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Recruitment, augmentation and apoptosis of rat osteoclasts in $1,25\text{-}(OH)_2D_3$ response to short-term treatment with 1,25-dihydroxyvitamin D_3 *in vivo*

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Abstract

Background: Although much is known about the regulation of osteoclast (OC) formation and activity, little is known about OC senescence. In particular, the fate of of OC seen after $1,25\text{-}(OH)_2D_3$ administration *in vivo* is unclear. There is evidence that the normal fate of OC is to undergo apoptosis (programmed cell death). We have investigated the effect of short-term application of high dose 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$) on OC apoptosis in an experimental rat model.

Methods: OC recruitment, augmentation and apoptosis was visualised and quantitated by staining histochemically for tartrate resistant acid phosphatase (TRAP), double staining for TRAP/ED1 or TRAP/DAPI, *in situ* DNA fragmentation end labelling and histomorphometric analysis.

Results: Short-term treatment with high-dose $1,25\text{-}(OH)_2D_3$ increased the recruitment of OC precursors in the bone marrow resulting in a short-lived increase in OC numbers. This was rapidly followed by an increase in the number of apoptotic OC and their subsequent removal. The response of OC to $1,25\text{-}(OH)_2D_3$ treatment was dose and site dependent; higher doses producing stronger, more rapid responses and the response in the tibiae being consistently stronger and more rapid than in the vertebrae.

Conclusions: This study demonstrates that (1) after recruitment, OC are removed from the resorption site by apoptosis (2) the combined use of TRAP and ED1 can be used to identify OC and their precursors *in vivo* (3) double staining for TRAP and DAPI or *in situ* DNA fragmentation end labelling can be used to identify apoptotic OC *in vivo*.

Background

1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$), the major pharmacologically active metabolite of vitamin D_3 plays a central role with regards to calcium homeostasis. It is known induce bone resorption in mouse and rat bone *in vitro*[20] and stimulates bone resorption *in vivo* in both intact and parathyroidectomized animals [42,35]. In short-term organ cultures of bone, the major effect of $1,25\text{-}(OH)_2D_3$ appears to be an increase in osteoclastic activity [20], without increasing osteoclast (OC] number. This

stimulation of activity is unlikely to be a direct effect as mature OC do not express the vitamin D receptor (VDR] [30] and in isolated OC systems, no direct effects of 1,25-$(OH)_2D_3$ on bone resorption has been seen [29]. In contrast, co-cultures of osteoblast-like cells and OC respond to 1,25-$(OH)_2D_3$ with an increase in resorption suggesting an indirect, osteoblast-mediated, effect [29]. Consistent with this, osteoblasts have been shown to express the VDR [40] and are known to respond to 1,25-$(OH)_2D_3$ treatment with an increase in the production of bone active cytokines such as IL-1 and IL-6 [12]. Cells of the monocyte-macrophage family and a number of lymphoid cell lines also express the VDR suggesting that 1,25-$(OH)_2D_3$ may also stimulate OC recruitment from haematopoietic precursors [26].

The origin of the OC has been much disputed, however, it is now widely accepted that OC are derived from mononuclear cells that originate in the bone marrow or other hematopoietic organs and migrate to the bone via vascular routes [13,23,27]; reviewed in [46,1]. The effect of 1,25-$(OH)_2D_3$ on OC recruitment and differentiation has been studied widely *in vitro*, but, due to the lack of suitable model systems, not *in vivo* (reviewed by [1,41,37]). The increased bone resorption in response to administration of 1,25-$(OH)_2D_3$*in vivo* is clearly associated with an increase in the number of OC [43], however, the low frequency of OC precursors in the bone marrow and the difficulty in unequivocally identifying these OC precursors greatly restricts their study *in vivo*. Thus, the precise pathway by which OC are recruited and differentiate *in vivo* has not been clearly demonstrated.

Although much is known about factors regulating OC formation and OC activity, little information is available about factors involved in OC senescence. In particular, the fate of the increased numbers of OC seen after administration of 1,25-$(OH)_2D_3$*in vivo* is unclear. There is growing evidence that the normal fate of OC is to undergo apoptosis (programmed cell death). Previous studies have indicated that apoptotic OC can be identified at reversal sites, however, because the activity of individual bone remodelling units is asynchronous, this is a difficult process to study *in vivo*.

We have developed a model system in which a rapid, predictable and reproducible increase in OC numbers is induced *in vivo* by the administration of high doses of 1,25-$(OH)_2D_3$. This increase in OC number is then followed by an equally rapid and reproducible apoptosis-mediated decrease in OC numbers. This model has allowed us to investigate the cellular events in the bone marrow and locally along the trabecular bone surface during the recruitment, differentiation and subsequent death of OC and their precursors.

