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Increased vitamin D-driven signalling and expression of the vitamin D receptor, MSX2, and RANKL in tooth resorption in cats

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Tooth resorption occurs in 20–75% of cats (*Felis catus*). The aetiology is not known, but vitamin D is suggested to be involved. Vitamin D acts through a nuclear receptor (VDR) and increases the expression of receptor activator of nuclear factor-κB ligand (*rankl*) and muscle segment homeobox 2 (*msx2*) genes. Mice lacking the muscle segment homeobox 2 (*msx2*) gene show decreased levels of *rankl*, suggesting an interaction among VDR, MSX2, and RANKL. Here, we investigated the expression of VDR, MSX2, and RANKL proteins, and the activity of the VDR-mediated signalling pathway (using the quantitative polymerase chain reaction on VDR target genes), in tooth resorption, and measured the serum levels of vitamin D metabolites in cats. Tooth resorption was categorized into either resorptive or reparative stages. In the resorptive stage, odontoclasts expressed MSX2 and RANKL (100% and 88%, respectively) and fibroblasts expressed VDR and MSX2 (both at 100%), whereas fibroblasts expressed RANKL in only 29% of the sites analysed. In the reparative stage, cementoblasts expressed VDR, MSX2, and RANKL, whereas fibroblasts expressed VDR and MSX2, but not RANKL. The vitamin D status did not differ between the groups, based on the serum levels of 25-hydroxycholecalciferol. However, increased expression of VDR protein, and the relative gene expression levels of *1α*-hydroxylase and the VDR-target gene, *24-hydroxylase*, indicated the involvement of an active vitamin D signalling in the pathophysiology of tooth resorption in cats.

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Tooth resorption is common in the permanent teeth of cats (*Felis catus*), and, depending on study methodology, 20–75% of cats have at least one resorptive lesion (1). The mandibular third molars are often mentioned as the most commonly involved teeth; however, any tooth (incisors, canines, other premolars, and molars) can be affected (2). The crowns of affected teeth are often missing on clinical examination, although the roots can sometimes still be recognized on dental radiographs. External tooth resorption is rare in humans, and, if present, seldom affects multiple teeth (3, 4), whereas tooth resorption in cats is a frequent diagnosis and usually involves multiple teeth (2). Neither the aetiology of tooth resorption in cats nor the mechanisms involved in its induction are known, but bacteria, viruses, and dietary components have been proposed to steer this process (1, 5). Bacteria in dental plaque may initiate the inflammatory resorption, or transform an initial non-inflammatory resorption into an inflammatory resorptive process. The study on a possible viral component lacks proper investigation methods to diagnose tooth resorption (5). Both dietary vitamin D excess (6) and deficiency (E. Servet *et al.*, 11th ESVCN Congress 2007) have been suggested to be involved in the pathophysiology of tooth resorption in cats. Vitamin D, and in particular its active metabolite, 1,25 dihydroxycholecalciferol [$1,25(OH)_2D$], stimulates osteoclastogenesis indirectly by up-regulating the expression of messenger RNA of the receptor activator of nuclear factor-κB ligand (*rankl*) by stromal cells (7, 8). The high level of expression of *msx2* mRNA levels in osteoclasts during tooth eruption and root elongation suggests that muscle segment homeobox 2 (MSX2) has a regulatory role in osteoclast differentiation and/or activation (9). This is supported by the decreased mRNA levels of *rankl* in the alveolar bone of $MSX2^{-/-}$ mice (9, 10), as well as by the induction of mRNA levels of *msx2* by $1,25(OH)_2D$ (11, 12).

$1,25(OH)_2D$ acts through a nuclear receptor, the vitamin D receptor (VDR) (13). Because $1,25(OH)_2D$ has the capacity to modulate the formation and activity

of osteoclasts and odontoclasts in resorptive processes, we hypothesized that vitamin D is involved in the pathophysiology of tooth resorption. To our knowledge, the expression of VDR, MSX2, and RANKL proteins, and serum levels of vitamin D metabolites, have not been analysed in relation to dental resorption and repair processes. In the present study, we analysed the expression of VDR, MSX2, and RANKL in feline teeth with tooth resorption by means of immunohistochemistry and measured the serum levels of the vitamin D metabolites, 25-hydroxycholecalciferol [25(OH)D], 1,25$(OH)_2$D, and 24,25-dihyroxycholecalciferol [24,25$(OH)_2$D], in cats with tooth resorption and in control cats. In addition, we measured the relative mRNA expression of *1α-* and *24-hydroxylase*, a VDR target gene, in cats with and without tooth resorption, using the reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Material and methods

