seifert et al. 1988). Recently it was shown that \( \text{\textit{t}l} \) bone particles implanted subcutaneously into normal littermates do not differ from normal bone in respect to the recruitment of giant cells and subsequent bone removal (Anderson et al. 1986). In addition, using the metatarsal culture system (Burger et al. 1982), it has been demonstrated that \( \text{\textit{t}l} \) metatarsals co-cultured with normal liver or spleen cells failed to develop normal osteoclasts or to show evidence of bone resorption (Osier et al. 1987). These data suggest that the resistance of \( \text{\textit{t}l} \) rats to cure by bone marrow transplantation is due to neither the production of unresorbable bone nor to a bone matrix deficient in its ability to recruit giant cells, but that the mutant skeletal environment is unable to
support the development and differentiation of normal osteoclasts. This conclusion is supported by the recent demonstration of aberrations in bone matrix in \( \text{il} \) rats (Lian and Marks, 1990; Shalhoub et al. 1991).

Osteopetrotic (op/op) mice, which also have few osteoclasts, monocytes and peritoneal macrophages (Marks & Lane 1976; Marks 1982; Wiktor-Jedrzejczak et al. 1982) and cannot be cured by transplants from normal spleen or bone marrow cells (Marks et al. 1984), have been shown to have a defect in the production of colony-stimulating factor-1 (macrophage colony-stimulating factor, hereafter denoted CSF-1) (Yoshida et al. 1990; Wiktor-Jedrzejczak et al. 1990; Felix et al. 1990). Furthermore, injections of CSF-1 not only elicited osteoclastic bone resorption in op/op mice (Felix et al. 1990b; Wiktor-Jedrzejczak et al. 1991), but also produced partial eruption of molars (Kodama et al. 1991).

We have recently shown that CSF-1 treatment of \( \text{il} \) rats corrects some skeletal, macrophage and dental defects in this mutation (Marks et al. 1992). The present study describes in detail the effects of such treatment on the dentition of \( \text{il} \) rats.

MATERIALS AND METHODS

Animals

Animals used in this study were offspring of tested breeders (\( \text{il}/+ \)) derived from stock originally donated by Dr. Carl Hansen of the Veterinary Resources Branch, Division of Research Services, N.I.H., Bethesda, MD. All animals were bred, maintained and used according to recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the ILAR, NRC (DHEW Publication No. NIH 86-23-1985) and guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School. Homozygous toothless (\( \text{il/il} \)) rats (hereafter referred to as \( \text{il} \) rats) were identified in litters at birth by radiography using hypothermic anesthesia.
Since heterozygous (+/1) and homozygous (+/+), normal rats are indistinguishable except by breeding, normal rats (+/?) used in this study were of either genotype.

**CSF-1 Administration**

Purified human recombinant CSF-1 was generously provided by Cetus Corporation (Ladner et al. 1987; Halenbeck et al. 1989). One million units of CSF-1 was subcutaneously injected daily into 25 11 rats, beginning at birth or varying times thereafter. After treatment, animals were anesthetized (7:1 Ketamine-Zylagine at 0.4 ml/kg intramuscularly) and killed at 1, 2, 3, 4, 6 and 8 weeks after birth. Untreated normal (13) and mutant (4) littermates served as controls.

**Evaluation of tooth eruption**

Eruption of incisors was determined by visual inspection daily. To observe the eruption of the molars, animals were lightly anesthetized and inspected on alternate days. Mandibles and maxillae were rapidly removed, radiographed and cut in the sagittal plane for histology. Half of each specimen was then fixed in 10% neutral buffered formalin, radiographed, demineralized in 10% EDTA, dehydrated via a graded series of ethanol, and embedded in paraffin. Serial sections were stained with hematoxylin and eosin. The other half of each specimen was fixed for 2 hours in 2.5% glutaraldehyde in 0.1M cacodylate buffer with 7% sucrose, demineralized in 10% EDTA, dehydrated in a graded series of ethanol, and embedded in resin for histochemical analyses of tartrate-resistant acid phosphatase (TrAP) and tartrate-resistant acid ATPase as previously reported (Cole and Waters 1987; Lindunger et al. 1990).
RESULTS

The effects of CSF-1 injections on tooth eruption in \( \text{II} \) rats are shown in Figure 1 and summarized in Table 1. The incisors in normal rats of this stock erupt at 9 to 10 days after birth and the first, second and third molars erupt at 17 to 18, 20 to 22, and 36 to 40 days after birth, respectively. In contrast, neither incisors nor molars erupted by 56 days in untreated \( \text{II} \) rats. However, in \( \text{II} \) rats treated with CSF-1, all molars erupted, albeit delayed when compared to normal littermates. In addition, one or more incisors erupted in about half of the treated \( \text{II} \) rats, again delayed compared to normal littermates. The eruption of incisors in CSF-1-treated mutants was markedly affected by the onset of treatment (Table 2). CSF-1 treatment begun at birth caused eruption of all incisors. Delaying CSF-1 treatment by a day or more greatly compromised incisor eruption and delayed molar eruption (Table 1).

Radiography confirmed these observations (Fig. 2). In untreated \( \text{II} \) rats unerupted molars exhibited poor root development. Furthermore, the posterior extension of the apex of mandibular incisors in these animals was abnormal, failing to extend past the first molars. In contrast, CSF-1 treated \( \text{II} \) rats displayed normal root formation for molars, but the incisor apices did not extend posterior to the first molars.

Microscopic examination of the dentition in normal rats revealed the usual growth of roots and the alveolar crest (Fig. 3a, 4a, 5a, 6a). In untreated \( \text{II} \) rats, developing roots of molars were severely distorted by compression against irregular bone trabeculae (Fig. 3b, 4b, 5b, 6b) and alveolar bone covered the occlusal surfaces of developing tooth crowns (2b). Few osteoclasts were observed on alveolar bone surfaces surrounding the developing teeth. Furthermore, proliferation of dental Issues in the absence of socket enlargement produced distortions or surface contour and areas of ankylosis of tooth to bone (Fig. 4b). Posterior extension of the mandibular incisor appeared to be prevented by failure of bone resorption near the apical end (Fig. 5b).
Continued growth of the apical end of these incisors produced odontoma-like masses consisting of distorted dentin and enamel matrices (Fig. 6b).

In \( \text{\textit{i}} \) rats treated with CSF-1, molars had well developed roots (Figs. 3c, 4c) similar to those found in normal rats. The presence of numerous osteoclasts on alveolar bone trabeculae, enlarged hemopoietic marrow spaces in the mandible and formation of an eruption pathway were features seen in normal rats and only in mutants treated with CSF-1. Although molars emerged into the oral cavity and possessed well developed roots and periodontal ligaments in CSF-1-treated mutants, tooth surface irregularities were observed in the cervical portion of some first molars. Numerous osteoclasts were present on alveolar bone surrounding the apical end of the incisors and there was no ankylosis of tooth to bone (Fig. 5c, 6c).

Histochemical studies of tartrate-resistant acid phosphatase (TrAP) and tartrate-resistant ATPase (TrATPase) expression in osteoclasts on alveolar bone surfaces surrounding the erupting molars are shown in Figs. 7 and 8, respectively. In normal rats, numerous osteoclasts stained intensely for TrAP and TrATPase (Fig. 7a, 8a). In untreated \( \text{\textit{i}} \) rats the few osteoclasts present stained weakly for TrAP and were unreactive for TrATPase (Fig. 7b, 8b). In \( \text{\textit{i}} \) rats treated with CSF-1, numerous osteoclasts stained positively for both enzymes (Fig. 7c, 8c), but the intensity was less than that seen in normal littermates.

**DISCUSSION**

These data show that daily injections of CSF-1 have a beneficial effect on tooth eruption in the toothless (\( \text{\textit{i}} \)) mutation in the rat and that the age at which these injections are begun is a major determinant of whether particular teeth will erupt. The effects on tooth eruption are clearly supported by our radiographic, histologic, and cytochemical studies. All molars and incisors erupted in \( \text{\textit{i}} \) rats treated from birth with
CSF-1. While molars in untreated 11 rats were poorly developed, the molars in treated 11 rats were not encased in alveolar bone and had fully developed roots without ankylosis. In addition, two enzymes linked to osteoclast function (Minkin 1982; Ek-Rylander et al. 1989), namely tartrate-resistant acid phosphatase (TrAP) and tartrate-resistant acid ATPase (TrATPase), were greatly elevated in treated mutants. This evidence of osteoclast function coincides with tooth eruption in treated mutants and illustrates the dependence of tooth eruption on bone resorption (Marks 1976; 1981).

We observed that CSF-1 treatment had to begin at birth in order to have a predictable effect on eruption of the incisors. Delaying treatment for only one day greatly compromised incisor eruption (Table 2). These data are understandable when viewed within the emerging concepts of tooth eruption as a localized process within alveolar bone in which formation of an eruption pathway is produced by bone resorption (Cahill and Marks 1982; Marks 1986). Formation of this eruption pathway is controlled locally, is linked to the dental follicle and results in a window for tooth eruption which occurs at a characteristic time for each tooth (Cahill and Marks 1980; Marks 1987; Cahill et al. 1988; Marks et al. 1988; Gorski et al. 1988). Because incisors are the first teeth to erupt in rodents, formation of the eruption pathway for them must occur earlier than molars. Our studies suggest that signals for the development of osteoclasts must be provided at or very soon after birth for incisor eruption to occur. Otherwise, with delayed development of the eruption pathway, ankylosis occurs and eruption becomes impossible. A corollary is that later erupting teeth have later critical periods for formation of the eruption pathway. This is shown in Table 1 and the work of Kodama et al. (1991) who demonstrated that supplying CSF-1 to op mice at 11 days after birth severely retards eruption of the first molar so that the second molar erupts before it. This suggests that the critical period for formation of the eruption pathway for the first molar by CSF-1 injections occurs before 10 days.
The present study confirms our earlier report (Marks et al. 1992) that CSF-1 treatment increases the osteoclast population in 11 rats. In addition, we show that this treatment has beneficial effects on the ability of these cells to synthesize two enzymes linked to bone resorption. However, these histochemical studies show that restoration of the enzymatic capacity of 11 osteoclasts by CSF-1 treatment is not complete (Fig. 7 and 8a, c), as has been shown earlier with respect to osteoclast number (Marks et al. 1992). The basis for these discrepancies deserves further study.

