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Increased migratory properties of aortal smooth muscle cells exposed to calcitriol in culture☆

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ABSTRACT

The aim of the present study was to examine the effect of $1,25(OH)_2D_3$ (calcitriol) on SMC (smooth muscle cell) migration, especially in the context to atherogenesis. SMCs were obtained from the aortas of newborn Wistar rats by enzymatic digestion. Different aspects of cell behavior during migration in culture were examined by phase contrast, fluorescence and electron microscopy (TEM, SEM) and supported by flow cytometric and biochemical analyses. Morphological studies revealed that supra-physiological (1–100 nmol/l) concentrations of calcitriol inhibit SMC differentiation, therefore these cells display several hallmarks of the synthetic state. Dynamic changes in actin cytoskeleton organization were a critical event in SMC shape, adhesion and spreading. Calcitriol diminished stress fibers assembly and focal adhesions formation. Reduced expression of β_1-integrin receptors on SMC surface after exposition to calcitriol coincided with increased proliferative and migratory activities of these cells. Moreover, after calcitriol stimulation, the ability of SMCs to the production of proinflamatory cytokines IFN-γ, TNF-α and IL-6 was inhibited. The results from these comparative investigations indicate that $1,25(OH)_2D_3$ inhibit differentiation and facilitate SMC migration in culture. It has been also suggested that such responses of SMCs to calcitriol play a beneficial role in fibrous cap formation during atherosclerotic process.

1. Introduction

The medial layer in healthy arteries mainly consists of highly specialized smooth muscle cells, which play a key role in the maintenance of vascular homeostasis. Although the principal function of SMCs in mature animals is contraction, they exhibit a high degree of phenotypic and functional plasticity [1]. In response to vascular injury, SMC can undergo a phenotypic modulation, proliferate as well as migrate from media to intima, secrete and deposit ECM, thereby contribute to the formation and progression of atherosclerosis [2]. Similar conditions occur when normal smooth muscle cells are established in culture. However, 1,25-dihydroxyvitamin D_3 causes many physiological responses in a variety of cells but its biological importance in vascular homeostasis is highlighted by the facts that SMC possess the vitamin D receptor (VDR) and are able to synthesize $1,25(OH)_2D_3$ [3,4]. In the migration of vascular smooth muscle cells an essential role plays dynamic remodeling of the cytoskeleton elements, but the actin cytoskeleton is the most important target of integrin-mediated signaling [5,6]. It has been shown recently that integrin signaling contributes not only to cell adhesion and motility, but also regulates cell proliferation, survival and differentiation [7]. The β_1-family of integrins represents the major class of cell-ECM adhesion receptors which connect the extracellular matrix to the actin cytoskeleton [8]. To gain more insight into biological responses of SMCs to calcitriol we investigated migration process due to its importance in the formation of vascular lesions. The high resolution of scanning and transmission electron microscopy makes possible the studies of the protrusive actin-based structures such as microvilli, filopodia, and lamellipodia, however preservation of fine surface structure was problematic.

2. Materials and methods

2.1. Cell culture

SMCs were obtained from the media of neonatal Wistar rat's aorta. Rats were purchased from a single animal breeding company. SMC isolation, purification and culturing were performed according to the method previously described [9]. The procedures were per-

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formed in accordance with institutional requirements of the Ethics Committee for Animal Care of Poland. $1,25(OH)_2D_3$ (Sigma) was dissolved in 95% ethanol and stored at −80 °C. The culture medium was changed every second day, and each time 10–100 nm/l of calcitriol was added. The purity and identity of the SMCs cultures was confirmed by immunocytochemical staining for α-smooth muscle actin (Sigma). Viable cells were identified by the exclusion of trypan blue and were higher than 95%. Primary cultures of SMCs and also passage 2 or 3 were used for experiments.

2.2. Immunofluorescence

The analysis of cytoskeleton elements of SMCs in primary culture was performed employing the direct immunofluorescence method. The cells attached to cover slips were fixed in absolute methanol at −20 °C, and air-dried. The cultures were incubated with the anti-α-tubulin monoclonal FITC-conjugated antibody or anti-α-actin monoclonal FITC conjugated antibody (Sigma, USA). Material was examined with a Nikon Eclipse 800 microscope equipped for epifluorescence using the appropriate filter set.

