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The growth and differentiation of aortal smooth muscle cells after calcitriol treatment are associated with microtubule reorganisation — an *in vitro* study

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The smooth muscle cells (SMCs) of the arterial media play a predominant role in functional and structural alterations of the arterial wall. The transition from the "contractile" to the "synthetic" phenotype appears to be an early critical event in the development of atherosclerotic disease. A number of observations suggest that 1,25(OH)$_2$D$_3$ (calcitriol) is of importance in maintaining normal cardiovascular function through its receptors in cardiac myocytes or aortal SMCs. The present study has focused on the microtubular (MT) network reorganisation after exposure to calcitriol. SMCs isolated by enzymatic digestion from the aortal media of neonatal rats were cultured on glass cover slips. 1 μM of 1,25(OH)$_2$D$_3$ was added to the culture medium every second day. The cytoskeletal features of SMCs after calcitriol were visualised by the immunofluorescence staining of α-tubulin. The alterations in α-tubulin expression and the distribution of microtubules related to the activities of the vascular smooth muscle cells, namely adhesion, migration, multilayer formation and cell division, were observed. A spindle shape, decreased cell adhesion, low expression of α-tubulin and a longitudinally arranged microtubular network manifested the high rate of SMC differentiation in the calcitriol-treated culture. A flat stellate morphology, high expression of α-tubulin and a radially distributed three-dimensional microtubular network were observed in the SMCs of the control culture. Destructive changes in the microtubular architecture which altered the cellular shape were evident in SMCs undergoing apoptosis. Cells with apoptotic features were more frequent in calcitriol-exposed culture. In contrast to the regular SMC divisions observed in the control culture, some of the mitotic cells exposed to calcitriol contained broader bipolar, multipolar or disordered spindles.

These alterations in the SMCs' microtubular cytoskeleton after calcitriol treatment were concomitant with changes in cell growth, differentiation and apoptosis, and may suggest a similarity to atherosclerotic plaque formation.

Key words: SMCs, 1,25(OH)$_2$D$_3$, cytoskeleton, tissue culture

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INTRODUCTION

In the early stages of atherosclerosis, the SMCs are modified from the differentiated "contractile" phenotype to the immature "synthetic" one. This phenotypic modulation allows SMCs to migrate into the intima, proliferate and secrete extracellular matrix components [1, 7]. This phenomenon, observed not only during atheromatous plaque formation but also in primary cultures of SMCs, is associated with the reorganisation of contractile and cytoskeletal proteins [13, 24, 28].

One of the main cytoskeletal components, together with actin microfilaments and intermediate filaments, is a highly organised network of microtubules composed of α and β-tubulin heterodimers. A number of studies in different cell types have shown that the microtubule cytoskeleton, which is very sensitive to differences in laboratory conditions, is essential for the polarisation of the motile cells and thus plays an important role in regulating cell adhesion/migration [17, 23]. Dramatic microtubule rearrangements take place during cell division and differentiation [6]. Apoptosis occurs in response to various stimuli under physiological and pathological circumstances, whereas loss of cell volume and degradation of the tubulin itself take place during the very early stages of apoptosis [8, 12]. It is not known how the disruption of microtubules leads to more advanced stages of apoptosis. It is well established, however, that, apart from its traditional role in calcium homeostasis, the active form of vitamin D3 — 1,25-$(OH)_2$cholecalciferol, has been shown to modulate different cellular activities and participate in such diverse functions as cellular proliferation and differentiation [19]. Recent studies suggest that 1,25-$(OH)_2D_3$ plays an important role in the cardiovascular system through its receptors in the heart and vascular smooth muscle cells. In particular, 1,25-$(OH)_2D_3$ has been shown to modulate growth, and increase calcification in smooth muscle cells [9, 11, 15, 16, 25, 26], although its effect on the microtubule network organisation has not been investigated.

MATERIAL AND METHODS

The investigation was carried out according to the requirements of the Ethics Committee for Animal Care of Poland.

