Immunohistochemical Detection and Distribution of Cornifin α and Vitamin D Receptor Expression in Vaginal Epithelium Treated With Vitamin D_3

Başak Nazan **YILDIRIM**[1], Gülçin **ABBAN**[2], Simin **ROTA**[3], Anton **JETTEN**[4]

[1]*Department of Obstetrics and Gynecology, Faculty of Medicine, Pamukkale University, Denizli, Turkey*
[2]*Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Turkey*
[3]*Department of Biochemistery, Faculty of Medicine, Pamukkale University, Denizli, Turkey*
[4]*Cell Biology Section, Laboratory of Pulmonary Pathobiology, NIEHS, NIH Research Triangle Park, NC 27709, USA*

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Abstract

Objective: In growth and differentation of keratinizing stratified squamous epithelium, 1.25-dihydroxyvitamin D_3 has an important role. Cornifin α as a marker of vaginal epithelial differentiation distribution has not been studied in the vaginal epithelium. We examined distribution of vitamin D receptor (VDR) and cornifin α in vagina epithelium in ovariectomized and vitamin D treated rats.

Materials and Methods: Bilateral ovariectomies were performed in 20 mature non-pregnant Wistar female rats. All the animals were divided into 2 groups; control group I untreated and group II i.m. 0.025 μg 1.25-dihydroxyvitamin D_3 injected for three days of a week for 2 weeks. In 15 days after the last injection, vaginas were removed and processed for immunohistochemistry.

Results: Epithelial differentiation, 1.25-dihydroxyvitamin D_3 receptor and cornifin α expression in vaginal epithelium of control and vitamin D_3 treated rats were recorded. In treated animals, vaginal epithelium changed into highly-stratified keratinizing layers. Cornifin α and 1.25-dihydroxyvitamin D_3 receptor as a marker of squamous differentiation is present in ovariectomized rats treated with 1.25-dihydroxyvitamin D_3. In contrast, cornifin α and 1.25-dihydroxyvitamin D_3 receptor were absent in all layers of vaginal epithelium in control group.

Discussion: The results indicate that 1.25-dihydroxyvitamin D_3 induced proliferation of vaginal epithelium which was consistent with the cornifin α expression. We demonstrated for the first time that 1.25-dihydroxyvitamin D_3 up-regulated 1.25-dihydroxyvitamin D_3 receptor expression in vaginal epithelium.

Keywords: vitamin D, vagina, cornifin α, VDR, immunohistochemistry

Özet

Vitamin D_3 Verilen Sıçanların Vajen Epitelinde Kornifin α ve Vitamin D Reseptör Ekspresyonun İmmünohistokimyasal Olarak Belirlenmesi

Amaç: Çok katlı yassı epitel çoğalmasında, farklılaşmasında ve keratinizasyonunda, 1.25 dihidroksivitamin D_3 önemli bir role sahiptir. Kornifin α, çok katlı yassı epitel farklılaşmasında belirteç olarak kullanılan bir proteindir. Bu çalışmada, overioktomize ve overioktomize+vitamin D tedavisi gören sıçan vajen epitelinde epitel farklılaşması, Vitamin D reseptör ve kornifin α dağılımı incelendi.

Materyal ve Metot: Çalışmada, çift taraflı overioktomize edilmiş 20 olgun Wistar tipi sıçan kullanıldı. Denekler iki gruba ayrıldı. Grup I'e hiçbir şey uygulanmadı. Grup II'ye ise i.m. olarak 0.025 μg 1.25 dihidroksivitamin D_3, haftada 3 kez olmak üzere iki hafta süreyle uygulandı. Son enjeksiyondan 15 gün sonra, bütün deneklerin vajinaları çıkarıldı ve immünohistokimyasal inceleme için hazılandı.

Sonuçlar: Overioktomize ve vitamin D tedavisi uygulanan sıçanların vajina epitelinin farklılaşması, vitamin D reseptör dağılımı ve kornifin α dağılımı açısından incelendi. Tedavi uygulanan grupta, az sıralı ve düzensiz olan epitelin çok katlı yassı epitele dö-

Corresponding Author: Dr. Başak Nazan Yıldırım
Pamukkale Üniversitesi Tıp Fakültesi,
Kadın Hastalıkları ve Doğum AD, 20100 Denizli, Türkiye
Phone : +90 258 213 40 30/1395
GSM : +90 532 694 95 26
E-mail : basakyildirim@hotmail.com

nüştüğü izlendi. Bu grupta epitel hücrelerinde vitamin D reseptörü ve kornifin α ekspresyonu pozitifti. Overioktomize grupta ise epitel düzensizdi ve yaklaşık iki sıralı idi. Epitel hücrelerinde vitamin D reseptörü ve kornifin α ekspresyonu negatifti.
Tartışma: Çalışmamızda vitamin D'nin, vajina epitelinde çoğalmayı ve farklılaşmayı sağladığını ve çok katlı yassı epitel belirleyicilerinden olan kornifin α'nın ekspresyonunu indüklediğini belirledik. Bu çalışmada ilk kez, vajina epitelinde 1.25 dihidroksivitamin D_3'ün, 1.25 dihidroksivitamin D_3 reseptör ekspresyonunu düzenlediği gösterildi.