Tissue collection

Client-owned cats were referred to the university clinic for specific dental treatment and screened for dental lesions. The ages of the cats – two male Main Coons and three Domestic Shorthairs (one male, two females) – ranged from 1 to 13 yr. Tooth resorption was diagnosed based on clinical and radiographic signs of resorption. Teeth were explored for resorption lacunae under general anaesthesia, and digital radiographs were obtained using parallel (mandibular premolars and molars) and bisecting angle (other teeth) techniques (Gendex AC90; Gendex Dental Systems, Milan, Italy and Vista-scan, Dürr Dental, Beuningen, the Netherlands). Radiolucent areas, not reflecting the pulpal cavity, in the teeth were identified as resorption. Treatment involved the extraction of teeth with tooth resorption under general anaesthesia; owners were informed that the teeth would be used for research purposes. The extracted teeth and adjacent tissue, including gingival and inflammatory soft tissue, were collected and fixed in buffered formaldehyde (4%) for 1–5 d at room temperature and were then demineralized in 10% EDTA for 3 wk at room temperature. Serum samples were taken after anaesthesia by jugular punction and stored at −20°C until further analysis. Three mandibles, including radiographically confirmed unaffected permanent teeth and partially resorbed deciduous teeth, were obtained from specimens made available by the pathology department and served as controls. Teeth were embedded in paraffin, and serial sections of 5 μm thickness were made for immunohistochemistry analyses. Dental samples (teeth + adjacent tissue) from cats with and without tooth resorption were snap-frozen in liquid nitrogen and stored at −70°C until required for molecular analysis.

Immunohistochemistry

The following primary antibodies were used: monoclonal mouse anti-human vimentin (MU074, 1:200 dilution; Biogenex, San Ramon, CA, USA), monoclonal rat anti-human VDR (MAB1360, 1:100 dilution; Chemicon International, Billerica, MA, USA), polyclonal chicken anti-human MSX2, and polyclonal goat anti-human RANKL (C-20, sc-7627, 1:5,000 dilutions; Santa-Cruz Biotechnology, Santa Cruz, CA, USA). The epitopes used for generating antibodies for VDR and RANKL were checked for specificity by their sequence alignment to the respective cat protein sequence. The respective cat sequences were obtained by sequencing the region around the epitope of each antibody (VDR accession number: GU138646; RANKL accession number: GU138645). The feline VDR protein showed 100% homology with several species in the region of the epitope, amino acids 89–105 of the chicken VDR (Fig. S1). According to the manufacturer the 20-amino-acid-long epitope used for the generation of the RANKL antibody maps within the range of amino acids 267–317 of RANKL of human origin (accession number O14788). Alignment of the protein of several species to the cat sequences showed a homology of 98% for this region (Fig. S1).

The polyclonal chicken anti-human MSX2 were generated using peptide mapping near the N-terminus of human MSX2 coupled to keyhole limpet haemocyanin (KLH). Human MSX2 (GPGPGGAEGAAEERR) has 100% homology with mouse, rat, and cat MSX2. Immunoglobulin Y (IgY) from egg yolk was prepared as described in a previous study (14). Affinity-isolated, antigen-specific antibody was obtained from IgY using an immunoabsorbent column consisting of glutathione-*S*-transferase–MSX2 (GST–MSX2) coupled to Sepharose 4B (Amersham Biosciences, Orsay, France). The specificity of the anti-MSX2 IgY was determined by immunoelectrophoresis. A specific band of 33 kDa was observed in nuclear extracts of cells transfected with MSX2. The specificity of the immunohistochemical reactions was assessed by performing the assays in the presence of an excess of relevant peptide vs. irrelevant peptide. The peptide used for immunization completely suppressed staining, whereas an irrelevant peptide at the same concentration did not. The chicken IgY anti-MSX2 was applied at a dilution of 1:40 in phosphate-buffered saline (PBS) containing 0.5 M NaCl, 0.1% Tween, and 10% non-immune horse serum (Vector, Burlingame, CA, USA). The secondary antibodies were horseradish peroxidase (HRP)-labelled goat anti-mouse ready to use (RTU) (Dako, Heverlee, Belgium) for vimentin, biotinylated goat anti-rat (Chemicon 21543, immunohistochemistry [IHC] select RTU) for VDR, rabbit anti-chicken IgY (whole molecule) peroxidase conjugate (A 9046; Sigma-Aldrich, St Louis, MO) at a 1:400 dilution in PBS containing 1% bovine serum albumin (BSA) fraction V (Euromedex, Souffleweyersheim, France) for MSX2, and donkey anti-goat (sc-2053 RTU) for RANKL. All rinsing steps and incubations were performed at room temperature, except for the primary antibody incubations for VDR, RANKL, and vimentin, which were performed overnight at 4°C.

