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MACROPHAGE COLONY STIMULATING FACTOR (M-CSF) IS ESSENTIAL FOR OSTEOCLAST FORMATION *IN VITRO*

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SUMMARY: The *op/op* mouse, in which the M-CSF gene is mutated, has greatly reduced numbers of macrophages and osteoclasts. We assessed the ability of M-CSF to induce osteoclast and macrophage formation in *op/op* hemopoietic cells *in vitro*. Osteoclast production was undetectable in *op/op* cell cultures, but was restored by M-CSF at concentrations approximately an order of magnitude higher than those that induced macrophages. In normal hemopoietic tissue M-CSF similarly increased macrophage numbers, but inhibited osteoclast formation. Despite cure of the macrophage defect, neither interleukin 3 nor granulocyte-macrophage CSF were able to induce osteoclastic differentiation in *op/op* cells. The results suggest that M-CSF induces osteoclastic differentiation but that macrophages, which are also induced by M-CSF, suppress osteoclast differentiation. Macrophages induced by other cytokines seem unable to contribute to osteoclast-formation.

The *op/op* variant of murine osteopetrosis is a recessive mutation characterised by impaired bone resorption due to a lack of osteoclasts (1). It has been shown that M-CSF is absent from *op/op* mice (2, 3) due to a mutation in the coding region of the M-CSF gene (4, 5). The osteoclastic defect can be corrected *in vivo* by administration of M-CSF (6, 7).

The mononuclear phagocyte system, the main target cell system for M-CSF (8) is also severely deficient in *op/op* mice, suggesting that this lineage may be directly or indirectly responsible for osteoclast formation. However, the relationship between the bone disorder, the macrophage deficiency and M-CSF remains uncertain. In two animals Kodama *et al* (7) found cure of osteopetrosis without monocyte recovery. However, monocyte numbers may not be a reliable guide to macrophage production, since monocytes will be depleted from blood by deficient target tissues. The half-life of M-CSF is measured in hours, *in vivo* (9), but despite this Felix *et al* (6) found that very large quantities of M-CSF were required for cure *in vivo*. Wiktor-Jedrzejczak *et al* (5) found that intraperitoneal implantation of L-cells in diffusion chambers induced many peritoneal macrophages, and restored circulating levels of M-CSF to near normal, but did not cure the osteopetrosis. The data from *in vivo* experiments do not distinguish between a physiological role for M-CSF in the induction of osteoclasts, and other possibilities such as an action by M-CSF as a weak agonist for an alternative product of the M-CSF gene, which is known to undergo alternative splicing (10), or the correction of some indirect disturbance caused by M-CSF deficiency (for example, *op/op* animals have increased levels of GM-CSF (5) which suppresses osteoclast formation (11)).

To determine the extent to which osteoclast-induction by M-CSF represents a physiological mechanism, it is necessary to assess the sensitivity of osteoclast formation to the presence of M-

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CSF. We therefore assessed the ability of M-CSF to induce osteoclast and macrophage formation from hemopoietic tissue of *op/op* animals *in vitro*. Because *op/op* animals do not have a marrow cavity, spleen cells were used as a source of hemopoietic cells, and calvarial cells substituted for bone marrow stromal cells to form an osteoclast-inductive microenvironment (12).

MATERIALS AND METHODS

Hepes-buffered medium 199 (Flow, Irvine, UK) was used for cell isolation. Incubations were performed in Eagle's Minimum Essential Medium (MEM), (Gibco, Paisley, UK) supplemented with glutamine (2mM) benzylpenicillin (100 IU/ml), streptomycin (100μg/l) and 10% fetal bovine serum (FBS) (all Gibco). All incubations were in 5% CO_2 in humidified air. 1,25$(OH)_2$ vitamin D_3 (1,25$(OH)_2D_3$) was supplied by Duphar, da Weesp, Holland. Salmon calcitonin (CT) was a gift from Sandoz (Basel, Switzerland). Murine recombinant GM-CSF, interleukin 3 (IL3) and human recombinant M-CSF was donated by Dr S Clark, Genetics Institute, Cambridge, USA. The colony forming ability of M-CSF was determined by semi-solid colony forming assay to be half-maximal at 0.5ng/ml. *op/op* mice and normal (+/?) littermates were obtained from the Jackson Laboratories (Bar Harbor, Maine). Bone slices (2x2x0.1mm) were prepared as previously described from cortical bovine bones (13). F4/80; a macrophage-specific monoclonal antibody, was supplied by Dr S Gordon, Oxford, UK. Antiserum to GM-CSF were donated by Dr G Seelig (Schering-Plough, Beaconsfield, NJ).