Anahtar sözcükler: vitamin D, vajina, kornifin α, VDR, immünohistokimya

Introduction

The biological effects of 1.25-dihydroxyvitamin D_3 ($1.25(OH)_2D_3$) are mediated by its nuclear receptor (1). The receptors are expressed in multiple tissues within the body such as liver, kidney, thyroid, adrenal gland, gastrointestinal tract, breast and skin (2). In our previous study, the presence of vitamin D receptor (VDR) in vaginal epithelium (3) was demonstrated for the first time. VDR was preferentially localized in basal and suprabasal layers, where cells undergo proliferation and differentiation. We demonstrated that, in vaginal epithelium VDR expression changed during the estrus cycle. In oopherectomized rats, VDR was absent in all layers of vaginal epithelium (3).

The physiological role of VDR and its relationship with $1.25(OH)_2D_3$ in vaginal epithelium is still unclear. Up-regulation of VDR is one of the best known effects of $1.25(OH)_2D_3$ in numerous cell models and several tissues and species: such as rat kidney, intestine (4-10), parathyroid gland (11), bone cell model (12).

Squamous differentiation is a multi-stage process characterized by the expression of specific genes in each step. According to some authors (13-15) morphological changes in vaginal epithelium were accompanied by the production of various keratins and proteins which are markers of epidermal and vaginal epithelial differentiation involving in the formation of cross-linked envelope. Cornifin α is one of the best known member of cross-linked envelope precursor protein family (16).

In the present study, we aimed to investigate the effect of $1.25(OH)_2D_3$ on vaginal epithelization. For this purpose, we analysed the expression of cornifin α in oopherectomized rat vaginal tissue deprived of functional VDR. We used cornifin α as a marker of vaginal epithelial differentiation. The other aim of the present study was to determine the respective role of $1.25(OH)_2D_3$ on VDR and cornifin α expression in vaginal epithelium.

Materials and Methods

Animals: Twenty adult non-pregnant Wistar female rats were used. The rats were housed in temperature controlled rooms (27±10°C) at light/dark 14/10 h regimen. Estrous cycle was monitored by cytological examination of vaginal smears taken between 08:30 and 09:30 a.m. 5 days a week. Only females which experienced two regular 4-day cycles consisting of 2 days diestrus, 1 day proestrus and 1 day estrus periods were used in the study. We followed the ethical guidelines for treatment of laboratory animals.

Tissue collection: Bilateral oopherectomy was performed in all mature non-pregnant Wistar rats. All the animals were divided into 2 groups consisting of 10 rats in each. Group I served as control. In group II, animals were injected with 0.025 μg vitamin D_3 (Calcitrol, Calsijex, Abbot) three times a week for 2 weeks. Two weeks after the last injections, vaginas of animals in group I and group II were removed under pentobarbital anesthesia and the rats were sacrificed. Tissues were fixed in 10% neutral buffered formalin for 72 h and then embedded in paraffin. Paraffin sections (5 μm) were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions.

Histochemistry: Three sections from each animal were processed by immunocytochemical assay for the expression of VDR and cornifin α. Human skin epidermis was used as positive control. Negative control was performed by omitting the primary antibody.

Antibodies and staining procedure for VDR: Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 minutes and the sections were incubated with saponin to facilitate the binding of primary antibody to the antigenic areas. Epitopes were stabilized by application of serum blocking solution (Goat serum, Lot# 20570999, Zymed Laboratories Inc., San Francisco, USA) for 20 min at room temperature. Sections were incubated overnight with VDR primary antibody (Lot# 1039, sc 1009 polyclonal antibody, Santa Cruz Biotechnology) 1:100 diluted in PBS at +4°C. After applying the secondary antibody anti-rabbit Ig, avidin-biotin-complex-peroxidase (ABC, Lot# 20570999, Zymed Laboratories Inc.) was applied to the slides. Diaminobenzidine (DAB, Lot# 10163354, Zymed Laboratories Inc.) was used as chromogen. Afterwards, slides were counterstained with hematoxylene for 1 minute, dehydrated in graded ethanol and mounted in conventional medium.