Sections were deparaffinized and subsequently rehydrated in a descending ethanol series. Antigen retrieval was used to localize the VDR (citrate buffer 10 mM, pH 6, for 30 min at 50°C) and RANKL [0.4% pepsin (S3002; Dako) for 15 min at 37°C]. Endogenous peroxidase was blocked using the peroxidase-blocking reagent, RTU (S2001; Dako). Serum blocks were performed using normal goat serum (X0907; Dako) for vimentin at a 1:10 dilution and for VDR at a 1:5 dilution for 30 min; for MSX2, 10% non-immune horse serum (Vector) was used in PBS containing 0.5 M NaCl and 0.1% Tween, for 30 min; and for RANKL, normal donkey serum was used from the kit (sc-2053) for 30 min. The antibodies and sera for the blocking step were diluted in PBS. After being rinsed in PBS containing 0.1% Tween, the sections were incubated with the secondary antibody. They were then rinsed with PBS and incubated with HRP-labelled streptavidin for VDR (Vector SA-5704) and RANKL (from the kit sc-2053) for 30 min. After another rinsing step, the enzyme substrate DAB (diaminobenzidine) (K3467; Dako)

was added. The incubation lasted for 2 min for VDR and vimentin, and for 7 min for RANKL. For the MSX2 Vector, the NovaRED substrate kit was applied for 5–15 min; this produces a red reaction product (SK-4800; Vector). Sections used for the detection of vimentin, VDR, and RANKL were counterstained with haematoxylin (H-3404; Vector) for 10 s. Sections for MSX2 were counterstained with hemalun de Mayer (Mayer's Hemalun 320550-100; Reactifs Ral, Bordeaux Technopolis, France).

Negative controls consisted of omission of the primary antibody (vimentin, VDR, MSX2, and RANKL), replacement of the primary antibody with an isotype control (vimentin (normal mouse IgG1; sc-3877), VDR (goat anti-rat IgG2b; CBL 606; Chemicon)), and RANKL (normal goat IgG) and blocking of the primary antibody (RANKL, sc-7627P).

Tartrate-resistant acid phosphatase analysis

Tartrate-resistant acid phosphatase (TRACP) activity was used to identify odontoclasts and osteoclasts. The staining was performed according to the manufacturer's instructions (387A-1KT; Sigma-Aldrich).

Vitamin D analysis

Serum levels of the vitamin D metabolites 25(OH)D, $1,25(OH)_2D$, and $24,25(OH)_2D$, from cats with ($n = 10$) and without ($n = 12$) tooth resorption, were measured at the Netherlands Organization for Applied Scientific Research (TNO) in Zeist, the Netherlands. 25(OH)D, $24,25(OH)_2D$, and $1,25(OH)_2D$ were extracted from serum using acetonitrile. The metabolites were separated using straight phase high-performance liquid chromatography (SP-HPLC). The concentrations of 25(OH)D and $24,25(OH)_2D$ were measured using a competitive protein-binding assay with rat vitamin D-binding globulin as a binder protein. $1,25(OH)_2D$ extract was measured using a radioreceptor assay.

1α- and 24-hydroxylase in dental samples

The relative mRNA levels of *1α-* and *24-hydroxylase* were determined in dental samples from cats with ($n = 35$) and without ($n = 43$) tooth resorption by qPCR, as described in a previous study (15, 16). Feline sequences were obtained by performing a BLAT http://genome.ucsc.edu/cgi-bin/hgBlat search on the cat genome using the canine sequence for the gene of interest as the query (17). The primers for the 1α-hydroxylase gene were 5′-ATGCCCATCCTTCAGC-3′ and 5′-ACACAAATGTCTTTGTCTGG-3′ (forward and reverse, respectively) at an optimal annealing temperature of 58°C, and for the 24-hydroxylase gene were 5′-GA-ACTGTATGCGGCTGTC-3′ and 5′-GGGATTACGGGA TAAATTGTAGAG-3′ (forward and reverse, respectively), with an optimal annealing temperature of 59°C. The PCR products were sequenced and the results were submitted to the NCBI gene bank with the accession numbers GQ247881 and GQ247880 for 1α-hydroxylase and 24-hydroxylase genes, respectively.

Histomorphometry

Because of the limited numbers of teeth, it was decided to study tooth resorption as an entity and to distinguish the phases of active resorption and repair, rather than dividing the lesions in the five different stages as followed by the American Veterinary Dental College. The tooth resorption was categorized in two groups, namely the active resorptive stage in which odontoclasts are present, and the reparative stage in which odontoclasts are absent and cellular cementum is deposited in the resorptive lacuna. Both stages could be present in adjacent areas in the same section, in which case we analysed the different stages separately. In total, 17 lesions in seven teeth from five different cats were analysed: nine resorptive lesions in six teeth from four different cats and eight reparative lesions in five teeth from four different cats. The expression of antigens in each lesion was assessed, as was the type of cell expressing the antigen (fibroblasts, odontoclasts, and cementoblasts). Expression was scored as positive when at least one cell showed expression. The results are presented as the percentage of the number of lesions in which expression was noted in fibroblasts, odontoclasts, and cementoblasts (and not as the percentage of cells per lesion that expressed the protein).

Statistical analysis

For statistical analyses of the serum levels of the vitamin D metabolites and relative mRNA levels of *1α-hydroxylase* and *24-hydroxylase*, a Student's *t*-test in SPSS 15.0 for Windows was used (SPSS, Chicago, IL, USA). Significance was set at $P < 0.05$.