Preparation of stromal cells

Calvaria from *op/op* and +/? mice were dissected free of adherent tissue and incubated in collagenase (1mg/ml) (Sigma, Poole, Dorset, UK) for 1.5hr at 37°. The cell suspension was washed twice in medium 199 and incubated in 75cm^2 tissue culture flasks (Falcon, NJ). After 5 days cells were removed from the flasks by trypsin/EDTA (Gibco), and resuspended in MEM/FBS at 5x10^4 cells/ml. 200μl of this suspension was added to the wells of a 96-well plate (Falcon) containing a bone slice and a 6mm thermanox coverslip (Lux, Flow), before incubation overnight. Spleen stromal cells were prepared by mechanically disaggregating spleens from 10-14 day old mice, by repeated passage through 21-gauge needles. The suspension was washed twice and incubated for 5 days as described above.

Preparation of hemopoietic cells and co-culture with stromal cells

Spleen cell suspensions were prepared from *op/op* mice as above, and added (1x10^6 cells/ml) to wells containing either *op/op* or +/? stromal cell layers with or without 1,25$(OH)_2D_3$, in the presence or absence of M-CSF, IL-3, GM-CSF, or a specific neutralising antibody against murine GM-CSF ($^1/_{100}$ dilution) for 14 days.

Cells from *op/op* long bones were mechanically disaggregated, and incubated with 1,25$(OH)_2D_3$ in the presence or absence of M-CSF for 14 days.

Bone marrow cells were obtained by flushing out the long bones of +/? adult mice with medium 199. A single-cell suspension was prepared by repeated passage through 21-G needles. The suspension was washed twice and resuspended in MEM/FBS at 2x10^6 cells/ml. The suspension was added (200μl/well) to a 96-well plate containing a bone slice and coverslip, incubated with or without 1,25$(OH)_2D_3$ in the presence or absence of M-CSF for 14 days.

Phenotypic assessment

Cells were removed from the bone slices by immersion in 10% NaOCl (BDH, Poole, Dorset, UK) for 10 min. The bone slices were then washed, dehydrated in ethanol, sputter-coated in gold and the entire surface of each bone slice was examined for evidence of bone resorption in a Cambridge S90 scanning electron microscope (SEM) (Cambridge Instruments, Cambridge, UK).

Differentiation of calcitonin receptors (CTR) was assessed by ^{125}I-labelled salmon CT as previously described (14). Salmon CT was iodinated by a modification of the chloramine T method (15) as previously described (14). Labelled CT (0.2nM) was incubated with cultures in medium 199 containing 0.1% bovine serum albumin (Sigma) for 1hr at 22°C. Non-specific binding was assessed by including excess (300nM) unlabelled CT in some wells. After incubation, coverslips were fixed in formalin before extensive washing in water. The coverslips were coated with K5 nuclear emulsion (Ilford, Ilford, UK), developed after 14 days at 4°C, and counterstained with Meyer's haematoxylin. CT receptor-positive (CTRP) cells were scored as

those that demonstrated sufficient grain density to clearly outline the cells. Macrophage differentiation was identified in glutaraldehyde-fixed preparations by binding to F4/80, using a standard immunoalkaline phosphatase staining technique (rat anti murine IgG; rabbit anti-rat IgG-alkaline phosphatase conjugate; both Serotec, Oxford, UK) followed by May-Grunwald Giemsa staining.