Isolation and culturing of cells

The cytoskeletal protein expression of SMCs was studied in cultured cells obtained from the media of the aorta of neonatal Wistar rats. The rats were purchased from a single animal breeding company. The aortas were immediately transferred into Eagle's Minimum Essential Medium (MEM, Biomed, Poland) enriched with 10% foetal bovine serum (FBS, Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin. After careful removal of the adventitia, the aortas were cut into 1 mm segments and digested for 1 hour with a 0.3% solution of collagenase 1A (Sigma). The resulting cell suspension was filtered through 50 μm nylon mesh, centrifuged three times (5 min $\times$ 100 g) and normally plated at a density of 2×10^5 viable cells/dish in plastic culture dishes or on glass coverslips. They were then incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. The culture medium was changed every second day, and each time 1 μM of 1,25$(OH)_2D_3$ (a gift from Prof. Kutner) in 95% ethanol was added to the medium. The same amount of ethanol was added to one of the two control cultures and the final concentration of ethanol both in the test and the control tissue culture medium was 0.1%. The second control culture was maintained without calcitriol or alcohol. The cells were examined from 5 to 12 days after inoculation, when they grew logarithmically in primary culture.

Examination of cell viability

Trypan blue (Sigma) was added to the culture of SMCs with the help of a haemocytometer, and the cells were assessed with an inverted microscope to count living and dead cells. About 95% of cells obtained by this method excluded trypan blue.

Immunocytochemical staining

The analysis of the cytoskeleton of *in vitro* cultured SMCs was performed employing the direct immunofluorescence method. The cells attached to the cover slips were washed with PBS, fixed by immersion in absolute methanol for 5 min at –20°C, and air-dried. Prior to incubation with antibodies, the specimens were washed with PBS and blocked for 2 min in PBS containing 1% foetal bovine serum. The culture was incubated for 30 min at room temperature with the anti-α-tubulin monoclonal antibody conjugated with FITC (Sigma, F2168) diluted 1:200 in PBS.

The cells were also stained with anti α-smooth muscle actin monoclonal antibody conjugated with FITC (Sigma, F3777, 1:500). After gentle washing in PBS the cells were mounted in glycerol and examined with a Nikon Eclipse 800 microscope equipped for epifluorescence using the appropriate filter set.

RESULTS

SMCs were attached to the glass cover slips and spread out within 48 h after seeding in culture. 95% of the cells were positively identified as SMCs by their reaction with antibodies against α-smooth muscle actin, which is specific to both contractile and synthetic phenotypes (Fig. 1).

The proliferating cells that formed the monolayer were large, well spread and polygonal in shape with lamellipodia which appeared as flap-like structures (Fig. 2). A clearly defined, three-dimensional cage of microtubules surrounded the cell nucleus. MTs of the control cells radiated from the centrosome, while in the cell body they were sinuous (Fig. 4, 8). During enhanced proliferation a significant proportion of SMCs spontaneously converted to a more mature phenotype (Fig. 6). In the later stages of control culture multilayered regions appeared focally as mounds surrounded by a monolayer (Fig. 8). After calcitriol treatment a large number of loosely arranged SMCs exhibited morphological and functional characteristics consistent with a differentiated phenotype. The SMCs were spindle-shaped with a well-defined long axis (Fig. 3, 5). Reduced cell spreading was associated with increased cell migration and decreased cell adhesion (Fig. 7). In older culture calcitriol-treated SMCs clearly changed their growth pattern forming more abundant multilayers (Fig. 9).

Dramatic changes in microtubule distribution, which altered cellular shape, were evident in SMCs undergoing apoptosis. The microtubular cytoskeleton was gradually disrupted, which correlated with alterations in the shape of apoptotic cells. The cells rounded up and became less adhesive to the substratum (Fig. 10, 11). The morphological features characteristics of apoptosis were more frequent in the cultures after calcitriol addition.

Some of the mitotic cells of calcitriol-treated asynchronous culture contained broader (polyploid?) bipolar, multipolar or disordered spindles (Fig. 14–17), whereas such abnormalities were not observed during the cell divisions in control culture (Fig. 12, 13).