Results

In the resorptive stage, odontoclasts were identified in the resorptive lacuna (Fig. 1A), based on their morphology (multinucleated) and positive TRACP activity (Fig. 1B). The number of nuclei per odontoclast in the plane of sectioning ranged from two to four. However, in some lesions giant odontoclasts were seen with up to 25 nuclei. In addition to odontoclasts, other cell types, in particular fibroblasts, partially covered the resorption lacunae. Inflammatory cells were often seen in the surrounding mesenchyme. In the reparative stage, cementoblasts were identified next to the reparative tissue and were incorporated as cementocytes in the cellular cementum layer. Abundant fibroblasts were detected, but few inflammatory cells were seen in the vicinity of the reparative lesion (Fig. 1C).

Protein expression in the resorptive stage

Odontoclasts expressed the VDR in 44% (4/9), MSX2 in 100% (6/6), and RANKL in 88% (7/8) of the lesions (Table 1, Fig. 2A–C). The VDR, MSX2, and RANKL were located in the cytoplasm and in the nuclei of the multinucleated cells. Expression of these proteins was variable within odontoclasts. Not all nuclei stained positive, and the staining intensity varied between nuclei within one cell. Fibroblasts expressed the VDR in 100% (8/8), MSX2 in 100% (6/6), and RANKL in 29% (2/7) of lesions (Table 1, Fig. 2D–F). The VDR was identified in the nucleus of the fibroblasts, whereas MSX2 and RANKL were identified in both the nucleus and the cytoplasm of fibroblasts.

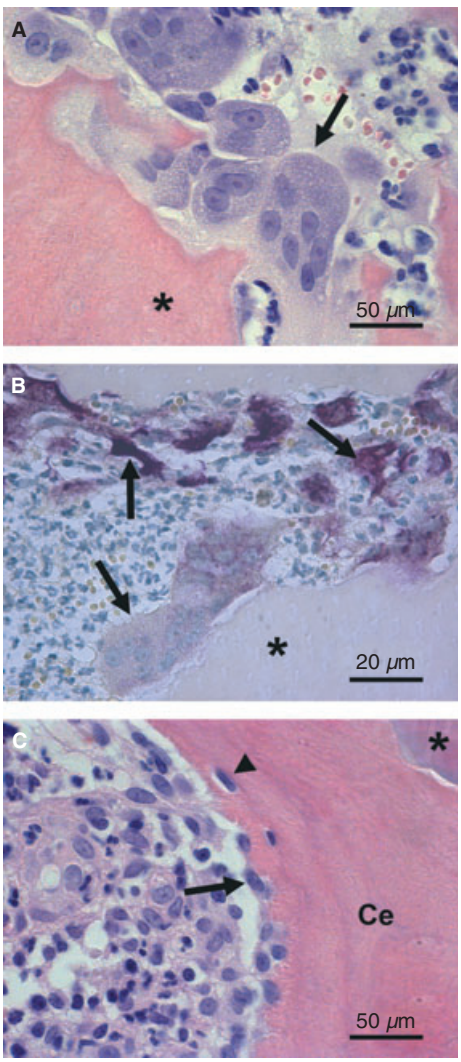

Fig. 1. Haematoxylin (A, C) and tartrate-resistant acid phosphatase (TRACP) staining (B) of different stages of resorption. (A) and (B) Dentin-resorbing odontoclasts (arrow) in the active resorptive stage. (C) Cellular cementum (Ce) with cementocytes (arrow head), deposited by cementoblasts (arrow) covering the resorbed dentin in the reparative stage. *Dentin.

Protein expression in the reparative stage

Cementoblasts expressed the VDR in the nucleus in 71% (5/7) of the lesions, MSX2 in the nucleus and cytoplasm in 100% (3/3) of the lesions, and RANKL in the nucleus in 50% (4/8) of the lesions (Table 1, Fig. 3A–C). Fibroblasts expressed the VDR in 71% (5/7) and MSX2 in 67% (2/3) of the lesions, but did not express RANKL (0/8) (Table 1, Fig. 3D–F). The VDR and MSX2 were identified in both the nucleus and the cytoplasm of fibroblasts.

Protein expression in control teeth

Fibroblasts expressed the VDR in two of five mandibles analysed but less intensely than in teeth with active resorption. Almost all fibroblasts at sites of physiological resorption of deciduous teeth were negative for the VDR (Fig. 4A), in contrast to the strong expression of the VDR that was observed in fibroblasts at sites of active pathological resorption of permanent teeth (Fig. 2D). Fibroblasts also expressed MSX2 in all mandibles investigated ($n = 3$) but RANKL was not detected in fibroblasts. Odontoclasts did not express the VDR but did express MSX2 in two of three mandibles analysed (Fig. 4B). There was a notable variation in MSX2 staining of the nuclei of odontoclasts (Fig. 4B). In one case, RANKL expression was clearly associated with the ruffled border (Fig. 4C), and this was also seen occasionally in the odontoclasts during the resorption of permanent teeth. Cementoblasts expressed the VDR in two of five mandibles analysed, expressed MSX2 in all mandibles analysed (3/3), and expressed RANKL in none (0/6).

