Original Article

Keloid-associated lymphoid tissues in keloid lesions express vitamin D receptor

Ethan J Kilmister¹, Kim H Lim¹, Tinte Itinteang¹, Bede van Schaijik¹, Helen D Brasch¹, Paul F Davis¹, Swee T Tan¹,²

¹Gillies McIndoe Research Institute, Wellington, New Zealand; ²Wellington Regional Plastic, Maxillofacial & Burns Unit, Hutt Hospital, Wellington, New Zealand

Accepted April 5, 2019; Accepted June 26, 2019; Epub August 1, 2019; Published August 15, 2019

Abstract: Objectives: Vitamin D receptor (VDR) may play a role in keloid disorder. This study investigated the expression of VDR by the embryonic stem cell (ESC)-like population within keloid-associated lymphoid tissues (KALTs) which expresses components of the renin-angiotensin system (RAS). Methods: 11 formalin-fixed paraffin-embedded sections of keloid lesions (KLs) underwent 3,3-diaminobenzidine (DAB) immunohistochemical (IHC) staining for VDR. Immunofluorescence (IF) dual IHC staining of CD34/VDR and OCT4/VDR was performed on two representative KLs. Transcriptional activation of VDR was investigated in four representative snap-frozen KLs using reverse-transcriptase-quantitative polymerase chain reaction (RT-qPCR). Results: DAB IHC staining demonstrated the presence of VDR on the KALTs within the keloid tissue samples. RT-qPCR confirmed transcriptional activation of VDR. IF IHC staining demonstrated expression of VDR on the CD34⁺ and the OCT4⁺ endothelium of the microvessels, and the OCT4⁺ perivascular cells, within the KALTs. Conclusions: This study demonstrated the expression of VDR by the ESC-like population within the KALTs in KLs. Further work is needed to elucidate the precise interaction between VDR and the RAS in regulating the primitive population within the KALTs.

Keywords: Keloid, vitamin D, vitamin D receptor, renin-angiotensin system, embryonic stem cells

Introduction

Keloid disorder (KD) is characterized by excessive dermal collagen deposition in response to wounding [1]. We have identified an embryonic stem cell (ESC)-like population within keloid-associated lymphoid tissues (KALTs) in keloid lesions (KLs) that expresses ESC markers OCT4, SOX2, NANOG and pSTAT3 [1]. This primitive population expresses components of the renin-angiotensin system (RAS) [2], and cathepsins B, D, and G, which constitute bypass loops of the RAS [3].

Increased prevalence and severity of KD in patients with hypertension compared with normotensive patients [4], and an inverse relationship between serum 25-hydroxyvitamin D and blood pressure have been observed [5]. Brittle hypertension is also implicated in KD [6].

Expression of vitamin D receptor (VDR) on the peripheral blood lymphocytes of KD patients is lower than in control individuals [7]. Its expression in the epidermis of KLs is lower than in normal skin [8]. Dark-skinned populations are more susceptible to vitamin D deficiency (VDD) with an increased prevalence of KD [4, 7]. VDD increases RAS activity which exacerbates fibroproliferative conditions [9]. Mouse podocytes from mutant VDR renal tissues in vitro and VDR knock-out mice in vivo demonstrate up-regulation of p53, which increases RAS activity by enhancing the expression of angiotensinogen, renin, and angiotensin II receptor 1 [5]. Increased expression of angiotensin II (ATII) induces hypertension, which is implicated in pathologic scarring [4]. ATII promotes cellular events in skin fibrosis, including extracellular matrix (ECM) deposition, fibroblast proliferation and migration [4], and increases inflammation by increased vascular permeability by increasing prostaglandins and vascular endothelial growth factor levels. High ATII levels prolong wound healing and alter pathological fibroblast behavior in KD [9].
Dysfunction of the RAS associated with KD, hypertension and VDD led us to investigate the expression of VDR by the primitive population within KALTs [1] that expresses the RAS [2].

**Materials and methods**

**Histology and immunohistochemical staining**

4 μm-thick formalin-fixed paraffin embedded sections of KLs from seven male and four female patients, aged 5-48 (mean, 24) years, included in our previous studies [1, 2] underwent hematoxylin and eosin (H&E) staining and 3,3-diaminobenzidine (DAB) IHC staining using primary antibodies for VDR (1:400; cat#137371, Abcam, Cambridge, MA, USA), OCT4, (1:30; Cell Marque, Rocklin, CA, USA), CD34 (ready-to-use; cat#PA0212, Leica, Newcastle-upon-Tyne, UK). All antibodies were diluted with BOND™ primary antibody diluent (cat#AR9352, Leica). DAB IHC-stained slides were mounted in Surgipath Micromount mounting medium (cat#38017322, Leica).

Immunofluorescence (IF) dual IHC staining of CD34/VDR and OCT4/VDR was performed on two representative KLs of the original cohort of 11 patients used for DAB IHC staining, using a combination of Vectafluor Excel anti-mouse 488 (ready-to-use; cat#VEDK2488, Vector Laboratories, Burlingame, CA, USA) and Alexa Fluor anti-rabbit 594 (1:500; cat#A21207, Life Technologies, Carlsbad, CA, USA) for the detection of CD34, OCT4 and VDR. All IHC staining was performed using the Leica BOND Rx autostainer (Leica) and mounted as previously described [2].

Normal human skin was used as the positive control for VDR. Negative controls were performed on sections of KLs using matched isotype control for mouse (ready-to-use; cat#DK2488, Abacus, Burlingame, USA) and rabbit (ready-to-use; cat#DK1594, Abacus) primary antibodies.