Materials and Methods

Animal procedures

All animals procedures were carried out according to current Home Office regulations and all necessary licenses had been obtained. Male wistar rats were allowed free access to a pelleted standard chow, containing 1.0% calcium and 0.65% phosphate and 4.5 IU Vitamin D_3/g, and tap water and the animals were acclimated for 1 week prior to the start of all experiments. In order to determine the effect of 1,25-$(OH)_2D_3$ on OC recruitment, differentiation and subsequent apoptosis, a series of 4 experiments were performed in which rats were treated with differing doses of 1,25-$(OH)_2D_3$ and then killed at various time-points afterwards. In experiment 1, six 200 g male Wistar rats were allocated into two groups and were injected subcutaneously (s.c.) with either vehicle or 10 μg 1,25-$(OH)_2D_3$ /kg body weight (BW) on day 1 of the study, and then killed on day 2 by cervical dislocation. In experiment 2, six 200 g male Wistar rats were allocated into two groups and injected s.c. with either vehicle or with 10 μg 1,25-$(OH)_2D_3$ /kg BW on days 1, 2 and 3 of the study and then killed on day 4. In experiment 3, thirty 200 g male Wistar rats were allocated into two groups and injected s.c. with either vehicle or 2 μg 1,25-$(OH)_2D_3$ /kg BW on days 1, 2 and 3 of the study. Three rats from each group were then killed on days 4, 6, 9, 13 and 17. In experiment 4, sixty male Wistar rats with a mean initial body weight of 285 g, were allocated into two weight-matched groups and were s.c. injected with either vehicle or 0.2 μg 1,25-$(OH)_2D_3$ / kg BW on days 1, 2 and 3 of the study. Five rats of each group were killed on days 4, 6, 8, 10, 12 and 14 by cervical dislocation. The reason for the small groups in Exp 1, 2 & 3 was that preliminary experiments had shown that these were sufficient provide statistically significant effects at these high doses.

Histology: Sample processing

After the rats were sacrificed the left proximal tibiae and the fourth lumbar vertebral bodies were removed (with the exception of Exp 1 where vertebrae were not sampled), dissected free of soft tissue and fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution) for 24 hr at 5°C as previously described [28]. After fixation, specimens were washed for 12 hours at 5°C in each of the following series of solutions: 0.01 M PBS containing 5% glycerol, 0.01 M PBS containing 10% glycerol, 0.01 M PBS containing 15% glycerol. The specimens were then decalcified in EDTA-G solution (14.5 g EDTA, 15 ml of glycerol, 85 ml distilled water, and solid sodium hydroxide added until a final pH of 7.3 was reached) for 10–14 days at 5°C as previously described [31]. Progression of decalcification was checked by micro-X ray. The decalcified tissues were washed sequentially at 5°C for 12 hr in (a) 15% sucrose and 15% glycerol in PBS, (b) 20% sucrose and 10% glycerol in PBS,

(c) 20% sucrose and 5% glycerol in PBS, (d) 20% sucrose in PBS; 10% sucrose in PBS, (e) 5% sucrose in PBS and (f) 100% PBS as previously described [5]. Tissues were dehydrated in graded alcohols and embedded in low-melting-point paraffin. 5 μm sections were cut at frontal for tibiae and at sagittal for vertebrae. The sections were stained with hematoxylin and eosin (HE), histochemically for tartrate resistant acid phosphatase (TRAP), immunohistochemically for ED1, double stained for TRAP/ED1 or TRAP/4',6-diamidino-2-phenylindole (DAPI) or by *in situ* DNA end-labelling of apoptotic OC using Klenow FragEL™ kit (ISEL).

Histology: Tartrate resistant acid phosphatase histochemistry

Osteoclastic cells were identified by staining histochemically for TRAP following a modified version of a previously described method. Briefly, the sections were deparaffinized and preincubated for 20 minutes in sodium acetate (50 mM) and potassium sodium tartrate (40 mM) buffer (pH 5.0) and then incubated for a further 15 minutes at room temperature (RT) in the same buffer containing naphthol AS-MX phosphate (2.5 mg/ml) (Sigma) and fast garnet GBC (0.5 mg/ml, Sigma). As a negative control, some sections were incubated in the above medium with fast garnet GBC but without naphthol AS-MX phosphate. After washing with distilled water, the sections were counterstained with Vector methyl green nuclear counterstain (Vector laboratories, Peterborough, UK) and mounted in Kaiser's glycerol jelly.

