Analysis of 1,25-Dihydroxyvitamin D₃ Receptors (VDR) in Basal Cell Carcinomas

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We have analyzed expression of 1,25-dihydroxyvitamin D₃ receptor (VDR) protein and mRNA in basal cell carcinomas (BCC) of human skin. VDR immunoreactivity in BCCs was compared with the staining pattern of the proliferation marker Ki-67 in the same tumors. Additionally, VDR staining was compared to staining pattern of apoptotic cells by terminal UTP nucleotide end labeling assay. Frozen sections of superficial type, nodular type, and fibrosing type BCCs were consistently immunoreactive for VDR (mAb 9A7γ) with almost every tumor cell labeled (*n* = 15). In general, VDR staining was pronounced in peripheral tumor cells. VDR immunoreactivity was consistently stronger in tumor cells than in adjacent or unaffected epidermis. No visual correlation was found in BCCs comparing labeling patterns of Ki-67-positive or apoptotic cells and mAb 9A7γ. VDR mRNA was increased in BCCs (*n* = 6) compared to normal human skin (*n* = 5), as revealed by reverse transcription-polymerase chain reaction analysis. Our findings indicate that VDR is strongly expressed in BCCs and may be involved in the growth regulation of this tumour, and VDR mRNA and protein are increased in BCCs as compared to normal human epidermis. (*Am J Pathol* 1999, 155:583–589)

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃ or calcitriol), the biologically active metabolite of vitamin D, has been shown to regulate the growth of various cell types, including human keratinocytes.^{1–3} This potent seco-steroid hormone acts via binding to a corresponding intranuclear receptor (VDR), present in target tissues.^{4, 5} VDR belongs to the superfamily of trans-acting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors as well as the retinoid-X receptors and retinoic acid receptors.^{6–8} Keratinocytes express VDR,^{2, 9} whose natural ligand, calcitriol, inhibits proliferation and induces differentiation of cultured human keratinocytes *in vitro*.^{1, 2} Recently, transcriptional regulation of the cell

cycle regulatory protein WAF-1 and other proteins involved in keratinocyte growth and differentiation, including β₃-integrin and fibronectin, by calcitriol has been shown.^{10–12}

Clinically, the antiproliferative and prodifferentiating effects of vitamin D analogues are used successfully in the treatment of the hyperproliferative skin disease psoriasis.^{13–15} Recently, combination of 1,25-dihydroxyvitamin D₃ and isotretinoin was reported to be effective in the chemotherapy of precancerous and cancerous skin lesions, including basal cell carcinomas (BCC).¹⁶ BCC is the most common malignant tumor of the skin.¹⁷ Most theories propose that BCCs arise from keratinocyte stem cells in the epidermal basal layer or from adnexal structures, but consensus concerning the origin and differentiation of this tumor is still lacking. BCCs are semimalignant skin tumors characterized by a locally aggressive and invasive growth pattern, but, except for very rare cases, have nonmetastasizing behavior.¹⁷ The biological mechanisms underlying these unique characteristics of tumor growth are completely unknown.¹⁸

The aim of this study was to analyze expression of VDR protein and VDR mRNA content in BCCs, using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). We addressed the following questions: i) Do BCCs express VDR? ii) Is VDR protein and mRNA in BCCs altered as compared to epidermis of normal human skin or hair follicle keratinocytes? iii) Can we correlate immunohistochemically the expression of VDR with proliferative activity or apoptosis of BCC cells, comparing staining for VDR protein with proliferation marker Ki-67 and terminal UTP nucleotide end labeling (TUNEL)?

Materials and Methods

Skin Specimens

Freshly excised BCC specimens (*n* = 15) and biopsies of normal skin (*n* = 5, healthy volunteers, no history of skin disease) were immediately embedded in OCT Tissue-Tek

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II (Miles Scientific, Naperville, IL) snap-frozen in liquid nitrogen, and stored at -80°C .

