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Activin A Is an Essential Cofactor for Osteoclast Induction

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Recently, receptor activator of NF-κB ligand (RANKL) was shown to be necessary for osteoclast formation. We now report that activin A, a cytokine enriched in bone matrix and secreted by osteoblasts and osteoclasts, powerfully synergized with RANKL for induction of osteoclast-like cells (OCL) from bone marrow precursors depleted of stromal cells. Moreover, OCL formation in RANKL was virtually abolished by soluble type II A activin receptors (ActR-II(A)), suggesting that activin A is essential for OCL formation. Activin A was most effective when precursors were exposed to RANKL and activin A simultaneously: resistance to OCL-induction that occurs when precursors are pre-incubated in M-CSF was reduced. Incubation on bone matrix also enhanced the sensitivity of precursors to OCL-induction by RANKL; and this was prevented by soluble ActR-II(A). Thus, activin A in bone matrix, or released from osteoblastic or other cells, enhances the osteoclast-forming potential of precursors and synergizes with RANKL in inducing osteoclastic differentiation.

Key Words: **RANKL; osteoclast; activin A; bone resorption.**

The osteoclast is the cell that resorbs bone. Osteoclasts form from a precursor shared with the macrophage, which differentiates into osteoclasts when precursors are incubated in contact with osteoblastic or bone marrow stromal cells (1, 2). It was recently found that receptor activator of NF-κB ligand (RANKL) (also called ODF, OPGL and TRANCE), originally identified as a member of the tumor necrosis factor (TNF) family that stimulates dendritic cells (3–5), is expressed by osteoblasts and substitutes for stromal cells in osteoclast formation and activation (6–10).

Osteoprotegerin (OPG) has also been identified as a novel soluble member of the TNF receptor family that binds to RANKL. This protein inhibits osteoclast formation and bone resorption *in vitro,* and appears to be a decoy receptor (6, 11–13). However, while hepatic over-expression of OPG in transgenic mice suppresses bone resorption in many sites, bone modeling and tooth eruption, both of which require bone resorption, are apparently unaffected (12). Moreover, while genetically modified soluble RANKL can substitute for stromal cells in osteoclastogenesis, only a minor proportion of precursors incubated in RANKL develop into osteoclasts, and the osteoclasts that do form are transient (14). Also, when hemopoietic cells are incubated in RANKL with M-CSF in semisolid cultures, osteoclasts develop in colonies mixed with macrophages (10, and personal observation). This suggests that the sensitivity of precursors to RANKL might be substantially modified in some sites by factors that synergize with RANKL. Responses to stimuli from the TNF superfamily are characteristically associated with critical inputs from cofactors.

There is evidence that activin A, a member of the transforming-growth factor (TGF)-β superfamily originally identified as an erythroid differentiation factor (15) and a protein that enhances follicle-stimulating hormone secretion in cultured pituitary cells (16, 17), is involved in bone cell physiology. Not only is activin A synthesized by bone marrow stromal cells, osteoblasts and osteoclasts, but it can also be extracted from bone matrix (18–22). Activin A has potent effects on bone cells *in vitro* and *in vivo. In vitro,* activin A stimulates the proliferation of osteoblastic cells, enhances matrix secretion by these cells (23, 24) and modulates chondrogenesis (25–27). *In vivo,* periosteal injection of activin A stimulates bone formation (28), ectopic bone formation induced by BMP is enhanced by activin A (18) and local administration of activin promotes fracture healing (29).

Activin A may also affect bone resorption. It has been reported to enhance osteoclast formation in organ culture and in murine bone marrow cultures incubated in the presence or absence of $1,25(OH)_2D_3$ or parathyroid hormone (30, 31). Moreover, activin A gene expression and secretion by bone marrow stromal cells is upregulated by bone-resorbing cytokines, including TNF-α and interleukin-1α (21, 32, 33).

