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# Cell Differentiation and Bone Protein Synthesis in the Lungs of Sheep with Spontaneous Calcinosis

**S. S. Barros and E. J. Gimeno***

*Department of Pathology, Veterinary Faculty, Federal University of Pelotas, P. O. Box 354, CEP 96010-900 Pelotas, Brazil and *Institute of Pathology, Faculty of Veterinary Sciences, National University of La Plata, P. O. Box 296, 1900 La Plata, Argentina*

**Summary**

The lungs of three sheep with spontaneous enzootic calcinosis induced by the calcinogenic plant *Nierembergia veitchii* (Nv) were examined electron microscopically and immunohistochemically. The main ultrastructural changes were activation of fibroblasts and modified smooth muscle cells (SMCs) in the pulmonary interstitium, with an increase in extracellular matrix and precipitation of calcium, either in a laminated pattern or as amorphous aggregates. Macrophages and multinucleated giant cells, some with calcium crystals in the cytoplasm, were found in areas of increased extracellular matrix. Thickening and replication of the basal lamina of capillaries were prominent. The bone proteins osteocalcin, osteopontin and osteonectin were detected immunohistochemically in the cytoplasm of activated fibroblasts, in modified SMCs and in the extracellular matrix. It is suggested that 1,25-dihydroxyvitamin $D_3$ in Nv induces cellular differentiation and the synthesis of a calcifiable matrix. 

## Introduction

Enzootic calcinosis (EC), caused by chronic plant poisoning in grazing livestock, is characterized by calcification of soft tissues and loss of body condition. Several calcinogenic plant species have been identified in different parts of the world (Worker and Carrillo, 1967; Dirksen *et al.*, 1970; Morris *et al.*, 1979; Riet-Correa *et al.*, 1987). *Nierembergia veitchii* (Nv) causes EC in sheep in southern Brazil (Riet-Correa *et al.*, 1987) and *Solanum glaucophyllum* (synonym *S. malacoxylon*) has been incriminated in parts of South America (Worker and Carrillo, 1967; Döbereiner *et al.*, 1971).

The gross and microscopical lesions of EC have been well described (Worker and Carrillo, 1967; Barros *et al.*, 1970; Döbereiner *et al.*, 1971; Morris *et al*, 1979; Puche *et al.*, 1980; Riet-Correa *et al.*, 1987). However, only a few ultrastructural investigations have been conducted on the tissues of animals affected by EC (Barros *et al.*, 1981; Moraña *et al.*, 1994; Marçolla *et al.*, 1997; Vasconcelos *et al.*, 1998; Barros and Rosa, 1999), and lung lesions have not been investigated electron microscopically.

Calcinogenic plants contain high concentrations of 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$] as glycoside derivatives (Wasserman *et al.*, 1976), as well as vitamin $D_3$ and $25(OH)_2D_3$ (Esparza *et al.*, 1982). The vitamin D endocrine system is important not only in calcium homeostasis but also in immune regulation, cell growth and cell differentiation (Suda *et al.*, 1986; Walters, 1992). Receptors for $1,25(OH)_2D_3$ have been found in a variety of cells and tissues (Stumpf *et al.*, 1979; Walters, 1992). The effects of vitamin D metabolites on growth and differentiation have been extensively studied in normal subjects and in those with diseases such as bone disease, psoriasis and cancer (Walters, 1992; Jones and Calverley, 1993). However, cell differentiation in plant-induced hypervitaminosis D has been evaluated only in the skin of cattle (Gimeno *et al.*, 1997).