Taken together these data show that daily injection of 11 rats with CSF-1 restores bone resorption and that the timing of these injections is critical for the eruption of different teeth.
TABLE 1: CHRONOLOGY OF TOOTH ERUPTION (DAYS) IN RATS OF TOOTHLESS STOCK

<table>
<thead>
<tr>
<th></th>
<th>Incisor</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
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<tr>
<td>NLM</td>
<td>9-10</td>
<td>17-18</td>
<td>20-22</td>
<td>36-40</td>
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<tr>
<td>ll/ll ntx</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ll/ll M-CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>start 0 or 1 day</td>
<td>14-16</td>
<td>20-22</td>
<td>22-29</td>
<td>43</td>
</tr>
<tr>
<td>3 day</td>
<td>-</td>
<td>25</td>
<td>30</td>
<td>56</td>
</tr>
<tr>
<td>11 day</td>
<td>-</td>
<td>-</td>
<td>31-44</td>
<td>-</td>
</tr>
</tbody>
</table>

- = No tooth eruption by 8 weeks
TABLE 2: EFFECT ON INCISOR ERUPTION OF DELAYING CSF-1 INJECTIONS IN \( \frac{11}{11} \) RATS

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of animals</th>
<th>0/4</th>
<th>1/4</th>
<th>2/4</th>
<th>3/4</th>
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<tbody>
<tr>
<td>NLM</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>( \frac{11}{11} ) untreated</td>
<td>4</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( \frac{11}{11} ) treated 0 day*</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1 day*</td>
<td>3</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 days*</td>
<td>6</td>
<td>84</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>3 days*</td>
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<td>100</td>
<td>0</td>
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</table>

* Age of first injection of CSF-1 (\( 10^6 \) units/day)

** Incisors erupt between 9-10 days after birth in normal rats of this stock.

Numbers under fractions represent the percentage of animals in which 0, 1, 2, 3 or all 4 incisors, maxillary and mandibular, were erupted by 17 days after birth.
Figure 1 - Photograph of incisor eruption in three 21-day-old littermate rats of toothless (null) stock. Incisors do not erupt in the untreated mutant (left) but are well erupted in the normal littermate (right). CSF-1 treatment from birth has caused a distinct but delayed eruption of the four incisors (arrow) in a mutant (center). bar = 10 mm.
Figure 2 - Radiographs of the maxilla and mandible from 24-day-old normal (2a), untreated mutant (2b) and CSF-1-treated mutant (2c) rats of 11 stock. Open arrows indicate the mandibular first molars and arrowheads the most posterior extension of the mandibular incisor roots of each rat.  bar = 10 mm.
Figure 3 - Photomicrographs of maxillary molars from 20 day-old rats of \( \text{II} \) stock. In untreated normal rats (3a) molars (1 and 2) are erupted and exhibit root elongation with open apices (asterisks). In untreated mutants (3b) molars form, do not erupt, are covered by alveolar bone (AB) and show areas of ankylosis (arrows). CSF-1 treatment of mutants from birth (3c) results in eruption of molars (1 and 2) in a manner indistinguishable from that in normal littermates (3a). \( \text{bar} = 0.25 \text{ mm} \).

Figure 4 - Higher power photomicrographs of the sections of root apices shown in figure 3. Note areas of extensive ankylosis (arrowheads) of root (R) dentin and alveolar bone (AB) in untreated mutants (4b) and absence of ankylosis in untreated normal (4a) and CSF-1-treated mutants (4c). \( \text{bar} = 0.1 \text{ mm} \).
Figure 5 - Photomicrographs of the apices of mandibular incisors from 20-24-day-old rats of 11 stock. In untreated normal rats (5a), the apex of the mandibular incisor extends beyond the third molar (M3) and has a well-developed pulp cavity (P) and dental epithelia distinctly separate from alveolar bone (AB). This region in untreated mutants (5b) is severely distorted with odontoma-like regions (asterisks) and areas of ankylosis (arrow). CSF-1 treatment of mutants from birth (5c) causes the apical region of the incisor to resemble that of normal littermates (5a) and prevents ankylosis and formation of odontomata. bar = 0.25 mm.

Figure 6 - Higher power photomicrographs of the apices of mandibular incisors shown in figure 5. In normal rats (6a) and CSF-1-treated mutants (6c) the apical region (A) is clearly separated from alveolar bone (AB) surfaces. In untreated mutants (6b), dentin (D) and enamel (E) matrices are fused with alveolar bone (AB), producing ankylosis and preventing eruption. bar = 0.1 mm.
Figures 7 & 8 - Photomicrographs of alveolar bone (AB) surfaces in 20-24-day-old rats of 11 stock stained for tartrate-resistant acid phosphatase (TrAP-Figure 7) and tartrate-resistant acid ATPase (TrATPase-Figure 8). Osteoclasts (arrows) in untreated normal rats stain heavily for both TrAP (7a) and TrATPase (8a). In untreated mutants osteoclasts stain weakly for TrAP (7b) and not at all for TrATPase (8b). After CSF-1-treatment from birth osteoclasts in mutants stain for both TrAP (7c) and TrATPase (8c) but the intensity for TrATPase (8c) is less than that in normal littermates (8a). bar = 0.05 mm.
Part C:
The Effects of CSF-1 on the Cellular Mediators of Bone Resorption During Tooth Eruption

Rationale:
CSF-1 promotes tooth eruption in toothless (I) rats, presumably by stimulating the bone resorption necessary for the formation of an eruption pathway during tooth eruption. Other studies have shown that CSF-1 promotes the development of osteoclasts from mononuclear precursors. Since the mononuclear cells appearing in the dental follicle early in eruption in dogs and rats have histochemical and ultrastructural features of preosteoclasts and their appearance coincides with the influx of osteoclasts on adjacent alveolar bone, we examined the cellular effects of CSF-1 administration on the eruption of mandibular first molars in mutant and normal rats of I stock with emphasis on the populations of osteoclasts and mononuclear cells around erupting teeth.

Summary of results and conclusions:
CSF-1 injections accelerated the eruption of first molars in normal rats and caused their eruption in toothless rats but this was delayed (2-4 days) compared to normal siblings. Eruption was accompanied by an influx of TRAP+ mononuclear cells in the dental follicle and TRAP+ osteoclasts on adjacent bone surfaces. CSF-1 treatment increased the number of these cell types in normal rats, but did not affect the timing of their appearance. In mutant rats, CSF-1 increased the numbers of these cell types, but the timing of their appearance was delayed compared to normal littermates. These data demonstrate that CSF-1 accelerates eruption in normal rats by increasing the number of osteoclasts and their precursors around erupting teeth. The delayed eruption of molars in treated I rats is identical to the length of the delayed appearance of these cell types during eruption. In addition, the presence of a few osteoclasts around erupting first
molars in untreated \#1\# rats, when they are very rarely seen throughout the remainder of the skeleton, indicates the strong influence of local factors in osteoclast recruitment and development during tooth eruption. These findings show that CSF-1 plays a role in the molecular events governing eruption and suggest that local administration of CSF-1 may be useful clinically in the movement and eruption of teeth.

**Publication:**

This study has been accepted for publication in: "The Biological Mechanisms of Tooth Eruption, Resorption, and Replacement by Implants", Z. Davidovitch, Editor, EBSCO Media, Birmingham, AL. and a reprint follows. Please refer to it for specific details.
Colony-Stimulating Factor-1 (CSF-1) is a Potent Stimulator of Tooth Eruption in the Rat

Accepted for publication in: The Biological Mechanisms of Tooth Eruption, Resorption, and Replacement by Implants, Z. Davidovitch, ed., EBSCO Media, Birmingham, AL (in press)

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Running title: CSF-1 stimulates tooth eruption

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SUMMARY

The congenital disease osteopetrosis is characterized by a systemic sclerosis due to absent or reduced bone resorption. The dental abnormalities caused by this disease, which range from lack of erupted teeth in severe mutations to delays in the eruption of incisors and/or molars in the milder mutations, provide unique opportunities for investigating the development and eruption of teeth.

Recently we have shown that systemic administration of colony-stimulating factor-1 (CSF-1 or M-CSF) to the osteopetrotic mutation toothless (tl) in the rat promotes tooth eruption, presumably by stimulating the bone resorption necessary for the formation of an eruption pathway. Other studies have shown that CSF-1 fosters the development of osteoclasts from their mononuclear precursors. Since the mononuclear cells which appear in the dental follicle early in eruption in dogs and rats possess histochemical and ultrastructural features of preosteoclasts and precede the influx of osteoclasts on adjacent alveolar bone, we decided to examine the effects of CSF-1 administration on the eruption of first molars in mutant and normal rats of the toothless stock with emphasis on the populations of osteoclasts and their precursors around the erupting tooth.

CSF-1 injections accelerated the eruption of first molars in normal rats and promoted eruption in toothless rats that was delayed (2-4 days) compared to normal littermates. These events were preceded by an influx of tartrate-resistant acid phosphatase-positive (TrAP+) mononuclear cells in the dental follicle early in eruption and TrAP+ osteoclasts on adjacent bone surfaces consistent with previous studies. Treatment with CSF-1 increased the number of these cell types in normal rats but did not affect the timing of their appearance. In mutant rats CSF-1 increased the numbers
of these cell types and the timing of their appearance was delayed in comparison with normal littermates. We believe that CSF-1 accelerates eruption in normal rats by increasing the number of TrAP+ osteoclasts and their precursors and promotes delayed eruption in CSF-1-treated toothless rats due to delays in the appearance of TrAP+ osteoclasts and their precursors. These findings indicate that CSF-1 plays a role in the molecular events regulating tooth eruption and suggest that local administration of CSF-1 may be useful in the movement and eruption of teeth clinically.

Key Words: Tooth eruption, colony-stimulating factor, bone resorption, osteoclast, dental follicle, monocyte, rat

Abbreviations: colony-stimulating factor-1 (CSF-1 or M-CSF); toothless (tl); tartrate-resistant acid phosphatase (TrAP)
INTRODUCTION

Teeth develop within bony crypts and, in order to assume their functional role in the oral cavity, must be translocated through alveolar bone by the process known as tooth eruption (Marks, 1987 and these proceedings). Although the mechanism(s) governing this process remain a matter of debate, it is becoming increasingly apparent that the dental follicle plays a central role in mediating tooth eruption. This was shown by Marks and Cahill (1980, 1982) who, using surgical techniques, demonstrated that tooth eruption is inhibited in the absence of the follicle and that the tooth itself plays a passive role in eruption. Additional histochemical and ultrastructural investigations of erupting teeth and surrounding tissues have revealed that a cellular infiltrate of mononuclear cells bearing features characteristic of preosteoclasts is present in the dental follicle of rats (Wise and Fan, 1989) and dogs (Marks, Cahill and Wise, 1983) early in eruption. These cells precede the appearance of osteoclasts on bone surfaces adjacent to the coronal portion of the dental follicle, suggesting that they are precursors to the osteoclasts responsible for producing the eruption pathway through bone.

That bone resorption is necessary for normal tooth eruption is exemplified by the various rat and mouse strains with congenital osteopetrosis, a disease characterized by a general skeletal sclerosis that is caused by a lack of or severely reduced bone resorption (Seifert et al., 1993). Among these animal mutations a spectrum of tooth eruption exists, ranging from lack of erupted teeth altogether to delays in incisor and/or molar eruption. One such mutant, toothless (tl) in the rat lacks erupted teeth, despite normal tooth formation and crown development (Cotton and Gaines, 1974). The teeth in tl mutant rats remain embedded within their bony crypts due to ankylosis of dentine and enamel matrices to alveolar bone (Iizuka et al., 1992).
Recently we have shown that daily injections of colony-stimulating factor-1 (CSF-1) alleviates most of the skeletal manifestations of osteopetrosis in \( \underline{\text{\#}} \) rats (Marks et al., 1992). In addition, CSF-1 treatment of \( \underline{\text{\#}} \) rats from birth promotes eruption of incisors and molars, albeit delayed compared to normal littermates (lizuka et al., 1992). To examine the mechanism(s) responsible for stimulating tooth eruption in toothless rats and the possible role of CSF-1 in normal tooth eruption, we performed histomorphometric analyses of erupting mandibular first molars and their normal littermates with emphasis on populations of tartrate-resistant acid phosphatase-positive (TrAP+) mononuclear cells in the dental follicle and TrAP+ osteoclasts on adjacent alveolar bone surfaces.
MATERIALS AND METHODS

Animals

The animals used in this study were derived from tested breeders (+/I1) from our inbred stock maintained to perpetuate the osteopetrotic mutation, toothless (I1) in the rat. All animals were housed, maintained, bred and used in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the ILAR, NRC (DHEW Publication No. NIH 86-23-1985) and guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School. Homozygous toothless (I1/I1) rats were identified at birth by radiography under hypothermic anesthesia (Schneider, Chenoud and Marks, 1979) and are hitherto denoted as I1 rats. The normal littermates used in this study were either homozygous (+/+) or heterozygous (+/I1), since the two genotypes are indistinguishable except by breeding (Cotton and Gaines, 1974).