Antibodies and staining procedure for cornifin α: Endogenous peroxidase activity was blocked in 3% hydrogen peroxidase for 10 minutes and the sections were incubated with saponin for binding of primary antibody to the antigenic areas. Epitopes were stabilized by application of serum blocking solution (Goat serum, Lot# 20570999, Zymed Laboratories Inc.) for 60 min at room temperature. Sections were incubated with 1:1000 diluted cornifin α primary antibody (SQ37A-Ab, Jetten Laboratories) in PBS at room temperature for 60 minutes. Then secondary antibody anti-rabbit Ig, avidin-biotin-complex-peroxidase (ABC, Lot# 20570999, Zymed Laboratories Inc.) were applied on tissue slides. Diamino-

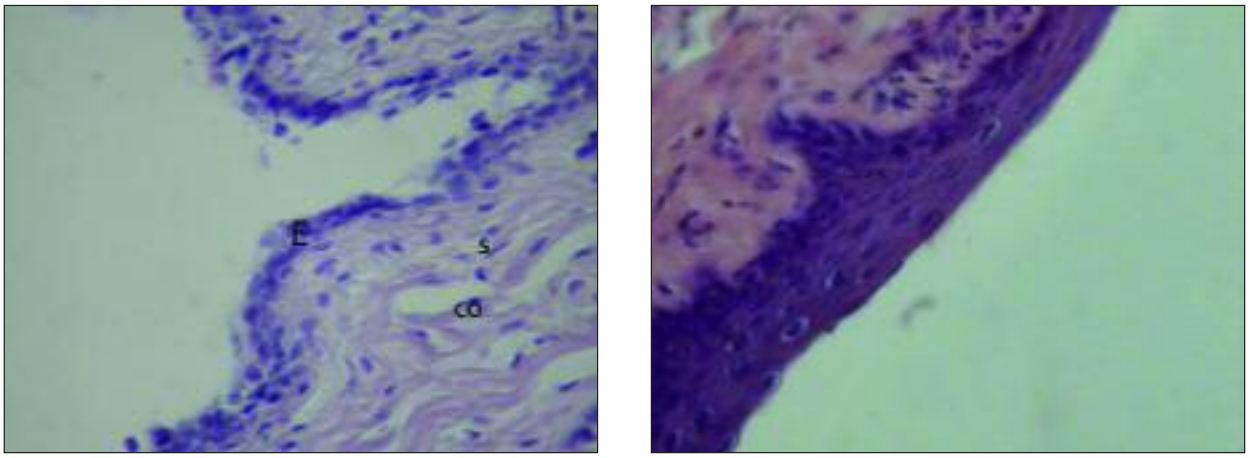

Figure 1. Histological changes in the vaginal epithelium upon treatment with vitamin D_3. **(a)** Oopherectomized vaginal epithelium; **(b)** vitamin D_3 treated vaginal epithelium.
E: Epithelium; co: collagen fibers; s: stromal cells
Hematoxyline-Eosin, magnification: x400

benzidine (DAB, Lot# 10163354, Zymed Laboratories Inc.) was used as chromogen. Afterwards, slides were counterstained with hematoxylene for 1 minute, dehydrated in graded ethanol and mounted in conventional medium.

Slides for VDR and cornifin α were examined by three experienced histologists. The intensity of immunoreaction was semiquantitatively evaluated which was expressed as intensive (+++), moderate (++), weak (+) and negative (-).

Results

Effect of 1.25(OH)$_2$D$_3$ on epithelial morphology: The atrophic vaginal epithelium of the oopherectomized rats was only 2-3 cell layers thick (Figure 1a). In response to 1.25(OH)$_2$D$_3$, basal epithelial cells proliferated rapidly, leading to the formation of a highly stratified epithelium (Figure 1b). The suprabasal cells, which did not exhibit mitotic activity underwent a well-characterized differentiative sequence as they moved up through the epithelium. They were transformed into a multilayer structure and underwent morphological changes towards cornification.