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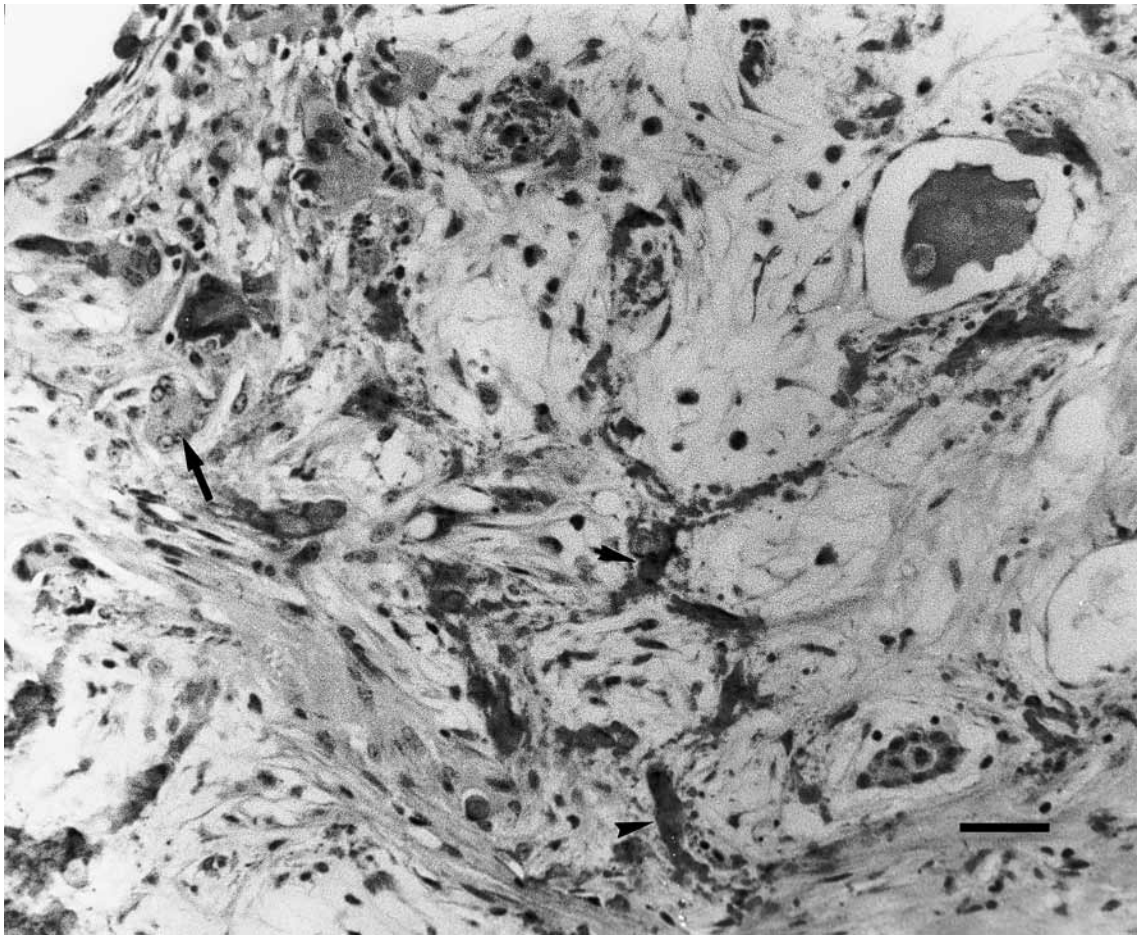

Fig. 1. Lung. Large areas of loose connective tissue with foci of mineralization, and infiltration of macrophages and multinucleated giant cell (arrow). HE. Bar, 70 μm.

The responses elicited by $1,25(OH)_2D_3$ are mediated mainly through the regulation of gene transcription (Brown *et al.*, 1992). Once bound to DNA, the $1,25(OH)_2D_3$-receptor complex alters the transcription of vitamin D response elements. As a consequence, a number of specific proteins are synthesized by the cell (Carlberg, 1997).

Soft-tissue mineralization in EC is preceded by changes in elastic fibres, extracellular accumulation of proteoglycans, and mesenchymal metaplasia (Puche *et al.*, 1980; Barros *et al.*, 1981).