DISCUSSION

1,25-dihydroxycholecalciferol, the active form of vitamin D_3, acts many physiological responses in a variety of cell types [19]. Our previous results demonstrated that calcitriol promoted the initial rate of phenotypic modulation, resulting in an increase in cell proliferation in log-phase [25, 26]. It is well established

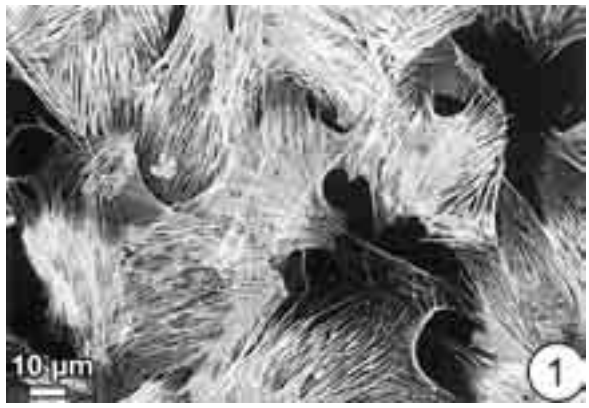

Figure 1. Identification of cultured cells as SMCs by positive reaction with anti a-smooth muscle actin monoclonal FITC conjugated antibody. An abundant three-dimentional network of microfilaments with stress fibres can be seen.

that mature contractile SMCs in culture can undergo phonotype modulation to the immature synthetic type, but in postconfluent primary culture they can spontaneously differentiate [2, 3, 18, 22, 24–27]. In the present study novel direct evidence was provided that noncontractile SMCs, after prolonged exposure to 1 μM of 1,25-$(OH)_2D_3$, developed quickly into a fully differentiated state. This high concentration, causing significant responses in our experiments, was not comparable with the physiological circulating level of calcitriol in the animal organism. Much higher local concentrations of 1,25-$(OH)_2D_3$ could, however, be reached under pathophysiological conditions [19]. A variety of cellular factors can modulate microtubule dynamics and recent publications have proposed that SMC phenotypic states be distinguished by alterations in the expression and distribution of cytoskeletal proteins [4, 21, 28]. Immunofluorescence study using a monoclonal antibody to α-tubulin indicated that calcitriol induced visible microtubule reorganisation. Our data showed that microtubule stability modulated by calcitriol allowed dramatic microtubule rearrangements concomitant with changes in SMC proliferation, migration, cell division and differentiation. The results are comparable with those obtained by Chitaley and Webb [5]. The authors drew the conclusion that the microtubular network was expressed in increased amounts in the synthetic phenotype as long as the SMCs received a stimulus to growth, whereas the contractile pathway was enhanced by microtubule depolymerisation. The cytoskeleton takes on a more active role in apoptosis, which occurs in response to various stimuli under physiological and pathological circumstances [8, 10, 12, 14]. In our studies calcitriol-