Primary Antibody

MoAb 9A7 γ

This rat monoclonal antibody (IgG_{2b}; MU 193-UC, Bio-Genex, CA) is directed against partially purified vitamin D receptor from chicken intestine and cross-reacts with human, mouse, and rat VDRs, but does not bind to glucocorticoid or estrogen receptors.¹⁹

PoAb Ki-67

A polyclonal rabbit antibody (A47, DAKO, Hamburg, Germany) is used to phenotype proliferating cells. This antibody has a reactivity similar to that seen with the monoclonal Ki-67, clone MIB-1.²⁰

Preparation of Sections and Fixation

Serial sections (5 μm) were cut on a cryostat (Reichert-Jung, Heidelberg, Germany) and mounted on pretreated glass slides. Pretreatment of slides with 2% aminopropylmethoxysilane (Sigma, München, Germany) in acetone for 5 minutes was performed to enhance sticking of sections during the staining procedure. Frozen sections to be stained for VDR were fixed in 3.7% paraformaldehyde (Merck 4005, Darmstadt, Germany) in phosphate buffered saline (PBS) for 10 minutes at room temperature (RT), incubated in methanol (Merck 6009, 3 minutes, -20°C) and acetone (Merck 22, 1 minute, -20°C), and transferred into PBS. Sections to be stained for Ki-67 were air-dried (2 hours, RT), followed by fixation in acetone (10 minutes, RT), air-drying, and rinsing in 0.19 mol/L Tris-buffered saline (TBS), pH 7.6 (10 minutes, RT).

In Situ Detection of Vitamin D Receptor and Ki-67 Antigen

Incubation steps were performed in a moist chamber at RT. The slides to be stained for VDR were incubated with the rat monoclonal antibody 9A7 γ (16 hours, 4°C) at a dilution of 1:500. The Ki-67 antibody was used at a dilution of 1:200 (30 minutes, RT). After intermediate washing steps (PBS/TBS, 2×5 minutes), the sections were incubated with biotin-labeled rabbit anti-rat IgG (DAKO) or goat anti-rabbit IgG (DAKO) at a dilution of 1:400 (30 minutes, RT), and then with streptavidin-peroxidase complexes (DAKO) at a dilution of 1:400 (30 minutes, RT) or streptavidin-Cy3 (Dianova, Hamburg, Germany). After rinsing, the sections were incubated with 3-amino-9-ethylcarbazole (AEC, Sigma A 5754) as a substrate for the peroxidase reaction, transferred into tap water, and mounted with AquaTex (Merck). Cy3-labeled specimens were mounted with glycerin/PBS (1:10) after a final washing step. For the simultaneous detection of VDR and Ki-67, a double staining procedure was developed. First VDR was labeled with Cy3 as described above (with the

exception that biotin-labeled swine anti-rat IgG (DAKO) was used as secondary antibody). Thereafter, Ki-67 antibody (1:20, 30 minutes, RT) was applied and detected by fluorescein isothiocyanate (FITC)-labeled mouse anti-rabbit IgG (Dianova).

In control sections, primary antibody was replaced with polyclonal rat IgG or rabbit IgG (DAKO). No immunoreactivity was observed in these control sections. Specimens were analyzed under a Zeiss microscope (Zeiss, Oberkochen, Germany) or by confocal laser scanning microscopy (Zeiss). Photographs were taken on Kodak Ektachrome 64 or Agfa CTX 100 film.

Semiquantitative Analysis of Immunolabeled Specimens

Microscopic analysis was performed by two independent observers (JK and JR). VDR staining intensity was assessed using a 5-point immunoreactivity scale (no, weak, moderate, strong, or very strong). Number of VDR-positive cells was assessed using a 4-point scale from, no positive cells to almost every cell positive (no, few, many, or almost every cell positive).

TUNEL of Apoptotic Cells in BCCs

For preparation of paraffin-embedded tissue, biopsies of BCCs ($n = 10$) had been fixed for 12 to 24 hours in 10% neutral buffered formalin and embedded in paraffin wax. Sections were cut at 5–7 μm , dried onto slides at 37°C , dewaxed by taking them through three changes of xylene, and then rehydrated by passing through three changes of alcohol, ending finally in water. We used the *In Situ* Cell Death Detection Kit AP (Boehringer cat. no. 1684809, Mannheim, Germany) according to product specifications as a modification of the original TUNEL technique²¹ to detect apoptotic cells. New fuchsin was used to visualize the alkaline phosphatase reaction and sections were counterstained with hematoxylin.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for VDR in Normal Human Skin and BCC