The localization of receptors for $1,25(OH)_2D_3$ in mesenchymal cells (Brown *et al.*, 1992) suggests that the changes observed in EC are the result of a vitamin D-mediated stimulus to differentiation. If so, hyaluronic acid and bone proteins such as osteocalcin, osteopontin and osteonectin, induced by $1,25(OH)_2D_3$ would be expected to form part of the calcifiable extracellular matrix (Butler, 1989; Kraichely and MacDonald, 1998).

The purpose of this study was to investigate the ultrastructural changes in the lungs of sheep with EC and to search for hyaluronic acid, osteocalcin, osteopontin and osteonectin by immunohistochemical methods.

## Materials and Methods

### *Tissue Samples and Histopathology*

Samples were obtained from the lungs of three sheep which had been showing clinical signs of EC after grazing on a pasture containing abundant quantities of Nv. Cases of EC in sheep from this farm had been studied previously by Vasconcelos *et al.* (1998). The sheep were killed by exsanguination under deep anaesthesia and lung samples were fixed in 10% neutral formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with haematoxylin and eosin (HE) and von Kossa stain.

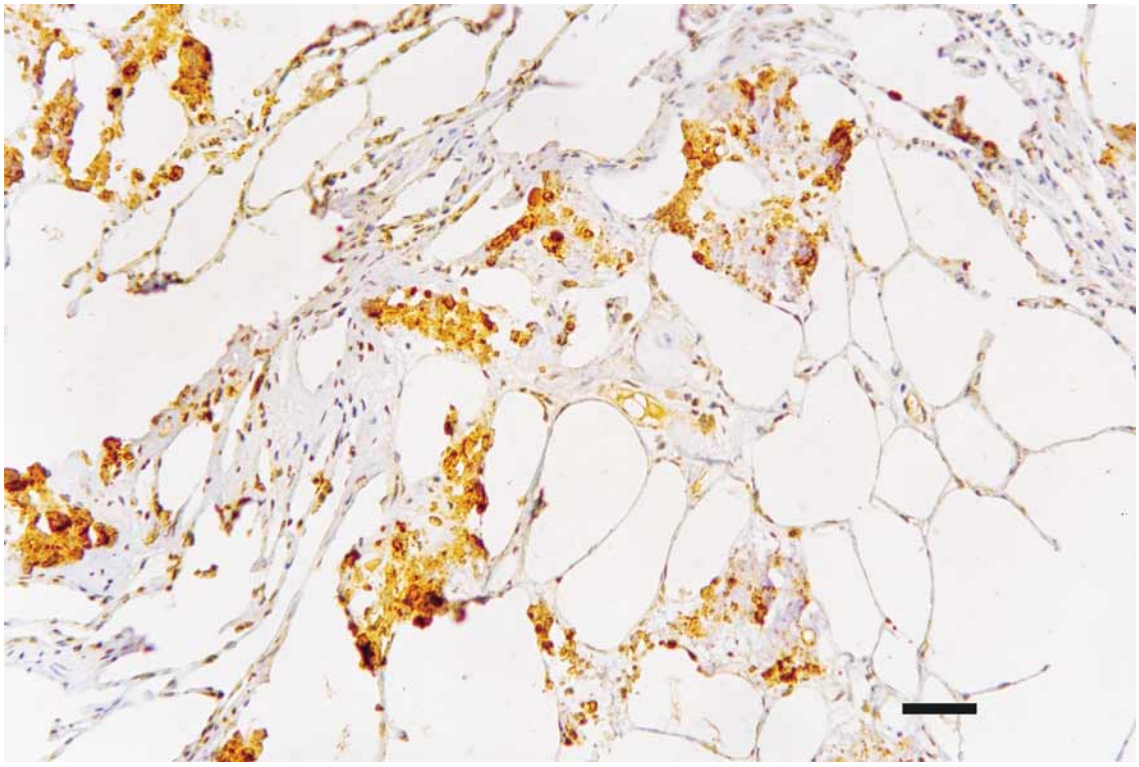

Fig. 2. Lung parenchyma. Distended alveoli and thickening of the interalveolar septa by loose connective tissue. Expression of osteocalcin in interstitial cells and extracellular matrix. Immunolabelling. Haematoxylin counterstain. Bar, 70 µm.