Treatment

Normal and mutant animals were treated from birth by daily subcutaneous injections of 10^6 units of purified human recombinant CSF-1, generously donated by Chiron Corporation (Ladner et al., 1987, Halenbeck et al., 1989). After the first seven postnatal days animals were treated as above, but on alternate days. The effects of CSF-1 injections given every other day or daily do not differ with respect to tooth eruption (unpublished data). Eruption of first molars was determined by daily inspection of anesthetized animals (6:1 Ketamine/Zylagin at 4 ml/kg body weight, intramuscularly) beginning on the 12th postnatal day. For histomorphometric analyses, anesthetized rats were killed at 1-2, 3, 5, 7, 10, 14 and 18 days after birth. Mandibles were then
rapidly removed, transected anterior and posterior to the gubernacular trough of the first molar and immersion-fixed in 2.5% gluteraldehyde in 0.1M cacodylate buffer with 7% sucrose for 2.5 hours at 4°C and demineralized in 10% EDTA. These blocks were then dehydrated in a graded ethanol series, transected through the center of the first molar and embedded in resin for histochemical analyses of tartrate-resistant acid phosphatase (TrAP) as reported previously (Cole and Walters, 1987).

The number of TrAP-positive (TrAP+) mononuclear cells in the dental follicle and TrAP+ osteoclasts on adjacent alveolar bone surfaces were enumerated at 100X in transverse sections of the first molar using the software package Osteomeasure (Osteometrics, Inc., Atlanta, GA). A representative section and corresponding Osteomeasure field are shown in Figure 1. Cell density was expressed as number per unit length of dental follicle (mononuclear cells) or alveolar bone surface (osteoclasts). The statistical significance of differences between groups at various ages was determined using a two-tailed student-test.

In some 10-day-old rats mandibles were removed for scanning electron microscopy (SEM) of the bony crypt during eruption (Boyde and Hobdell, 1969). Briefly, the lingual cortical plate and the underlying first molar were removed and the exposed crypt was subjected to organic digestion by immersion in 2% sodium hypochloride and prepared for SEM as described previously (Marks and Cahill, 1986).
RESULTS

The effects of CSF-1 injections on tooth eruption are shown in Table 1. As previously reported, treatment of toothless rats from birth promotes eruption of the first molar (Iizuka et al. 1992), but it is delayed compared to untreated normal littermates. Treatment of normal littermates with CSF-1 from birth accelerated eruption of first molars by 3-4 days.

Histomorphometric analyses of the dental follicle and the adjacent alveolar bone surfaces revealed that TRAP+ mononuclear cells and osteoclasts could be found in all 4 groups, but their time of appearance or numbers differed. These data are shown in Figures 2 (normal animals ± CSF-1) and 3 (T rats ± CSF-1).

TRAP+ mononuclear cells (Fig. 2a) are present in the dental follicles of first molars in normal rats at 1-2 days after birth and reach their highest population density at 3 days postnatally. After falling to low levels at 5 days, TRAP+ mononuclear cells are present in greater numbers at 7 days, declining thereafter. Treatment with CSF-1 caused a similar mononuclear cell profile, but the number of TRAP+ mononuclear cells per length dental follicle was generally greater compared to untreated normal littermates. A similar pattern was seen for TRAP+ osteoclasts on alveolar bone surfaces (Fig. 2b). In untreated normal littermates, osteoclasts were present early in eruption around first molars, attained their highest population density at 3 days and declined thereafter. Treatment of normal animals with CSF-1 caused a dramatic increase in populations of TRAP+ osteoclasts on bone surfaces. As with mononuclear cell density, the greatest number of TRAP+ osteoclasts on alveolar bone surfaces was attained at 3 days after birth and was followed by a secondary peak at 7 days, declining thereafter.
These cell populations around first molars of untreated and CSF-1-treated \( \text{\textdollar}\) rats are shown in Figure 3. CSF-1 treatment of \( \text{\textdollar}\) rats (Fig 3a) caused a dramatic influx of TrAP+ mononuclear cells into the dental follicle. While untreated \( \text{\textdollar}\) rats possess few TrAP+ mononuclear cells during the period examined, treatment with CSF-1 caused the appearance of TrAP+ mononuclear cells between the third and fifth postnatal day. Mononuclear cells reach their highest levels at 7 days, decline at 10 days, attain a secondary elevation at 14 days and decrease thereafter. CSF-1 treatment also increases the number of TrAP+ osteoclasts on alveolar bone surfaces around erupting first molars (Fig. 3b). Untreated \( \text{\textdollar}\) rats possess few osteoclasts during the period examined, but CSF-1 treatment of toothless rats results in a substantial increase in osteoclast numbers, which peaks at 5 days after birth and then steadily decline.

Scanning electron microscopy of 10-day-old crypts from mutant and normal rats CSF-1 revealed differences in crypt size and shape and resorptive activity as assayed by surface topology (Fig. 4). In normal rats, the first molar occupied a large crypt that, when examined at higher magnification, contained large areas with surface characteristics of active bone resorption (Fig. 4 a,b). However, in untreated toothless rats the first molars rested within smaller crypts (Fig. 4c) which lack the remodeling seen in normal littermates and had surface topologies characteristic of forming bone (Fig. 4d). Treatment of \( \text{\textdollar}\) rats with CSF-1 produced a larger mandibular first molar crypt (Fig. 4e) that possessed regions of active bone resorption, similar to the surface contours seen in normal littermates (Fig. 4b).
DISCUSSION

These data show that CSF-1 treatment from birth causes early eruption of first molars in normal rats and promotes tooth eruption in toothless littermates. These observations are consistent with previous work which has shown that CSF-1 fosters osteoclast development by accelerating the differentiation of osteoclast precursors (Tanaka et al., 1993, Udagawa et al., 1990).

Analyses of the populations of both TrAP+ mononuclear cells within the dental follicle and TrAP+ osteoclasts on adjacent bone surfaces shows that the accelerated eruption in normal littermates is preceded by significant increases in these cell types early in the eruptive process. Similarly, CSF-1 treatment of mutant (I) rats causes an early influx of TrAP+ mononuclear cells in the dental follicle of first molars and greater numbers of TrAP+ osteoclasts per unit length of the adjacent alveolar bone surface. However, in treated I rats the time of the appearance of these cell types is delayed in comparison with normal littermates and first molar eruption is delayed an identical period. TrAP+ mononuclear cells are present at 1-2 days in normal littermates, but do not appear in the dental follicle of treated toothless rats until 3-5 days. Osteoclasts are present in follicles of normal littermates as early as 1 day and appear on bone surfaces around erupting first molars of I rats between the 3rd and 5th postnatal days. In addition, while peak population density for both TrAP+ mononuclear cells and osteoclasts is reached at day 3 for normal rats, the corresponding peaks for I littermates are not attained until 7 days for mononuclear cells and 5 days for osteoclasts. The numbers of mononuclear cells in I rats never reach the levels seen in normal littermates. The delays in the appearance of these cell types together with reduced numbers of TrAP+
mononuclear cells are likely to account for the delayed eruption of first molars in \( \downarrow \) rats treated with CSF-1.

Our data agree with previous studies describing the influx of TrAP+ mononuclear cells early in the eruptive process in the beagle dogs (Marks and Grolman, 1987) and in rats (Wise and Fan, 1989) and imply that the bone resorption necessary for the formation of the eruption pathway is mediated by the supply to the surrounding bony crypt of TrAP+ mononuclear precursors in the dental follicle. That bone resorption is necessary for the eruption of teeth is evident in the various animals with congenital osteopetrosis, a disease characterized by severely reduced or absent bone resorption (Seifert et al., 1993). All of these animal mutations possess dental abnormalities ranging from complete lack of erupted teeth to delayed eruption of incisors and/or molars. In certain mutations treatments which restore bone resorption in the neonatal period, such as stem cell replacement (Marks, 1981) or CSF-1 injections (Iizuka et al., 1992), also permit tooth eruption. However, the timing of the restoration of bone resorption has different implications for different teeth (Iizuka et al., 1992).

The powerful influence of local factors in the recruitment of osteoclasts and their precursors is supported not only by the influx of these cell populations around erupting teeth during the eruptive process in dogs and rats (Figs. 2, 3; Marks et al., 1983; Wise and Fan, 1989; Marks and Grolman, 1987) but also by the timing of their appearance around first molars in untreated \( \downarrow \) rats (Fig. 3), in which very few osteoclasts can be found in the remainder of the skeleton. Since CSF-1 is produced by cells of the dental follicle (Wise these proceedings, Wise and Lin, 1994) it is likely to be involved in the molecular signaling which governs the eruptive process. Furthermore, two proteins produced by cells in the dental follicle and stellate reticulum have been shown to delay tooth eruption in postnatal rats (Lin, Fan and Wise, 1992).
Taken together these data show that systemic administration of CSF-1, a factor that causes a local increase in TrAP+ mononuclear cells, osteoclasts and bone resorption around erupting teeth, can accelerate eruption of a tooth after this process has begun. This suggests that bone resorption is a rate-limiting step of the early phase of eruption and that its increase accelerates this process. Whether local applications of CSF-1 can produce the same effect, whether eruption can be initiated by CSF-1 much earlier than it would normally occur, and whether CSF-1 can accelerate orthodontic tooth movement are three potential clinical applications of this work which need to be examined.

**TABLE 1**

The Effects of CSF-1 on Eruption of First Molars in Rats of t1 Stock

<table>
<thead>
<tr>
<th>Groups:</th>
<th>NLM untx</th>
<th>NLM +CSF-1</th>
<th>t1/t1 untx</th>
<th>t1/t1 +CSF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of Eruption: (days)</td>
<td>17-18</td>
<td>13-14</td>
<td>never</td>
<td>20-22</td>
</tr>
</tbody>
</table>
FIG. 1: Histological sections (Fig. 1a and 1b) of transverse sections of the mandibular first molar of a 7-day-old rat and the histomorphometric representation (Fig. 1c) of part of the section shown in Fig. 1b, using the Osteomeasure software package. Osteoclasts (large arrows, black circles in b and c) on alveolar bone (AB, dark line) and TrAP+ mononuclear cells (small arrows, white circles) in the dental follicle (DF, white line) were enumerated and expressed as number of cells/mm bone or follicle surface, respectively. D = dentine, E = enamel, P = pulp, open arrow = gubernaculum, large arrow = direction of eruption. 1A = 70X, 1B = 160X, 1C = 105X.
Populations of TrAP+ mononuclear (a) and osteoclasts (b) around erupting first molars in normal rats of 11 stock.

a. Mononuclear cells were present early in the eruptive process and reached peak values at 7 days in both CSF-1 treated normal rats (closed circles) and controls (open squares). CSF-1 treatment caused a general increase in the numbers of TrAP+ mononuclear cells within the dental follicle early in eruption.

b. Osteoclasts were present early in eruption and peaked at 3 days in normal rats treated with CSF-1 (closed circles) and controls (open squares). CSF-1 caused a significant increase in the numbers of TrAP+ osteoclasts on alveolar bone surfaces adjacent to the erupting first molar in rats.