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The target cell for the action of activin A on osteoclast formation has not been identified. The systems previously used for the analysis of osteoclastic responsiveness are complex: cells expressing RANKL have needed to be present, exposing cultures to the potential for indirect or confounding influences. Indeed activin A has effects on both the osteoblastic/bone marrow stromal cells that stimulate osteoclast formation and resorption, and on immature cells of the mononuclear phagocyte system from which osteoclasts derive (34–36). We therefore exploited the opportunity provided by the availability of soluble recombinant RANKL, to analyze the role of activin A in osteoclastic differentiation from hemopoietic precursors.

MATERIALS AND METHODS

Media and reagents. Cells were incubated in minimum essential medium (MEM) with Earles' salts, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml benzylpenicillin, and 100 μg/ml streptomycin (all Imperial Laboratories, Andover, Hants, UK). Recombinant human M-CSF was provided by Genetics Institute (Cambridge, MA); soluble recombinant human RANKL was from Insight Biotechnology (Wembley, Middlesex, UK); recombinant human activin A, soluble recombinant type IIA activin receptors (ActR-II(A)) and recombinant human inhibin A were from R & D (Abingdon, Oxon, UK). All incubations were performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

Isolation and culture of bone marrow precursors. Bone marrow cells were isolated from 5- to 8-week-old MF1 mice as previously described (14). Mice were killed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out into a petri dish by slowly injecting phosphate-buffered saline (PBS) (Imperial) at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single cell suspension. The bone marrow cells were washed, resuspended in MEM containing 10% FBS, and incubated for 24 h in M-CSF (5 ng/ml) at a density of 3×10^5 cells/ml in a 75 cm^2 flask (Helena Biosciences, Sunderland, Tyne & Weir, UK). After 24 h, nonadherent cells were harvested, washed and resuspended (3×10^5/ml) in MEM-FBS. This suspension was added (100 μl/well) to the wells of 96-well plates (Helena Biosciences) containing a 6 mm Thermanox coverslip (Gibco BRL, Paisley, UK) or a slice of bovine cortical bone (37). To each of these wells an additional 100 μl medium containing M-CSF, RANKL, and/or cytokines or soluble receptors were added. Cultures were fed every 2–3 days by replacing 100 μl of culture medium with an equal quantity of fresh medium and reagents. Absence of contaminating stromal cells was confirmed in cultures in which M-CSF was omitted. Such cultures showed no cell growth. Coverslips and bone slices were assessed for tartrate-resistant acid phosphatase (TRAP) or bone resorption as previously described (14).

Statistical analysis. Differences between groups were analyzed using unpaired Student's t-test.

RESULTS

Activin A induced a substantial increase in the number and degree of multinuclearity of TRAP-positive osteoclast-like multinuclear cells (OCL) formed in response to soluble recombinant RANKL in non-adherent M-CSF-dependent bone marrow cells (Fig. 1). Precursors incubated on bone slices also showed augmentation by activin A of RANKL-induced resorptive function (Fig. 1). Addition of activin A at 10 ng/ml significantly reduced total cell numbers in cultures incubated with M-CSF + RANKL; lower concentrations of activin A were without effect (total number of nuclei/cm^2 (mean ± SEM, $\times 10^{-3}$): M-CSF + RANKL, 51.2 ± 1.7; M-CSF + RANKL + activin A (10 ng/ml), 43.6 ± 1.2*; *$p < 0.01$ vs M-CSF + RANKL). Activin A did not itself induce OCL, but enhanced the ability of RANKL to do so (Fig. 2). This augmentation was substantial on plastic. Although precursors incubated on bone slices also showed some augmentation by activin A of RANKL-induced OCL formation, basal levels of OCL were higher on bone and the extent of stimulation by activin A was less than that seen on plastic (Fig. 2). These effects of activin A do not seem to be mediated by stroma since incubating bone marrow cells at low density in M-CSF for 24 h depletes the cell preparations of stroma (14). Activin A thus powerfully synergizes with RANKL in OCL-induction through a direct effect on precursors.