Histology: ED1 Immunostaining

Cells of the monocytic lineage were identified by staining for ED1 using the avidin-biotin-peroxidase complex (ABC) immunoperoxidase technique. The tissue sections were preincubated for 30 minutes at 37°C with 2% Bovine testicular hyaluronidase in PBS. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 10 min, followed by washing in running water for 5 min and then flooding with TBS for 5 min. The sections were then incubated with 20% normal goat serum for 30 min at RT to block non-specific binding. The sections were incubated overnight at RT with the mouse anti-rat monocytes/macrophages monoclonal antibody (ED1, Serotec Ltd, Oxford, England) diluted 1: 100 in 1% BSA. As a negative control, Mouse IgG1 negative control (DAKO, Denmark) was substituted for the primary antibody. After washing, the sections were incubated with secondary antibody (biotinylated goat anti-mouse IgG, Fab special, Sigma, diluted 1:300 TBS) for 30 min at RT. The sections were then washed as above and incubated with Vectastain *Elite* ABC reagent (Vector laboratories, Peterborough, UK) for 30 min at RT. After washing as above, grey pigmentation was produced by 5–10 min treatment with Vector SG substrate (Vector laboratories, Peterborough, UK). After washing with distilled water, the sections were counterstained with methyl green and mounted with Kaiser's glycerol jelly.

Histology: Identification of apoptotic osteoclasts

Apoptotic OC were identified morphologically by the following features: 1) detachment from resorption surface; 2) chromatin condensation and nuclear fragmentation; 3) cytoplasmic condensation; 4) strong TRAP staining to confirm osteoclastic origin [21]. Using these criteria, apoptotic OC in TRAP stained sections of could easily be distinguished from viable OC (fig. 1a,1b,1c,1d). In order to confirm the presence of apoptotic OC, they were also assessed for nuclear fragmentation after DAPI staining and by *in situ* DNA end-labelling. The sections were stained with DAPI after TRAP histochemistry by incubating the sections in 1 μg/ml DAPI in TBS for 10 minutes. After washing with distilled water the sections were air dried and mounted in citifluor. Free 3'-OH groups generated by endonucleases released during apoptosis were end-labelled using a Frag EL™ DNA fragmentation detection Kit (Calbiochem-Novabiochem LTD, UK) according to the manufacturers instructions with the exception that Vector SG substrate (Vector laboratories, Peterborough, UK) was substituted for diaminobenzidene. The sections were counterstained with methyl green and mounted in Kaiser's glycerol jelly. All three methods produced comparable results in terms of changes in the numbers of apoptotic OC in response to 1,25-$(OH)_2D_3$ and so for quantitative purposes, apoptotic OC were identified according to their morphological characteristics.

Histology: Quantitative histomorphometry

Numbers of TRAP positive (+ve) bone marrow cells (BMC) and ED1 +ve BMC were counted in the bone marrow immediately below the tibial secondary spongiosae. OC parameters were measured in the secondary spongiosae of the left proximal tibia and the fourth lumbar vertebral body using a semiautomatic system (Osteomeasure, OsteoMetrics, Inc. GA, USA) and an Olympus microscope with a drawing attachment. Static histomorphometric parameters were measured in sections stained for TRAP. The following primary parameters were determined in five fields of view (×200) in one section per rat: tissue area, bone perimeter, number of TRAP +ve BMC, number of ED1 +ve BMC, OC number, OC perimeter and the number of apoptotic OC. The values obtained from these 5 fields were then averaged and used as the value for that particular animal. From the primary data, the following static parameters were calculated:-

Number of TRAP +ve BMC/tissue area = N.TRAP +ve BMC/T.Ar (#/mm^2)