Populations of TrAP+ mononuclear cells (a) and osteoclasts (b) around erupting first molars in 1/11 rats.

a. Few mononuclear cells were observed in the dental follicle in untreated 1/11 rats (open squares). CSF-1 treatment of 1/11 rats (closed circles) stimulated the appearance of TrAP+ mononuclear cells in the dental follicle between 3 and 5 days. Peak values were reached at 7 days, delayed 4 days compared to normals (Fig. 2a).

b. Few osteoclasts were observed on alveolar bone surfaces in control 1/11 rats (open squares). CSF-1 induced osteoclastogenesis in treated 1/11 rats (closed circles) between 3 and 5 days, and peak numbers of osteoclasts were observed at 5 days, delayed 2 days compared to normal rats (Fig. 2b).
Mononuclear Cells Around Erupting Osteoclasts

Mononuclear Cells Around Erupting Mandibular First Molars

Osteoclasts Around Erupting Mandibular First Molars

2a

2b

3a

3b
FIG. 4: Scanning electron micrographs of the mineralized surfaces of bony crypts of first mandibular molars from 10-day-old rats.

In normal rats (a,b) the first molar crypt is large with scalloped surface features characteristic of resorbing bone. The crypt of untreated ♂♀ rats (c,d) was smaller and had a surface topology with knobby projections (large arrows) indicative of forming bone (d). CSF-1 treatment enlarged the crypt of treated ♂♀ littermates (e) and stimulated bone resorption of crypt surfaces (f), as evidenced by scalloped surfaces (small arrows) and numerous osteoclast lacunae (open arrows). a,c,e = 18X, b,d,f = 180X
Part D:

The Contrasting Effects of CSF-1 and EGF on Tooth Eruption in Normal Rats

Rationale:

Cohen (1962) demonstrated that systemic doses of EGF cause precocious eruption of incisors in rodents. Although the mechanism by which EGF influences incisor eruption remains a mystery, it is possible that EGF stimulates bone resorption through a second factor. Because EGF stimulates expression of CSF-1 mRNA and release of CSF-1 protein by cultured murine marrow stromal cells (Abboud 1992), it is possible that EGF mediates eruption via CSF-1. Therefore, we explored the effects of CSF-1, EGF, and a combination of both factors on incisor and first molar eruption, with an emphasis on the populations of TRAP+ mononuclear cells and osteoclasts around the erupting molar.

Summary of Results and Conclusions:

Systemic administration of CSF-1 accelerated molar eruption, but not incisor eruption, in normal rats. As with the above study, CSF-1 increased osteoclasts and their precursors around erupting first molars. Treatment of normal rats with EGF accelerated incisor eruption, but molar eruption was unaffected. Populations of osteoclasts and their precursors around erupting first molars were not influenced by injections of EGF. These data indicate that incisor and molar eruption in rodents respond to different molecular signals or events. EGF plays a major role in incisor eruption, while CSF-1 contributes to the molecular cascade of molar eruption.

Publication:

This study will be presented at The Vth International Conference on Tooth Morphogenesis and Differentiation in Kerkrade, The Netherlands in May 1994, and submitted for publication in Connective Tissue Research. The key figures follow; please refer to them for specific details.
Table 1

The Effects of CSF-1 and/or EGF on Tooth Eruption in the Rat

<table>
<thead>
<tr>
<th>Group</th>
<th>Incisors</th>
<th>First Molars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10.25 ± 0.7</td>
<td>17.3 ± 0.4</td>
</tr>
<tr>
<td>+ EGF</td>
<td>7.42 ± 0.9</td>
<td>16.7 ± 0.6</td>
</tr>
<tr>
<td>+ CSF-1</td>
<td>9.21 ± 1.3</td>
<td>14.6 ± 0.5</td>
</tr>
<tr>
<td>+ EGF and CSF-1</td>
<td>7.70 ± 1.1</td>
<td>14.5 ± 0.5</td>
</tr>
</tbody>
</table>

Average Eruption Age ◊ Range of Eruption Age
FIG. 1: Populations of TrAP+ mononuclear cells (left) and osteoclasts (right) around erupting first molars in mutant (top) and normal (bottom) rats of $tl$ stock.

Left Top: Mononuclear cells were present early in the eruptive process and reached peak values at 7 days in both CSF-1 treated normal rats and controls. CSF-1 treatment caused a general increase in the numbers of TrAP+ mononuclear cells in the dental follicle early in eruption.

Right Top: Osteoclasts were present early in eruption and peaked at 3 days in normal rats treated with CSF-1 and controls. CSF-1 caused a significant increase in the numbers of TrAP+ osteoclasts on alveolar bone surfaces adjacent to the erupting first molar in rats.

Left Bottom: Few mononuclear cells were observed in the dental follicle in untreated $tl/tl$ rats. CSF-1 treatment of $tl/tl$ rats stimulated the appearance of TrAP+ mononuclear cells in the dental follicle between 3 and 5 days. Peak values were reached at 7 days, delayed 4 days compared to normals.

Right Bottom: Few osteoclasts were observed on alveolar bone surfaces in control $tl/tl$ rats. CSF-1 induced osteoclastogenesis in treated $tl/tl$ rats between 3 and 5 days, and peak numbers of osteoclasts were observed at 5 days, delayed 2 days compared to normal rats.
Mononuclear Cells Around Erupting Mandibular First Molars in Normal Rats

Osteoclasts Around Erupting First Mandibular Molars in Normal Rats
The effects of EGF-treatment of normal rats on the cellular mediators of bone resorption during tooth eruption. EGF had no significant effects on TrAP+ osteoclasts (bottom) during eruption in normal rats.
Mononuclear Cells Around Erupting Mandibular First Molars

Osteoclasts Around Erupting Mandibular First Molars
**FIG. 3:** Scanning electron micrographs of the mineralized surfaces of bony crypts of first mandibular molars in 14 day old rats of \( \uparrow \) stock.

In normal rats (a, b) the first molar crypt is large with scalloped surface features and numerous osteoclast lacunae (arrows) characteristic of bone resorption. CSF-1 treatment enhanced bone resorption as evidenced by numerous scalloped surfaces with abundant osteoclast lacunae (arrows d).

The crypt of untreated \( \uparrow \) rats (e, f) was smaller and had a surface topology with areas of knobby projections (bold arrow) indicative of forming bone, and very few scalloped surface features (open arrow) characteristic of reduced bone resorption. CSF-1 treatment enlarged the crypt of treated \( \uparrow \) littermates (g) as evidenced by scalloped surfaces with osteoclast lacunae (arrows h).

\( 2 = \) second molar, a, c, e, g = 18x, b, d, f, h = 180x.
SPECIFIC AIM 2

Part E:

The General and Skeletal Manifestations of Osteopetrosis in a New Mutation (microphthalmia blanc) in the Rat

Rationale:
Osteopetrotic mutations interrupt the development and/or function of osteoclasts and represent reproducible models in which to study the systemic and local factors essential for osteoclast differentiation and function. Recently, a new osteopetrotic mutation (microphthalmia blanc) in the rat was described (Moutier et al. 1989). These mutants have small eyes, lack skin and fur pigmentation, and inherit a typical skeletal sclerosis at birth, which diminishes by 8 weeks. The limited data about the skeletal manifestations of osteopetrosis in the microphthalmia blanc mutation and the central role animal mutations have played in our understanding of osteoclast development and function have motivated us to characterize the osteopetrotic manifestations in the microphthalmia blanc mutation.

Summary of Results and Conclusions:
Neonatal mutant (mib) rats exhibit the typical skeletal deformities and sclerosis which are reduced significantly during the first postnatal month. Osteoclast numbers, staining for TRAP and TrATPase, and bone resorption are reduced in mutants during the first two postnatal weeks, but all of these elements improve by one month. Serum concentrations of calcium and phosphorous are normal in mib rats, but 1,25-
dihydroxyvitamin D levels are higher at one week than in normal littermates. Neonatal mib rats also exhibit extramedullary hemopoiesis in the spleen. These results indicate that the transient perinatal skeletal sclerosis in mib rats is caused by reduced production and function of osteoclasts during this period.

**Publication:**

This study has been submitted for publication in Bone, and a copy of the manuscript with original Figures follows. Please refer to it for specific details.
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Running Title: Osteopetrosis in microphthalmia blanc rats

Key words: Osteopetrosis, Osteoclast, Microphthalmia blanc, Rat, Mutation, Skeleton, Bone resorption
ABSTRACT

We have examined the general and skeletal manifestations of osteopetrosis in a new, mild osteopetrotic mutation in the rat, microphthalmia blanc (mib). Newborn mutant (mib) rats exhibit the typical skeletal deformities and sclerosis at birth which are reduced significantly during the first postnatal month but don't disappear entirely up to 8 months later. Osteoclast numbers, staining for TRAP and TrAPase, and bone resorption are reduced in mutants during the first two postnatal weeks but improve by one month. In mutants, serum concentrations of calcium and phosphorus are normal but 1,25(OH)2 D levels are higher at one week than those in normal littermates. Neonatally, mutants exhibit extramedullary hemopoiesis in the spleen. These results are interpreted to mean that the transient perinatal skeletal sclerosis in mib rats is caused by reduced production and function of osteoclasts in this period. The recent description of transient, perinatal osteopetrosis in a child suggests that analyses of the early differences between mild and severe animal mutations might distinguish those children with osteopetrosis who need treatment from those who do not.
INTRODUCTION

Osteopetrosis is a rare metabolic bone disease distinguished by a generalized skeletal sclerosis caused by reduced bone resorption (Marks 1987, Osier and Marks 1992). While spontaneous mutations have been documented in cattle (Gopal et al. 1980, Zetterholm 1972), dogs (Lees and Sautter 1979), deer (Smits and Bubenik 1990), and humans (Loria-Cortes et al. 1977), the nine distinct mutations found in common laboratory animals (mice, rats, and rabbits) have been most studied (Marks and McGuire 1988, Marks 1984, Seifert et al. 1993). These mutations are congenital, inherited as autosomal recessive traits. All mutants possess a characteristic skeletal sclerosis at birth, which persists in the severe forms of the disease, but may gradually diminish in the milder mutations. Because these mutations interrupt the development and/or function of osteoclasts, they represent reproducible models in which to study the systemic and local factors essential for osteoclast differentiation and function (Marks and Walker 1976, Marks 1992). In addition, because osteoclasts fail to develop in certain skeletal sites in different mutations, these mutants offer models in which to explore the emerging site-specificities of osteoclast development and function (Marks et al. 1993).