Activin A is present in serum and is expressed by bone marrow monocytes, macrophages and osteoclasts (22, 38–41). Therefore, to test whether 'basal' OCL formation by RANKL in cultures to which no activin A was added could be attributed to these sources, precursors were incubated in RANKL, with or without soluble ActR-II(A). The soluble receptors neutralized the effects of exogenous activin A on OCL and macrophage numbers (Fig. 3). The soluble receptors also virtually abolished OCL formation by RANKL, suggesting that activin A is an important co-factor for osteoclast formation. This could not be attributed to any toxic effect of soluble ActR-II(A), since macrophage numbers did not show a similar reduction (Fig. 3).

Although activin A strongly potentiated osteoclast formation on plastic substrates, we have noted that its effects were less marked on bone slices. Furthermore, the number of OCL induced on bone slices by RANKL alone was far greater than that seen on plastic coverslips under the same conditions. Given that activin A may be an important cofactor for RANKL and because activin A is enriched in bone matrix, it seemed possible that the bone slices were substituting as a source of activin A in these cultures. To test this we incubated precursors in M-CSF (30 ng/ml) and RANKL (10 ng/ml) on bone slices for 6 days, with or without soluble ActR-II(A) (2.5 μg/ml). The number of OCL formed by RANKL was suppressed by soluble receptors (OCL/cm^2 (mean ± SEM, $\times 10^{-2}$): M-CSF + RANKL, 11.6 ± 1.0; M-CSF + RANKL + ActR-II(A), 3.4 ± 0.6*; *$p < 0.01$ vs M-CSF + RANKL; 8 bone slices per treatment).

We have previously noted that precursors become resistant to OCL-induction by RANKL when preincubated in M-CSF (14). Maturation of macrophages to an

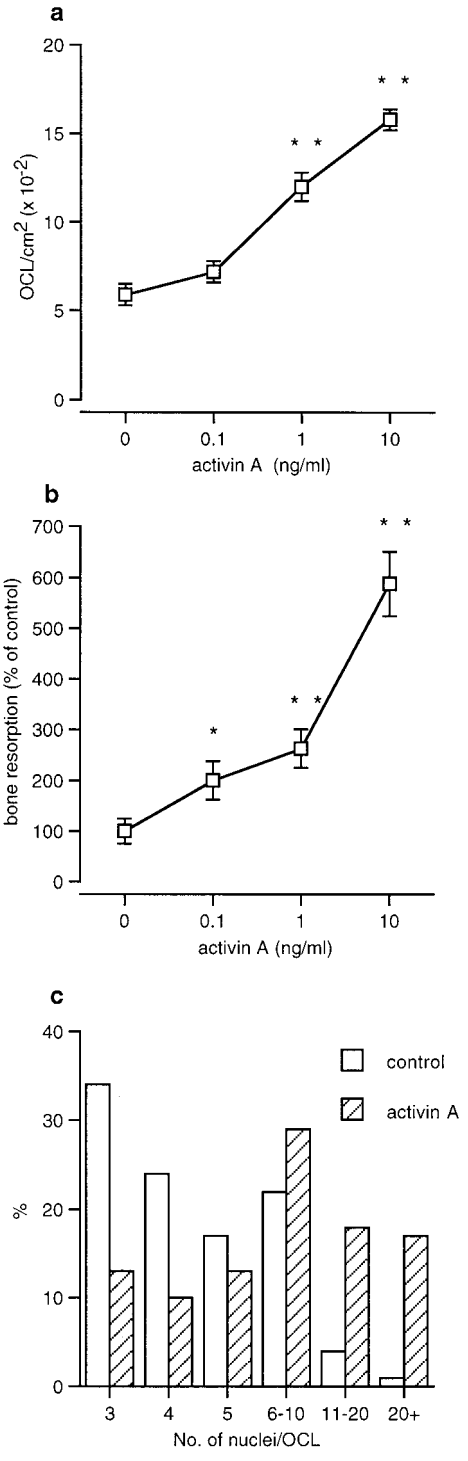

FIG. 1. Activin A enhances osteoclast formation from bone marrow cells. (a) Non-adherent M-CSF-dependent bone marrow cells were incubated on plastic coverslips in soluble RANKL (10 ng/ml), M-CSF (30 ng/ml) and activin A for 6 days. n = 10 cultures per variable. Data shown as mean ± SEM. (b) Bone resorption in parallel cultures incubated for 12 days on bone slices. n = 8 cultures per variable. Data shown as mean ± SEM. (c) Distribution of nuclei per OCL in cultures incubated in RANKL (10 ng/ml) and M-CSF (30 ng/ml) with vs without activin A (10 ng/ml) for 6 days on plastic coverslips. % of total cells in each group that had a particular nuclear number. n = 200 cells per treatment. *$p < 0.05$; **$p < 0.01$ vs cultures incubated without activin A.

