Brief Definitive Report

Congenital Osteoclast Deficiency in Osteopetrotic (*op/op*) Mice Is Cured by Injections of Macrophage Colony-stimulating Factor

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Summary

Osteopetrotic (*op/op*) mice have a severe deficiency of osteoclasts, monocytes, and peritoneal macrophages because of a defect in the production of functional macrophage colony-stimulating factor (M-CSF) resulting from a mutation within the M-CSF gene. In this study, we examined whether daily 5-μg injections of purified recombinant human M-CSF (rhM-CSF) for 14 d would cure these deficiencies in the mutant mice. Monocytes in the peripheral blood of the *op/op* mice were significantly increased in number after subcutaneous injections of the factor two or three times a day. In contrast, osteopetrosis in the long bones of *op/op* mice was completely cured by only one injection of rhM-CSF per day. Bone trabeculae in the diaphyses were removed. Many osteoclasts were detected on the surface of bone trabeculae in the metaphyses. Although development of tooth germs of uninjected *op/op* mice was impaired, rhM-CSF injection restored the development of molar tooth germs and led to tooth eruption as a consequence of the recovery of bone-resorbing activity. These results demonstrate that M-CSF is one of the factors responsible for the differentiation of osteoclasts and monocyte/macrophages under physiological conditions.

Mice homozygous for the recessive osteopetrosis mutation (*op/op*) have a severe deficiency of osteoclasts, monocytes, and peritoneal macrophages (1–3). These defects of the *op/op* mice can be neither transferred into irradiated normal mice (3), nor cured by transplants of normal spleen or bone marrow cells (4). Recently, *op/op* mice have been reported to have a defect in the production of functional macrophage colony-stimulating factor (M-CSF) (5–7), and Yoshida et al. (5) found that the mutant mice have a 1-bp insertion within the coding region of the M-CSF gene on chromosome 3. Furthermore, Wiktor-Jedrzejczak et al. (6) found that the macrophage deficiency in the peritoneal cavity of *op/op* mice is partially corrected by implantation of diffusion chambers containing M-CSF-producing L-929 cells. These findings strongly suggest that lack of functional M-CSF is the primary cause of the deficiency of monocyte/macrophages and osteoclasts in the *op/op* mice.

However, the role of M-CSF in osteoclast differentiation has been controversial. Although M-CSF has been reported to stimulate osteoclast formation in vitro (8, 9), other reports have been contradictory (10–13). Therefore, we examined whether the osteoclast deficiency in *op/op* mice could be cured by an exogenous supply of purified human recombinant M-CSF (rhM-CSF). We found that this deficiency, as well as that of monocytes in the peripheral blood, was completely cured by daily injections of the rhM-CSF for 14 d.

Materials and Methods

Mice. F_2 hybrid mice of +/+, *op*/+, and *op/op* genotypes were raised in our laboratory from breeding pairs of B6C3F1-*a/a*, *op*/+ mice obtained from The Jackson Laboratory, Bar Harbor, ME. The mutant *op/op* mice could be clearly recognized by 10 d of age by failure of eruption of the incisors and by a domed skull.

Injection of Hemopoietic Factors into op/op Mice. Purified rhM-CSF was generously provided by Morinaga Milk Industry Co. Ltd. (Kanagawa, Japan). 5 μg of rhM-CSF was subcutaneously injected one to three times a day into *op/op* mice that were 11 d old at the start of the treatment. After consecutive injections of the factor for 14 d, the mice were killed at 25 d of age.

Hematological Measurements. Peripheral blood of the mice was obtained by retroorbital puncture under anesthesia. Nucleated cells were counted in a hemocytometer. Cellularity of the spleen was

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determined from counts of single cell suspensions from the organ. Differential cell counts were performed on May-Grünwald/Giemsa–stained smears, and the results were confirmed by staining for nonspecific esterase.

Morphological Observations. Tibias, femurs, and mandibles were fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C. After having been rinsed in the buffer, some specimens were dehydrated in ethanol and embedded in JB-4 medium. 4-μm-thick sections were stained with toluidine blue or for tartrate-resistant acid phosphatase (TRACP) activity (14). Other specimens were decalcified in 5% EDTA (pH 7.2), post-fixed in 1% osmic acid, and embedded in Epon 812. Ultrathin sections were stained and observed under the electron microscope. Whole calvariae were fixed in 10% phosphate-buffered formalin for 30 min at room temperature and stained for TRACP activity.