Microphthalmia blanc (mib) is a new osteopetrotic mutation in the Norway rat discovered by Moutier and others (1989). Affected mutants, hitherto designated mib rats, lack body and fur pigmentation, have small eyes and abnormal incisors, and inherit a mild form of osteopetrosis at birth, which significantly improves by the sixth postnatal week. The limited data concerning the skeletal manifestations of osteopetrosis in the microphthalmia blanc mutation and the central role animal mutations have played in our understanding of osteoclast development and function have motivated us to characterize the osteopetrotic manifestations in the microphthalmia blanc rat.
MATERIALS AND METHODS

Animals: Breeding stock for the mib mutation was generously donated by Dr. René Moutier, Centre de Sélection et d'Elevage d'Animaux de Laboratoire, CNRS, Orléans, France. Animals used in this study were offspring of tested females (+/mib) and mutant males (mib/mib) derived from Dr. Moutier's stock and maintained at the University of Massachusetts according to the NIH Guide for the Care and Use of Laboratory Animals (1985) and the guidelines of the University of Massachusetts Animal Advisory Committee. Homozygous microphthalmia blanc (mib/mib) rats were visually identified at birth by lack of pigmentation.

All animals were weighed prior to use and their body weight recorded at regular intervals. At 1, 7, 14, 21, and 28 days after birth, a minimum of 4 normal and 4 mutant animals were killed at each age and the following tissues removed for analysis. Blood was removed via cardiac puncture of anesthetized animals (7:1 Ketamine: Zylagin at 0.4 ml/Kg intramuscularly); serum concentrations of calcium were measured by atomic absorption spectroscopy (Trudeau and Freier 1967) and phosphorous was determined by a colorimetric method (Chen et al 1956). Serum 1,25-dihydroxyvitamin D levels were determined for 3 +/mib and 2 mib/mib rats by Dr. Joe Zerwekh using the calf thymus receptor assay as previously described (Zerwekh et al. 1987; Reinhardt and Horst, 1990). Tibiae and femora were removed bilaterally and those on one side were used for high resolution radiography (Schneider et al. 1979) or scanning electron microscopy, while those on the opposite side were processed for histochemical and histological analyses. Marrow cavity size as a percent of bone length was measured in high resolution radiographs of tibiae from at least 5 animals at each age. For light microscopy and histochemical analyses, long bones were rapidly removed, cleaned of excess soft tissue, bisected longitudinally, and fixed for 2 hours in 2.5%
glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) containing 7% sucrose as previously described (Cole and Walters 1987). Fixed specimens were then demineralized in 10% EDTA in 0.1M Tris buffer, pH 7.0 and embedded in methyl methacrylate or epoxy resin. Sections cut at 0.6 μm were reacted for tartrate-resistant acid phosphatase (TRAP) or tartrate-resistant acid ATPase (TrATPase) by methods previously described (Cole and Walters 1987; Andersson and Marks 1989; Lindunger et al., 1990). These sections were used for general skeletal surveys and for quantitation of osteoclasts in metaphyseal bone (Marks et al. 1984). Osteoclasts were identified as large, multinucleated cells on bone surfaces and their density in tibial metaphyses was expressed as number of cells per unit area. Histochemical reactivity of mutant osteoclasts at various ages was evaluated and compared to that in normal littermates. Negative and positive controls for these histochemical analyses included incubation without substrate or with 10 mM sodium-orthovanadate, and incubation of sections from normal rats of a different stock known to possess reactivity for TRAP/TrATPase. Negative controls demonstrated neither TRAP nor TrATPase reactivity.

Scanning electron microscopy of the mineralized surfaces of tibiae was performed to examine and compare the metabolic state of surface bone in mutant and normal siblings of mib stock (Boyde and Hobdell, 1969). Briefly, tibiae were rapidly removed, carefully cleaned of excess soft tissue, and fixed in 10% neutral buffered formalin (NBF) for 4 hours. Remaining soft tissue and non-mineralized bone was removed in 1.5% sodium hypochloride, and the specimens were dehydrated in a graded ethanol series. Tibiae were then mounted on aluminum stubs with the anterior surface exposed, sputtercoated, and observed using an ETEC scanning electron microscope as previously described (Marks et al. 1994).

In order to screen for possible extraskeletal manifestations or complications of osteopetrosis in mib rats, the following soft tissues were histologically examined in 0.5
RESULTS

The body weights of normal and mutant rats of mib stock are shown in Figure 1 for the first four postnatal weeks. There are no significant differences in weight between the two groups until weaning (three weeks), when mutants weigh less than their normal littermates. As is typical in most osteopetrotic mutations, mib rats continue to weigh less than normal rats throughout life.

Radiographs of mutants and normal littermates from birth to four weeks at weekly intervals are shown in Figure 2. Notice that at each age shown the skeleton of mutants exhibits greater sclerosis than that in normal rats of this stock. At birth, in contrast to normal littermates, there are no marrow cavities in mib rats, but these appear during the first week. However, metaphyseal sclerosis remains prominent in some long bones and caudal vertebrae. Sclerosis in caudal vertebrae is present even eight months after birth (Fig. 3). Direct measurement of marrow cavity size in radiographs confirms that significant metaphyseal sclerosis persists in mutants four weeks after birth (Fig. 4).

Examination of anorganic preparations of tibial surfaces by scanning electron microscopy revealed reduced resorption in mib rats (Fig. 5). This is especially evident in the proximal tibia where the prominent flare, a product of local resorption in normal rats (Fig. 5a), is poorly developed in mutants (Fig. 5b), producing a bulbous convexity rather than a concavity at this site. At higher magnification (Fig. 5c-f) sites exhibiting the characteristic scalloping seen at resorbing surfaces in normal rats (Fig. 5c,e) are
replaced in many areas in mutants by surfaces with impressions of new osteocyte lacunae (Fig. 5d,f), characteristic of bone formation.

Histological analyses of the skeleton, as expected, showed no obvious morphological differences between the osteoblast populations in mutants and normal rats. However, quantitation of osteoclasts in these two phenotypes during the first postnatal month (Fig. 6) showed significant reductions in osteoclast profiles in mutants through the second postnatal week. Histochemical studies of staining for TRAP (Fig. 7) and TrATPase (not illustrated) demonstrated that all osteoclasts in mib rats up to 14 days after birth stained less intensely for these enzymes than osteoclasts in normal littermates. By 30 days after birth, the staining intensity was not different between these two groups (Fig. 7g,h).

Analyses of the blood levels of calcium and phosphorus showed no significant differences between mutants and normal littermates during the first postnatal month (Fig. 8). Analyses of circulating levels of 1,25-dihydroxyvitamin D in pooled samples demonstrated that this hormone in mib rats was elevated (90 and 55 pg/ml vs. 40,41 and 35 pg/ml in normal littermates) at one week after birth.

To determine the extent of the extramedullary manifestations of osteopetrosis in the microphthalmia blanc mutation during the first month, we examined the microscopic anatomy of selected tissues. Relative enlargement of the red pulp in the spleen of neonatal mutants was suggestive of extramedullary hemopoiesis but there were no hepatic foci of hemopoiesis in either phenotype. The kidney, spleen and liver of mutants showed no cytoarchitectural abnormalities, and mutant thymus and lymph nodes contained well-developed B and T-cell-dependent zones. Thus, with the exception of splenic hemopoiesis, no morphological differences between mutants and normal littermates were noted in the spleen, liver, thymus, lymph nodes, kidney and parathyroid glands.
DISCUSSION

We have described the general skeletal manifestations of a new osteopetrotic mutation, microphthalmia blanc (mib), in the rat. As reported by Moutier et al. (1989) the mib mutation exhibits a mild, transient osteopetrosis in which the skeletal sclerosis present at birth is gradually reduced by the 6th postnatal week. However, the disease is not completely resolved in mib rats because residual metaphyseal sclerosis persists in the long bones and axial skeleton up to 8 months after birth. Mineral homeostasis is not compromised in mib/mib rats, even at birth, when the disease is most acute. Our data show that serum calcium and phosphorous levels are normal from birth to the fourth postnatal week in mutant rats. This is in contrast to other animal mutations including three mouse mutations (mi, oo, q1) and the os rabbit which exhibit hypocalcemia and hypophosphatemia and two rat mutations (op, ja) which are hypophosphatemic at least early in life (Seifert et al., 1993).

Elevations of serum 1,25-dihydroxyvitamin D [1,25(OH)2D] at some point in life have been detected in all animals with congenital osteopetrosis (Popoff et al. 1992). Our data show this to be the case for 1-week-old mutant (mib) rats. These data support the concept of interdependence of the skeleton and the vitamin D-endocrine system and suggest that elevations in serum [1,25(OH)2D] are a consequence of the reduced bone resorption caused by osteopetrosis (Popoff et al., 1992).

Our data indicate that aberrations in osteoclast development and function early in life account for the early and transient sclerosis in mib rats. First, scanning electron microscopy (SEM) of tibial bone surfaces reveals little bone resorption in 1 week old mib rats. Second, marrow cavity space increases dramatically in mib rats between birth and 10 days. Third, osteoclast numbers are severely reduced in mutant mib rats.
at birth (48% compared to normal littermates), but increase to normal levels by four weeks. Fourth, histochemical analysis of tartrate-resistant acid phosphatase (TRAP) reveals little reactivity in mutant osteoclasts between birth and the first postnatal week. However, osteoclasts contain nearly normal levels of this functional enzyme by four weeks. These data taken together suggest that within the limitations of the methods we used: (1) decreased osteoclast numbers combined with reduced osteoclast function early in life bring about the severe sclerosis present at birth in \textit{mi}b rats, (2) the gradual improvement of osteoclast numbers and function leads to reduced skeletal sclerosis, and (3) residual metaphyseal sclerosis present up to 8 months after birth is caused by a persistent reduction in osteoclast function. It is interesting that osteoclasts in two mild osteopetrotic mutations in the rat, \textit{mi}b and incisors-absent (ia) exhibit minimal and excessive numbers and reactivity for TRAP, respectively (present data, Marks 1973). This underscores the remarkable variety of cellular manifestations even in two mutations with early, improving skeletal sclerosis.

Studies on osteopetrotic mutations have shed light on the remarkable heterogeneity of the disease in mammals and provided insights concerning bone metabolism in general. The pathogenetic mechanisms of osteopetrosis, whether due to deviations in osteoclast origin, structure, or function serve as models for understanding normal osteoclast biology. This is exemplified by the pioneering work of Walker, in which he cured \textit{mi/mi} and \textit{gal/gal} mice via parabiotic union (Walker 1972; 1973) and subsequently cured mice of the disease using normal spleen and marrow transplantation (Walker 1975a; 1975b). These studies lead to the first successful clinical treatment of human infantile osteopetrosis and fostered our current understanding of the hemopoietic origin of osteoclasts. Recent investigations with the \textit{il} rat and \textit{op} mouse have illustrated the importance of local factors or cytokines in supporting osteoclast development,
recruitment, function, and maintenance (Yoshida et al. 1990; Felix et al., 1990; Marks et al. 1992; 1993; Sundquist et al. 1994; Wiktor-Jedrzejczak et al., 1990).