2.3. Ultrastructural studies

2.3.1. TEM

The cells were fixed directly in Petri dishes in 2.5% glutaraldehyde (GA) with 0.15% picric acid, and with 15% OsO_4 in 0.1 M Na–cacodylate buffer at pH 7.4. Fixation was carried out at 4 °C for 2 h and then cell culture was rinsed three times in the same buffer. Following fixation the cells were treated with 1.5% tannic acid, dehydrated with graded series of ethanol, immersed in propylene oxide, embedded in Epon 812 and polymerized. Semi-thin sections (1.5 μm) were stained with toluidine-blue and examined by light microscopy. The ultra-thin sections were collected on formvar coated cooper grids, double-stained with lead citrate and uranyl acetate and examined with the aid of a JEM 1200EX II electron microscope at an accelerating voltage of 80 kV.

2.3.2. SEM

The SMC grown on slides were fixed in 2.5% glutaraldehyde in 0.1% cacodylate buffer, postfixed in 2% OsO_4, dehydrated in alcohol–acetone series and dried by the critical point method using

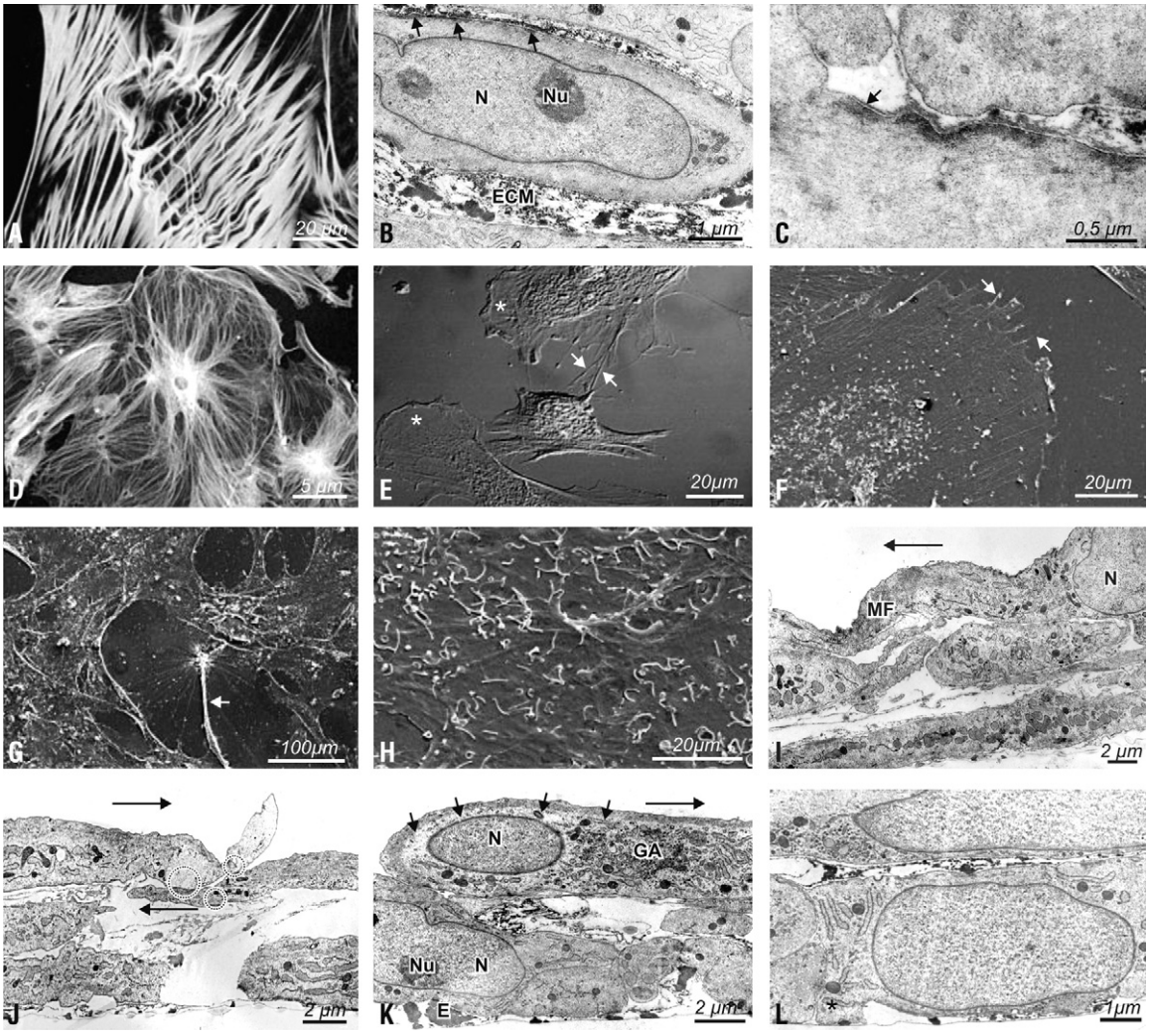