Controls were negative when the primary antibody was omitted (VDR, MSX2, and RANKL), an isotype control was used (VDR and RANKL), and when a blocking peptide was present (RANKL) (Fig. 5).

Vitamin D metabolites and expression of 1α-hydroxylase and 24-hydroxylase genes

The levels of the metabolites 25(OH)D, $1,25(OH)_2D$, and $24,25(OH)_2D$ were measured in the available sera of cats, with and without tooth resorption (Table 2). The level of $1,25(OH)_2D$ was significantly higher in cats without tooth resorption than in cats with such lesions (132 nmol l^{-1} vs. 103 nmol l^{-1}, respectively; $P = 0.04$), whereas the levels of 25(OH)D and $24,25(OH)_2D$ did not differ significantly between the two groups (Table 2). Relative gene expression levels of *1α-hydroxylase* were 5.0-fold up-regulated ($\Delta Ct = 1.39$; $P = 0.002$) and those of the VDR target gene *24-hydroxylase* were

Table 1

Percentage of sites where the protein is expressed (number of lesions with positive expression/total number of sites analysed for the protein expression)

	Active resorption stage			Reparative stage		
	VDR	MSX2	RANKL	VDR	MSX2	RANKL
Fibroblast	100% (8/8)	100% (6/6)	29% (2/7)	71% (5/7)	67% (2/3)	0% (0/8)
Odontoclast	44% (4/9)	100% (6/6)	88% (7/8)	NP	NP	NP
Cementoblast	NP	NP	NP	71% (5/7)	100% (3/3)	50% (4/8)

MSX2, muscle segment homeobox 2; NP, not present; RANKL, receptor activator of nuclear factor-κB ligand; VDR, vitamin D receptor.

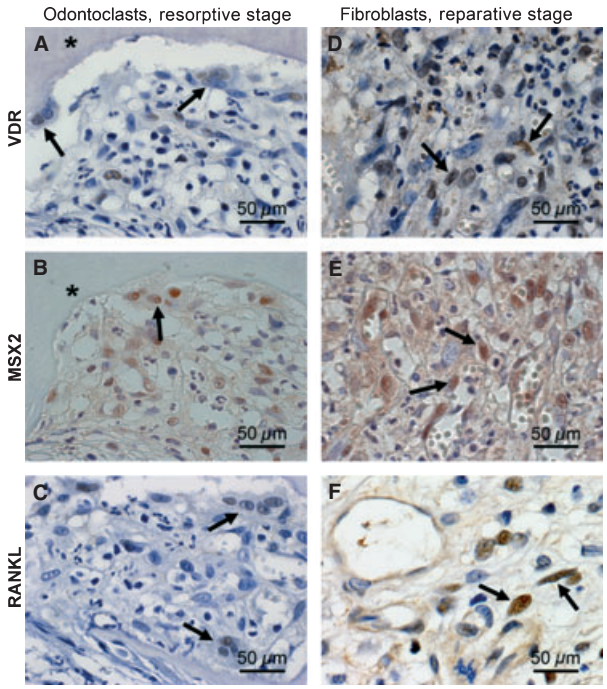

Fig. 2. Expression of the vitamin D receptor (VDR) (A, D), muscle segment homeobox 2 (MSX2) (B, E), and receptor activator of nuclear factor-κB ligand (RANKL) (C, F) by fibroblasts and odontoclasts in the active resorptive stage. Dentin-resorbing odontoclasts (arrows) show expression of the VDR (A), MSX2 (B), and RANKL (C). Fibroblasts (arrows) show expression of the VDR (D), MSX2 (E), and RANKL (F). *Dentin.