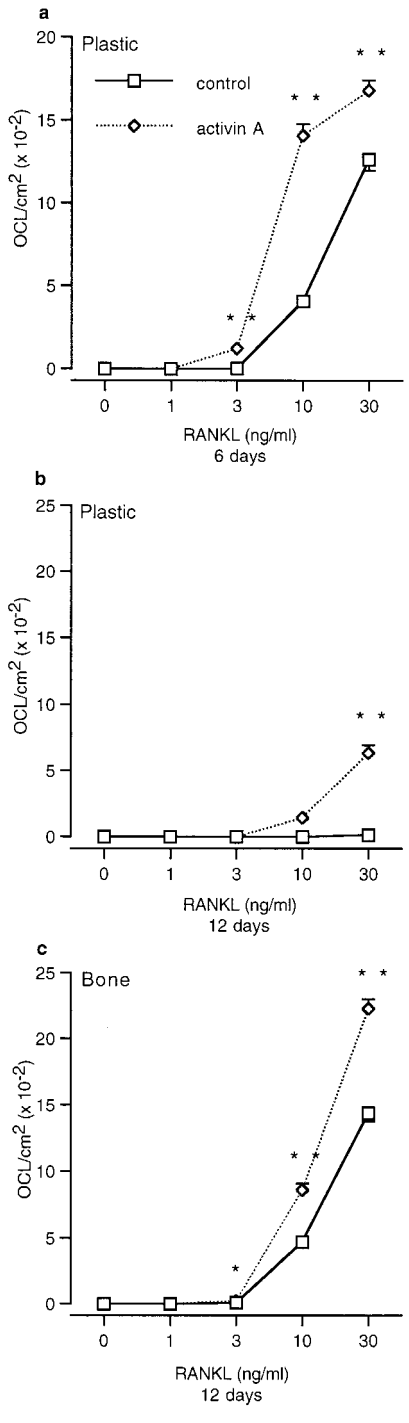

FIG. 2. Activin A synergizes with RANKL in osteoclast induction. Bone marrow cells were incubated on plastic coverslips (a & b) or bone slices (c) in M-CSF (30 ng/ml) with or without RANKL and activin A (10 ng/ml) for 6 or 12 days. n = 12 cultures per variable. Data shown as mean ± SEM. *$p < 0.05$; **$p < 0.01$ vs control cultures.

unresponsive state occurs for other cytokines also. We therefore tested the extent to which activin A might facilitate osteoclast induction through suppression of the maturation step in macrophages. While precursors

