

Delayed Hematopoietic Development in Osteopetrotic (*op/op*) Mice

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Summary

Changes in structure, cellularity, hematopoietic progenitor cell and macrophage content, and osteoclast activity were investigated in the hematopoietic organs of the colony-stimulating factor 1 (CSF-1)-less osteopetrotic (*op/op*) mouse. The data indicated that *op/op* mice undergo an age-related hematopoietic recovery and resolution of osteopetrosis, suggesting that the hematopoietic system has the capacity to use alternative mechanisms to compensate for the absence of an important multifunctional growth factor, CSF-1. In young animals, *op/op* femurs were heavily infiltrated with bone, and marrow cellularity was significantly reduced. After 6 wk of age, there was an increase in the marrow space available for hematopoiesis. The femoral cavity of *op/op* mice progressively enlarged, and by 22 wk of age its appearance and marrow cellularity was comparable to that of controls. The percentage of *op/op* mononuclear phagocytes, defined by F4/80 antigen expression, progressively increased to normal levels by 35 wk of age. There was no difference in the incidence of both primitive and mononuclear phagocyte-committed, CSF-1-responsive progenitor cells in *op/op* marrow, but their femoral content was significantly reduced in young mice. During the period of reduced hematopoiesis in the marrow of young *op/op* mice, splenic hematopoietic activity was elevated. This mutant mouse represents a system for the study of the CSF-1-independent regulatory mechanisms involved in hematopoietic regulation.

CSF-1 stimulates the survival, proliferation, and differentiation of mononuclear phagocytes and their precursors (1–3). Osteopetrotic (*op/op*) mice are characterized by an autosomal recessive inactivating mutation in the CSF-1 gene resulting in the absence of CSF-1 (4–7). As a consequence, young *op/op* mice have impaired mononuclear phagocyte development (1, 2) characterized by a deficiency of both macrophages and osteoclasts. Bone density is increased, marrow cavities are occluded (8–11), and it has been reported that marrow cellularity of *op/op* mice is 10%, and marrow macrophage content is 1%, that of control mice (12).

Previous studies have generally been restricted to analysis of young *op/op* mice (6, 7, 9, 12, 13). In these mice, bone matrix formation is elevated, but by 6 wk of age there is evidence of remodeling in the long bones (8, 10). This raises the possibility that in older *op/op* mice there are significant changes in the organization and regulation of hematopoiesis associated with a substantial correction of the osteopetrosis.

In the present study, hematopoietic and mononuclear phagocyte development was investigated in *op/op* mice from 2 wk to 5 mo of age. Progressive changes in the structure of the femoral marrow space were accompanied by a normalization of marrow cellularity and macrophage content,

demonstrating that as *op/op* mice age they correct hematopoietic deficiencies in the absence of CSF-1.

Materials and Methods

Mice. Mice of *op/op* and wild-type control (+/+, *op/+*) genotypes were raised as previously described (14). Osteopetrotic (*op/op*) mice were distinguished from normal siblings at 10 d of age by the absence incisor eruption, and separated from wild-type mice at weaning. They were fed a pureed mixture of mouse food (Barastoc, St. Arnaud, Victoria, Australia) and infant formula (Ensure; Ross Laboratories, Columbus, OH) twice daily to optimize their nutritional status. Control mice received mouse chow ad libitum.

Growth Factors. Human rCSF-1 (8×10^7 U/mg protein) was a gift from Chiron Corp. (Emeryville, CA). Human rIL-1 α (2.5×10^9 U/mg protein) was a gift from Hoffman-La Roche Inc. (Nutley, NJ). rIL-3 was prepared as conditioned medium from the genetically altered mouse mammary cell line (C127) expressing mouse cDNA (15). Factors were used at optimal concentrations (16).

Preparation of Microscopic Sections. Anesthetized (Penthrane; Abbott Laboratories, North Chicago, IL) mice were perfused with 4 ml of 2.5% glutaraldehyde at 0.3 ml/min into the descending aorta (17). Femurs were immersion fixed for a further 3 h before decalcification in 10% EDTA (pH 7.0) for 5–7 d. Postfixation with

2% osmium tetroxide (80 mM Sorensen's phosphate buffer, pH 7.4) for 1 h was followed by dehydration in graded ethanol, and 100% acetone for 45 min at each step in a vacuum. Femurs were placed in a 1:1 mixture of Spurr's resin and 100% acetone in a vacuum overnight, then for 6 h in fresh solution. The femurs were embedded in 100% Spurr's resin in a vacuum for 3 h followed by incubation at 60°C overnight. For light microscopy, 0.5 μ m transverse sections were cut and stained with methylene blue. For EM, 0.9-nm sections were cut and stained using uranyl acetate and lead citrate.