Results and Discussion

After consecutive injections of purified rhM-CSF into *op/op* mice, we compared their hematological profile with that of uninjected littermates (age matched). As shown in Table 1, we could not find monocytes in the peripheral blood of uninjected *op/op* mice by counting 200 nucleated cells. The number of monocytes in the peripheral blood of the mutant mice was increased by injections of rhM-CSF two or three times a day for 14 d, but not by a single daily injection, and the monocyte counts were significantly higher than those in phenotypically normal +/? mice (Table 1). Monocyte counts in spleens were not significantly different in any of the mice (Table 2). A significant number of erythroblasts was detected in the peripheral blood of uninjected *op/op* mutants, and the erythroblast number decreased after the rhM-CSF injections (Table 1). This increase in erythroblast number in the uninjected mutants seems to reflect the extramedullary hemopoiesis that occurs in these animals, as well as the drop in this number after the rhM-CSF treatment and the recovery of bone marrow hemopoiesis (see below). These results demonstrate that injection of purified rhM-CSF is enough to cure the monocyte deficiency in *op/op* mice.

By contrast, cure of osteopetrosis and development of red marrow in the long bones of *op/op* mice injected with rhM-CSF once a day for 14 d was readily noticeable by macroscopic observation. In the long bones of uninjected *op/op* mice, development of the bone marrow cavity was prevented by an excessive amount of bone trabeculae, and osteoclasts were very rarely detected (Fig. 1 *a*) (1, 2, 4). In the bones of rhM-CSF-injected *op/op* mice, however, the bone trabeculae in the diaphysis were mostly replaced by bone marrow showing active hemopoiesis. Furthermore, many osteoclasts were observed on the surface of bone trabeculae in the metaphyseal region (Fig. 1 *b*). No obvious abnormality was found in these osteoclasts (Figs. 1, *b* and *c*), except that the cells tended to be smaller in size and to contain a smaller number of nuclei than the osteoclasts found in normal mice; whereas the osteoclasts in *op/op* mice have been reported to have an abnormal cytoplasmic distribution of acid phosphatase, toluidine blue–positive and electron-dense cytoplasmic inclusions, and hypertrophy of clear zones and ruffled borders (1, 2). TRACP-positive mononucleated cells were found in the bone marrow cavity of the rhM-CSF-injected *op/op* mice (Fig. 1 *b*), consistent with the finding of Wiktor-Jedrzejczak et al. (6) that TRACP-positive cells were detected in the bone marrow of *op/op* mice implanted with diffusion chambers containing L-929 cells. These observations demonstrate that a single injection of rhM-CSF once a day for 14 d is sufficient to cure the osteoclast deficiency in the long bones of *op/op* mice.

Failure of tooth eruption due to reduced bone resorption is a common feature of mammalian osteopetrotic mutants. As shown in Fig. 2 *a*, the molar teeth of uninjected 25-d-old *op/op* mice remained within mandible. The tooth germs were

Table 1. *Hematological Parameters of Peripheral Blood of Uninjected and M-CSF-injected op/op Mice and Uninjected Normal Littermates*

Mouse number	Genotype	No. of rhM-CSF injections/d*	No. of nucleated cells	Differential counts‡				
				Neu	Eo	Lym	Mono	Ebl
			$10^{-3}/\mu l$			%		
1	*op/op*	–	5.7	28	1	67	0	4
2	*op/op*	–	6.3	34	0	64	0	2
3	*op/op*	1	7.2	21	0	79	0	0
4	*op/op*	1	7.6	30	0	70	0	0
5	*op/op*	2	6.3	14	8	70	8	1
6	*op/op*	3	6.0	20	0	75	4	1
7	*op/op*	3	4.2	34	2	57	6	1
8	+/?	–	6.3	7	1	90	2	0
9	+/?	–	12.0	14	0	85	1	0

All mice were killed at 25 d after birth. Neu, neutrophils; Eo, eosinophils; Lym, lymphocytes; Mono, monocytes; Ebl, erythroblasts.
* 5 μg of M-CSF was subcutaneously injected once to three times a day for 14 d.
‡ 200 cells were counted in each sample.

Table 2. *Hematological Parameters of Spleen of Uninjected and M-CSF-injected op/op Mice and Uninjected Normal Littermates*

Mouse number	Genotype	No. of rhM-CSF injections/d	No. of nucleate cells/spleen	Differential counts*				
				Neu	Eo	Lym	Mono	Ebl
			$\times 10^{-8}$			%		
1	*op/op*	–	1.0	9	3	13	0	75
2	*op/op*	–	1.4	5	0	24	2	69
3	*op/op*	1	0.96	6	2	13	1	78
4	*op/op*	1	1.1	8	1	11	1	79
5	*op/op*	2	0.57	4	1	16	1	78
6	*op/op*	3	1.9	8	0	18	2	72
7	*op/op*	3	0.90	20	1	24	3	52
8	*+/?*	–	1.1	6	0	26	2	66
9	*+/?*	–	1.9	5	1	22	0	72

Mice used in this experiment and in Table 1 were the same.
Abbreviations used in this Table are the same as those used in Table 1.
* 200 cells were counted in each sample.

completely ankylosed to bone trabeculae, and the periodontal ligament failed to develop. TRACP-positive cells were hardly detectable in the mandibles. In contrast, in the mandibles of the *op/op* mice injected with rhM-CSF once a day for 14 d, the mucous membrane covering the second and third molars was broken down in places, and the crown of these molars emerged through these perforations into the oral cavity (Fig. 2 *b*). Osteoclasts were detected on the surface of alveolar bone, and the roots and periodontal ligament of the molars had apparently formed normally. These observations demonstrate that an injection of rhM-CSF once a day for 14 d restored the development of molar tooth germs in *op/op* mice and led to tooth eruption as a consequence of the recovery of bone resorption.