Effect of 1.25(OH)$_2$D$_3$ on VDR Expression: In oopherectomized rat vaginal epithelium the effect of 1.25(OH)$_2$D$_3$ on VDR expression was investigated. Figure 2a and 2b demonstrates the absence of VDR and cornifin α in oopherectomized rat epithelium before 1.25(OH)$_2$D$_3$ treatment. VDR regulation was investigated after 1.25(OH)$_2$D$_3$ injection. The distribution and localization of VDR in 1.25(OH)$_2$D$_3$ treated rat vaginal epithelium was shown in Figure 3. Prominent nuclear and cytoplasmic immunostaining of VDR was observed in vaginal epithelium. Basal, suprabasal and apical cell layers exhibited a

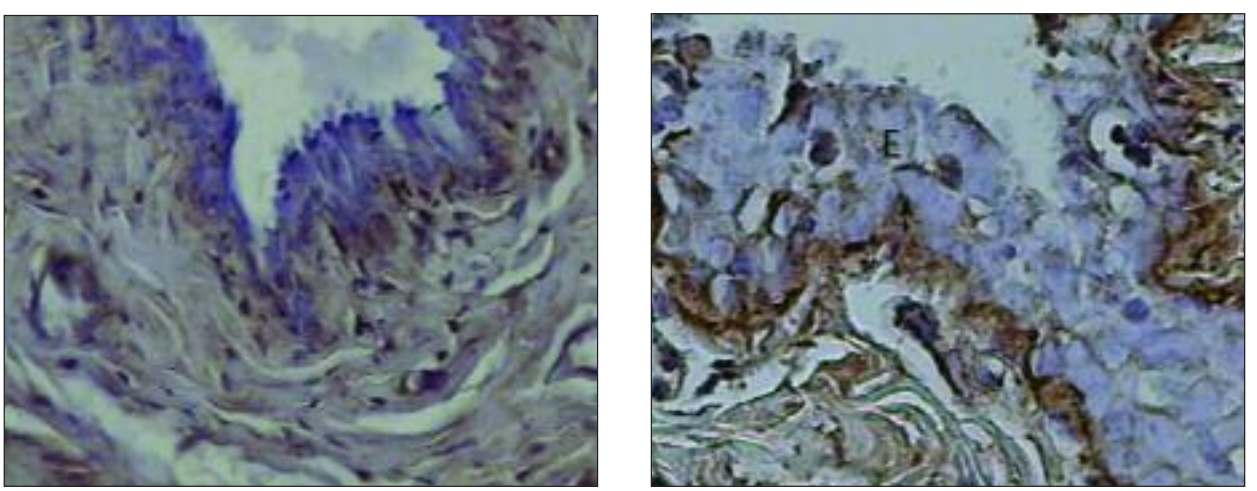

Figure 2. Negative immunostaining of **(a)** VDR; **(b)** cornifin α in the vaginal epithelium of oopherectomized rats.
E: Epithelium
Immunoperoxidase, magnification: x400

Table 1. Localization and distribution Vitamin D receptor in vaginal epithelium

	Cornified layer	Apikal cells	Suprabasal cells	Basal cells	Basal membran	Nucleus
Overectomized	-	-	-	++	-/+	-
Vitamin D treated	-	+++	+++	+++	+++	++

Table 2. Localization and distribution cornifin α in vaginal epithelium

	Cornified layer	Apikal cells	Suprabasal cells	Basal cells	Basal membran	Nucleus
Overectomized	-	-	-	-	+++	-
Vitamin D treated	-	+++	++	+	++	+++

positive VDR reaction, but suprabasal and apical cells of vaginal epihelium demonstrated more diffuse and stronger immunostaining for the VDR compared to basal cells (Figure 3). While nuclei of epithelial cells were VDR positive, negative immunoreaction was noticed in basal membrane.

Effect of 1.25(OH)$_2$D$_3$ on Cornifin α Expression: In vaginal epithelium of oopherectomized rats, two weeks after 1.25(OH)$_2$D$_3$ injection stratification and cornification occured (Figure 1b). Immunohistochemical staining for cornifin α was very intense in the suprabasal and apical layers (Figure 4). Only a few immunopositive cells were present in basal cell layer. In addition, in basal membrane moderate immunoreaction for cornifin α was observed. While nuclei of apical cells showed strong immunostaining for cornifin α, no immunostaining was observed in the nuclei of basal and suprabasal cells. Cornified layer was negative for cornifin α (Figure 4).

Discusssion

The present results indicated that vaginal response to 1.25(OH)$_2$D$_3$, including epithelial proliferation, stratification, cornification occurred in atrophic vaginal epithelium. It is consistent with the immunohistochemical staining of cornifin α. Cytoplasmic and nuclear VDR immunostaining was also detected in basal, suprabasal and apical cell layers. The proliferative response to 1.25(OH)$_2$D$_3$ might be mediated through VDR besides other receptors or some nonreceptor-mediated pathways.

