Journal of Steroid Biochemistry & Molecular Biology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Cell cycle arrest and apoptosis induced by 1α,25(OH)$_2$D$_3$ and TX 527 in Kaposi sarcoma is VDR dependent

Verónica González-Pardo[a,*], Alejandra Suares[a], Annemieke Verstuyf[b], Pierre De Clercq[c], Ricardo Boland[a], Ana Russo de Boland[a]

[a] Departamento de Biología, Bioquímica & Farmacia, Universidad Nacional del Sur-CONICET, 8000 Bahía Blanca, Argentina
[b] Laboratory of Clinical and Experimental Endocrinology, KU Leuven, B-3000 Leuven, Belgium
[c] Vakgroep Organische Chemie, Universiteit Gent, Krijgslaan 281 S4, B-9000 Gent, Belgium

ARTICLE INFO

Article history:
Received 27 June 2013
Received in revised form
15 November 2013
Accepted 23 November 2013

Keywords:
1α,25(OH)$_2$D$_3$
TX 527
VDR
Apoptosis
Kaposi sarcoma

ABSTRACT

We have previously shown that 1α,25(OH)$_2$-Vitamin D$_3$ [1α,25(OH)$_2$D$_3$] and its less calcemic analog TX 527 inhibit the proliferation of endothelial cells transformed by the viral G protein-coupled receptor associated to Kaposi sarcoma (vGPCR) and this could be partially explained by the inhibition of the NF-κB pathway. In this work, we further explored the mechanism of action of both vitamin D compounds in Kaposi sarcoma. We investigated whether the cell cycle arrest and subsequent apoptosis of endothelial cells (SVEC) and SVEC transformed by vGPCR (SVEC-vGPCR) elicited by 1α,25(OH)$_2$D$_3$ and TX 527 were mediated by the vitamin D receptor (VDR). Cell cycle analysis of SVEC and SVEC-vGPCR treated with 1α,25(OH)$_2$D$_3$ (10 nM, 48 h) revealed that 1α,25(OH)$_2$D$_3$ increased the percentage of cells in the G0/G1 phase and diminished the percentage of cells in the S phase of the cell cycle. Moreover, the number of cells in the S phase was higher in SVEC-vGPCR than in SVEC due to vGPCR expression. TX 527 exerted similar effects on growth arrest in SVEC-vGPCR cells. The cell cycle changes were suppressed when the expression of the VDR was blocked by a stable transfection of shRNA against VDR. Annexin V-PI staining demonstrated apoptosis in both SVEC and SVEC-vGPCR after 1α,25(OH)$_2$D$_3$ and TX 527 treatment (10 nM, 24 h). Cleavage of caspase-3 detected by Western blot analysis was increased to a greater extent in SVEC than in SVEC-vGPCR cells, and this effect was also blocked in VDR knockdown cells. Altogether, these results suggest that 1α,25(OH)$_2$D$_3$ and TX 527 inhibit the proliferation of SVEC and SVEC-vGPCR and induce apoptosis by a mechanism that involves the VDR.

Contents

1. Introduction 00
2. Materials and methods 00
 2.1. Cell lines and transfections 00
 2.2. Cell cycle analysis 00
 2.3. Annexin V-PI 00
 2.4. Western blot analysis 00
 2.5. Statistical analysis 00
3. Results and discussion 00
4. Conclusions 00
 Acknowledgements 00
 References 00

1. Introduction

The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor (vGPCR) is a key molecule in the pathogenesis of Kaposi sarcoma. Persistent expression and activity of vGPCR is

* Corresponding author at: Departamento Biología Bioquímica & Farmacia, Universidad Nacional del Sur. San Juan 670, 8000 Bahía Blanca- Argentina.
Tel.: +54 291 4595101x2430; fax: +54 291 4595130.
E-mail address: vgpardo@criba.edu.ar (V. González-Pardo).

0960-0760/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jsbmb.2013.11.014

required for tumor maintenance [1]. At the molecular level, the angiogenic and paracrine transforming effect of vGPCR involves the activation of multiple mitogen activated protein kinases and small GTPases of the Rho family whose activities converge in the nucleus to control transcription factors such as hypoxia-inducible factor 1a, AP-1, and NF-kB, thereby promoting the expression and secretion of growth factors such VEGF and proinflammatory cytokines such as IL-6, IL-8/CXCL8, and MIP-1/CCL3 [2–6]. Therefore, controlling vGPCR signaling pathway is important for the treatment of Kaposi's sarcoma. 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the hormonally active form of vitamin D, in addition to its classical effects maintaining calcium homeostasis, exerts anti-proliferative, pro-apoptotic, and pro-differentiating actions on various malignant cells [7,8]. Most of its activity is mediated by the vitamin D receptor (VDR) [7,9]. Because of its calcemic effects, the use of 1α,25$(OH)_2D_3$ for therapeutic purposes is limited. The analog TX 527 [19-nor-14,20-bisepi-23-yne-1,25$(OH)_2D_3$] has been shown to possess markedly diminished *in vivo* calcemic effects in combination with enhanced antiproliferative and prodifferentiating capacities on normal and malignant cell types when compared with 1α,25$(OH)_2D_3$ [10,11]. In addition, TX 527 has enhanced immune regulatory capacities when compared to the parental compound which makes TX 527 a suitable candidate to treat hyperproliferative and inflammatory disorders [12]. For instance, TX 527 has ameliorated disease symptoms in a chemically induced model of inflammatory bowel disease in the absence of side effects [13]. We have previously demonstrated that 1α,25$(OH)_2D_3$ and its less calcemic analog TX 527 have antiproliferative effects on endothelial cells transformed by vGPCR *in vitro* and *in vivo* by a mechanism that depends on VDR expression [14]. Furthermore, down regulation of the NFκB pathway by 1α,25$(OH)_2D_3$ in vGPCR cells was found to be part of the mechanism of inhibition [15]. In addition, TX 527 similarly to 1α,25$(OH)_2D_3$ inhibited the NFκB pathway and controlled the expression of inflammatory genes and the proliferation of endothelial cells transformed by vGPCR in a VDR-dependent manner [16]. In this work, we further explored the mechanism of action of both vitamin D compounds studying whether they induce cell cycle arrest and subsequent apoptosis of endothelial cells and transformed by vGPCR through the VDR.