On the inner surface of calvariae of *op/op* mice injected with rhM-CSF two or three times a day for 14 d, we found more osteoclasts than in age-matched *+/?* mice, and their distribution was similar to that in *+/?* mice (data not shown).

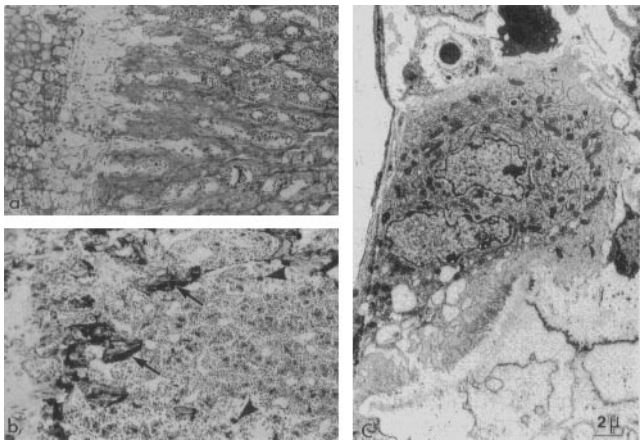

Figure 1. Micrographs of proximal tibias. The proximal epiphyseal plate is at the left in *a* and *b*. (*a*) TRACP-stained longitudinal section from an uninjected *op/op* mouse. Large amount of bone trabeculae and reduced marrow space are noted. No TRACP-positive cells are found in this micrograph (×100). (*b*) TRACP-stained longitudinal section from an rhM-CSF-injected *op/op* mouse. A large part of bone trabeculae is replaced by marrow cavity, and a considerable number of TRACP-positive osteoclasts (*arrows*) are arranged along the surface of remaining bone trabeculae. TRACP-positive mononucleated cells (*arrowheads*) are found in the marrow cavity (×100). (*c*) Electron micrograph of tibial tissue from an rhM-CSF-injected *op/op* mouse shows a giant cell displaying cytological characteristics of a typical osteoclast, such as well-developed ruffled border, clear zone, and lysosomal structures (×2,000).

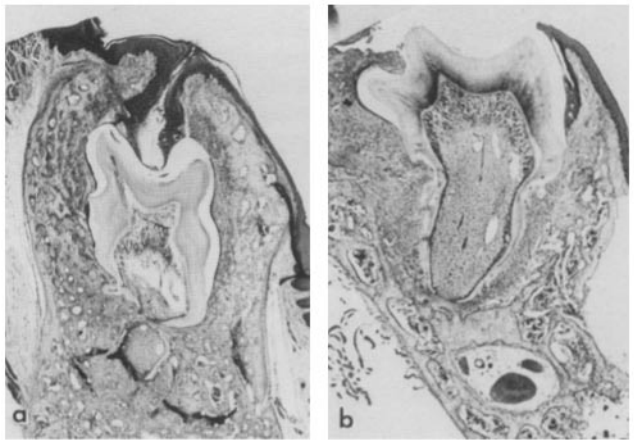

Figure 2. Toluidine blue–stained bucco-lingual sections of mandibles. (*a*) Second molar of an uninjected *op/op* mouse is completely embedded in the mandible, and its root is severely distorted (×31). (*b*) Tooth crown of second molar of an rhM-CSF-injected *op/op* mouse has emerged into oral cavity, and its root and periodontal ligament appear to be developing normally (×31).

Our present study demonstrates that the deficiency in osteoclasts and monocytes in *op/op* mice is caused by the absence of functional M-CSF. Interestingly, osteoclasts in different bones and monocytes in peripheral blood showed different responsiveness to the rhM-CSF injections. Osteoclasts appearing in response to the rhM-CSF injections showed a distribution similar to that observed in normal mice and actively participated in physiological bone remodeling. These observations strongly suggest that M-CSF plays an essential role in the differentiation of osteoclasts and monocyte/macrophages under physiological conditions. Since our present experimental system does not allow us to further analyze the precise role of M-CSF in osteoclast and macrophage differentiation, and since some stromal cell lines derived from newborn mouse calvaria or adult mouse bone marrow can support osteoclast differentiation (15), we have recently established stromal cell lines from newborn *op/op* mouse calvariae. We found that some of the cell lines can support the differentiation of macrophages and osteoclasts only when M-CSF is exogenously supplied (manuscript in preparation), confirming our present results. Availability of the M-CSF-deficient mice and stromal cell lines derived from them will provide a unique opportunity to clarify the physiological role of M-CSF.

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