Cells Suspensions. Marrow from control mice was collected by flushing femoral shafts with cold HEPES-buffered balanced salt solution (BSS) supplemented with 2% newborn bovine serum (NBBS). Femurs from *op/op* mice were routinely ground using a mortar and pestle because <20% of marrow cells could be recovered by flushing alone. The suspension was gently vortexed to free cells adhering to bone fragments, and the supernatant decanted and pooled, together with three washings of the bone fragments.

Spleen cell suspensions were prepared by mincing in BSS supplemented with 2% NBBS, and dispersed through a mesh sieve. Cell counts were performed using a hemocytometer.

Hematopoietic Progenitor Cell Assays. Marrow and spleen cell suspensions were assayed for low and high proliferative potential colony-forming cells (LPP-CFC and HPP-CFC), using a double-layer nutrient agar culture system (16).

Flow Cytometric Analysis. Bone marrow macrophage content was quantitated on the basis of F4/80 cell surface Ag expression (18). Cells were labeled with F4/80 Ab, followed by FITC-conjugated goat anti-rat F(ab')₂ IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD); each for 30 min at 4°C; and were analyzed using a FACStar Plus[®] cell sorter (Becton Dickinson & Co., Mountain View, CA). A forward vs. perpendicular light scatter window was set to exclude lymphocytic and granulocytic cells that may be nonspecifically labeled with F4/80 Ab (19). Background fluorescence (cell surface binding of FITC-conjugated second Ab) was not >5%.

Osteoclast Staining. Mice were anesthetized and perfused with 2 ml of 1% glutaraldehyde and 1% formaldehyde into the descending aorta. Femurs were immersion fixed at 4°C for 1 h before decalcification in 5% EDTA in cacodylate buffer at 4°C for 5 d. The femurs were dehydrated in graded ethanol and xylene for 90 min at each step and embedded in paraplast. Sections (10 μ m) were cut and stained using tartrate-resistant ATPase histochemistry.

Resorption Pit Analysis. Femurs were excised using blunt dissection. After immersion fixation in 2.5% glutaraldehyde overnight at 4°C and 8 h in a vacuum, they were placed in 4% (wt/vol) NaOCl to remove remaining tissue. Femurs were dehydrated in graded ethanol for 125 min in a vacuum, and, after critical point drying, were sputter coated with 30 μ m of gold and examined by scanning electron microscopy.

Statistical Analysis. Differences between means were evaluated by one-way analysis of variance (ANOVA).

Results

Changes in Bone Structure, Marrow Cellularity, and Hematopoietic Activity of *op/op* Mice. The femoral marrow cellularity of control mice progressively increased postnatally, attaining adult levels by 6–8 wk of age. In contrast, *op/op* marrow cellularity remained stable at $\sim 2.5 \times 10^6$ cells per femur during this period, and by 8 wk had only attained 16% of the cellularity of control femurs (Fig. 1). This decreased cel-

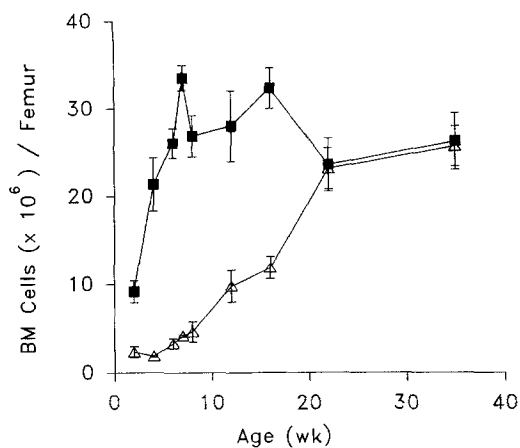


Figure 1. Age-related changes in the femoral bone marrow content of *op/op* (Δ) and control (\blacksquare) mice. Values are the means \pm SEM of between three and eight determinations per time point.

lularity in young *op/op* mice reflected occlusion of the marrow space by bone: there was no obvious central marrow cavity at 4 wk of age (Fig. 2 A). By 6 wk of age, formation of a central marrow cavity resulted in an increase in the space available for hematopoiesis (Fig. 2, B and C). Thereafter, the