Freshly excised normal human skin ($n = 5$, healthy volunteers with no history of skin disease) and BCC specimens ($n = 7$) were immediately embedded in OCT-Tissue-Tek II (Miles Scientific) snap-frozen in liquid nitrogen, and stored at -80°C . RNA was isolated using GITC as described previously.²²

Two micrograms each of total RNA from human basal cell carcinomas and normal human skin was reverse transcribed according to the protocol for BRL's superscript preamplification system (GIBCO BRL, Gaithersburg, MD) for first-strand cDNA synthesis. Ten percent of each cDNA reaction was used as template in each sample. PCR sequence-specific primers for hVDR are: forward (located in exon 1), 5'ACTTCCTGCCTGAC-CCTGG3'; reverse (located in exon 4), 5'GTCTTATGGT-

Figure 1. Immunohistochemical detection of VDR in normal human skin adjacent to a hair follicle. Note VDR-positive cells in all cell layers of the viable epidermis (arrows).

GGTGGGCGTCCAG3'. Primers for hGAPDH are forward, 5'TCCCATCACCATCTTCCAGGA3' and reverse, 5'GTCCACCACCCTGTTGCTGTA3'. Final reaction concentration was 0.2 μ mol/L for each primer 0.2 μ mol/L dNTPs, 1 $\times$ standard PCR buffer 2.5 μ mol/L $MgCl_2$, and 0.75 units of AmpliTaq DNA polymerase in a final reaction volume of 30 μ l. Thermocycling conditions in a GeneAmp PCR System 9600 (Perkin-Elmer) were as follows: 2 minutes' initial denaturation at 94°C, followed by 30 cycles of denaturing at 95°C 20 seconds, annealing at 60°C 30 seconds, and extension at 72°C 30 seconds. A 10-minute incubation at 72°C after 30 cycle concluded the extension. PCR products were separated on 1.2% agarose gel. The gel was denatured in 0.5 mol/L NaOH-1.5 mol/L NaCl for 30 minutes and neutralized in 1.5 mol/L NaCl-0.5 mol/L Tris HCl (pH 7.5) for 30 minutes RT-PCR products were transferred to nylon membrane and fixed to the membrane using UV cross-linker. Membranes were hybridized in Rapid-hyb buffer (Amersham) with a partial VDR cDNA probe or a partial GAPDH probe labeled with (32p)deoxy-CTP using random primer method. The blot was washed in 1 $\times$ SSC and 0.1% SDS at 65°C for 30 minutes and subsequently exposed to X-ray film overnight.

Results

Expression of 1,25-Dihydroxyvitamin D₃ Receptors (VDR) in Normal Human Skin

All analyzed biopsies ($n = 5$) revealed nuclear VDR immunoreactivity consistently in all cell layers of the viable epidermis (Figure 1). In 3 of 5 biopsies analyzed, intensity of VDR immunoreactivity was stronger in keratinocytes of the basal layer as compared to upper layers. In the hair follicle VDR was most markedly expressed in keratinocytes of the outer root sheath, whereas staining of cells of the inner root sheath was heterogeneous. Single scattered VDR-positive fibroblasts (identified by their shape) were found as well.

Expression of 1,25-Dihydroxyvitamin D₃ Receptors (VDR) in BCC

VDR immunoreactivity was observed in all BCCs analyzed ($n = 15$). There was no visual difference comparing staining pattern for VDR in the different types of BCCs (nodular type, superficial type, fibrosing type). Almost every tumor cell revealed consistently strong nuclear immunoreactivity for VDR (Figures 2 and 3, Table 1). In general, VDR immunoreactivity was pronounced in the palisaded array of peripheral tumor cells (Figures 2 and 3). This staining pattern was detected focally in 8 specimens and continuously in 7 biopsies. VDR staining intensity was markedly stronger in BCCs as compared to adjacent epidermis or to distant unaffected epidermis of the same section (Figures 1–3, Table 1).

Expression of Ki-67 Antigen in BCCs

All BCCs analyzed were immunoreactive for Ki-67 antigen. Heterogeneous Ki-67 immunoreactivity with no visual differences between central and peripheral areas was found in most tumor specimens (11 of 15), although some BCCs revealed pronounced labeling for Ki-67 antigen in palisades of peripheral tumor cells (4 of 15). Confocal laser scanning microscopy confirmed these results, showing that double-stained sections for VDR and Ki-67 revealed no visual correlation of labeling patterns (Figure 3).