Recently, Monoghan and others (1991) reported a patient with transient infantile osteopetrosis in which neonatal, asymptomatic, biopsy-proven osteopetrosis resolved by 28 months. Infantile osteopetrosis is usually a malignant, autosomal recessive disease distinguished clinically by anemia, compressive cranial neuropathies, skeletal fractures, upper airway obstruction, and a progressively fatal course if left untreated (Reeves et al. 1981, Cook and Moore 1992). Given the availability and risk associated with several experimental treatments for patients with infantile osteopetrosis (van Lie Peters et al. 1993, Coccia 1993), identifying those infants with osteopetrosis destined to recover spontaneously is a principal concern. Our studies indicate that the mib mutation in the rat is similar to the patient reported by Monoghan et al. Additional studies of the differences between mib rats and more severe mutations (Il and op rats) could lead to clinical tests to distinguish neonates with a sclerotic skeleton who need treatment from those who do not.
Body weights of normal littermates (NLM) and \textit{mib} rats at selected intervals during the first four weeks of postnatal life. For each time point the mean weight and standard deviation for at least 10 animals of each phenotype is shown. Mutants weigh significantly less ($p \leq 0.05$) than normal littermates by two weeks and older.
FIGURE 2: Radiographs of representative normal (left column) and mutant rats of mib stock at birth (0 days), 7, 21 and 28 days. At birth mutant long bones have no marrow spaces (arrows) but they develop by the first week. At all ages mutants can be distinguished from normal littermates by increased metaphyseal sclerosis in long bones and caudal vertebrae (arrows) reproduced actual size.
FIGURE 3: X-Rays of the caudal skeleton of normal (A) and mutant (B) rats of mib stock at 8 months. Sclerosis persists in the metaphyses of the caudal vertebrae and the distal tibia (arrows).
FIGURE 4: Marrow cavity size in normal (NLM) and mib rats expressed as a percentage of the distance between tibial growth plates during the first four postnatal weeks. The marrow cavity of mutants is significantly smaller than normal ($p \leq 0.05$) through the first four postnatal weeks.
Scanning electron micrographs of the anorganic (mineralized) anterior surface of the proximal tibia of one-week-old normal (nlm-Fig. 4a,c,e) and mutant (mib-Fig. 4b,d,f) rats. Areas of bone resorption are irregular and scalloped and areas of bone formation are smooth. There is more resorption in normal rats than mutants (compare Fig. 4a and b) which can be confirmed at higher magnification (Figs. 4c-f). Fl = flare; c = anterior tibial crest; v = vascular space; r = scalloped area of resorption; f = areas of formation. a,b = 20X; c,d,e,f = 150X.
FIGURE 6: Number of osteoclast profiles per unit area (mm$^2$) of proximal tibial metaphysis from normal and mib rats at various postnatal ages. Data are presented as mean counts per unit area from at least three non-adjacent sections from at least three rats of each phenotype at each age. Osteoclasts in the mutant skeleton are significantly reduced ($p<0.05$) during the first two postnatal weeks.
Osteoclast Populations in Rats of mib Stock

Number per Unit Area

AGE (days)

0 10 20

Normal
Mutant

*
FIGURE 7: Photomicrographs of the proximal tibial metaphysis from a 7-day-old normal rat (a and c) and its mib littermate (b and d) reacted to show tartrate-resistant acid phosphatase (TRAP) activity histochemically. In normal rats at this age (Fig. 7a) the proximal metaphysis (double-ended arrow) is short, situated between the proximal epiphyseal plate (E) and the marrow cavity (M). Osteoclasts (arrowheads) are numerous and stain intensely for TRAP (Fig. 7c). In mib rats at this age (Fig. 7b) the metaphysis is extensive (long arrow), there is no marrow cavity visible at this magnification and fewer, weakly staining osteoclasts (arrowheads) are present (Fig. 7b and d). Lightly counterstained with toluidine blue. a,c = 115X; b,d = 300X.
**FIGURE 8:** Photomicrographs of TRAP-stained sections of tibial metaphysis from one-day-old (Fig. 8a,b) and 30-day-old (Fig. c,d) normal (a and c) and *mib* (b and d) littermates. At birth osteoclasts in normal rats (arrowheads, Fig. 8a) stain much more intensely for TRAP than those in *mib* littermates (Fig. 8b). By 30 days the staining intensity of normal (Fig. 8c) and *mib* (Fig. 8d) osteoclasts (arrowheads) does not differ. Lightly counterstained with toluidine blue. X300.
FIGURE 9: Serum phosphorus and calcium concentrations in normal and mutant rats of mib stock. Data are mean values from triplicate determinations from at least three rats of each phenotype at each age.
Part F:
Dental Manifestations of the \textit{mib} Rat

\textbf{Rationale:}

Tooth eruption is the bilaterally symmetrical, highly coordinated biological event whereby a tooth is translocated through alveolar bone to its functional position in the oral cavity. It has been documented that bone resorption is a necessary and rate-limiting component of eruption. This concept is exemplified among the congenital mammalian osteopetroses, where a veritable spectrum of tooth eruption exists, ranging from complete lack of erupted dentition in the most severe forms of the disease to delays in incisor and/or molar eruption in the milder ones. As shown above, bone resorption is reduced in \textit{mib} rats in the perinatal period. We have examined the dental consequences of this transient reduction in bone resorption.

\textbf{Summary of Results and Conclusions:}

The mild osteopetrosis in \textit{mib} rats caused incisor defects and delayed the eruption of all teeth. Radiography and histological analyses revealed that in \textit{mib} rats the apical end of the incisor failed to extend posterior to the third molar as in normal siblings. However, molar root and crown development was normal in \textit{mib} rats. The temporary encroachment of bone on incisor matrices leads to malformed and maloccluded incisors in \textit{mib} rats. These data support the hypothesis that each tooth has a "window of opportunity" in which bone resorption must occur during tooth eruption, since incisors--the first teeth to erupt in rodents--are most affected by the disease in \textit{microphthalmia blanc}.

\textbf{Publication:}

This study has been submitted for publication in Archives in Oral Biology. A copy of the manuscript with original Figures follows. Please refer to it for specific details.
DENAL ABNORMALITIES IN THE OSTEOPETROTIC RAT MUTATION MICROPHTHALMIA BLANC

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Running Title: Dental Abnormalities in microphthalmic rats

Key words: Dentition, tooth eruption, osteopetrosis, rat
We have examined the dental manifestations of the mild, transient osteopetrosis in the rat mutation microphthalmia blanc (mib). Eruption of all teeth was delayed in mib rats compared to normal littermates. These delays ranged from 5 days for incisors to 3 and 2 days for the first and second molars. Normal rats had straight incisors which exhibited signs of wear, but in mib littermates the incisors were maloccluded, distorted in shape, and showed no signs of wear. Radiographic and histologic examination of the dentition of 1 and 4 week old rats revealed that the apical end of incisors in mib rats failed to extend posteriorly to the third molar region as in normal siblings, but ended at the first molar region. Histologic examination of longitudinal sections of mandibles through the incisors of neonatal normal and mib rats showed that in 1 day old mutants the incisor was closely surrounded by alveolar bone to which it was ankylosed. In addition, the incisor body in mib rats was malformed, with an indented apical end. This ankylosis was temporary, being resolved by 3 days. These data demonstrate that neonatal reductions in bone resorption cause incisor defects and delay the eruption of all teeth in mib rats. The maloccluded and distorted incisors of mib rats are likely caused by temporary ankylosis of incisor matrices to alveolar bone. Taken together, these data illustrate that bone resorption is an essential and rate-limiting element of tooth eruption.
INTRODUCTION:

Osteopetrosis is a rare inherited metabolic bone disease in mammals marked by a generalized skeletal sclerosis caused by reduced bone resorption (Marks, 1987; Osier and Marks, 1992). The pathogenesis of the disease varies among the mutations, and includes interceptions in osteoclast development and/or function; however, osteopetrosis invariably involves reduced osteoclastic bone resorption (Marks, 1984; 1989). Although spontaneous mutations have been documented for a variety of mammals, the nine distinct congenital osteopetroses occurring in common laboratory animals are best characterized (Seifert et al., 1993). Common features of the disease include a characteristic skeletal sclerosis at birth, which persists in the severe forms of the disease but gradually diminishes in the less severe mutations, reduced or absent marrow cavities, abnormal bone modeling and remodeling, extramedullary hemopoiesis, and abnormal tooth eruption.

A spectrum of aberrant tooth eruption exists among the animal osteopetroses, from complete absence of an erupted dentition in the most severe forms of the disease (Cotton and Gaines, 1974), to delays in eruption of incisors and/or molars in the less severe mutations (Marks, 1976). These observations suggest that tooth eruption depends on bone resorption, a concept supported by abundant experimental evidence. Cahill (1970; 1974) has shown that the radiographic and histologic hallmark of tooth eruption is the timely formation of an eruption pathway, and conditions such as osteopetrosis, which inhibit the formation of the eruption pathway, compromise eruption. Tooth eruption is inhibited when bone resorption is blocked by local infusions of bafilomycin A1, an antagonist to the osteoclastic proton pump (Sundquist and Marks, 1994). Tooth eruption is restored in osteopetrotic animals with treatments that increase bone resorption, such as stem cell replacement or administration of colony-
stimulating factor-1 (CSF-1 or M-CSF), respectively (Marks, 1981; Izuka et al., 1992). In addition, tooth eruption is accelerated in normal rats after injections of CSF-1 (Cielinski et al., 1994).

Microphthalmia blanc is a new osteopetrotic mutation in rats discovered by Moutier and colleagues (1989). Mutant rats, hitherto referred to as mib rats, are distinguished at birth by lack of pigmentation and a typical skeletal sclerosis, which diminishes during the first postnatal month. We have recently reported that osteoclast numbers are reduced in mib rats at birth and contain little of the functional enzymes tartrate-resistant acid phosphatase (TRAP) and tartrate-resistant acid ATPase (TrATPase) (Cielinski and Marks, 1994). However, by the fourth postnatal week osteoclast numbers and function, as assayed by these functional enzymes, approaches those observed in normal siblings. We describe here the dental consequences of this temporary reduction in bone resorption in mib rats.

MATERIALS AND METHODS:

Animals:

Animals used in this study were offspring of tested females (+/mib) and mutant males (mib/mib) derived from breeding stock donated by Dr. René Moutier, Centre de Sélection et d'Elevage d'Animaux de Laboratorie, CNRS, Orléans, France. The rats were maintained and bred at the University of Massachusetts according to the NIH Guide for the Care and Use of Laboratory Animals, 1985 and the guidelines of the University of Massachusetts Animal Advisory Committee. Homozygous microphthalmia blanc rats (mib/mib) were identified at birth by lack of skin pigmentation. After weaning at 21 days, mib rats were provided with powdered food ad libitum, as previously described for toothless (tl, osteopetrotic) rats (Cotton and Gaines, 1974).
Tooth eruption:

Tooth eruption was defined as the age at which the given tooth first pierced through the oral epithelium. For incisors, eruption was monitored by daily visual inspection beginning seven days after birth. Molar eruption was determined by palpation of anesthetized animals on alternate days (7:1 Ketamine : Zylagone at 0.4 ml/Kg, intramuscularly) beginning fourteen days after birth.

Histology and Histochemistry:

At birth and weekly intervals thereafter, a minimum of 5 normal and 5 mutant animals were killed by decapitation under anesthesia (see above) for histological and histochemical analyses. The mandibles were rapidly removed, cleaned of excess soft tissue, and transected posterior to the third molar to facilitate penetration of fixatives. For histological examination, mandibles were fixed in 10% neutral-buffered formalin at room temperature for 24 hours, demineralized in 10% EDTA in 0.1M Tris buffer, dehydrated in a graded ethanol series, embedded in paraffin, and 5 μm sections were stained with hematoxylin and eosin. Alternatively, isolated mandibles were fixed in 2.5% gluteraldehyde in 0.1M cacodylate buffer (pH 7.2) containing 7% sucrose as previously described (Cole and Walters, 1987), demineralized in 10% EDTA in 0.1M Tris buffer, pH 7.0, and embedded in methyl methacrylate. Sections cut at 0.6 μm were examined via light microscopy.