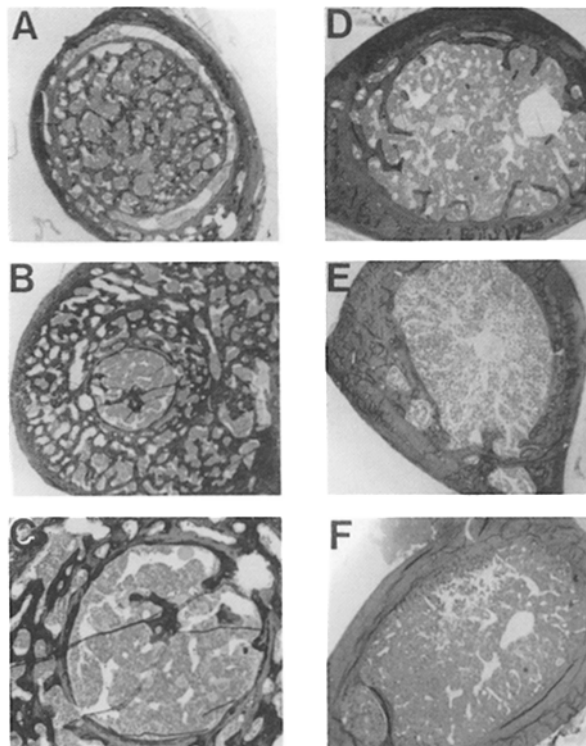


Figure 2. Age-related changes in the *op/op* femoral bone marrow cavity. Transverse sections of the diaphyseal region. (A) *op/op* femur at 4 wk ($\times 26$); (B) *op/op* femur at 6 wk ($\times 29$); (C) an enlargement of B, demonstrating the development of the *op/op* marrow cavity ($\times 66$); (D) *op/op* femur at 22 wk ($\times 28$); (E) control littermate femur at 4 wk ($\times 26$); (F) control littermate femur at 22 wk ($\times 25$).

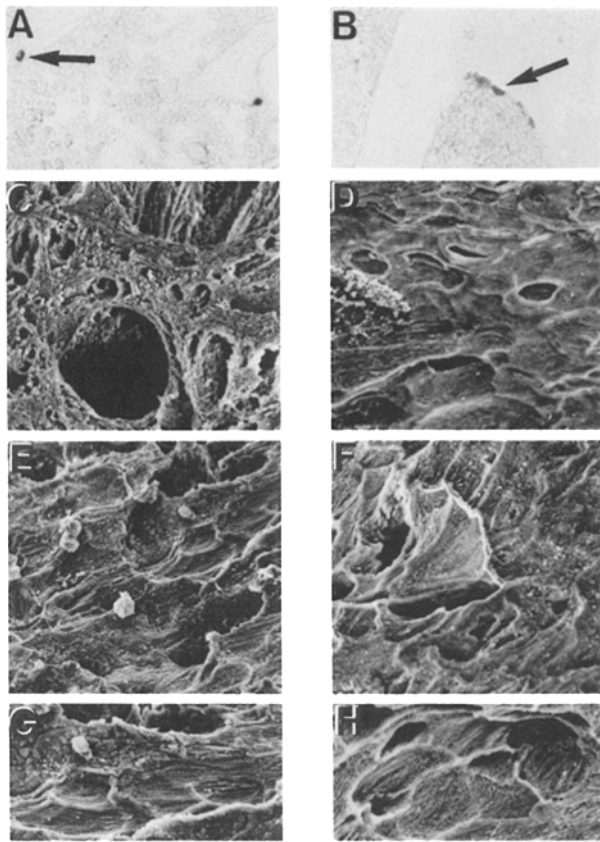


Figure 3. Evidence of osteoclast activity in *op/op* femoral bone. Longitudinal sections of femurs from 22-wk-old *op/op* (A, $\times 126$) and control (B, $\times 131$) mice. Arrows indicate ATPase-labeled osteoclasts. Scanning electron micrographs of the femoral surface from 4-wk-old *op/op* (C, $\times 377$); 4-wk-old control mice (D, $\times 204$); 22-wk-old *op/op* (E, $\times 406$; G, $\times 765$); 17-wk-old control mice (F, $\times 447$; H, $\times 594$).

marrow cavity progressively enlarged, and by 22 wk of age (Fig. 2 D), its appearance was comparable to that of control mice (Fig. 2, E and F). However, the femurs of *op/op* mice remained shorter and more stunted than those of control mice at all ages examined.