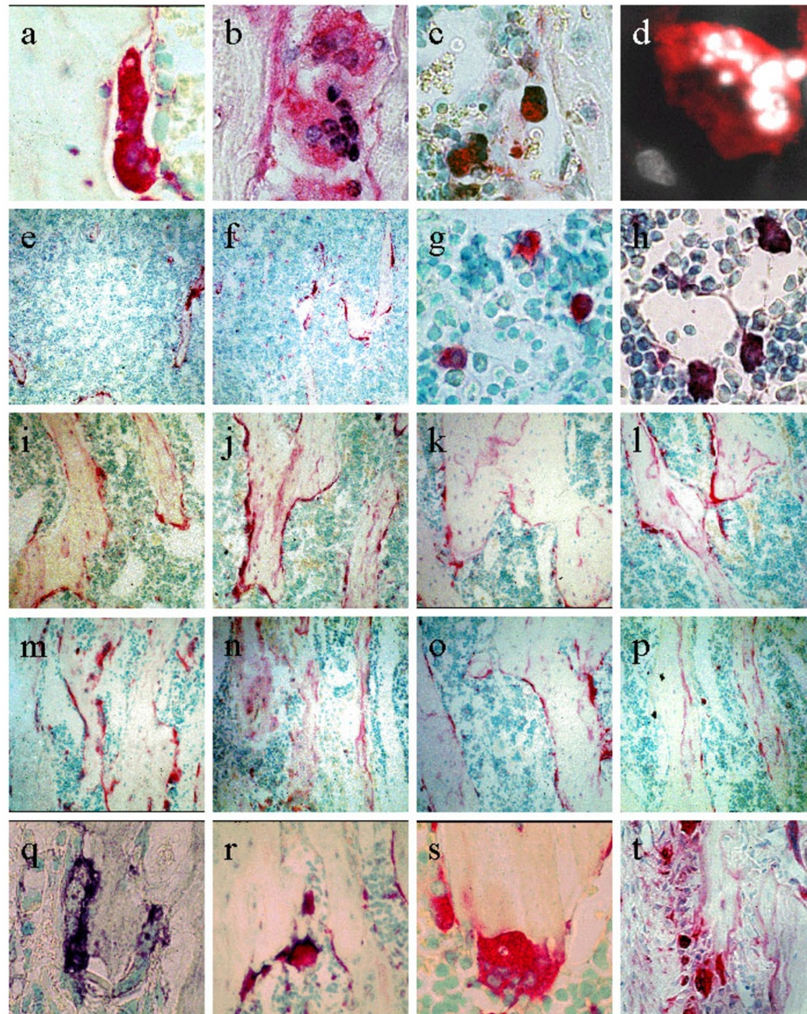

Figure 1

Apoptotic OC (a-d). (a) An early stage TRAP +ve apoptotic OC that has detached from the resorption surface. Its cytoplasm is condensed and it is surrounded by space due to cell shrinkage (×1000). (b) An early stage apoptotic OC with cytoplasmic TRAP (red) and nuclear ISEL (grey) staining (×1000). (c) Late stage apoptotic OC with intense cytoplasmic TRAP staining and condensed, fragmented nuclei which are positive for ISEL (×1000). (d) A typical apoptotic OC with intense cytoplasmic TRAP staining, a condensed cytoplasm and condensed positive nuclei stained with DAPI (×1000). **Expression of TRAP/ED1 in bone marrow cells (e-h).** (e) In the tibia of vehicle treated rats there were only occasional TRAP +ve BMC (×200). (f) A single treatment with 10 μg/kg 1,25-$(OH)_2D_3$ gave rise to a significant increase in the number of TRAP +ve BMC (×200). (g) TRAP +ve BMC located in the bone marrow (×1000). (h) TRAP/ED1 +ve BMC located in the bone marrow (×1000): **TRAP expression in the secondary spongiosa (day 4) (i-l).** Tibiae from (i) vehicle and (j) 1,25-$(OH)_2D_3$ treated rats (2 μg/kg) and vertebrae from (k) vehicle and (l) 1,25-$(OH)_2D_3$ treated rats (2 μg/kg) (magnification: ×200). **Expression of TRAP in the secondary spongiosae (day 6) (m-p).** Tibiae from vehicle (m) and 1,25-$(OH)_2D_3$ treated rats (2 μg/kg) (n) or vertebrae from vehicle (o) and 1,25-$(OH)_2D_3$ treated rats (2 μg/kg) (p) (magnification: ×200): **Expression of TRAP/ED1 in bone tissue (q-t).** ED1 immunostaining show (q) ED1 +ve viable OC (×1000). Double staining for TRAP and ED1 show (r) both positive viable OC for TRAP (red) and ED1 (grey). ED1 is richest in the clear zone of OC (×400). (s) TRAP +ve viable OC (×1000), and (t) TRAP +ve OC together with periosteal cells weakly +ve for TRAP (×400).

Number of ED1 +ve BMC/tissue area = N.ED1 +ve BMC/T.Ar (#/mm^2)

Number of OC/tissue area = N.OC/T.Ar (#/mm^2)

Number of OC/bone perimeter = N.OC/B.Pm (#/mm)

OC perimeter/bone perimeter = OC.Pm/B.Pm, (%)

Number of apoptotic OC/tissue area = Ap.OC/T.AR, (#/mm^2)

Statistical analysis

Statistical comparisons were made using the non-parametric Mann-Whitney test. P values of less than 0.05 were considered significant. The data are presented as means ± S.E.

Results

Changes in ED1 +ve and TRAP/ED1 +ve bone marrow cell numbers

In the bone marrow, ED1 staining was found to be localised to large macrophages and small monocyte-like cells. The numbers of ED1 +ve BMC were increased significantly in 1,25-$(OH)_2D_3$ treated rats on day 2 in Exp 1, day 4 in Exp 2 and on day 6 in Exp 3 (table 1). TRAP +ve BMC were found only occasionally in the bone marrow of control animals however, in Exp 1 on day 2 of the study their numbers were increased in the 1,25-$(OH)_2D_3$ treated rats 10 fold compared to vehicle controls. No significant difference in the numbers of TRAP +ve BMC was observed between vehicle and 1,25-$(OH)_2D_3$ treated rats in Exp 2 and Exp 3 (table 1). It was found that all TRAP +ve BMC were also ED1 +ve and morphologically appeared to be large macrophage-like cells. However, not all ED1 +ve BMC were also TRAP +ve and apart from day 2 in Exp 1, the number of ED1 +ve cells always exceeded the number of TRAP +ve cells (table 1). There appeared to be more TRAP +ve BMC in the bone marrow at a distance from the metaphysis than in the bone marrow of the secondary spongiosae. A number of these TRAP +ve mononuclear cells were found around or within bone marrow sinusoids or capillaries (fig. 1e,1f,1g,1h). Treatment with 3×0.2 μg/kg 1,25-$(OH)_2D_3$ (Exp 4) had no significant effect on the numbers of ED1 +ve and TRAP/ED1 +ve bone marrow cell numbers (data not shown).