Autoradiography:

To study the formation rate of alveolar bone and dentin, at least 4 animals at 1, 7, 14, 21, and 28 days of each genotype (+/mib, mib/mib) were given 5.0 μCi 3H-proline (New England Nuclear) per gram body weight and killed 6 hours later (Walker, 1966; Marks, 1969). Serial sections were cut from paraffin-embedded mandibles prepared as above, dipped in Ilford-L4 emulsion, and alternate slides were placed in dry
chambers for 1 or 2 week exposures, respectively. Exposed slides were developed in D-19 (Kodak) for 4 minutes, rinsed in water for 1 minute, fixed in 30% sodium thiol sulfate for 5 minutes, and rinsed in tap water. Some sections were then counterstained with hematoxylin and eosin.

Radiology:

For radiographic examination of the dentition, mutant and normal siblings of mib stock were killed by decapitation at 1 and 4 weeks after birth, and the heads bisected into hemisections through the sagittal plane prior to radiography as previously described (Iizuka et al., 1992).

RESULTS:

The effects of the mild, transient osteopetrosis on tooth eruption in mib rats are shown in Figure 1 and summarized in Table 1. In normal rats of this stock, the incisors erupted 11 days after birth, and the first, second, and third molars erupted 19, 21, and 34 days after birth respectively. The eruption of all teeth was delayed in mib rats, and, with the exception of the third molar, earlier erupting teeth were most affected by the disease. Incisors erupted in mib rats between 13-21 days after birth, on average 5 days later than incisor eruption in normal siblings. Molars erupted between 21-25 days, 23-25 days, and 38-40 days for first, second and third molars, respectively. Therefore, the first molars were delayed an average of 3 days and the second molars 2 days compared to normal littermates. Figure 1 demonstrates the dramatic effect of osteopetrosis on eruption of incisors in mib rats. While the incisors of normal rats are well worn at 4 weeks after birth, the incisors of mib rats are maloccluded and distorted, lacking any signs of wear.
These observations were confirmed by radiologic and histologic analyses. Radiologic examination of animals at 1 and 4 weeks (Figure 2) revealed that in mib rats, the apical end of the incisor failed to extend past the first molar (Fig. 2b, d), while in normal siblings the apex of the incisor extends posterior to the third molar (Fig. 2a, c). Molars in mutants were indistinguishable from normal in radiographs at 4 weeks, with well developed crowns and roots. Low magnification histomorphology (not shown) confirmed these observations.

To investigate the cause of the incisor abnormalities present in mib rats, we examined longitudinal histologic sections of the lower mandible through the incisor of young animals. At birth, the incisors of mib rats (Fig. 3b) exhibited abnormal modeling compared to normal littermates (Fig. 3a), including an irregularly shaped incisor body and indented incisor apex. Higher magnification of the apical end of these teeth revealed encroachment of incisor tissue by bone matrix in mib rats (Fig. 3d), while incisor tissue and bone matrix were clearly separated in normal siblings (Fig. 3c). This ankylosis of bone with incisor enamel and dentin matrices was temporary, being resolved by the 3rd postnatal day (data not shown).

Bone and dentine formation were not different in mib rats and normal siblings, as revealed by autoradiography of mineralizing matrices (Figure 4). Radiolabeled proline was incorporated equally into newly formed alveolar bone and dentine matrices in both normal (Fig. 4a, c) and mib rats (Fig. 4b,d).

**DISCUSSION:**

We demonstrate that the mild form of osteopetrosis in microphthalmia blanc rats causes severe distortion of incisor shape and position and delays the eruption of all teeth. Radiographic and histologic analyses revealed deficient posterior extension of the incisor apex of mib rats and temporary ankylosis of enamel and dentin matrices to mineralized
bone surfaces at birth. This ankylosis in \textit{mib} rats is similar to the odontoma-like regions produced by permanent ankylosis of incisors in two other osteopetrotic rat mutations, incisor-absent (\textit{ia}) (Schour et al., 1949) and toothless (\textit{tl}) (Iizuka et al., 1992). In \textit{tl} rats, neonatal injections of the growth factor CSF-1 stimulate bone resorption, which corrects the apical incisor defects and allows the teeth to erupt (Iizuka et al., 1992). We believe that the natural resolution of the ankylosed incisor by 3 days in \textit{mib} rats is similar to the condition brought about by CSF-1 treatment of \textit{tl} rats. In addition, this temporary ankylosis likely accounts for the occlusal defects which persist after eruption.

It is noteworthy that the eruption and development of incisors are more affected than other teeth by the mild and transient osteopetrosis present in \textit{mib} rats. This is understandable given the mild nature of the disease in these rats (Cielinski and Marks, 1994) and in light of the emerging concept of tooth eruption as a biological event dependent upon bone resorption for the timely formation of an eruption pathway (Marks et al., 1994). Pathway formation is regulated by the dental follicle and is precisely timed according to each tooth (Cahill and Marks, 1980; Marks, 1987; Cahill et al. 1988; Marks et al. 1988). Since incisors are the first teeth to erupt in rodents, the "window of opportunity" for the formation of their eruption pathway must precede that of molars. This is supported by the recent report that treatment of \textit{tl} rats from birth promotes eruption of all teeth, but delaying treatment by only 1 day compromises incisor eruption (Iizuka et al. 1992). These data suggest that signals for the development and activation of osteoclasts must be provided by the dental follicle soon after birth for incisors to erupt. Incisors-absent (\textit{ia}) is another mild osteopetrotic mutation in the rat characterized by a neonatal sclerosis with dental abnormalities confined to lack of erupted incisors. The work of Kodama et al. (1991) has shown that in the CSF-1 deficient \textit{op} (osteopetrotic) mouse, exogenous administration of CSF-1, when
postponed 11 days after birth, causes second molars to erupt before the first molars. These results indicate that the critical periods for the formation of an eruption pathway in rodents is birth for incisors, and prior to the 10th postnatal day for first molars.

Taken together, our data show that the mild and transient osteopetrosis present in mib rats delays the eruption of all teeth and causes severe occlusal defects in incisors. The observation that incisors are more severely affected by this mutation than later-erupting molars is consistent with their respective critical periods for the eruption pathways. The temporary encroachment of the mineralized matrices of the apical incisor by bone matrix can account for both the delays in the eruption of these teeth and the persistent deformities after eruption.
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<td>M1</td>
<td>n=3</td>
<td>11</td>
<td>16.3±2.8</td>
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<td></td>
<td></td>
<td>13-21</td>
<td>22.5±1.2</td>
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<tr>
<td>M2</td>
<td>n=6</td>
<td>21-25</td>
<td>23.3±0.8</td>
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<tr>
<td></td>
<td></td>
<td>34</td>
<td>39.3±1.0</td>
</tr>
<tr>
<td>M3</td>
<td>n=3</td>
<td>38-40</td>
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Figure 1 - Photograph of a normal (nlm) 5-week old rat and its \textit{mlp} littermates. Note straight, worn ends of the incisors in the normal rat (white arrowhead) in contrast to the rotated, malpositioned incisors in the mutant, with little evidence of attrition. Actual size, bar 3mm.
Figure 2 - Original radiographs of normal (a, c) and mib (b, d) rats at 7 and 28 days after birth. Notice that in mib rats (b, d) the apical incisor (arrowheads) extends posterior to the first molar region, but not to the third molar as in normal rats (a, c). Normal root and crown development are evident in older mib first molars (arrow, d).
Figure 3 - Photomicrographs of mandibular incisors from normal (a, c) and mutant (b, d) rats of mib stock at birth.  

a, b:  Low power (35x) photomicrographs illustrate the regular shape of the incisor of normal rats, with a characteristic apical end (open arrow, a). The mutant incisor (b) is deformed with an abnormally indented apical end (open arrow) and bulb-shaped incisor body (asterisks).  

c, d: Higher power photomicrographs of the apical incisor regions indicated by open arrows in a, b. The incisor apex in the normal rat is surrounded by undifferentiated mesenchyme and separated from alveolar bone (AB). In mutants, the incisor apex is ankylosed (arrows) to alveolar bone (AB).  

bar (a, b) = 0.5mm; bar (c, d) = 0.1mm.
Figure 4 - Autoradiograms of mandibular first molars (M1) and second molars (M2) in normal (a) and \textit{milb} (b) rats, aged 3 weeks. Newly formed dentin (small arrows) and alveolar bone matrices (large arrows, AB) are qualitatively similar in these rats, which were prelabeled with $^3$H-proline for 6 hours. bar = 0.5mm.
Part G:

Bone Metabolism in the Osteopetrotic Rat Mutation microphthalmia blanc (mib)

Rationale:

We have described the dental and skeletal manifestations of osteopetrosis in the rat mutation microphthalmia blanc and shown that the transient, mild nature of the disease in these rats is due to neonatal reductions in osteoclast number and function. Calcium and phosphate homeostasis are normal in mib rats, but 1,25-(OH)\textsubscript{2}D3 levels are elevated at one week. Although reductions in osteoclast number and function in neonatal mib rats can account for the recovering neonatal sclerosis, other possibilities may exist. These include increased bone formation, which has been demonstrated to compound osteopetrosis in the four mouse mutations gl, mi, oc, and op (Marks 1989), and deficiencies in ruffled border formation, which are common in congenital osteopetrosis (Marks 1984). In this study we measured bone formation and evaluated osteoclast ultrastructure and gene expression as reflected by mRNA levels of TrATPase and carbonic anhydrase II (CAII).

Summary of Results and Conclusions:

The rate of bone formation in mib rats and normal littermates at 1, 7, 14, 21, and 28 days after birth by quantitative analysis of the incorporation of radiolabeled proline into newly formed bone matrix (Walker 1966, Marks 1969). Bone formation falls within normal ranges for all ages observed, with the exception of 21 days, when bone formation is reduced to 56-60% compared to normal littermates. We interpret this result to be a consequence of the nutritional disadvantage in mib rats at a time when they are weaned and must switch to a diet of ground food with maloccluded incisors. These data show that increased bone resorption does not complicate the skeletal sclerosis in this mutation.
Ultrastructural features of osteoclasts in tibiae of normal and mutant rats of mib stock were analyzed at birth and 7 weeks. Osteoclasts of normal rats at birth and 7 weeks had the classical features of active osteoclasts including numerous mitochondria, vacuolated cytoplasm, clear zones and elaborate ruffled borders. Ruffled border were absent or poorly developed in osteoclasts from neonatal mib rats. By 7 weeks, mutant osteoclasts exhibit the distinct features of active osteoclasts including extensive membrane rufflings flanked by clear zones. These results indicate that neonatal abnormalities in osteoclast function in mib rats include inability to form a well-developed ruffled border, similar to the situation in oc and mi mice, the os rabbit, and ia rat mutations (Marks 1984). This defect is also resolved by 4 weeks, similar to derangements in osteoclast numbers and function described earlier.

Osteoclastic gene expression is also deficient in neonatal mib rats, as evidenced by significant reductions in mRNA encoding TrATPase and carbonic anhydrase II. These abnormalities normalize by 4 weeks. Therefore, mib rats exhibit neonatal reductions in both numbers and function of osteoclasts which resolve during the first postnatal month. These include reduced message encoding the functional enzymes TrATPase and carbonic anhydrase II, reduced amounts of the functional enzymes TRAP and TrATPase as shown by histochemical staining, and inability of osteoclasts to form ruffled borders. All of these abnormalities are improved dramatically by 4 weeks or later.