*Immunohistochemistry*

Sections were mounted on slides coated with 3-aminopropyltriethoxy-silane (Sigma Diagnostics, St Louis, MO, USA), dewaxed with xylene, passed through a graded alcohol series and rinsed three times in deionized water and phosphate-buffered saline (PBS). A microwave method (Portiansky *et al.*, 1997) was used for antigen retrieval.

The primary monoclonal antibodies used were as follows: osteocalcin (clone OC1; Biodesign International, Kennebunk, ME, USA) diluted 1 in 400, osteonectin (clone N50; Biodesign International) 1 in 800, osteopontin (clone MPIIIB10; Developmental Studies Hybridoma Bank, Iowa, USA) 1 in 500, and chondroitin sulphate (clone CS-56; Sigma) 1 in 400.

The immunohistochemical detection system was a streptavidin–biotin-complex technique (StreptABComplex/HRP Duet; Dako A/S, Glostrup, Denmark). The horseradish peroxidase was activated by incubation for 10 min with a buffered Tris-HCl 0·05 M solution, pH 7·6, containing diaminobenzidine (DAB) 0·02% and $H_2O_2$ 0·03%. Positively labelled cells showed a golden dark brown 3,3′-DAB tetrahydrochloride-$H_2O_2$ reaction product. After counterstaining with haematoxylin, the slides were dehydrated and mounted for examination.

*Electron Microscopy (EM)*

Small fragments of tissue were immersed in 0·3 M sodium cacodylate solution (pH 7·4) containing glutaraldehyde 2% and paraformaldehyde 2%. They were then post-fixed in osmium tetroxide, dehydrated in a graded ethanol series and embedded in Epon.

Thick sections were stained with methylene blue. From these sections, appropriate areas were selected and cut with a diamond knife. The ultrathin sections were mounted on copper grids and stained with lead citrate and uranyl acetate. The sections were examined with a transmission electron microscope.

## Results

*Gross Lesions and Histopathology*

Gross lesions in all sheep were characterized by severe mineralization, mainly in the arteries and valves on the left side of the heart. The lungs were mildly emphysematous and small areas of