As well as macrophages, monocytes and TRAP +ve BMC, most OC also expressed ED1. It was found that the OC expressed ED1 more frequently in the primary spongiosa than in the secondary spongiosa and that the ED1 staining was most intense in the clear zone of the OC.

Changes in osteoclast numbers

The changes in OC-related parameters are summarised in tables 2 & 3. The changes in OC numbers were dependent on the dose of 1,25-$(OH)_2D_3$ used with higher doses producing both stronger and quicker responses. The administration of 3 daily injections of 10 μg 1,25-$(OH)_2D_3$/kg BW had no significant effect on OC numbers in the tibiae, but caused an increase in the number of vertebral OC by 125% on day 4 of the study. The administration of 3 daily injections of 2 μg 1,25-$(OH)_2D_3$/kg BW resulted in a 48% increase in the number of OC in the tibiae and 81% in the vertebrae on day 4 of the study, and a decrease in the number of OC by 61% in the tibiae and 52% in vertebrae on day 6 of the study (fig. 1i,1j,1k,1l). The administration of 3 daily injections of 0.2 μg 1,25-$(OH)_2D_3$/Kg BW resulted in an increased in the number of the OC by 37% in the tibiae on day 6 of the study, and a decrease in the number of OC by 48% in the tibiae on day 8 of the study (fig. 1m,1n,1o,1p). At this dose however, no effect was seen in the vertebrae. In general, it was found that the response of the tibiae to 1,25-$(OH)_2D_3$ was stronger and quicker than that of the vertebrae. In all cases, OC parameters were increased for only a short time and rapidly returned to basal levels.

Changes in numbers of apoptotic osteoclast

As shown above, the number of viable OC increased after treatment with 1,25-$(OH)_2D_3$ but then decreased within a short period of time. This appeared to be due to the rapid onset of OC apoptosis after 1,25-$(OH)_2D_3$-induced recruitment (tables 2 & 3, fig. 1a,1b,1c,1d). In Exp 3, the number of apoptotic OC was increased significantly in both vertebrae and tibiae on day 4 of the study and remained above control values for a further two days, but returned to control values in both the vertebrae and tibiae by day 9 of the study. As with OC recruitment, the numbers of apoptotic OC was also dependent on the dose of 1,25-$(OH)_2D_3$administered with higher doses of 1,25-$(OH)_2D_3$ induced more OC to enter apoptosis more rapidly. It was also noted that fewer apoptotic OC number appeared in vertebrae than in tibiae, and that apoptosis was delayed compared to tibiae.

Comparison of sections stained for TRAP activity with those stained using ISEL revealed that fewer apoptotic OC were identified using ISEL than with the classical morphological features of apoptosis. This is presumably because ISEL identifies OC at an earlier stage of apoptosis before they detached from the resorption surface (fig. 1b). On the other hand, some apoptotic bodies of OC are not identified by ISEL because of their lack of condensed nuclei. This characteristic of late stage apoptotic OC was confirmed by double staining for TRAP and DAPI. This shows that in the later stages of OC apoptosis, the nuclei and cy-

Table 1: Effect of 1,25-$(OH)_2D_3$ on the number of TRAP +ve and ED1 +ve Bone Marrow Cells

Exp	Dose	Time	TRAP +ve BMC (#/mm²)		ED1 +ve BMC (#/mm²)	
			Vehicle	1,25-$(OH)_2D_3$	Vehicle	1,25-$(OH)_2D_3$
1	1 × 10 μg/kg	2 d	1.60 ± 0.41	16.37 ± 4.53*	1.66 ± 0.33	16.67 ± 5.09*
2	3 × 10 μg/kg	2 d	0.33 ± 0.16	0.44 ± 0.19	2.08 ± 0.45	4.58 ± 1.25*
3	3 × 2 μg/kg	4 d	0.74 ± 0.13	0.67 ± 0.24	0.83 ± 0.33	1.46 ± 0.60
		6 d	0.63 ± 0.21	0.56 ± 0.19	1.25 ± 0.33	11.04 ± 4.00*
		9 d	0.59 ± 0.27	0.59 ± 0.19	0.83 ± 0.30	1.36 ± 0.28
		13 d	0.56 ± 0.24	0.59 ± 0.24	1.36 ± 0.28	2.50 ± 1.18
		17 d	0.74 ± 0.19	0.78 ± 0.14	1.25 ± 0.33	2.92 ± 0.79*

All values are means ± SE, *: $P < 0.05$.