Fig. 1. SMCs on day 7 in primary culture (A–C). Highly expressed network of actin stress fibers stabilize "contractile" phenotype of SMC in control culture (A). Electron micrograph illustrating the ultrastructural features of differentiated SMC. Note: densely packed actin filaments in focal adhesions to ECM indicated by arrows (↓). Extracellular matrix (ECM) elements are visible between neighboring cells (B). Basement membrane is partially rebuilt after the differentiation of cells (C). SMCs on 7 day in primary culture exposed to calcitriol (D–L). Cytoskeleton visualized by reaction with anti-α-tubulin FITC (D). Cells viewed under phase-contrast light microscope (E) and scanning electron microscope (F–H). Sheet-like protrusive structures – lamellipodia are indicated by asterisk (*), finger-like protrusions – filopodia by arrow (↓). Spread SMC with numerous microvilli (H). Ultrastructure of migrating SMCs exposed to calcitriol (I–L). Focal contacts (Fc) stabilizing lamellipodium are indicated by circle (J). Thick arrows mark direction of cell movement (→). MF, myofilaments. Cortical array of actin filaments is clearly visible beneath the plasma membrane in part of the cell passively moving forward (K ↓). Nucleus (N). Golgii apparatus (GA). Elastin (E). Invadopodium (L*).