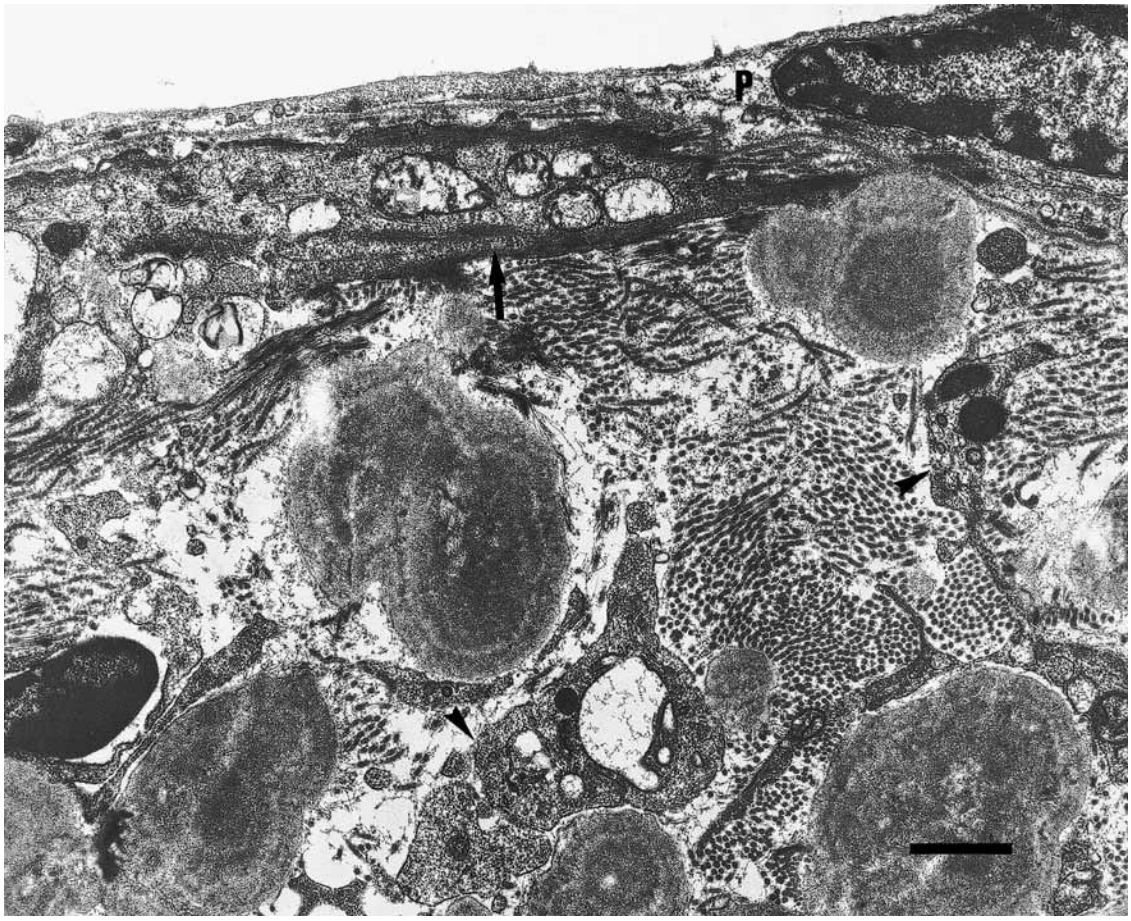


Fig. 3. Lung. Modified smooth muscle cell of synthetic type (arrow) with reduction and peripheral distribution of myofilaments and increased number of synthetic organelles. Bundles of collagen fibres are prominent. Profiles of mesenchymal cells (arrowheads) are in close contact with calcium deposits giving a laminated appearance. Pneumocyte (P). EM. Bar, 1 μm.

calcification were detected in the diaphragmatic lobes by palpation.

Microscopically, areas of distended alveoli with thickened walls caused by an increase in loose connective tissue were observed. Large areas of myxoid appearance were common. In such areas, spindle-shaped, branched or stellate cells, macrophages and giant cells were seen within a faintly eosinophilic extracellular matrix, with some dense bundles of collagen fibres and calcium precipitation (Fig. 1). Mineralization of the muscle layer of the bronchi and the bronchial cartilage, identified by von Kossa stain, were observed. The peribronchial tissue was infiltrated by mast cells, lymphocytes, macrophages, and multinucleated giant cells. Macrophages and multinucleated giant cells were also seen in the interstitium and within alveoli. Osseous metaplasia was observed in some heavily calcified areas.

*Immunohistochemistry*

Osteocalcin, osteonectin and osteopontin were detected in the cytoplasm of mesenchymal cells and also in the extracellular matrix (Fig. 2). Chondroitin sulphate immunoreactivity was observed only in hyaline cartilage of the bronchi.

