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.

Materials and Methods

Mice. F2 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...
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 un.injected mutants seems to reflect the extramedullary hemopoiesis that occurs in these animals, as well as the drop in this

| Mouse number | Genotype | No. of rhM-CSF injections/d | No. of nucleated cells | Differential counts¹ |
|--------------|----------|-----------------------------|------------------------|----------------------|
| 1            | op/op    | –                           | 5.7                    | 10⁻¹/μl              |
| 2            | op/op    | –                           | 6.3                    | 28                   |
| 3            | op/op    | 1                           | 7.2                    | 1                    |
| 4            | op/op    | 1                           | 7.6                    | 21                   |
| 5            | op/op    | 2                           | 6.3                    | 14                   |
| 6            | op/op    | 3                           | 6.0                    | 20                   |
| 7            | op/op    | 3                           | 4.2                    | 34                   |
| 8            | +/±      | –                           | 6.3                    | 7                    |
| 9            | +/±      | –                           | 12.0                   | 14                   |

All mice were killed at 25 d after birth. Neu, neutrophils; Eo, eosinophils; Lym, lymphocytes; Mono, monocytes; Ebl, erythroblasts.

¹ 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 1. Hematological Parameters of Peripheral Blood of Uninjected and M-CSF-injected op/op Mice and Uninjected Normal Littermates
Table 2. Hematological Parameters of Spleen of Uninjected and M-CSF-injected op/op Mice and Uninfected Normal Littermates

| Mouse number | Genotype | No. of rhM-CSF injections/d | No. of nucleate cells/spleen | Differential counts* |
|--------------|----------|----------------------------|-----------------------------|---------------------|
|              |          |                            |                             | × 10^-8            | Neu | Eo | Lym | Mono | Ebl |
| 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. 2b). 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).
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 calvaria. 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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References

1. Marks, S.C., Jr., and P.W. Lane. 1976. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. J. Hered. 67:11.

2. Marks, S.C., Jr. 1982. Morphological evidence of reduced bone resorption in osteopetrotic (op) mice. Am. J. Anat. 163:157.

3. Wiktor-Jedrzejczak, W., A. Ahmed, C. Szczyluk, and R.R. Skelly. 1982. Hematological characterization of congenital osteopetrosis in op/op mouse. J. Exp. Med. 156:1516.

4. Marks, S.C. Jr., M.F. Seifert, and J.L. McGuire. 1984. Congenitally osteopetrotic (op/op) mice are not cured by transplants of spleen or bone marrow cells from normal littermates. Metab Bone Dis. & Relat. Res. 5:1883.

5. Yoshida, H., S. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okumura, T. Sudo, L.D. Shultz, and S. Nishikawa. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature (Lond.). 345:442.

6. Wiktor-Jedrzejczak, W., A. Bartocci, A.W. Ferrante, Jr., A. Ahmed-Ansari, K.W. Sell, J.W. Pollard, and E.R. Stanley. 1990. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc. Natl. Acad. Sci. USA. 87:4828.

7. Felix, E., M.G. Cecchin, W. Hofstetter, P.R. Elford, A. Stutzer, and H. Fleisch. 1990. Impairment of macrophage colony-stimulating factor production and lack of resident bone marrow macrophages in the osteopetrotic op/op mouse. J. Bone Miner. Res. 5:781.

8. MacDonald, B.R., G.R. Mundy, S. Clark, E.A. Wang, T.J. Kuehl, E.R. Stanley, and G.D. Roodman. 1986. Effects of human recombinant CSF-GM and highly purified CSF-1 on the formation of multinucleated cells with osteoclast characteristics in long-term bone marrow cultures. J. Bone Miner. Res. 1:227.

9. Hagenaaars, C.E., A.A.M. van der Kraan, E.W.M. Kawilarang-de Haas, J.W.M. Visser, and P.J. Nijweide. 1989. Osteoclast formation from cloned pluripotent hemopoietic stem cells. Bone Miner. 6:179.

10. van de Wijngaert, F.P., M.C. Tas, J.W.M. van der Meer, and E.H. Burger. 1987. Growth of osteoclast precursor-like cells from whole mouse bone marrow: Inhibitory effect of CSF-1. Bone Miner. 3:97.

11. Kurihara, N., T. Suda, Y. Miura, H. Nakachi, H. Kodama, K. Hiura, Y. Hakeda, and M. Kumegawa. 1989. Generation of osteoclasts from isolated hematopoietic progenitor cells. Blood. 74:1295.

12. Dickson, I.R., and B.A.A. Scheven. 1989. Regulation of new osteoclast formation by a bone cell-derived macromolecular factor. Biochem. Biophys. Res. Commun. 159:1383.

13. Shinar, D.M., M. Sato, and G.A. Rodan. 1990. The effects of hemopoietic growth factors on the generation of osteoclast-like cells in mouse bone marrow cultures. Endocrinology. 126:1728.

14. Hammerström, L.E., J.S. Hanker, and S.U. Toverud. 1971. Cellular differences in acid phosphatase isozymes in bone and teeth. Clin. Orthop. Relat. Res. 78:151.

15. Udagawa, N., N. Takahashi, T. Akatsu, T. Sasaki, A. Yamaguchi, H. Kodama, T.J. Martin, and T. Suda. 1989. The bone marrow-derived stromal cell lines MC3T3-G2/Pa6 and ST2 support osteoclast-like cell differentiation in cocultures with mouse spleen cells. Endocrinology. 125:1805.