Table 2: Effect of 1,25-$(OH)_2D_3$ on Osteoclastic parameters in rat tibial trabecular bone

Exp	Dose	Time	N.Oc/T.Ar (#/mm²)		N.Oc/B.Pm (#/mm)		N.ApOc/T.Ar (#/mm²)	
			Vehicle	1,25-$(OH)_2D_3$	Vehicle	1,25-$(OH)_2D_3$	Vehicle	1,25-$(OH)_2D_3$
1	1 × 10 μg/kg	1 d	63.1 ± 2.0	61.7 ± 2.9	6.0 ± 0.5	6.4 ± 0.8	0.7 ± 0.3	1.0 ± 0.6
2	3 × 10 μg/kg	4 d	76.7 ± 2.4	65.7 ± 3.8	7.5 ± 0.1	6.3 ± 0.3	1.0 ± 0.6	11.7 ± 0.9*
3	3 × 2 μg/kg	4 d	75.0 ± 6.3	111.0 ± 2.0*	7.0 ± 0.3	9.7 ± 5.3*	0.3 ± 0.3	10.3 ± 0.7*
		6 d	85.1 ± 9.5	41.3 ± 12.9*	8.4 ± 0.5	3.2 ± 1.1*	1.0 ± 0.6	5.0 ± 0.6*
		9 d	91.9 ± 10.	98.4 ± 9.8	8.8 ± 0.7	8.8 ± 0.6	0.7 ± 0.3	0.7 ± 0.3
		13 d	9	81.1 ± 4.5	8.9 ± 1.2	6.7 ± 0.7	0.7 ± 0.3	0.3 ± 0.3
		17 d	71.0 ± 5.3	73.9 ± 2.6	6.5 ± 0.2	6.5 ± 0.4	0.7 ± 0.3	1.0 ± 0.6
			73.5 ± 2.2					
4	3 × 0.2 μg/kg	4 d	39.2 ± 4.9	33.4 ± 3.0	3.5 ± 0.3	3.9 ± 0.2	0.4 ± 0.2	0.6 ± 0.2
		6 d	32.9 ± 2.4	45.1 ± 4.1*	3.8 ± 0.3	5.1 ± 0.7*	0.6 ± 0.2	1.0 ± 0.3
		8 d	37.6 ± 5.6	23.4 ± 2.1*	5.2 ± 0.7	3.1 ± 0.2*	0.4 ± 0.2	1.6 ± 0.2*
		10 d	35.5 ± 3.4	40.9 ± 3.5	5.2 ± 0.5	4.6 ± 0.6	0.4 ± 0.2	0.8 ± 0.4
		12 d	36.9 ± 5.0	39.0 ± 7.9	4.6 ± 0.3	4.5 ± 0.4	0.4 ± 0.2	0.6 ± 0.2
		14 d	41.1 ± 3.9	37.5 ± 1.3	5.7 ± 0.5	4.9 ± 0.4	0.2 ± 0.2	0.4 ± 0.2

All values are mean ± SE, *: $P < 0.05$

toplasm may split into fragments of various sizes, which may or may not contain nuclear fragments.

Discussion

It has been shown i*n vitro* that the increase in OC seen after treatment with 1,25-$(OH)_2D_3$ is due to the differentiation of monocytic OC precursors and the activation of mature OC. As mature OC do not possess a VDR this is unlikely to be a direct effect and is probably mediated osteoblastic cells or marrow stromal cells [1,41,37]. The study of these events *in vivo* has been hampered by a lack of specific markers which can identify OC and their precursors *in vivo*. OC are usually identified histologically by staining for TRAP, however, as other cells, e.g. osteoblasts and osteocytes, also express TRAP activity *in vivo* this is not 100% specific [3]. This means that OC can only be identified using a combination of morphology, e.g. multinuclearity, and TRAP staining. To investigate the effects of 1,25-$(OH)_2D_3$ on OC recruitment *in vivo*, we have used double staining for both TRAP and ED1, a monoclonal antibody ED1 directed against rat CD68 which reacts with peripheral monocytes and macrophages [8,2,22,8] and also OC and their precursors [38]. Using this approach, it was possible to follow both the increase in monocytic cells in the

Table 3: Effect of 1,25-$(OH)_2D_3$ on osteoclastic parameters in fourth lumbar vertebral trabecular bone