*Electron Microscopy*

The ultrastructural changes included irregularly contoured, well demarcated structures in the interalveolar septa and in the myxoid areas. In these structures, calcium precipitates were found as amorphous or acicular crystals arranged in concentric bands of electrolucent and electrodense material (Fig. 3). Fibroblasts with hyperplastic rough endoplasmic reticulum and modified smooth muscle cells (SMCs) were associated with these

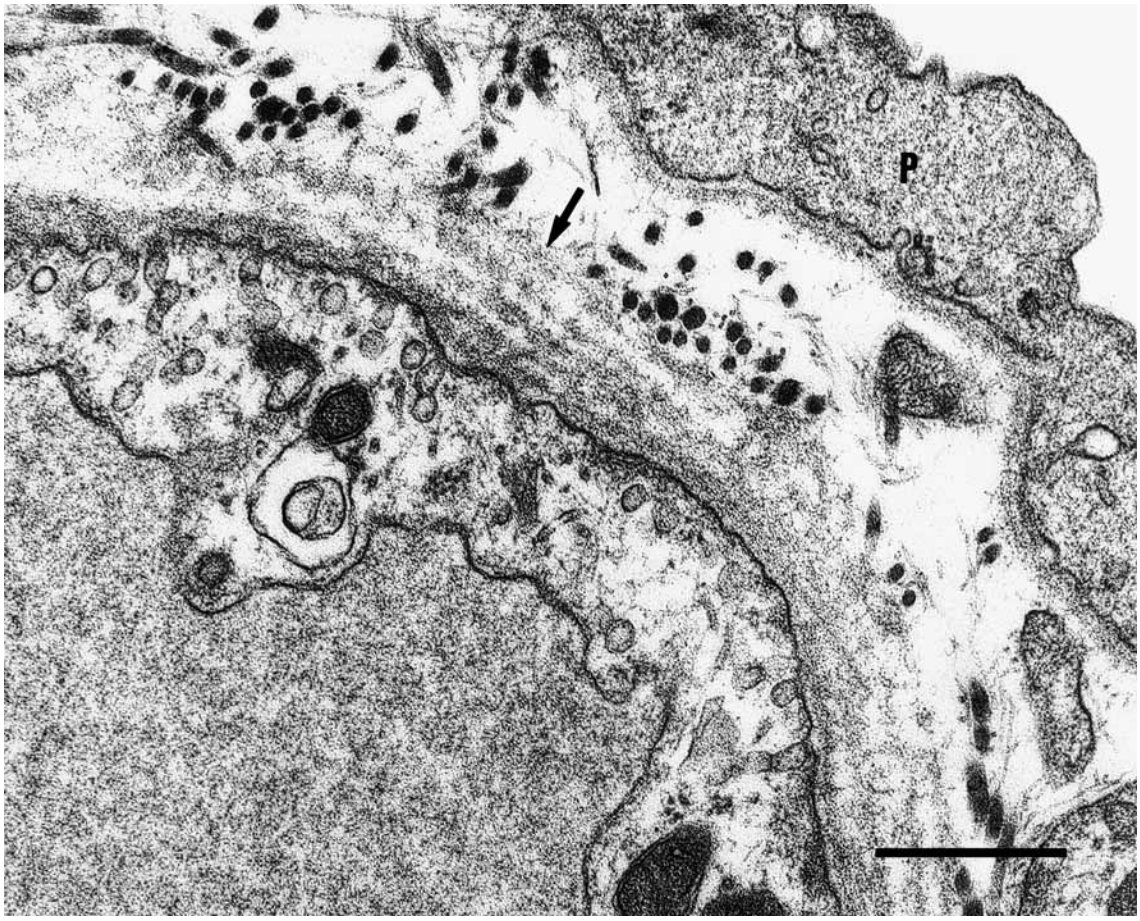


Fig. 4. Lung. Replication of the basal lamina (arrow) of capillary endothelial cell. Pneumocyte (P). EM. Bar, 1 μm.

mineralized areas and, in some places, with abundant collagen fibres. The modified SMCs showed conspicuous rough endoplasmic reticulum, increased numbers of mitochondria, free ribosomes, decreased numbers of myofibres and dense bodies. In most cases they had lost the basal lamina. Thickening and replication of the basal lamina were observed in some capillary endothelial cells (Fig. 4). Macrophages and multinucleated giant cells, some with free calcium crystals in the cytoplasm (Fig. 5), were seen in the interstitium or in the alveoli.