The increase in marrow space in *op/op* femurs was accompanied by an increase in cellularity, which also became comparable to that of control femurs by 22 wk of age (Fig. 1). This progressive increase in space available for hematopoiesis was indicative of increased osteoclast activity. Very few osteoclasts were observed along the edge of the marrow cavity in young *op/op* mice (data not shown), but by 22 wk of age these cells were easily detected along the femoral surface (Fig. 3 A). Osteoclast activity was confirmed by examination of the femoral surface in young and old *op/op* mice using scanning electron microscopy. Resorptive pits are indicative of osteoclast activity, but these were absent in young (4 wk) *op/op* mice (Fig. 3 C). As well, the rugged irregular edges of the femoral surface were suggestive of bone formation (Fig. 3 C). However, by 22 wk of age, the femoral surface of *op/op* mice resembled that of control mice, with resorptive pits being clearly distinguishable (Fig. 3, E-H).

Table 1. F4/80 Ag Expression on Marrow Mononuclear Cells

Age	Percentage F4/80 ⁺ cells	
	<i>op/op</i>	Control
wk		
3	22.7 \pm 3.2	42.4 \pm 5.0
8	35.4 \pm 6.4	54.7 \pm 3.0
35	36.7 \pm 8.5	34.8 \pm 6.2

Values are the mean \pm SEM of analyses in three individual mice.

Flow cytometric analysis of F4/80 surface Ag expression on mononuclear cells with defined light scattering properties (see Materials and Methods) demonstrated an age-related correction of the marrow macrophage deficiency observed in young *op/op* mice. At 3 wk of age the proportion of F4/80⁺ cells was $\sim 50\%$ that in control marrow (Table 1). By 35 wk of age the proportion and number of F4/80⁺ cells in the *op/op* mouse was comparable to that of controls (Table 1). The presence of macrophages in *op/op* marrow was confirmed by electron microscopy. No morphological differences were observed between macrophages detected in *op/op* and control mice at any age examined, and paracrystalline inclusions (20) were present in macrophages of normal and *op/op* mice of comparable age (data not shown).

Changes in Hematopoietic Progenitor Cell Populations in *op/op* Mice. The hematopoietic status of *op/op* mice was evaluated by in vitro clonal assay of LPP-CFC and HPP-CFC. LPP-CFC are CSF-1-dependent, lineage-restricted mononuclear phagocyte progenitors. HPP-CFC with a synergistic requirement for IL-1, IL-3, and CSF-1 are closely related to primitive hematopoietic cells with long-term repopulating ability in

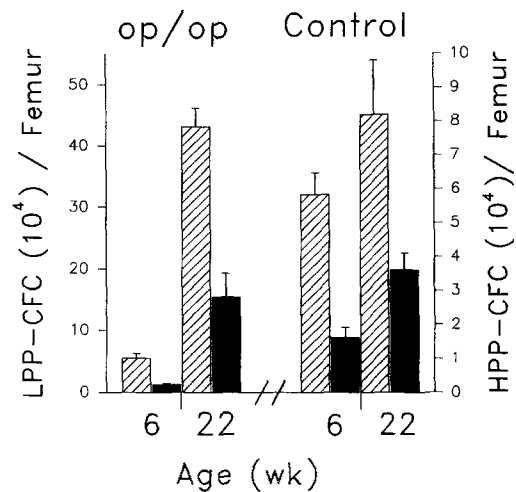


Figure 4. Total femoral LPP-CFC (hatched bars) and HPP-CFC (filled bars) content of young and old *op/op* mice.

Table 2. Progenitor Cell Incidence in Spleens of *op/op* and Control Mice

Progenitor	Age	CFC/2,500 cells	
		<i>op/op</i>	Control
	<i>wk</i>		
LPP	6	29 ± 3.6	6.8 ± 1.3
	27	6 ± 1.2	6.3 ± 1.1
HPP	6	1.8 ± 0.4	0.1 ± 0.05
	27	0.2 ± 0.1	0.1 ± 0.05

Values are means ± SEM of three replicate dishes for each of three mice.

vivo (21). There were no differences in the incidence of LPP-CFC or HPP-CFC in the marrow of *op/op* and control mice at any age examined (data not shown). However, the total femoral content of these progenitors (Fig. 4) reflected the age-related changes observed in marrow cellularity (Fig. 1). LPP-CFC and HPP-CFC levels in 6-wk-old *op/op* mice were ~10-fold less than that of control mice. By 22 wk of age, the femoral progenitor cell content in *op/op* and control mice was comparable (Fig. 4).