Exp	Dose	Time	N.Oc/T.Ar (#/mm²)		N.Oc/B.Pm (#/mm)		N.ApOc/T.Ar (#/mm²)	
			Vehicle	1,25-$(OH)_2D_3$	Vehicle	1,25-$(OH)_2D_3$	Vehicle	1,25-$(OH)_2D_3$
2	3 × 10 μg/kg	4 d	33.9 ± 5.0	66.4 ± 5.5*	3.9 ± 0.7	7.7 ± 1.0*	0	4.6 ± 1.3*
3	3 × 2 μg/kg	4 d	31.0 ± 1.7	56.3 ± 3.3*	3.5 ± 0.4	6.9 ± 0.4*	0.5 ± 0.3	3.1 ± 0.4*
		6 d	35.0 ± 4.2	16.7 ± 0.6*	4.1 ± 0.5	3.3 ± 0.1*	0.7 ± 0.2	3.5 ± 0.3*
		9 d	30.6 ± 7.3	33.9 ± 4.7	3.8 ± 0.8	3.9 ± 0.7	0.7 ± 0.2	0.7 ± 0.2
		13 d	26.7 ± 2.4	46.9 ± 4.8*	2.9 ± 0.4	5.1 ± 0.3*	0.7 ± 0.2	1.2 ± 0.4
		17 d	30.3 ± 5.0	44.0 ± 4.5	3.5 ± 0.3	4.6 ± 0.6	0.5 ± 0.2	1.0 ± 0.3
4	3 × 0.2 μg/kg	4 d	28.8 ± 1.9	27.3 ± 1.9	3.0 ± 0.2	2.7 ± 0.2	0.2 ± 0.2	0
		6 d	28.2 ± 1.5	27.1 ± 2.6	3.0 ± 0.1	3.0 ± 0.2	0.2 ± 0.2	0
		8 d	26.9 ± 2.8	29.2 ± 2.2	2.9 ± 0.2	3.2 ± 0.4	0	0.4 ± 0.2
		10 d	29.4 ± 2.4	31.7 ± 2.3	3.7 ± 0.1	3.1 ± 0.3	0.2 ± 0.2	0.4 ± 0.2
		12 d	31.5 ± 2.7	32.5 ± 3.3	3.3 ± 0.3	3.6 ± 0.3	0.2 ± 0.2	0.2 ± 0.2
		14 d	30.6 ± 3.2	29.8 ± 2.7	3.5 ± 0.3	3.5 ± 0.3	0	0.2 ± 0.2

All values are mean ± SE, *: $P < 0.05$

bone marrow and their subsequent differentiation to OC after treatment with 1,25-$(OH)_2D_3$ (fig. 1q,1r,1s,1t).

A 1,25-$(OH)_2D_3$ mediated transient increase in OC numbers and recruitment of OC precursors has been reported previously [6], however, the temporal and dose dependent effects of 1,25-$(OH)_2D_3$ on OC recruitment and differentiation have never been studied in detail *in vivo*. In this study we have used high doses of 1,25-$(OH)_2D_3$ to induce a rapid and reproducible increase in the numbers of OC precursors and OC followed by their subsequent apoptosis mediated death. The results here show that treatment with 1,25-$(OH)_2D_3$ leads to an increase in OC numbers on the surface of trabecular bone which is preceded by an increase in the number of ED1 positive mononuclear cells increased in the bone marrow. In addition, in rats given a single, very high, dose of 1,25-$(OH)_2D_3$ (10 μg/kg), significant numbers of ED1/TRAP +ve cells were seen in the bone marrow and some distance from the bone surface. These ED1/TRAP +ve bone marrow cells could be sub-divided into monocyte/macrophage-like BMC, located mainly in the bone marrow at a distance from metaphysic and apoptotic bodies of OC which had previously died with condensed nuclei or without nuclei. These results together, are consistent with the *in vitro* findings that the first stage of 1,25-$(OH)_2D_3$ mediated osteoclastogenesis is the recruitment of monocytic bone marrow cells. The appearance of TRAP +ve/ED1 +ve bone marrow cells is also consistent with the suggestion that the presence of bone surfaces are not absolutely necessary for subsequent osteoclastic differentiation [34].

The various *in vitro* models developed over the last decade to study OC differentiation have led to three alternative hypotheses for OC differentiation: (1) independent development from a specialised progenitor originating in the GM-CFU compartment [17,18]; (2) divergence from the M-CFU compartment [10,11]; or (3) late monocytes or macrophages are direct precursors of OC [44,39]. The results here show that the increase in OC numbers was always associated with, or preceded by, an increase in the number of ED1 +ve BMC. Due to the subsequent apoptotic death of the newly induced OC, this increase in OC numbers is transient, however, the increase in ED1 +ve BMC numbers is maintained after the disappearance of OC. The temparospatial relationship between the increased numbers of ED1 +ve BMC, the TRAP +ve/ED1 +ve BMC and the newly recruited OC, together with the finding that most OC were also ED1 +ve, tends to support the 3rd hypothesis, that the newly recruited OC are derived from monocytic BMC and that the increase in OC numbers is due to a general increase in monocyte numbers.