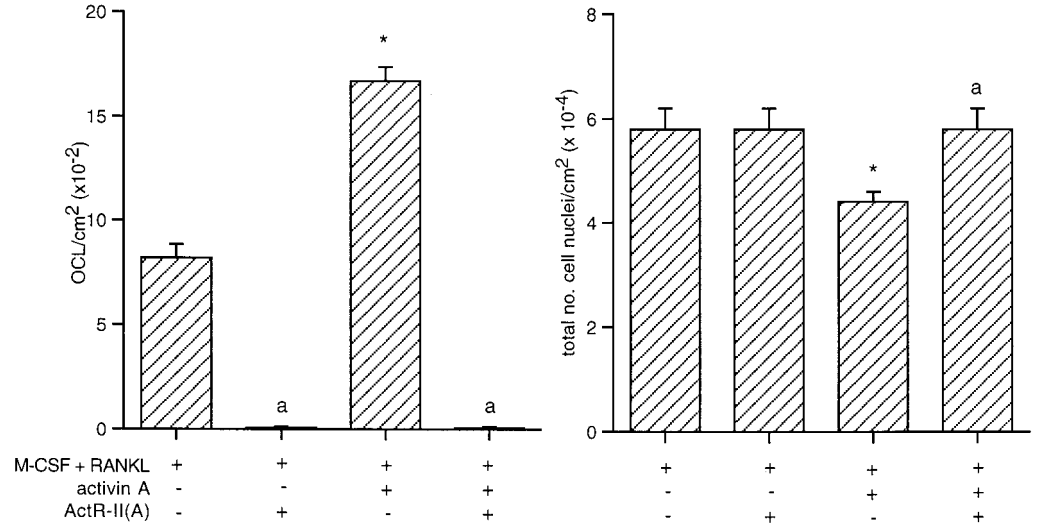

FIG. 3. OCL formation and total number of nuclei formed in cultures of bone marrow cells incubated with RANKL (10 ng/ml), M-CSF (30 ng/ml) and ± activin A (10 ng/ml) and soluble ActR-II(A) (2.5 μg/ml) for 6 days. n = 8 cultures per variable. Data shown as mean ± SEM. *$p < 0.01$ vs control cultures; [a]$p < 0.01$ vs no soluble receptors.

preincubated in M-CSF alone were able to produce very few OCL, activin A dramatically increased the proportion of cells that developed osteoclastic characteristics when added with RANKL for the last 6 days (Table 1). Furthermore, OCL formation was similar when activin A was added in the last 6 days to cultures preincubated in M-CSF or M-CSF and RANKL. OCL production was also enhanced in cultures preincubated in M-CSF and activin A. However, OCL formation in these cultures was only restored to the level seen in cultures incubated in M-CSF, RANKL and activin A throughout, when activin A was additionally present during the second period of incubation (Table 1). This suggests that although activin A may enhance the sensitivity of precursors to subsequent RANKL exposure, its major effect is as a synergist with RANKL. This conclusion is supported by the observation that, when precursors were preincubated in M-CSF with vs without soluble ActR-II(A) for 7 days on bone slices or plastic coverslips before washing and incubating with RANKL for a further 6 days, OCL formation was unaffected (Table 2).

Finally, activin and inhibin often exert opposing effects. We found that inhibin A (up to 100 ng/ml) had no effect on the number of OCL formed from precursors incubated in RANKL (data not shown).

DISCUSSION

Recently, RANKL has been found to be necessary for osteoclast formation. We have found that activin A, a cytokine generated by bone marrow stromal cells, osteoblasts and osteoclasts and abundant in bone matrix, strongly synergizes with RANKL in osteoclastogenesis and causes increased fusion. Inhibin A, a protein that often has opposing actions to activin A, was without effect. Although activin A was unable to induce osteoclast formation in the absence of RANKL, production of OCL in cultures without exogenous activin A was virtually abolished by soluble receptors to activin A, suggesting that activin A may be an essential costimulator for osteoclast formation.

Although activin A has been known for some time to have effects on bone cell physiology, its effects on osteoclast precursors are unclear. This is because the experimental systems used previously have been dependent upon the presence of RANKL-expressing cells, introducing the potential for indirect or confounding influences. Although previous studies have found that activin A promotes osteoclast formation *in vitro* (30, 31) and activin is known to induce the monocytic differentiation of the human promyelocytic leukemia cell line HL-60 (42), it also has actions on cells of the osteoblastic lineage (23, 24): these cells are known to be important in osteoclast differentiation. We have now shown that activin A acts directly on precursors to enhance osteoclast-differentiation.