The diminished space for marrow hematopoiesis and the reduced hematopoietic activity in young *op/op* mice were correlated with an increase in splenic hematopoiesis. At 6 wk of age, the spleens of *op/op* mice were significantly enlarged, and the incidence and content of both LPP-CFC and HPP-CFC were significantly higher than those of control mice (Table 2, Fig. 5). By 22 wk of age, at a time when *op/op* marrow cellularity had normalized (Fig. 1), the incidence and content of progenitor cells in the spleen had declined to values equivalent to those in control mice (Table 3, Fig. 5). Neither

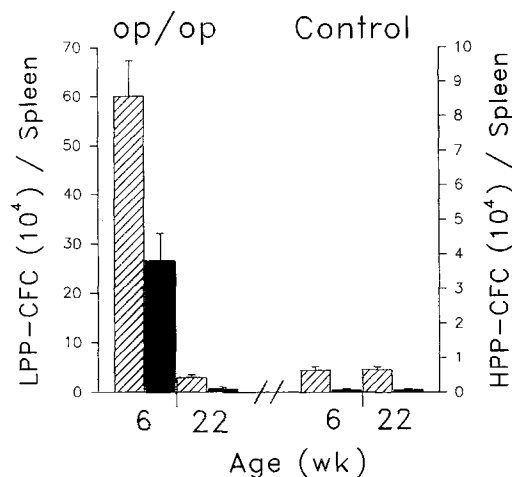


Figure 5. Total splenic LPP-CFC (hatched bars) and HPP-CFC (filled bars) content of young and old *op/op* mice.

progenitor cell type could be detected in the livers of normal or *op/op* mice at any age examined (3–27 wk) (data not shown).

Discussion

Previous studies of *op/op* mice, which are largely restricted to the biology of macrophages and osteoclasts in young mice, indicate that occlusion of the marrow cavity by bone is associated with a severe decrease in cellularity, and a deficiency of osteoclasts and mononuclear phagocytes (11, 12, 22–26). These findings were confirmed, however, it appears that the decrease in marrow cellularity is less severe than previously reported, and the osteopetrosis and hematopoiesis deficiencies in *op/op* mice are not permanent.

In contrast to previous studies of *op/op* mice up to 4 mo of age, indicating no increase in marrow cellularity or frequency of F4/80⁺ cells (25, 27), the present study showed a progressive age-related increase in marrow cellularity and macrophage content. This increase in cellularity was apparent by 8 wk, and continued until normal levels were reached by 22 wk. By 35 wk of age the frequency and total number of F4/80⁺ cells was also normal.

The normalization of marrow cellularity in *op/op* mice was associated with remodeling of the marrow cavity, reduction of bone matrix formation, and the appearance of resorptive pits on the femoral surface. During this period, there was a slow resolution of the osteopetrosis with the formation of a marrow space comparable to that seen in control mice. In agreement with our data, other workers (8, 10, 26) have also noted evidence of the beginnings of the formation of a femoral shaft in 6-wk-old *op/op* mice.

Variations in the data from different laboratories appear to be due to differences in bone sampling methodology and nutritional status of the mice. As to the latter, we have noticed that the combination of an enriched diet and early separation of *op/op* mice from their normal littermates greatly improves the rate of hematopoietic recovery.

Although there were comparable proportions of HPP-CFC and LPP-CFC in the marrow of young *op/op* and control mice, the absolute number of these progenitors was reduced. The normal frequency of LPP-CFC in *op/op* marrow from young mice was unexpected, since *in vitro* it has been shown that generation of LPP-CFC from HPP-CFC is CSF-1 dependent (16, 28). In *op/op* mice it is possible that both the rate of generation of LPP-CFC as well as their CSF-1-induced differentiation are slowed, resulting in the same concentration of LPP-CFC as in normal mice. Alternatively, the normal concentration of LPP-CFC (and HPP-CFC) could be due to the presence of a growth factor with a CSF-1-like effect.

Interestingly, during the period of suppressed marrow hematopoiesis in young *op/op* mice, there was an increase in splenic hematopoiesis and cellularity. Splenic granulocytopenia and megakaryocytopenia was elevated (data not shown), as well as the frequency and number of LPP-CFC and HPP-CFC.

Other studies have indicated that daily administration of CSF-1 to newborn *op/op* mice is required to prevent osteopetrosis and accompanying hematopoietic deficiencies (29).

However, our data suggest that the hematopoietic system has the capacity to use alternative mechanisms to compensate for the absence of CSF-1 in *op/op* mice. Two growth factors that may be involved, GM-CSF and IL-3, are normal or elevated in *op/op* mice (6). However, administration of GM-CSF to

young *op/op* mice does not resolve osteopetrosis (30). Investigations of possible compensatory mechanisms in *op/op* mice should contribute to our understanding of hematopoietic regulation and bone remodeling.

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