Previous studies have shown conflicting results *in vivo*, finding that 1,25-$(OH)_2D_3$ results in an increase [47], a decrease [14,36] or no change in bone resorption [33,15]. These inconsistencies may reflect the different doses administered or the different time points and the sites observed. Our results have shown that short-term treatment with high dose 1,25-$(OH)_2D_3$ (2 μg/kg and above) resulted in an increase in OC parameters in both tibiae and vertebrae whereas short-term treatment with a lower dose of 1,25-$(OH)_2D_3$ (0.2 μg/kg) increased OC parameters in tibiae but not in vertebrae. Short-term treatment with

high dose 1,25-$(OH)_2D_3$ resulted in a transient increase in OC numbers followed by a decrease relative to control animals. As most previous studies have only observed one time point after treatment, it is quite possible that they failed to observe key events in the recruitment and differentiation of OC after treatment. These data are consistent with those of Boyce & Weisbrode [6], in that treatment with 1,25-$(OH)_2D_3$ resulted in a transient augmentation followed by a decrease in OC parameters with the exception that they observed an earlier increase in OC parameters than ours. This difference may be the result of different levels of dietary calcium with high levels of dietary calcium being used in the experiments of Boyce and Weisbrode and normal dietary calcium in our experiments. In clinical studies assessing the effects of short-term 1,25-$(OH)_2D_3$ treatment on calcium and bone metabolism, no initial resorptive phase was observed [15,4,16]. It is likely that this is due to the much lower doses of 1,25-$(OH)_2D_3$ used in human studies.

The ultimate fate of OC after treatment with 1,25-$(OH)_2D_3$ has never been studied in detail *in vivo*. In this study we have shown that short-term treatment with high-dose 1,25-$(OH)_2D_3$ induces an increase in OC number which is subsequently followed by a rapid decrease due to death by apoptosis. As with OC recruitment, higher doses of 1,25-$(OH)_2D_3$ resulted in an earlier onset of apoptosis and a higher percentage of apoptotic OC. Similarly, OC apoptosis occurred earlier and in higher numbers in tibiae compared to vertebrae. This site specific effect of 1,25-$(OH)_2D_3$ may be due to differences in the blood supply leading to a higher effective concentration of 1,25-$(OH)_2D_3$ arriving at the tibiae or it may be due to differences in the numbers of OC precursors being present in the marrow. To identify apoptotic OC we used a combination of histochemistry for TRAP and double staining for TRAP and DAPI or ISEL. TRAP staining is essential to identify apoptotic OC as it is difficult to identify apoptotic OC in HE or Giemsa stained sections and double staining for TRAP and DAPI could be used to demonstrate chromatin condensation and nuclear fragmentation. ISEL was used to visualise DNA fragmentation, however, ISEL and morphological studies identified slightly different populations of apoptotic OC, due to the presence of some late stage apoptotic bodies without nuclei.

The mechanism of action underlying this increase in OC numbers followed by their subsequent apoptosis remains to be resolved and may involve systemic factors, local factors or a combination of the two. It is well established that 1,25-$(OH)_2D_3$ can induce osteoclast formation and activity *in vitro*[20,29,24] and it would seem likely that this is also the case *in vivo*. Calcium homeostasis *in vivo* is thought to be governed mainly by interactions between 1,25-$(OH)_2D_3$ and parathyroid hormone (PTH) with PTH being the major factor responsible for maintaining osteoclastic activity. This may explain the transient nature of the osteoclastic response to 1,25-$(OH)_2D_3$ as we have previously shown that short-term treatment with 1,25-$(OH)_2D_3$ is followed by a transient decrease in serum PTH [9]. This down-regulation of serum PTH may in turn be due to either a direct effect of 1,25-$(OH)_2D_3$ or the hypercalcaemia seen shortly after treatment with 1,25-$(OH)_2D_3$ [9].

The relationship between OC apoptosis and 1,25-$(OH)_2D_3$ treatment is unclear. To date there have been no studies published detailing the effects of 1,25-$(OH)_2D_3$, PTH or their withdrawal on OC apoptosis suggesting that they do not play a major role in the process. Consistent with this, it has been shown previously that OC numbers decrease after 1,25-$(OH)_2D_3$ treatment even when 1,25-$(OH)_2D_3$ is not withdrawn [6]. The data here show that OC apoptosis tended to closely parallel the 1,25-$(OH)_2D_3$-induced OC activity suggesting that the degree of OC apoptosis was related to the OC activity itself. A great many growth factors including TGF-β, IGF, PDGF, FGF and the BMPs are known to be stored in the bone matrix and released during bone resorption [19] and it would seem likely that the 1,25-$(OH)_2D_3$-induced apoptosis may well be mediated by the release of one of these. To support this, it has been shown that TGF-β can induce apoptosis in OC *in vitro* and the induction of OC apoptosis by oestrogen *in vitro* is also mediated by TGF-β [21]. Furthermore it has recently been shown that the OC apoptosis associated with periodontal tooth movement *in vivo* is also associated with an increase in TGF-β expression[32].

Further study is required to elucidate the biochemical pathway that leads to apoptosis of OC following exposure to 1,25-$(OH)_2D_3$. Nevertheless, the administration of 1,25-$(OH)_2D_3$ can induce a predictable and reproducible increase in the number of apoptotic OC. This may be a useful model for studying the regulation of OC apoptosis in vivo.

Competing interests

None declared.

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