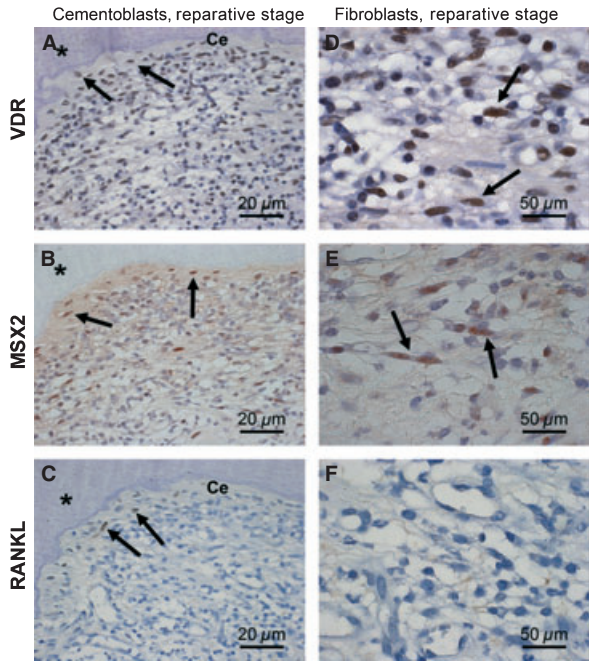

Fig. 3. Expression of the vitamin D receptor (VDR) (A, D), muscle segment homeobox 2 (MSX2) (B, E), and receptor activator of nuclear factor-κB ligand (RANKL) (C, F) by cementoblasts and fibroblasts in the reparative stage. Cellular cementum (Ce), covering the resorbed dentin, is shown by the asterisk. Cementoblasts (indicated by arrows) show expression of the VDR (A), MSX2 (B), and RANKL (C). Fibroblasts (indicated by arrows) show expression of the VDR (D) and MSX2 (E), but not of RANKL (F).

7.4-fold up-regulated (ΔCt = 2.08; P = 0.04) in dental samples with tooth resorption compared to those without tooth resorption.

Discussion

This is the first study to evaluate the expression of VDR, MSX2, and RANKL proteins in feline tooth resorption in conjunction with serum levels of vitamin D metabolites and relative levels of expression of genes for *1α-* and *24-hydroxylase* (readouts for increased VDR-mediated signalling). The VDR is essential for $1,25(OH)_2D$ to exert its function in the cell, and the receptor is up-regulated by $1,25(OH)_2D$ (13, 18). The finding that the receptor is expressed in resorptive lesions of permanent teeth of cats suggests that $1,25(OH)_2D$ plays a role in feline tooth resorption. This corroborates previous findings showing that $1,25(OH)_2D_3$ increases the expression of *rankl* and subsequently stimulates osteoclastogenesis (7, 8). This view is also supported by the observation that VDR and RANKL were expressed in fibroblasts in the resorptive stage, whereas RANKL was not detected in fibroblasts present in the reparative stage. Interestingly, cementoblasts and fibroblasts also expressed VDR in the reparative stage. Taken together, we suggest that $1,25(OH)_2D$ has a dual role: induction of resorption by odontoclasts in the active resorptive stage and pro-reparative effects in the cementoblasts and fibroblasts in the reparative stage.

Fibroblasts, and in particular those associated with the tooth, appear to have the capacity to induce osteoclast formation (7, 19). Because the latter type of fibroblasts share many similarities with osteoblasts (20), and it is known that $1,25(OH)_2D$ stimulates osteoblast growth and differentiation (21), our findings suggest that fibroblasts associated with feline teeth respond similarly to $1,25(OH)_2D$. Within the limitations of this study, we propose that the expression of the VDR by fibroblasts suggests that $1,25(OH_2)D$ has an indirect role in the formation of odontoclasts, presumably by stimulating RANKL and MSX2 production.

The present study reports, for the first time, the expression of MSX2 protein in pathological tooth resorption in mammalian permanent teeth. Previous reports identified *msx2* expression either by *in situ* hybridization or in transgenic mice where β-galactosidase expression was driven by the MSX2 promoter (9, 10, 22). We found MSX2 to be highly expressed in the active resorptive phase in both fibroblasts and odontoclasts in affected feline teeth. MSX2 is involved in epithelial–mesenchymal interactions and is expressed in many tissues, including tooth buds and the dento–alveolar bone complex during their allometric growth (22). *Msx2* is expressed in a subpopulation of osteoclasts during tooth eruption and root elongation processes (9, 10), and mice lacking the *msx2* gene show decreased osteoclast activity (9). We also found that odontoclasts

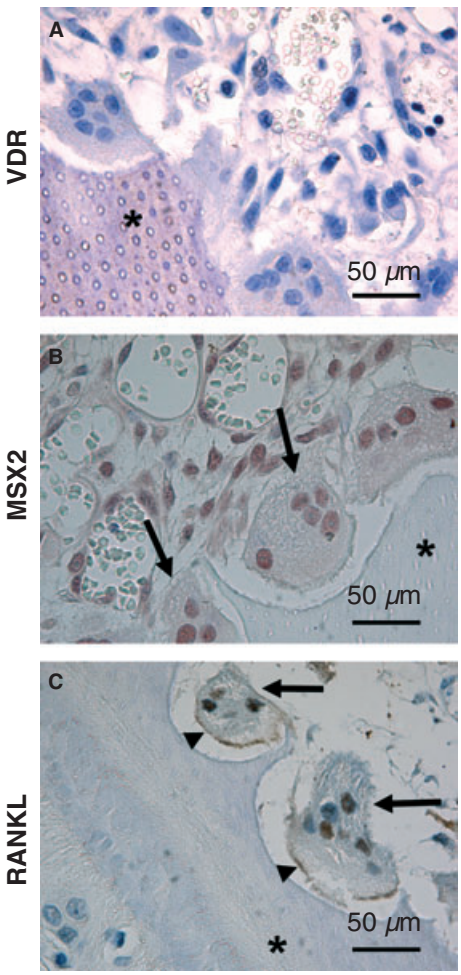

Fig. 4. Expression of the vitamin D receptor (VDR) (A), muscle segment homeobox 2 (MSX2) (B), and receptor activator of nuclear factor-κB ligand (RANKL) (C) in control teeth. Fibroblasts show no or weak expression of the VDR (A). Dentin-resorbing odontoclasts (arrows) show expression of MSX2 (B) and RANKL (C). RANKL expression was noted at the ruffled border (arrowheads). Staining intensity for RANKL was variable both within and between odontoclasts (C). *Dentin.