RESULTS

No bone resorption, and no F4/80-positive cells were seen in co-cultures of *op/op* calvarial cells with *op/op* spleen cells. Parallel co-cultures of +/? calvarial cells and *op/op* spleen showed extensive resorption of bone slices and macrophage formation (see Table 1). M-CSF induced osteoclast and macrophage formation in co-cultures of *op/op* calvaria and *op/op* spleen cells (see Table 1 and Figure1), in a dose-dependent manner, with 5-10-fold greater responsiveness for formation of macrophages than for induction of resorptive function by M-CSF. Although macrophage formation was restored to normal levels, bone resorption was significantly less than was observed in cultures of +/? bone marrow cells (see Table 1). M-CSF similarly induced osteoclast and macrophage differentiation in cultures of *op/op* long bones (see Table 1).

In experiments in which *op/op* calvarial cells were replaced by *op/op* spleen stromal cells, and co-cultured with *op/op* spleen cell suspensions, M-CSF induced macrophagic differentiation similar to that observed in *op/op* calvarial cell-*op/op* spleen cell co-cultures, but no osteoclastic differentiation was detected (Table 1).

Table 1. Effect of M-CSF, GM-CSF and anti GM-CSF antiserum on the generation of resorptive function and macrophages from cells from *op/op* and +/? mice

Cell types incubated (no. of bone slices/coverslips per variable)		Cytokine (ng/ml)	F4/80 positive cells/mm^2 (±SEM)	Bone resorption (per cent of plan area resorbed ± SEM)
+/? calvarial + *op/op* spleen	(5)	0	112 ± 36	7.1± 2.6
op/op calv + *op/op* spleen	(6)	0	0	0
		M-CSF 50	740 ± 129	1.5± 0.8
op/op long bone	(5)	0	0	0
		M-CSF 50	513 ± 117	2 ± 0.8
		5	24 ± 24	0.7± 0.2
op/op spleen stroma + *op/op* spleen cells	(12)	0	0	0
		M-CSF 500	715 ± 196	0
		50	298 ± 155	0
		5	62 ± 31	0
		0.5	18 ± 3	0
		GM-CSF 10	215 ± 35	0
op/op calvaria + *op/op* spleen cells	(12)	0	0	0
		GM-CSF 50	322 ± 107	0
		10	498 ± 276	0
		1	ND	0
		+ anti GM-CSF	ND	0

ND = not done.

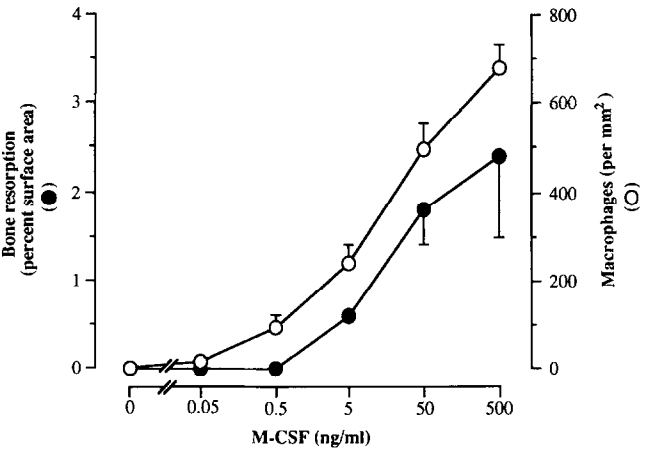

Figure 1.
Bone resorption and macrophage formation by co-cultures of *op/op* calvarial cells and *op/op* spleen cells incubated in the presence of M-CSF and $1,25(OH)_2D_3$ for 14 days. Mean ± SEM of 5 experiments, each with 5 cultures per variable.

GM-CSF induced macrophage differentiation in *op/op* calvarial cell-*op/op* spleen cell co-cultures but we observed no evidence of osteoclastic differentiation (Table 1). Antiserum to GM-CSF, which enhances osteoclast formation in cultures of bone marrow from normal mice (16), did not induce osteoclastic differentiation. IL3, despite induction of similar numbers of F4/80 positive cells, also did not induce resorption (data not shown).

Similar to our previous observations, we found that M-CSF inhibited osteoclast formation in bone marrow from +/? mice, although macrophage formation was stimulated (Table 2). We also found inhibition of resorptive function by M-CSF in co-cultures of +/? calvarial cells and *op/op* spleen cells, while in the same experiment *op/op* calvarial cells co-cultured with *op/op* spleen cells showed induction of osteoclastic function by M-CSF (Table 2).