## Discussion

Calcification and osseous metaplasia of soft tissues are hallmarks of EC. Morphological, morphometrical and histochemical analyses have demonstrated changes in the elastic fibres, accumulation of proteoglycans, and deposits of calcium salts. Additionally, the development of cartilage and bone tissue has been reported frequently (Worker and Carrillo, 1967; Döbereiner *et al.*, 1971; Puche *et al.*, 1980). These metaplastic changes are preceded by proliferation of mesenchymal cells (Hänichen *et al.*, 1970; Döbereiner *et al.*, 1971; Barros *et al.*, 1981). Ultrastructural changes in SMCs of the arteries and stomach of spontaneous and experimental cases of EC were attributed to the action of $1,25(OH)_2D_3$ (Barros *et al.*, 1981; Moraña *et al.*, 1994; Vasconcelos *et al.*, 1998). However, little is known regarding the pathogenetic mechanisms in plant-induced calcinotic lesions.

The present investigation demonstrated early changes in the fibroblasts and SMCs of the interalveolar septa. Hyperplasia of the rough endoplasmic reticulum, and increased numbers of mitochondria and free ribosomes were observed in these secretory-type cells, which also synthesized collagen fibres and extracellular matrix. Immunohistochemically, osteocalcin, osteopontin and osteonectin were detected in both the mesenchymal cells and extracellular matrix.

Abe *et al.* (1984) reported that $1,25(OH)_2D_3$ induced *in vitro* the differentiation of monocytes to macrophages and their fusion to form