and osteoclasts in control teeth expressed MSX2 in areas of resorption associated with tooth eruption and shedding. On the basis of these data, we suggest that the MSX2 protein modulates the resorptive activity of odontoclasts, which are responsible for feline tooth resorption. Our findings support a functional association between high regional MSX2 expression and osteoclast activity in the periodontal microenvironment during normal and pathophysiological growth.

Lastly, both fibroblasts and odontoclasts expressed RANKL in their nuclei as well as in the cytoplasm during active resorption. As indicated by its name, the ligand RANKL induces activation of NF-κB in RANK-expressing cells. Therefore, nuclear RANKL expression came as a surprise because most previous reports indicate membranous and cytoplasmic expression but do not describe a specific expression in the nucleus (23–25). The specificity of the RANKL staining was confirmed by a series of negative controls: both replacement of the first antibody and incubation with neutralized antibody (by pre-incubation with a blocking peptide) resulted in the absence of staining. Moreover, the antibody was raised against a RANKL epitope with 98% homology to the feline RANKL sequence, thus making specific recognition of the used antibody highly likely. We therefore assume that the nuclear RANKL staining was probably caused by the presence of RANKL in the nucleus. A cytoplasmic localization of RANKL has been previously noted in periodontal ligament cells with a fibroblast-like appearance during physiological root resorption of human deciduous teeth (23, 24), in the gingival connective tissue of human patients with chronic periodontitis (26), in odontoblasts, ameloblasts, and pulp cells (27), and in various skeletal and extra-skeletal tissues and cells, including osteoblasts and osteoclasts (25). It thus appears that a relatively wide variety of cell types express RANKL in the cytoplasm. We also noted that some, but not all, odontoclasts expressed RANKL in their nuclei, although nuclear staining was not present in all nuclei of the same odontoclast. The intensity of expression also appeared to vary among different odontoclasts and among different odontoclast nuclei. These observations are in line with an earlier study in which some, but not all, nuclei of osteoclasts and chondroclasts stained positive for RANKL (28). It would seem that also in this respect odontoclasts resemble osteoclasts. The findings regarding cytoplasmic RANKL staining are consistent with those of a previous study (24) that also reported considerable differences in the staining intensity of RANKL among odontoclasts. It was suggested that the variability in expression could be a result of different phases of resorptive activity in resorbing cells (24, 28). In line with the latter, we have reported relative gene-expression levels of *rankl* to be indifferent in teeth with or without resorptive lesions (16), reflecting more advanced and combined lesions in one sample (i.e. not distinguishing different stages of resorptive activity). Accordingly, in the current study RANKL is more often encountered in the lesion in the resorptive stage (80%) and less often the reparative stage (50%), supporting our view on the role of RANKL in the first stages of tooth resorption in cats.

Are odontoclasts with a nuclear RANKL expression more active than those without such expression, or are they about to become active? Further studies are needed to establish whether the cytoplasmic or nuclear localization of RANKL reflects cell activity. It is of interest that RANKL is expressed by both osteoclasts and odontoclasts. The protein is expressed under normal conditions by osteoblasts and binds to receptor activator of nuclear factor-κB (RANK), which is expressed by osteoclast/odontoclast precursors. Our findings, and those of others, suggest that RANKL is internalized in target cells (28). This would occur in combination with RANK to which it is initially bound (29). Collectively, the data may suggest a hitherto unknown role of RANKL in odontoclast function.

The expression of VDR, MSX2, and RANKL is up-regulated by $1,25(OH)_2D$ (7, 8, 11, 13, 18). We