The mechanism by which activin A synergizes with RANKL in osteoclast induction is uncertain. It is unlikely to act by inducing proliferation of osteoclast precursors, since we found high concentrations of activin A suppressed total cell numbers in cultures incubated in M-CSF and RANKL. We have previously noted that bone marrow macrophages become resistant to OCL-induction by RANKL during culture (14). This is consistent with the ability of M-CSF to induce terminal macrophage differentiation in mononuclear phagocytes (43–45). Maturation of mononuclear phagocytes to an

unresponsive state occurs for other cytokines also. We found that pre-incubation of precursors in activin A before RANKL-addition did sensitize the precursors to RANKL action. However, the co-stimulatory activity and synergy of osteoclast-forming potential are unlikely to be explained through suppression by activin A of macrophage commitment alone, since this early action was less marked than that seen when activin A was incubated simultaneously with RANKL.

Although activin A strongly potentiated osteoclast formation on plastic substrates, we noticed that its actions were less marked when precursors were incubated on bone slices. We also noted that the numbers of osteoclasts formed on bone was substantially greater than on plastic. Because activin A is enriched in bone matrix, it seemed possible that the bone slices were substituting as a source of activin A in these cultures. This interpretation is consistent with soluble ActR-II(A) abrogating the ability of bone to enhance osteoclastic differentiation.

Bone is not a static tissue, but is continuously remodeled throughout life. It would seem appropriate if one of the roles of activin A in bone matrix were to facilitate the differentiation of the cell responsible for its removal. This would provide a model in which the optimal conditions for osteoclast formation are RANKL expression by osteoblastic cells and the activin A present in bone. The activin A might be released from bone by osteoclast precursors, which have been shown to release growth factors from extracellular matrix during migration (46), or by mature osteoclasts during resorption: activin A has been detected immunohistochemically in osteoclastic lacunae (22). In addition, activin A secreted by osteoblasts, osteoclasts or their precursors may contribute to osteoclastic differentiation.

TABLE 1

Activin A Maintains Sensitivity of Precursors to RANKL, and Synergises with RANKL in OCL Formation

Incubation conditions			OCL per cm^2 (mean ± SEM)
M-CSF + RANKL			63 ± 17
M-CSF + RANKL + activin A			1703 ± 59*
7 days in:		*6 days in:*	
M-CSF	then	M-CSF RANKL	9 ± 5
M-CSF	then	M-CSF RANKL activin A	561 ± 47[a,b]
M-CSF activin A	then	M-CSF RANKL	194 ± 26[a]
M-CSF activin A	then	M-CSF RANKL activin A	1358 ± 83[b]
M-CSF RANKL	then	M-CSF RANKL activin A	477 ± 50*

Note. OCL formation by bone marrow cells incubated for 13 days in M-CSF (30 ng/ml), RANKL (10 ng/ml) with vs without activin A (10 ng/ml), compared with OCL formation from bone marrow cells incubated for 7 days in M-CSF in the presence or absence of activin A or RANKL before removal of medium and replacement with M-CSF and RANKL ± activin A. n = 12 cultures per variable. *$p < 0.01$ vs M-CSF + RANKL d 0–13; [a]$p < 0.01$ vs group incubated in M-CSF alone in the first period and M-CSF + RANKL in the second period; [b]$p < 0.01$ vs group incubated in M-CSF + activin A in the first period and M-CSF + RANKL in the second period.

TABLE 2

Activin A is Most Effective When Precursors Are Simultaneously Exposed to RANKL

Incubation conditions			OCL per cm^2 (mean ± SEM)	
			Bone	Plastic
7 days in:		*6 days in:*		
M-CSF	then	M-CSF RANKL	1006 ± 66	40 ± 13
M-CSF ActR-II(A)	then	M-CSF RANKL	1088 ± 75	28 ± 9

Note. OCL formation by bone marrow cells incubated for 7 days in M-CSF (30 ng/ml) in the presence or absence of soluble ActR-II(A) (2.5 μg/ml) before removal of medium and replacement with M-CSF and RANKL. n = 8 cultures per variable.

In conclusion, our results show that while RANKL is necessary for osteoclast differentiation and activation, activin A strongly synergizes with RANKL in osteoclast formation and may be essential. Synergy with activin A and similar cofactors might provide for diversity and complexity in the cellular and hormonal regulation of bone resorption, such that regulatory inputs from morphogenetic, mechanical, calcium-regulating and inflammatory stimuli can be made.

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