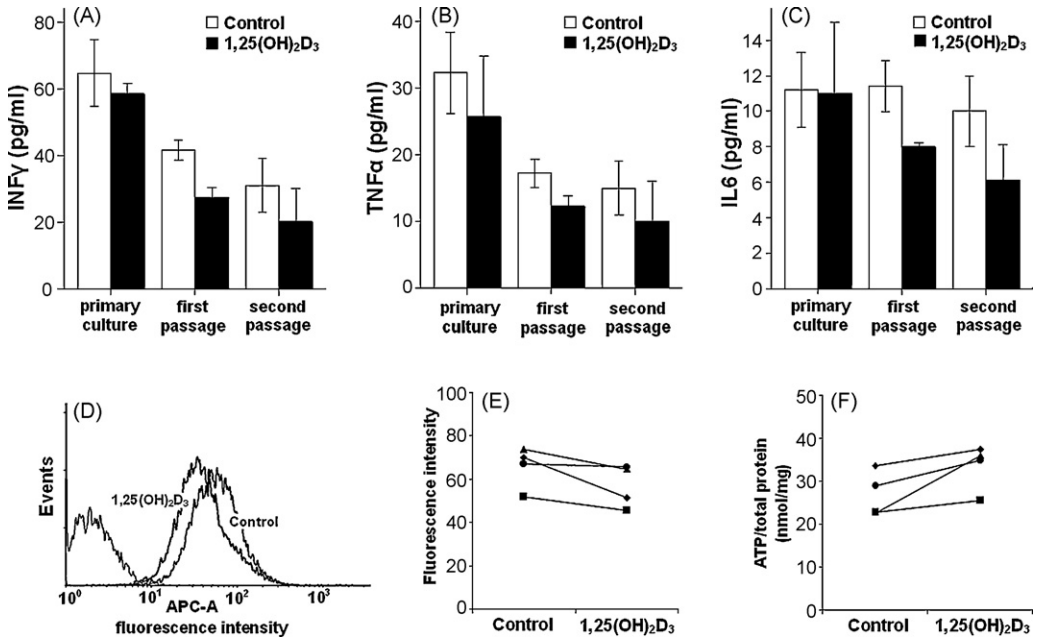

Fig. 2. The effects of 1α,25(OH)$_2$D$_3$ (10 nmol/l) on proinflammatory cytokine production by SMCs in culture (A–C). Calcitriol decreased the levels of all proinflammatory cytokines (TNF-α, IFN-γ, and IL-6) examined in the culture supernatants. All the experiments were performed in triplicate and repeated at least three times. Values are given as mean ± SD and statistically significant ($P < 0.01$) differences were assessed using the unpaired Student's *t*-test. Expression of β_1-integrin receptors in plasma membrane of SMCs from calcitriol-treated (10 nmol/l) and control culture (D). Effects of calcitriol on β_1-integrin receptors level (E). Data were averaged from at least four independent experiments in each group. Statistical significance was assessed by an impaired Student's *t*-test. *P*-value of <0.01 was considered significant. Effects of calcitriol on ATP content in SMC cultured for 9 days (F). All the experiments were performed in triplicate and repeated at least four times. Statistical significance was assessed by an impaired Student's *t*-test. *P*-value of <0.047 was considered.

liquid CO_2. Finally, specimens were coated with gold in a sputter-evaporator (Balzers) and stored under vacuum until they were examined and imaged in a Philips XL 30 scanning electron microscope operated at an accelerating voltage of 10 kV.

2.4. Flow cytometric analyses

2.4.1. β_1-Integrin expression

The attached cells were collected (5×10^5 cell), suspended with monoclonal anti-β_1-inegrin antibody (I G1, clone MAR4, BD) conjugated with allophycocyanine (APC) and incubated in the dark. After fixation the cells were analyzed by flow cytometry (Becton Dickinson, LSR II).

2.4.2. Cytokine production

Cell culture supernatants were collected and cytokines (IFN-γ, TNFα and IL-6) were measured by the flow Cytometric Bead Array (CBA), using Rat Flex Set BD^{TM} according to the manufacturer's instructions.

3. Results

Aortal SMC modulated from the "contractile" to the "synthetic" phenotype in culture. Such transition between phenotypes resulted in high rate of proliferation and production of EMC. On the other hand, differentiation of SMCs (phenotypic reversion) has been also observed (Fig. 1B). Immunofluorescence analysis for SM α-actin revealed that stress fibers were more prominent in differentiated stationary stage of smooth muscle cells, especially in control variant of primary culture (Fig. 1A). Actin filaments terminated at focal adhesions were predominantly found in tightly adherent, resting cells (Fig. 1C). Cytoplasmic protrusions including both sheet-like structures such as lamellipodia or raffles, and finger-like extensions such as fillopodia or microvilli were frequently visible in migrating SMC exposed to calcitriol in culture (Fig. 1E, F, I, and J). Lamellipodia protruded at the front of the cell weakly attached to the underlying cell or substratum (Fig. 1I and J). Small adhesions, mediating attachment of the cell membrane to the extracellular matrix, known as focal contacts (Fc) were observed at the leading edge of migrating cells (Fig. 1J). In addition to actin filament remodeling, cell migration also revealed remodeling of microtubules. As cells migrated, the nucleus moved towards the rear of the cell (Fig. 1K) but microtubules radiated from the microtubule-organizing center (MTOC) to both the front and the rear of the cell (Fig. 1D). Diminished surface expression of β_1-integrin on SMC exposed to calcitriol (Fig. 2D and E) was associated with increased proliferative and migratory activities of these cells. Concentration of ATP after the exposition of SMCs to 10 nmol/l of calcitriol was significantly increased (Fig. 2F). Moreover, the ability of SMCs to produce proinflamatory cytokines such as IFN-γ, TNF-α and IL-6 after calcitriol stimulation was reduced (Fig. 2A–C).