2. Materials and methods

2.1. Cell lines and transfections

SV-40 immortalized murine endothelial cells stably expressing vGPCR full-length (SVEC- vGPCR), or empty vector pCEFL (SVEC) as a control, were used as experimental model of Kaposi sarcoma [14,17] and were cultured as reported before [17]. Stable SVEC-vGPCR endothelial cells targeted with small hairpin RNA against mouse vitamin D receptor (vGPCR-shVDR) or control shRNA (vGPCR-shCtrl) were obtained by transduction of lentiviral particles and cultured according to previous work [14].

2.2. Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. The cells were incubated with 1α,25$(OH)_2D_3$ and TX 527 (10 nM, 48 h) or vehicle (0.01% ethanol, ctrl). Cells were trypsinized, washed, and fixed. Cells were then stained with propidium iodide following the protocol as described earlier [16]. Stained cells were analyzed in a FACS Calibur flow cytometer (Becton Dickinson; NJ, USA). The program used for the acquisition and analysis of the samples was the CellQuest Pro.

2.3. Annexin V-PI

Apoptosis was measured using annexin V kit (Annexin V-FITC Apoptosis Detection Kit, Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were collected after treatment, washed twice with PBS and incubated with 5 μl of FITC-conjugated annexin V and PI for 15 min. Finally, cells were analyzed by flow cytometry (FACScan, BD FACSCalibur).

2.4. Western blot analysis

Western blot analyses were performed as reported before [15]. Antibodies used include monoclonal anti-VDR (1:1,500), from Affinity Bioreagents (Golden, CO, USA); rabbit anti-cleaved caspase-3 (1:500), from Cell Signalling Technology, Danvers, MA, USA; and tubulin (1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) combined with anti-rat (1:5000), rabbit (1:10,000) and anti-mouse (1:5000) horseradish peroxidase-conjugated secondary antibodies respectively (Santa Cruz Biotechnology).

2.5. Statistical analysis

Data are shown as mean ±SD. Results were analyzed by the two-tailed *t*-test to evaluate differences between control (vehicle) and treated conditions (1α,25$(OH)_2D_3$, TX 527). A p-value<0.01 (**) and <0.05 (*) were considered highly statistically significant and statistically significant, respectively.

3. Results and discussion

In most cell types, the antiproliferative effect of 1α,25$(OH)_2D_3$ results in an accumulation of cells in the G0/G1 phase of the cell cycle. 1α,25$(OH)_2D_3$ inhibits the growth of many malignant cells by inducing cell cycle arrest and stimulating apoptosis [7]. In this work, cell cycle analysis of SVEC and cells transformed by vGPCR (SVEC-vGPCR) treated with 1α,25$(OH)_2D_3$ (10 nM, 48 h) revealed that 1α,25$(OH)_2D_3$ induced a statistically significant increase in the percentage of cells in the G0/G1 phase and a reduced percentage of cells in the S phase of the cell cycle (Fig. 1A and B). Moreover, the number of cells in the S phase, when comparing under basal conditions, was higher in SVEC-vGPCR than in SVEC probably due to vGPCR expression (4.4%, $p < 0.05$) (Fig. 1B). As shown in Fig. 1C, in SVEC-vGPCR, TX 527 (10 nM, 48 h) induced cell cycle arrest in G0/G1 phase comparable to 1α,25$(OH)_2D_3$. When VDR expression was blocked in stable SVEC-vGPCR cells targeted with small hairpin RNA against mouse VDR (vGPCR-shVDR), the effect of both vitamin D compounds on the cell cycle was suppressed.

Next, we used Annexin V-PI to identify changes in the plasma membrane with externalization of membrane phospholipid phosphatidylserine (PS), one of earliest feature of cells undergoing apoptosis. As shown in Fig. 2, both, 1α,25$(OH)_2D_3$ and TX 527, at 10 nM for 24 h treatment increased the amount of Annexin positive cells in both SVEC (Fig. 2A and C) and SVEC-vGPCR (Fig. 2B and D) and these effects were blocked when the VDR was knocked down. It has been reported that 1α, 25$(OH)_2D_3$ can induce apoptosis in cancer cells by caspase-3 dependent and independent mechanisms [18–21]. Therefore, we investigated whether 1α, 25$(OH)_2D_3$ and TX 527 induced apoptosis by activation of caspase-3 through the participation of VDR. VDR knock-down (vGPCR-shVDR) or control (vGPCR-shCtrl) cells were cultured and treated as shown in Fig. 3. Cleavage caspase-3 fragment of 17–19 kDa (indicator of caspase-3 activation) detected by Western blot analysis increased significantly in both, SVEC and SVEC-vGPCR. Furthermore, cleavage caspase-3 was found to be more increased in SVEC than in

Please cite this article in press as: V. González-Pardo, et al., Cell cycle arrest and apoptosis induced by 1α,25$(OH)_2D_3$ and TX 527 in Kaposi sarcoma is VDR dependent, J. Steroid Biochem. Mol. Biol. (2013), http://dx.doi.org/10.1016/j.jsbmb.2013.11.014