**Imaging**

DAB and IF IHC-stained slides were imaged using an Olympus BX53 light microscope fitted with an Olympus SC100 digital camera and processed with the CellSens 2.0 Software (Olympus, Tokyo, Japan); and an Olympus FV1200 biological confocal laser-scanning microscope and subjected to 2D deconvolutal processing with CellSens Dimension 1.11 software, respectively (Olympus, Tokyo, Japan).

**Reverse transcriptase-quantitative polymerase chain reaction**

RNA was extracted from four snap-frozen KLs from the original cohort included in the DAB IHC staining. 20 mg of tissue underwent RNA extraction by lysis, homogenization, and extraction, as previously described [2]. RNA quantity and quality were determined using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific). Transcriptional expression was analyzed using the Roto-Gene Q (Qiagen) and the Roto-Gene Multiplex RT-PCR Kit (Qiagen) using the primer probe VDR (Hs01045843_m1) (cat#4448892, ThermoFisher Scientific). Gene expression was determined against the housekeeping gene GAPDH (Hs99999905_m1, cat#4351370, ThermoFisher Scientific). End-point amplification products were checked for the presence of bands of the correct size by 1% agarose gel electrophoresis (cat#G402001, ThermoFisher Scientific).

**Results**

**Histology and immunohistochemistry**

H&E staining of the keloid tissue samples showed the typical architecture with the KALTs underneath the epidermis (Figure 1A and 1B). DAB IHC-staining demonstrated expression of VDR (Figure 1C, brown) on the endothelium of the microvessels and perivascular cells within the KALTs in all 11 keloid samples. Positive staining was demonstrated on sections of human skin (data not shown). Matched isotype control for both mouse and rabbit primary antibodies showed minimal staining on sections of KLs (data not shown).

IF IHC staining demonstrated abundant nuclear expression of VDR (Figure 2A and 2B, red) on the CD34+ (Figure 2A, green) and the OCT4+ (Figure 2B, green) endothelium of the microvessels (arrows), and both nuclear and cytoplasmic expression of VDR on the OCT4+ perivascular cells within the KALTs (arrowheads). The negative control showed minimal staining (data not shown).
Reverse transcriptase-quantitative polymerase chain reaction

RT-qPCR demonstrated the presence of mRNA transcripts for VDR, against GAPDH, in all the keloid samples examined (Figure 3). Specific amplification of the primers was demonstrated by electrophoresis of PCR products on 1% agarose gels (data not shown).

Discussion

The number of cells in the epidermis in KLs with nuclear expression of VDR is significantly lower than normal skin [8]. VDD causes over-activation of the RAS, which stimulates ECM deposition and fibrosis through increased activation of TGF-β [9].

Vitamin D has an anti-fibrotic effect by inhibiting the RAS and attenuates inflammatory responses by inhibiting the NF-κB and Wnt/β-catenin pathway [10]. VDD in mice causes damage to lung parenchyma with progressive fibrosis and ECM deposition through the over-activation of the RAS [9]. It is known that hypertension induced fibrosis is influenced by the RAS [6].

Wounding is characterized by inflammatory, proliferation, and remodeling phases. Pathologic wounding exhibits a stronger inflamma-
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Hypertension also alters proliferation and remodeling phases of wound healing, as hypertensive rats demonstrate increased capillary proliferation following wounding compared to normotensive rats [12]. Hypertension can induce functional changes in cells within KLs, including endothelial cells, pericytes/myofibroblasts, and mast cells [6]. Microvessels within KLs have multiple surrounding pericytes; these differ from normal capillaries, as they have either one, or no pericytes. Inner pericytes closely resemble myofibroblasts and are found in around half of KLs. Their sensitivity and consequent contraction in response to hypoxia may impair blood flow [13] which may contribute to fibroproliferation [6]. Mast cells are also implicated in hypertension-induced fibrosis. Mast-cell activation results in the release of tryptase, chymase and histamine which increase pro-collagen synthesis in normal dermal fibroblasts, and collagen synthesis in keloid fibroblasts [11]. Patients with hypertensive nephropathy have five-fold more mastcellsthancontrolnormalfibroblasts[14]. Additionally, in vitro treatment with tryptase of cardiac fibroblasts isolated from spontaneously hypertensive rats demonstrates increased fibroblast proliferation and collagen synthesis [15]. It is also known that hypertension increases RAS activity [16]. We have reported expression of components of the RAS by the ESC-like population [2] on the endothelium of the microvessels and the perivascular cells within the KALTs [1]. The identification and localization of VDR to the ESC-like population is novel.

Mutant VDR renal tissues and VDR knock-out mice exhibit up-regulation of p53 which increases RAS activity [5]. This may account for dark-skinned individuals, who are at risk of VDD [8], having a higher incidence of KD. It is also exciting to speculate that decreased VDR expression causes heightened activity of the RAS, which stimulates proliferation and differentiation of the primitive population within the KALTs.

Further investigation into the role of VDR in the KALTs with a larger sample size and functional work is needed to unravel the precise interaction between VDR and the RAS and their effect on the ESC-like populations within the KALTs.

Acknowledgements

We thank Ms. Liz Jones of the Gillies McIndoe Research Institute for her assistance in IHC staining. EK was supported by a summer scholarship from the Deane Endowment Trust. This research did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure of conflict of interest

TI, PD and ST are inventors of a PCT application Treatment of Fibrotic Conditions (PCT/NZ-2016/050187). The authors are otherwise not aware of any commercial associations or financial relationships that might pose or create a conflict of interest with information presented in any submitted manuscript.

Address correspondence to: Swee T Tan, Gillies McIndoe Research Institute, PO Box 7184, Newtown
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