4. Discussion

Here, we investigated the migration of vascular SMCs due to its importance in the formation of vascular lesions. Considerable evidence suggests that modifications in the matrix deposition maybe responsible for an increased rate of SMCs proliferation and migration. It has been also implicated that elastin is a negative regulator of SMCs activity within the arterial wall and promotes quiescent, contractile phenotype of these cells [6,10]. Our previous report demonstrated that accelerated proliferation of aortal SMCs exposed to active metabolite of vitamin D [1,25(OH)$_2$D$_3$] coincided with impaired formation of elastic fibers in vitro [9]. In the current study we have found marked morphological changes in adhesion and spreading of SMCs after exposition to supraphysiological concentrations of calcitriol which well correlated with their intensive migration. Cell migration was sensitive to the proliferative state of SMCs, nonproliferating cells were not motile. Our study supports the notions that dynamic remodeling of the actin cytoskeleton

plays an essential role in the migration of vascular smooth muscle cells [11–13]. Down-regulation of the β_1-integrin in SMC exposed to calcitriol determined their phenotypes, and therefore these cells displayed several hallmarks of the synthetic, migratory state. Diminished surface expression of β_1-integrin on SMC most likely reduced binding of the cells to the matrix [5–7]. It is known that SMCs in vitro have the potential to express reactive mediators like cytokines involved in inflammation [14]. In our study the ability of SMCs to produce proinflamatory cytokines such as IFN-γ, TNF-α and IL-6 was reduced after calcitriol stimulation. This data confirmed the results obtained by others that calcitriol is a potent modulator of inflammation involved in all stages of atherosclerosis [15].

In conclusion, $1,25(OH)_2D_3$—an active form of vitamin D applied in supraphysiological concentrations of 10 nmol/l inhibited differentiation and facilitated migration of aortal smooth muscle cells in culture. It has been also suggested that observed physiological responses of SMCs to calcitriol play a beneficial role in fibrous cap formation during atherosclerotic process.

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References

[1] R. Ross, The pathogenesis of atherosclerosis: a perspective for the 1990s, Nature 362 (1993) 801–809.
[2] A.W. Clowes, M.M. Clowes, J. Fingerle, M.A. Reidy, Regulation of smooth muscle cell growth in injured artery, J. Cardiovasc. Pharmacol. 14 (1989) S12–S15.
[3] D. Somjen, Y. Weisman, F. Kohen, B. Gayer, R. Limor, O. Sharon, N. Jaccard, E. Knoll, N. Stern, 25-Hydroxyvitamin D_3-1α hydroxylase is expressed in human vascular smooth muscle cells and is up regulated by parathyroid hormone and estrogenic compounds, Circulation 111 (2005) 1666–1672.
[4] J. Merke, W. Hofmann, D. Goldschmidt, E. Ritz, Demonstration of $1,25(OH)_2$ vitamin D_3 receptors and actions in vascular smooth muscle cells in vitro, Calcif. Tissue Int. 41 (1987) 112–114.
[5] S. Abraham, N. Kogata, R. Fässler, R. Adams, Integrin beta-1 subunit controls mural cell adhesion, spreading, and blood vessel wall stability, Circ. Res. 102 (2008) 562–570.
[6] W.T. Gerthoffer, Mechanisms of vascular smooth muscle cell migration, Circ. Res. 100 (2007) 607–621.
[7] C. Brakebusch, R. Fässler, The integrin–actin connection, an eternal love affair, EMBO J. 15 (2003) 2324–2333.
[8] S. Wiesner, K.R. Legate, R. Fässler, Integrin–actin interactions, Cell Mol. Life Sci. 62 (2005) 1081–1099.
[9] C. Tukaj, Enhanced proliferation of aortal smooth muscle cells treated by $1,25(OH)_2D_3$ in vitro coincides with impaired formation of elastic fibers, Int. J. Exp. Pathol. 89 (2008) 117–124.
[10] S.R. Peyton, A.J. Putnam, Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion, J. Cell Physiol. 204 (2005) 198–209.
[11] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (2003) 1704–1709.
[12] S. Pellegrin, H. Mellor, Actin stress fibers, J. Cell Sci. 120 (2007) 3491–3499.
[13] D.D. Tang, Y. Anfinogenova, Physiologic properties and regulation of the actin cytoskeleton in vascular smooth muscle, J. Cardiovasc. Pharmacol. Ther. 13 (2008) 130–140.
[14] C.A. Singer, S. Salinthone, K.J. Baker, W.T. Gerthoffer, Synthesis of immune modulators by smooth muscles, Bioessays 26 (2004) 646–655.
[15] A. Zittermann, S. Schleithoff, R. Koerfer, Putting cardiovascular disease and vitamin D insufficiency into perspective, Br. J. Nutr. 94 (2005) 483–492.