Publication:

This study is being prepared for publication in Bone. The key figures follow; please refer to them for specific details.
**BONE METABOLISM IN RATS OF mib STOCK--**
Expressed as Ratios of Values of Mutants to Values of Normals

<table>
<thead>
<tr>
<th>AGE</th>
<th>Osteoclast Population*</th>
<th>Bone Formation**</th>
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</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>48%</td>
<td>85% (74-99)</td>
</tr>
<tr>
<td>7 Days</td>
<td>55%</td>
<td>104% (99-112)</td>
</tr>
<tr>
<td>14 Days</td>
<td>46%</td>
<td>111% (103-119)</td>
</tr>
<tr>
<td>21 Days</td>
<td>60%</td>
<td>58% (56-60)**</td>
</tr>
<tr>
<td>28 Days</td>
<td>66%</td>
<td>100%</td>
</tr>
</tbody>
</table>

* Osteoclast population quantitated as number per unit area of tibial metaphysis and expressed as ratio of values in mutants compared to normal littermates. At least 3 rats of each phenotype were examined at each age.

** Calculated by 6-hr incorporation of 3H-proline into matrix of calvarial bone measured by scintillation counting and expressed as mean (+ range) for mutants compared to normal rats.

*** This low value may reflect the nutritional disadvantage of mutants at weaning when they have to switch to a ground diet with delayed eruption and malocclusions.
FIG. 1: Transmission electron micrographs of osteoclasts from 1-day-old normal (A) and mib (B) rats. Normal osteoclasts exhibit characteristics of active osteoclasts, including mitochondria (m), vacuoles (v), and a well-developed ruffled border (RB) surrounded by an organelle-free clear zone (cz). Mutant osteoclasts (B) possess mitochondria (m), vacuoles (v), and clear zones (cz), but membrane ruffling is limited (arrows). N = nucleus, n = nucleolus, b = bone, c = cartilage, 9200x.
FIG. 2: Transmission electron micrographs of osteoclasts from 1-day-old mib rats. Mutant osteoclasts lack ruffled borders (A) or exhibit limited membrane ruffling (B, arrow). v = vacuoles, N = nucleus, b = bone, c = cartilage, cz = clear zone, 7450x.
FIG. 3: Transmission electron micrographs of the ultrastructural features of active osteoclasts from 7-week-old normal (A) and \textit{mib} (B) littermates. In normal rats the cytoplasm of osteoclasts possess numerous mitochondria (m) and vacuoles (v) near the ruffled border. In the mutant osteoclast (B), a distinct ruffled border (R) is present adjacent to mineralized cartilage (c). \textit{cz} = clear zone, \textit{b} = bone, \textit{N} = nucleus, \textit{n} = nucleolus, 4000x.
FIG. 4: Total cellular RNA from 1-day-old mib and normal (n) rat longbones was isolated and processed as described in the text.

Top: Autoradiogram of a northern blot hybridized with $^{32}$P-dCTP labeled probe against tartrate-resistant acid ATPase (1 week exposure).

Notice the difference in relative abundance of TrATPase message in normal rats (n, lanes 5, 6, 10, 11, 12) and mib littermates (lanes 1, 2, 3, 4, 7, 8, 9).

Bottom: Autoradiogram of the same northern blot probed for 18S ribosomal RNA as an internal standard.
Results from northern analyses of osteoclast-specific gene expression in rat longbones. Data are presented in densitometric units generated by scanning laser densitometry of blots from at least 5 litters (1-day) or 4 rats of each genotype (28-days).

Notice that at birth, normals have greater amounts of both TrATPase and CAII mRNAs. No differences between normals and mibs are observed at 28 days.
Northern Analysis of Osteoclast-Specific Gene Expression in Rat Long Bones

- NLM
- mib

Densitometric Units

mRNA and Age (days)

ATPase 1 ATPase 28 CA II 1 CA II 28
CHAPTER 5

SUMMARY AND DISCUSSION

In view of the fact that the significance of all these results have already been discussed in each of the publications above, this chapter will be mercifully brief. Please refer to each publication for more wide-ranging discussion of these data.

Part A:

Significant contributions of this work

1. We have shown that in the toothless rat, a mutation lacking erupted dentition due to severely reduced bone resorption, CSF-1 promoted tooth eruption that was delayed compared to normal rats. Eruption was accompanied by changes in the populations of TRAP+ mononuclear cells in the dental follicle and TRAP+ osteoclasts on adjacent alveolar bone surfaces. These cell populations were dramatically increased in treated mutants compared to untreated II rats, but the timing of their appearance was delayed compared to normal littermates. This lag in the appearance of osteoclasts and their precursors corresponded to the delays in eruption of first molars observed in treated II rats. These data show that increased bone resorption--provided by CSF-1 injections--promotes eruption in II rats and affirm the role of bone resorption in tooth eruption.

2. We have demonstrated that CSF-1 accelerates the eruption of molars in normal rats. CSF-1 increased the number of TRAP+ mononuclear cells in the dental follicle and TRAP+ osteoclasts on adjacent alveolar bone surfaces, but had no effect on the timing of their appearance in normal rats. These data suggest that CSF-1 stimulates eruption of molars in rats by influencing the cellular mediators of the bone resorption
necessary for tooth eruption. In addition, our data support the concept that bone resorption is a necessary and rate-limiting element of tooth eruption (Marks et al. 1993, Marks 1981), agree with the proposed role of the dental follicle in regulating the resorption of eruption (Cahil and Marks, 1980), and together with the observation that CSF-1 is produced and secreted by cultured dental follicle cells (Wise et al. 1994), indicate that CSF-1 plays a role in the molecular cascade which regulates eruption. The potential clinical uses of CSF-1 in the movement and eruption of teeth must be explored.

3. Our data have revealed a differential effect on tooth eruption of the growth factors CSF-1 and EGF. CSF-1 accelerated eruption of molars in normal rats, but had no effect on incisor eruption. On the other hand, EGF accelerated incisor eruption, but did not effect molar eruption in normal rats. These results agree with the original description of EGF by Cohen (1962) as a factor causing precocious incisor eruption. Our data show that this effect is not extended to molars and indicate that incisor and molar eruption in rodents are governed by different molecular signals. Therefore, data generated by observing incisor eruption as an assay for tooth eruption must be reevaluated, and exclusive use of only this method to identify factors affecting tooth eruption must be avoided.

4. We have also detailed for the first time the mechanism for the transient, mild form of osteopetrosis inherited by mib rats. Mutant animals possessed a typical sclerosis at birth, which diminished--but was not resolved--during the first postnatal month. We believe that these characteristics are caused by early reductions in osteoclast number and function which improve to normal levels by 4 weeks. Osteoclast numbers were severely reduced in mib rats between birth and 2 weeks, but improved to near normal levels by 4 weeks. Neonatal abnormalities in osteoclast function included reduced staining for the functional enzymes TRAP and TrATPase, decreased levels of mRNA for both TrATPase and CAII, and inability to form a well-developed ruffled border.
None of these defects were apparent after the first postnatal month. It is interesting that two mild osteopetrotic mutations in the rat, \textit{mib} and \textit{ia} (incisors-absent) exhibit minimal and excessive osteoclast numbers and reactivity for TRAP, respectively (present study, Marks 1973). This underscores the remarkable variety of cellular manifestations of osteopetrosis—even in two mutations with early, improving sclerosis.

5. We have shown that the dental abnormalities caused by the mild, transient form of osteopetrosis in \textit{mib} rats are limited to incisor defects and delays in the eruption of all teeth. Histologic and radiographic examination of mutant incisors revealed that, contrary to the situation in normal rats, the apex of the incisor of \textit{mib} rats failed to extend past the first molar region to the third molar. The incisor apex of newborn \textit{mib} rats was misshaped due to ankylosis of incisor matrices with alveolar bone. This ankylosis was temporary, being resolved by the third postnatal day. We believe that the delayed eruption of incisors in \textit{mib} rats and abnormal shape and occlusion of these teeth in older animals is a consequence of the temporary ankylosis in newborn rats. It is noteworthy that the \textit{ia} rat is another mild osteopetrotic mutation in the rat with neonatal reductions in bone resorption and dental abnormalities limited to delayed eruption and incisor defects. In addition, the early resolution of incisor ankylosis in \textit{mib} rats is similar to the situation in \textit{il} rats treated with CSF-1. Incisors are permanently ankylosed and fail to erupt unless CSF-1 treatment is started at or soon after birth. These observations are understandable when viewed within the emerging concept of tooth eruption as a localized process within alveolar bone in which formation of an eruption pathway is produced by bone resorption (Cahill and Marks 1982, Marks 1986). Formation of this eruption pathway is controlled locally, is linked to the dental follicle, and results in a window of opportunity for tooth eruption which occurs at a characteristic time for each tooth.
Part B

Future Directions

1. Can prenatal administration of CSF-1 resolve the characteristic skeletal defect of mib rats? The hemopoietic growth factor CSF-1 has been shown to increase osteoclast numbers and activity in both il rats and op mice. CSF-1 also accelerates eruption of molars in normal rats by increasing the number of TRAP+ osteoclasts and their putative precursors around the erupting dentition. These data indicate that exogenously supplied CSF-1 has a positive effect on osteoclastogenesis and function. The ability to visually identify mib rats at birth, compared to radiographic identification of mutants in other stocks, provides the unique opportunity to study the prenatal effects of CSF-1 on the skeletal defects of newborn mib rats. Given the mild, transient osteopetrosis in mib rats, exogenously supplied CSF-1 may stimulate early recovery of the disease as determined by skeletal radiographs and determination of osteoclast number, histochemistry, ultrastructure, and gene expression.

2. We have show that CSF-1 promotes eruption of teeth in il rats and accelerates eruption in normal rats by increasing bone resorption and formation of an eruption pathway. However, the mechanism by which EGF influences incisor eruption remains a mystery. EGF has been shown to stimulate production of CSF-1 mRNA and secretion of CSF-1 protein in cultured marrow stromal cells (Abboud 1992), suggesting that EGF may influence bone resorption during incisor eruption by upregulating local secretion of CSF-1. Our observations on the differential effects of CSF-1 and EGF on molar and incisor eruption in rats does not support this concept and suggests that the eruption of these two types of teeth are different. Another way to test the hypothesis that EGF plays a role in eruption by augmenting bone resorption is to treat newborn il rats with EGF and
examine its effects on incisor eruption. Eruption of incisors in these bone resorption-deficient rats would support this concept.

3. In the mib rat, we have identified numerous transient osteoclast defects including reduced number and abnormal function as evidenced by histochemistry, ultrastructure and gene expression. In the other rat mutations with congenital osteopetrosis (ja, j, and op), abnormal osteoblastic gene expression has been documented (Shalhoub et al. 1991). These abnormalities are understandable in light of the concept that the development and function of both osteoblasts and osteoclasts is coordinated or coupled to maintain skeletal homeostasis. However, are extracellular matrix abnormalities in osteopetrotic rats universal, or confined to more sclerotic mutations? Examining osteoblastic gene expression in the mib rat will facilitate a better understanding of this question and provide insights regarding the interactions between the two major cellular constituents of the skeleton.

These avenues of research are logical progressions given our current understanding of osteoclast ontogeny.