TUNEL Assay in BCCs

All BCC specimens ($n = 10$) revealed numerous terminal UTP nucleotide end-labeled apoptotic cells with considerable variation in their number. Distribution of apoptotic cells within the tumor was heterogeneous, with only 4 of 10 specimens showing focally pronounced labeling of peripheral tumor cells (Figure 4). Number of apoptotic cells was markedly less than number of VDR-positive cells.

RT-PCR Analysis of VDR in BCCs and Normal Human Skin

The RT-PCR product of the VDR gene was a 600-bp fragment, RT-PCR product of GAPDH was 720 bp. We used the equal amount of total RNA for reverse transcription. The VDR gene was highly expressed in BCCs. VDR mRNA expression was consistently increased in BCCs compared to normal human skin. There was no significant difference in the GAPDH gene expression between normal human skin and BCC. Southern blot analysis of the RT-PCR products from the agarose gel were transferred to the nylon membrane, then hybridized with the VDR cDNA probe and GAPDH cDNA probe. The Southern blot showed VDR gene expression in BCC that was approximately 10-fold higher than normal human skin (Figure 5).

Figure 2. Immunohistochemical detection of VDR (A–C) and Ki-67 antigen (D) in basal cell carcinomas (streptavidin-peroxidase technique with AEC as substrate). Note increased nuclear immunoreactivity for VDR in BCCs as compared to adjacent unaffected (A) or overlying (B) epidermis. Note also strong expression of VDR in tumor cells of palisaded array (arrow) compared to central parts of the tumor (arrowhead). In contrast, Ki-67 staining was heterogeneous in most specimens analyzed (D).

Discussion

The monoclonal antibody 9A7 γ has been used successfully in the immunohistochemical investigation of VDR in various tissues such as chicken intestine, rat brain, disaggregated rat bone cells, rat osteosarcoma 17/2.8 cells,²³ fibroblasts,^{24, 25} and normal and psoriatic human skin.^{9, 25–27} Our evaluation of 9A7 γ immunoreactivity in normal human skin is in agreement with previous observations.^{9, 25–27} To our knowledge, this is the first report demonstrating VDR expression in BCCs. Interestingly, VDR staining intensity in all specimens analyzed was

much stronger in BCCs than in either unaffected epidermis of the same section or normal human skin, indicating increased levels of VDR protein in these tumors. This was consistent with our observation that there was a marked increase in VDR mRNA in BCCs compared to normal skin (Figure 5).

Labeling pattern of VDR in most analyzed tumors was not concordant with the staining pattern of Ki-67 antigen. Our findings are in agreement with previous immunohistochemical investigations that have demonstrated peripheral or random distribution of Ki-67-positive cells in BCCs of all different histological types.²⁸ The actual pro-

Figure 3. Immunohistochemical detection of VDR and Ki-67 antigen in basal cell carcinomas using double-staining technique (confocal laser scanning microscopy). Note increased nuclear fluorescence for VDR in BCCs (arrows) as compared to adjacent unaffected epidermis (arrowheads). No visual correlation was observed comparing staining patterns of exclusively Ki-67-(red) or VDR-(green) labeled tumor cells. Double-labeled nuclei (yellow) were heterogeneously distributed in the tumors with no pronouncement of peripheral array.

liferative rate of BCC cells is somewhat unclear, but it appears to be in the range of that of normal keratinocytes.¹⁷ It is still unknown what signals determine the extent of proliferative activity in BCCs.²⁸ Our findings indicate that VDR expression is not exclusively a function of cellular proliferation in these tumor cells, but may be determined by additional, different mechanisms.

Figure 4. Immunohistochemical detection of apoptotic cells in BCC by TUNEL. Note heterogeneous distribution of stained cells (red-brown label) within the tumor and only focally pronounced labeling of peripheral tumor cells (arrows).

It appears that VDR expression may be a function of the state of differentiation. In various cell cultures, including human keratinocytes, VDR displays highest maximal ligand binding in the early logarithmic phase of cell growth and diminishes as cells reach confluence.²⁹ The BCC cell has been shown histologically to be highly undifferentiated.¹⁸ Our observation of increased VDR expression in BCCs could reflect the altered differentiation pattern in these tumor cells.