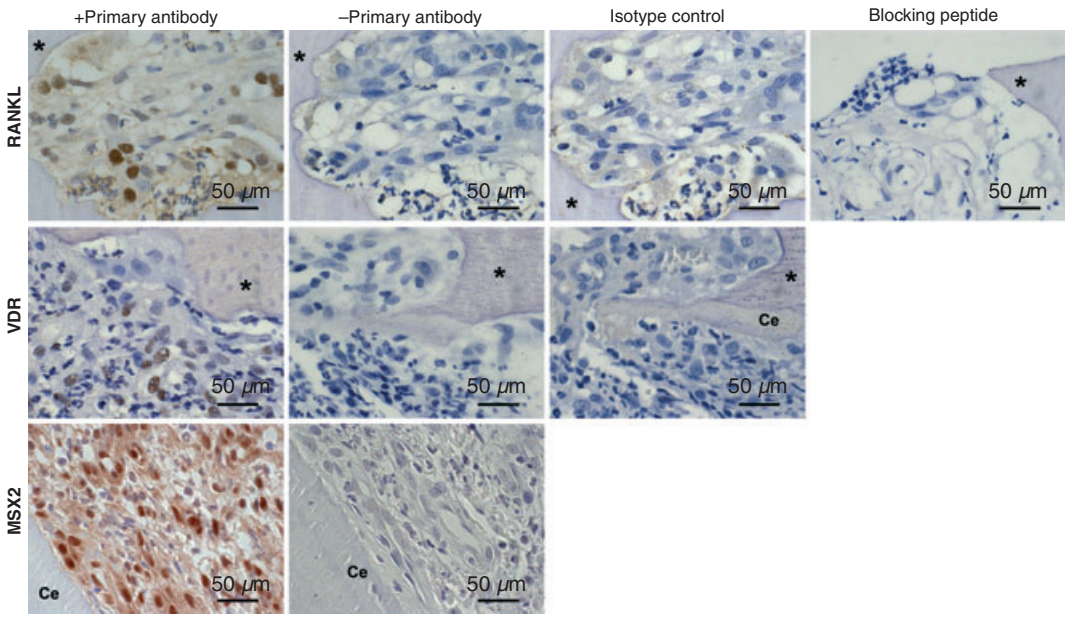

Fig. 5. Control sections for receptor activator of nuclear factor-κB ligand (RANKL), the vitamin D receptor (VDR), and muscle segment homeobox 2 (MSX2). *Dentin. Ce, cementum.

Table 2

Levels of serum vitamin D metabolites

	Cats with tooth resorption (n = 10)	Cats without tooth resorption (n = 12)
25(OH)D	132.8 (± 20.9)	134.8 (± 10.6)
$1,25(OH)_2D$	103.1 (± 12.3)	132.3 (± 6.7)*
$24,25(OH)_2D$	49.5 (± 9.6)	69.5 (± 9.6)

Levels of the serum vitamin D metabolites (± SEM) 25-hydroxycholecalciferol [25(OH)D; nmol l^{-1}], 1,25 dihydroxycholecalciferol [$1,25(OH)_2D$; pmol l^{-1}], and 24,25-dihyroxycholecal ciferol [$24,25(OH)_2D$, nmol l^{-1}].
*$P < 0.05$.

therefore expected that serum levels of 25(OH)D and $1,25(OH)_2D$ would be higher in cats with tooth resorption, whereas we found $1,25(OH)_2D$ levels to be higher in control cats and the levels of 25(OH)D and $24,25(OH)_2D$ to be similar in cats with and without such lesions. It is questionable whether the lower serum levels of $1,25(OH)_2D$ in cats with tooth resorption is of biological significance, because there are no differences in the vitamin D status between the two groups based on the 25(OH)D serum levels (30–32). Cells expressing higher levels of VDR may be more responsive to $1,25(OH)_2D$ (33), and in an earlier study we found the expression of nuclear *vdr* mRNA to be significantly higher in cats with tooth resorption than in those without (16). Likewise, in the current study, we found a higher expression of VDR protein in fibroblasts from teeth with tooth resorption than in fibroblasts from teeth with reparative lesions (100% and 71%, respectively). The notion that vitamin D plays a role in tooth resorption in cats, as reflected by the expression of VDR protein in odontoclasts and fibroblasts in the active resorptive stage, is further supported by the local up-regulation of *1*α-hydroxylase and *24-hydroxylase* mRNA levels. The active metabolite $1,25(OH)_2D$ can be produced locally in cells that express 1α-hydroxylase, such as vascular cells and macrophages (32, 34, 35). The increased expression of *1*α-hydroxylase mRNA in dental samples from cats with tooth resorption supports the view that the local production of $1,25(OH)_2D$ is up-regulated, resulting in an active vitamin D pathway, as reflected by the up-regulated VDR levels, as well as *24-hydroxylase*, a target gene of the vitamin D pathway (36). Therefore, we propose that fibroblasts expressing VDR are more responsive to local $1,25(OH)_2D$ production and may play a role in the formation and/or activity of the odontoclasts.

In conclusion, we showed that in the active resorptive stage of tooth resorption, fibroblasts express high levels of VDR and MSX2, whereas odontoclasts express high levels of MSX2 and RANKL. We suggest that fibroblasts indirectly modulate odontoclast activity in tooth resorption in cats and propose that vitamin D might be a stimulatory paracrine or autocrine factor in the pathophysiology of tooth resorption in cats by activating the odontoclasts via the fibroblasts at these sites.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of the cat VDR protein sequence to human, rat, mouse and chicken protein sequences.