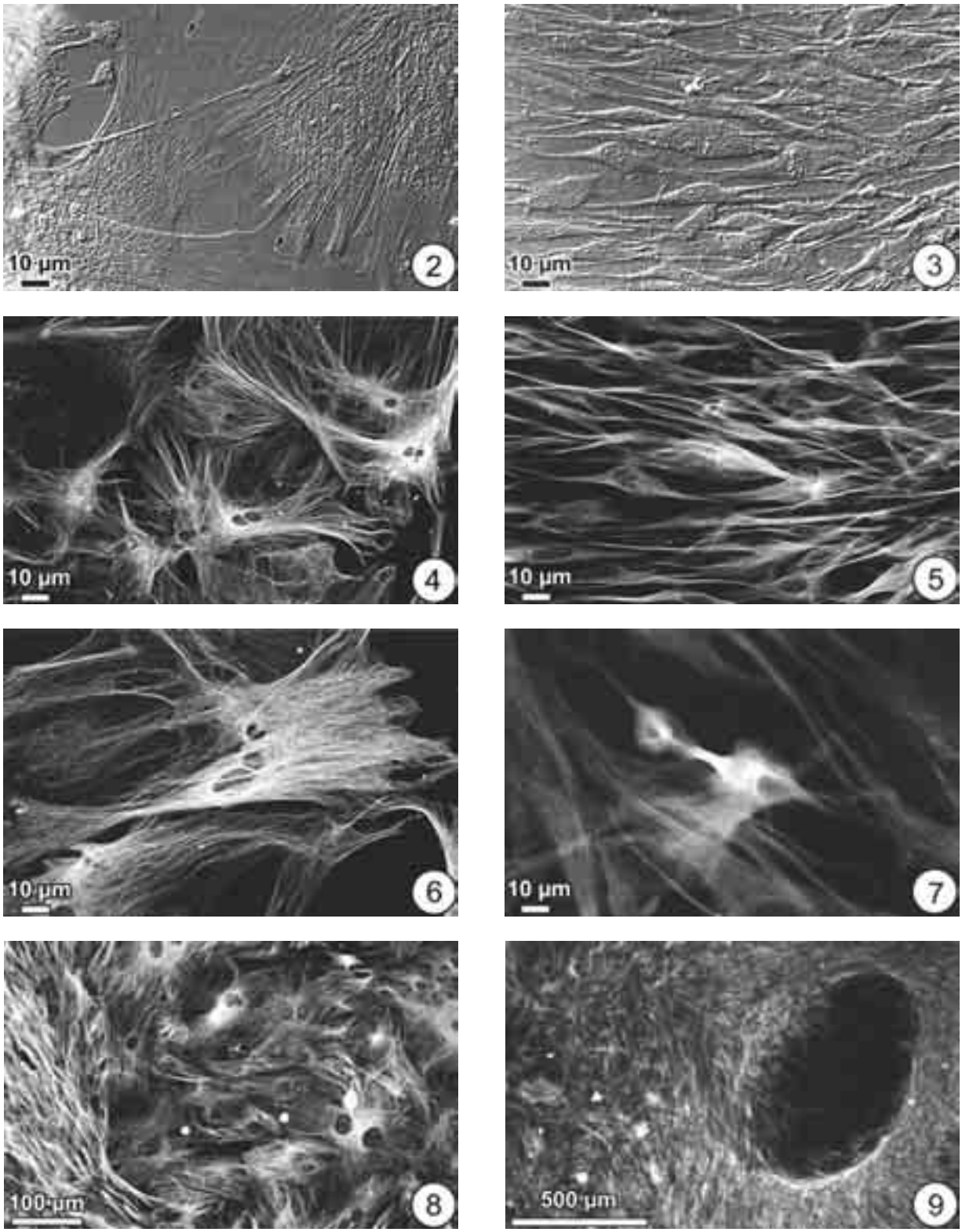

Figure 2–9. SMCs in primary culture fixed and immunostained with anti-α-tubulin monoclonal FITC conjugated antibody; Fig. 2, 4, 6, 8 — control culture; Fig. 3, 5, 7, 9 — calcitriol-treated culture; Fig. 2, 3 — SMCs viewed under Nomarski differential interference contrast; arrows point to lamellipodia.