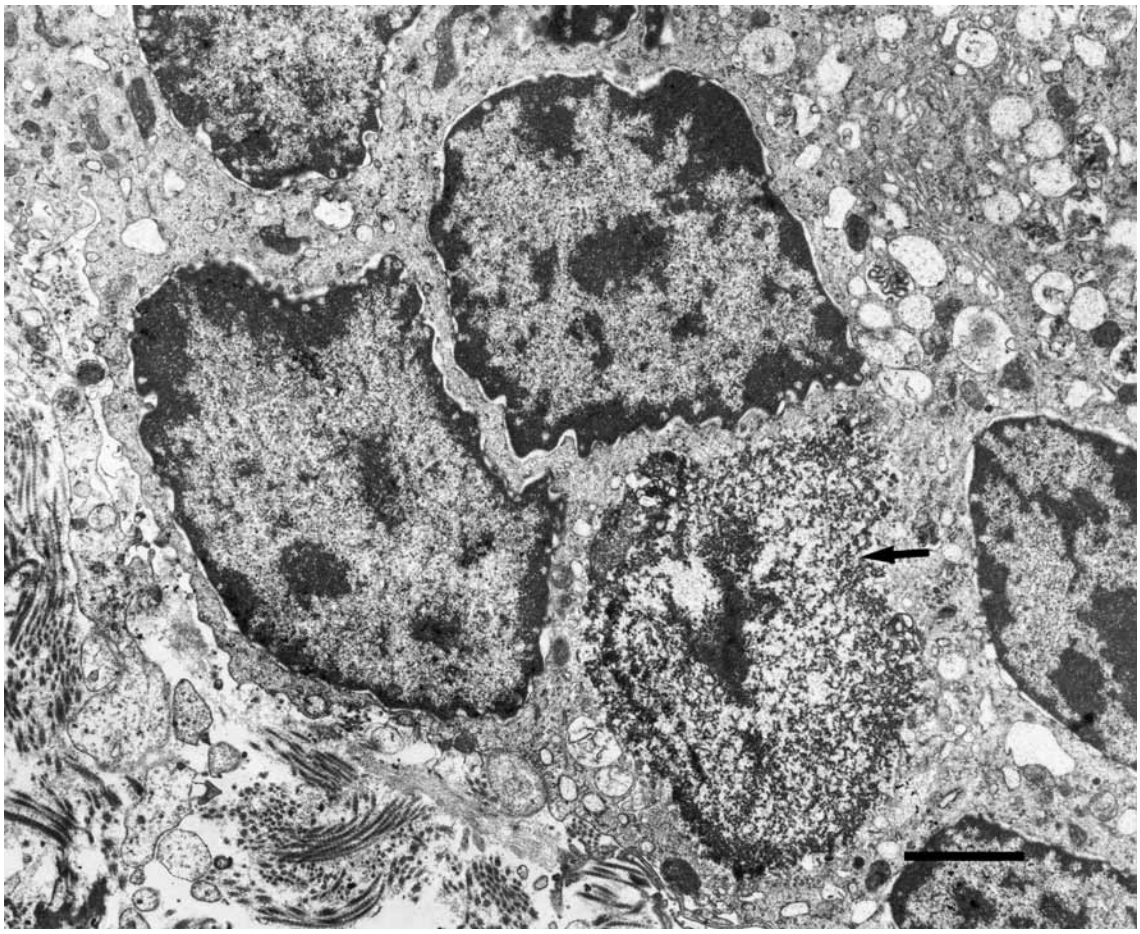

Fig. 5. Lung. Multinucleated giant cell with calcium crystal free in the cytoplasm (arrow). EM. Bar, 2 μm.

multinucleated giant cells. In the present study, macrophages and multinucleated giant cells, some with calcium crystals in their cytoplasm, were seen in the interstitium of the lungs of sheep with EC. Similar cells were reported previously in the lamina propria of the stomach (Moraña *et al.*, 1994) and peribronchial tissue (Barros and Rosa, 1999) of rabbits intoxicated experimentally by the calcinogenic plant *Solanum malacoxylon*, and in the arteries of spontaneous cases of EC in sheep (Vasconcelos *et al.*, 1998). The presence of large numbers of cells of the mononuclear phagocytic system in the lungs of sheep with EC may have resulted from the action of $1,25(OH)_2D_3$, as previously proposed in relation to the arteries of sheep with EC (Vasconcelos *et al.*, 1998). The giant cells may have been a response to mineralized tissue, but the presence of some such cells in areas without calcium precipitation reinforced the hypothesis of possible participation of $1,25(OH)_2D_3$ in their formation. The thickening and replication of the basal lamina of capillary endothelial cells seen in this study were also observed by Vasconcelos *et al.* (1998) in endothelial cells and modified SMCs of arteries of spontaneous cases of EC in sheep. This change may be a cellular response to the $1,25(OH)_2D_3$ activity but is not specific for any disease state (Ghadially, 1997).

The non-collagenous bone matrix proteins, osteocalcin, osteonectin and osteopontin, play a pivotal role in the processes of cell differentiation, cell activation and normal tissue mineralization (Sommer *et al.*, 1996; Cowles *et al.*, 1998). However, there are few references concerning the participation of these proteins in pathological calcification (Mohler *et al.*, 1997; Ohtsuki *et al.*, 1998), and such participation has not been studied in relation to EC lesions.

The results of this study indicate a possible specific differentiation effect of $1,25(OH)_2D_3$ on mesenchymal cells, as has been demonstrated in different cell types previously (Suda *et al.*, 1986; Brown *et al.*, 1992; Carlberg, 1997). Fibroblasts and SMCs became secretory cells, a modification

also observed in the arteries of animals with experimental (Barros *et al.*, 1981) and spontaneous (Vasconcelos *et al.*, 1998) EC. The expression of non-collagenous matrix proteins by activated fibroblasts or SMCs is partly coincidental with membranous ossification (Furusawa *et al.*, 1996).

Our results suggest that the morphological and biochemical modifications observed in the lungs of sheep with EC are attributable to specific genomic effects of $1,25(OH)_2D_3$.

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