In our study, both VDR expression and vaginal epithelial cell differentiation was demonstrated in response to 1.25(OH)$_2$D$_3$ stimulation. Our results showed that VDR epitopes are not present in vaginal epithelium of oopherectomized rats which is not the case in the presence of estradiol. By some other authors, in the absence of estradiol, atrophic, and 2-3 layers of squamous epithelial cells were reported in vaginal epithelium (17,18) as we demonstrated experimentally in this study. Also, in an *in vivo*

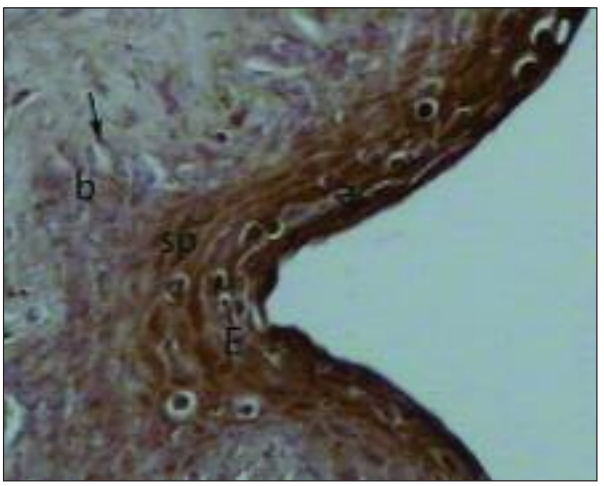

Figure 3. Immunohistochemical detection of VDR in vaginal epithelium treated with vitamin D$_3$. Positive VDR immunostaining in suprabasal and apical cells.
E: Epithelium; arrow: basal membrane; b: basal cells; sp: suprabasal cells; ac: apical cells
Immunoperoxidase, magnification: x200

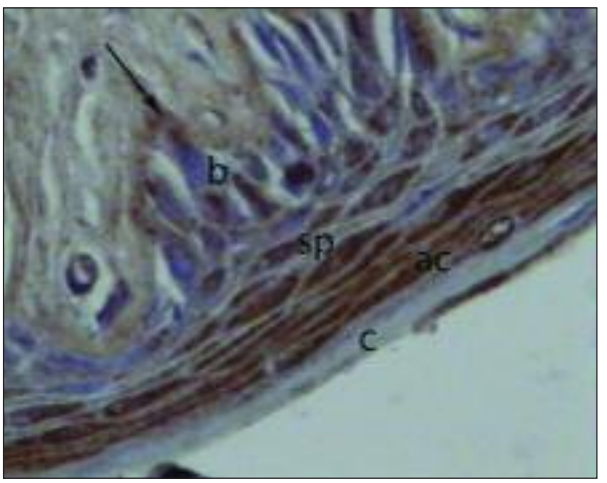

Figure 4. Immunohistochemical detection of cornifin α in vaginal tissue treated with vitamin D$_3$. Positive cornifin α immunostaining in suprabasal and apical cells.
Arrow: basal membrane; b: basal cells; sp: suprabasal cells; ac: apical cells; c: cornified layer
Immunoperoxidase, magnification: x200

study, induction of the proliferation and thickening of vaginal epithelium with flattened cells that accumulated in the superficial layers, as well as stimulation of VDR expression were demonstrated after $1.25(OH)_2D_3$ treatment (18). These results were the first showing the *in vivo* up-regulation of VDR expression in vaginal epithelium in response to $1.25(OH)_2D_3$. The demonstration of induced VDR expression by $1.25(OH)_2D_3$ raised a question concerning about the role of VDR in vaginal differentiation and stratification. Thus, it can be hypothesized that VDR might be a mediator during the induction of proliferation and differentiation of the vaginal epithelium by $1.25(OH)_2D_3$.

The mechanism of $1.25(OH)_2D_3$-induced epithelial interactions may have some clinical implications. In our previous clinical study, $1.25(OH)_2D_3$ supplementation resulted with squamous maturation in vaginal epithelium (19). Though estrogen is routinely used for vaginal atrophy in clinical practice, estrogen treatment is within the initiation and progression of neoplasias of estrogen target organs as mammary gland, endometrium, vagina, and cervix (7,20). In this regard, $1.25(OH)_2D_3$ may potentially be a new and safe therapeutical agent for vaginal atrophy.

To the best of our knowledge, in vaginal epithelium this is the first study showing the proliferative effect of $1.25(OH)_2D_3$ and VDR up-regulation. Our results demostrate that, $1.25(OH)_2D_3$ might play a key role in the regulation of differentiation and cornifin a expression in vaginal epithelium. The mechanisms underlying vaginal epithelium differentiation and VDR up-regulation by $1.25(OH)_2D_3$ remains to be further evaluated.

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