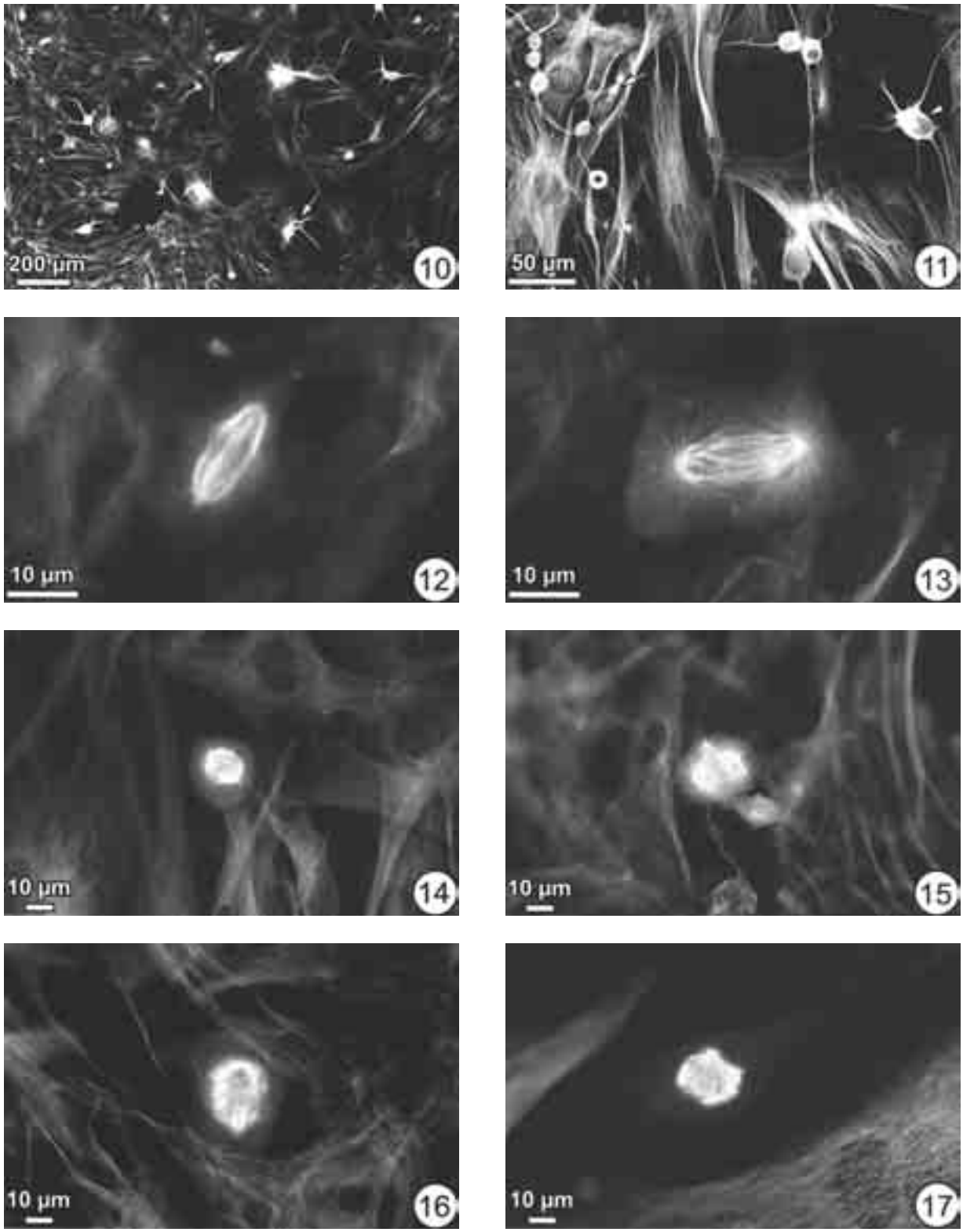

Figure 10–17. SMCs in primary culture fixed and immunostained with anti-α-tubulin monoclonal FITC conjugated antibody; Fig. 10 — calcitriol treated culture; arrow points to SMC undergoing apoptosis; Fig. 11 — SMCs during apoptosis in calcitriol-treated culture; arrowhead points to SMC rounding-up and losing adhesion at an early stage of apoptosis; the asterisk marks apoptotic cell; Fig. 12, 13 — mitoses in control culture; typical metaphasic and anaphasic cells, respectively; Fig. 14, 17 — abnormal spindles (broader bipolar, tripolar, multipolar and tetrapolar, respectively) in dividing cells from culture exposed to calcitriol.

induced tubulin polymerisation took place, although the microtubular network was gradually disrupted, which correlated with apoptotic morphology. We observed dramatic changes in microtubule disorganisation which altered cellular shape. Cells were rounded up, shrank and became less adhesive to the substratum. These characteristics were similar to those observed during apoptosis following treatment with other drugs that do not interact directly with tubulin [8]. It is known that apoptosis takes place when the structural integrity of the cytoskeleton is compromised and cell shape is altered. The mechanisms responsible for cell shrinkage during this process are, however, poorly understood [12]. Our notions are also in agreement with others which suggest that adhesion-dependent changes in tubulin structure may play a supporting role in both the induction and execution of the apoptotic program [23]. We also observed an increased incidence of atypical mitotic figures of calcitriol-treated asynchronous culture. These abnormalities in mitotic spindles, organised by multiple poles, can lead to chromosome segregation errors. Such defective mitoses are a common feature of human cancers but the mechanisms leading to these abberations are still unclear [20].

In conclusion, the findings indicate that microtubule alterations in aortal SMCs exposed to calcitriol in primary culture were concomitant with changes in cell growth, differentiation and apoptosis.

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