Table 2. Effect of M-CSF (50ng/ml) on macrophage production, CTRP cell production and resorptive function in hemopoietic cultures after 14 days of incubation

	+/? bone marrow			+/? calv +*op/op* spl	*op/op* calv + *op/op*spl
	F4/80 positive cells/mm²	CTRP cells/mm²	% bone surface resorbed	% bone surface resorbed	
Control	464 ± 200	0	0	ND	0
M-CSF	904 ± 186	0	0	ND	0
$1,25(OH)_2D_3$	374 ± 152	24 ± 7	28 ± 8	2.0 ± 0.7	0
$1,25(OH)_2D_3$ + M-CSF	624 ± 126	3 ± 2	1.4 ± 0.7	0.4 ± 0.2	1.5 ± 0.8

n = 6 for each variable.

DISCUSSION

We have found that both macrophages and osteoclasts are severely deficient in cultures of *op/op* hemopoietic tissue, but that both can be induced by the addition of M-CSF. The sensitivity of both osteoclasts and macrophages to induction by M-CSF suggests that the product of the M-CSF gene which accounts for the osteoclastic and macrophage deficiency is likely to be M-CSF itself.

Although M-CSF cured the macrophage defect in spleen cell cultures, osteoclast formation required the additional presence of calvarial cells. This result is consistent with previous studies which have shown that contact with calvarial or bone marrow stromal cells is required for osteoclastic differentiation, and that other types of stromal cells, including spleen stromal cells, do not support osteoclast formation from hemopoietic precursors (12, 17). Our results also show, because macrophage formation was similar for spleen and calvarial stromal cells, that osteoclast formation was not attributable to generally-superior hemopoiesis in cultures with calvarial cells, but was due to an additional osteoclast-inductive signal which, with M-CSF, induces osteoclast formation. The osteoclast-inductive capacity of tissues clearly differs. In the current experiments bone marrow was superior to calvarial cells in osteoclast-induction. However, the similar ability of *op/op* calvarial cells and +/? calvarial cells to generate osteoclasts, in the presence of M-CSF, suggests that M-CSF has restored the capacity of *op/op* cultures to generate osteoclasts to normal.

The similar direction of effect of M-CSF on osteoclastic and macrophagic induction contrasts with the inverse relationship observed in normal hemopoietic cultures, where very small concentrations of M-CSF strongly inhibit osteoclast formation (11, 16). This was originally attributed to lineage divergence of shared precursors by M-CSF towards macrophages, but this view is not consistent with the similar sensitivities of macrophages and osteoclasts to induction by M-CSF seen in the current experiments. The observation is more in keeping with the suggestion (18) that macrophages produce an inhibitor of osteoclastic differentiation, and that M-CSF, which increases not only the number but the functional activity of macrophages (see 19), increases the osteoclast-suppressive activity of macrophages, which are likely to be present throughout the incubation period in cultures of normal bone or bone marrow cells, but are present only after a delay in *op/op* hemopoietic cultures. This view is also consistent with our experiments, in which, although M-CSF restored macrophage numbers to those seen in cultures of normal hemopoietic tissue, bone resorption remained suboptimal. Inhibition of osteoclast formation by GM-CSF in both normal (11, 16) and M-CSF-cured *op/op* cell cultures is also consistent with this explanation. We are currently investigating the mechanism by which macrophages inhibit osteoclast formation, a process that may be crucial in lineage regulation.

It is intriguing that neither GM-CSF nor IL3 induced osteoclast formation, despite macrophage induction. Together with the inhibitory effect of macrophages, this makes an accessory cell role for macrophages less likely. It also suggests that if M-CSF generates osteoclasts through the provision of mononuclear phagocytes as osteoclastic precursors, then the macrophages induced by GM-CSF and IL3 cannot substitute.

M-CSF receptors are not limited to the MPS. They have also been detected on placental epithelial cells, and hemopoietic stem cells (20) and on cells that form granulocyte colonies (21). Thus, whether the osteoclast derives from the MPS, or from some other, related or unrelated cell type that also possesses M-CSF receptors, remains uncertain.

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