Table 1. VDR Immunoreactivity in BCCs (*n* = 15)

	Intensity of VDR immunoreactivity				
	no	weak	moderate	strong	very strong
BCC specimens					
central areas	0	0	6	8	1
palisaded peripheral cells	0	0	2	6	7
unaffected epidermis	0	5	9	1	0
	Number of VDR-positive cells				
	no cells	few cells	many cells	almost every cell	
BCCs	0	0	4	11	
epidermis	0	2	13	0	

Figure 5. Southern hybridization analysis of VDR expression in basal cell carcinomas and normal human skin. RT-PCR product of basal cell carcinomas in lane 1 (B9), lane 2 (B2), and in lane 3 (B1), and normal human skin in lane 4 (H2) and lane 5 (H1).

Our results concerning apoptosis in BCCs are in agreement with previous studies demonstrating that BCCs are characterized by a high percentage of apoptotic cells.³⁰ Apoptosis is an asynchronous cellular process with cytoplasmic and nuclear condensation, disruption of the cytoskeleton, and condensation of intermediate filaments around the nucleus. Recently it has been shown that calcitriol induces apoptosis in various tumor cells.³¹ It can be speculated whether increased levels of VDR and subsequent interaction with 1,25(OH)₂D₃ in BCCs may be a reason for the high percentage of apoptotic cells. However, comparison of staining patterns in our study revealed no substantial evidence for correlation of VDR expression and apoptosis.

In various cell types, calcitriol has been shown to enhance VDR expression at the mRNA and protein levels *in vitro*.^{32, 33} Interestingly, this elevation of the VDR protein following calcitriol administration is due to an increased transcription of the VDR gene³⁴ and/or an increased receptor protein lifetime.³³ A recent study indicated that human keratinocytes may have the capacity to hydroxylate vitamin D at the C-1 and C-25 positions.^{35, 36} Therefore, keratinocytes are able not only to produce vitamin D by the action of UV light, but also to synthesize the biologically active vitamin D metabolite, 1,25(OH)₂D₃. It is not known whether basal carcinoma cells are able to synthesize calcitriol from vitamin D₃ as well. However, it can be speculated whether the increased content of VDR on the mRNA and protein levels that we have shown in this study is due to an increased formation of calcitriol in these tumor cells.

We and others have shown that cytokines and other factors that stimulate transmembrane-signaling pathways alter VDR levels in keratinocytes and other cell types *in vitro*.^{37, 38} Surrounding stroma of BCCs often contains an increased number of inflammatory cells. Excreted cytokines and inflammatory peptides of these cells may be a reason for increased VDR expression in adjacent tumor cells.

Our results do not allow any conclusion on the function of VDR in BCCs. It is not known if the VDR in BCCs exhibits a functionally inactivating mutation and whether

the increased VDR presence in BCCs may be due to a feedback loop coupled with an expression by the tumor cells of a defective VDR.

The steroid hormone responsiveness is directly proportional to the number of corresponding receptors.³² Because VDR mediates the biological effects of calcitriol and analogues on proliferation and differentiation in target cells,^{7, 39} increased VDR content in BCCs may indicate a sensitivity to endogeneous or therapeutically applied calcitriol. A great deal of work has been done evaluating the chemotherapeutic and/or chemopreventive effects of retinoids on BCCs.^{40, 41} These effects are thought to be mediated via binding to the corresponding intranuclear receptors, retinoic acid receptors (RAR) - α , - β , and - γ . Recently, it has been demonstrated that VDR require heterodimerization with auxiliary proteins for effective DNA interaction.^{42, 43} These auxiliary proteins have been identified as the retinoid-X receptors (RXR) - α , - β , and - γ .^{6, 42, 43} Heterodimerization of VDR with auxiliary proteins has been shown to increase the transcriptional function and DNA binding to the respective response elements in target genes.^{6, 42, 43} We have recently shown that RXR- α is strongly expressed in BCCs.⁴⁴ Combination of 1,25-dihydroxyvitamin D₃ and retinoids has been reported to be effective in the treatment of precancerous and cancerous skin lesions, including actinic keratoses, squamous cell carcinomas, cutaneous T-cell lymphomas, and BCCs.¹⁶ These early results are encouraging; however, much more work is required to establish a role for vitamin D analogues and retinoids in the chemoprevention of these tumors.

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