Characterization of a vitamin D receptor knockout mouse as a model of colorectal hyperproliferation and DNA damage

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The vitamin D receptor knockout (VDR-KO) mouse presents with a skeletal phenotype typical for complete lack of genomic 1,25-dihydroxycholecalciferol effects. Our previous data from human colorectal tissue suggest that the steroid hormone and its receptor may have protective function against tumour progression. In order to investigate the relevance of the vitamin D system for pre-malignant site-directed changes in the colon, we characterized the amount and site-specific distribution of the VDR along the large intestine in wild-type (WT), heterozygote (HT) and KO mice. We also evaluated expression of proliferating cell nuclear antigen (PCNA), of cyclin D1 and the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative stress. In colon ascendent, proliferative cells were dispersed all along the crypt and expression levels of all three markers were high in WT mice. A decrease of VDR expression did not affect expression significantly. In colon descendens, however, fewer proliferative cells were solely located in the lower third of the crypt, and an inverse relationship between VDR reduction, PCNA positivity and cyclin D1 expression was found in HT and KO mice. In parallel to enhanced proliferation a highly significant increase of 8-OHdG positivity occurred. Therefore, the sigmoid colon of VDR-KO mice, fed on an appropriate lactose/calcium-enriched diet to alleviate impaired calcium homeostasis-related phenotypic changes, is an excellent model for investigating induction and prevention of pre-malignant changes in one of the hotspots for human colorectal cancer incidence.

Introduction

1,25-(OH)² vitamin D₃ (1,25-D₃), the major regulator of calcium homeostasis, is critically important for normal mineralization of bone. In addition to these classical effects of the steroid hormone on small intestine, bone and kidney, a multitude of other action sites have been discovered. This insight resulted mainly from the observation that the vitamin D receptor (VDR), which mediates the hormone’s genomic activity, is expressed in almost all tissues of the human body (1).

Colorectal cancer is an extremely common malignant condition that is second only to lung malignancies as a cause of cancer deaths in the USA (2). Several pieces of evidence suggest that 1,25-D₃ is involved in the pathogenesis of colon tumours: colon cancer is prevalent in areas of the world with low exposure to sunlight (3). In some epidemiological studies, low serum levels of 25-OH-D₃ suggest a predisposition to epithelial cancers (3). In vitro, vitamin D concentrations lower than physiological ones increase proliferation of Caco-2 cells, a human colon adenocarcinoma-derived cell line (4) which suggests that low serum levels of the steroid hormone could indeed be a contributing factor to colon pathogenesis. However, pharmacological amounts of 1,25-D₃ (>1 nmol) inhibit cell proliferation and/or stimulate cell differentiation in many cell types derived from non-classical tissues (5–7).

The VDR was found to be expressed in colon cancer cell lines (8), in primary cultures derived from human non-malignant and malignant colon tissues (9) and also in surgically removed colon cancer specimens (10–12). In addition, we were able to demonstrate that VDR expression is increased during early stage, low-grade malignancy, whereas cytokeratin 20, a marker of differentiated colonocytes, is decreased (11,13).

Although the kidney was long assumed to be the main site of 1,25-D₃ synthesis, other cell types have recently been shown to possess 1α-hydroxylase activity (for review see ref. 14). We were able to demonstrate for the first time that also colon cancer cells can produce significant amounts (in the nanomole range) of the active metabolite by 1α-hydroxylation of the precursor 25-D₃ (15) and that the 25-D₃-1α-hydroxylase mRNA is increasingly expressed during colorectal tumour progression (12). This led us to suggest that enhanced expression of VDR and tumour-localized production of 1,25-D₃ could be evidence for autocrine/paracrine growth control during early stages of colon tumour progression (12). However, the physiological relevance of this purported physiological defence system for slowing down or possibly even stopping human colon tumour progression is still elusive.

Gene targeted knockout mice provide an excellent possibility to study complex regulatory systems that cannot be modelled in isolated in vitro systems. Two independent groups using homologous gene targeting have generated VDR-deficient mice (16,17). Yoshizawa et al. (16) ablated exon 2 of the VDR gene, which encodes the first zinc finger of the DNA-binding domain, whereas Li et al. (17) ablated a VDR fragment spanning exons 3–5, which encode, among others, the second zinc finger of the DNA-binding domain. From ~3 months after weaning, both animal models display a phenotype that closely resembles human vitamin D dependent rickets type II, including 1,25-D₃ resistance, hypocalcaemia, secondary hyperparathyroidism and osteomalacia. This phenotype progressively worsens unless VDR-knockout (KO) mice are fed a special

Abbreviations: HT, heterozygote; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PCNA, proliferating cell nuclear antigen; VDR-KO, vitamin D receptor knockout; WT, wild-type.

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rescue diet (18), whereas heterozygote (HT) mice do not develop any outward physical signs of rickets. The aim of our study was to characterise the VDR-KO mouse of Yoshizawa et al. (16) with respect to its relevance as a model for colorectal pre-malignancy. For this purpose we examined quantity and distribution of the VDR protein in all three genotypes in relation to known ‘hotspots’ for human colon tumour incidence. We also evaluated three markers of human colorectal hyperproliferation: recent studies have implicated loss of cell cycle control in early tumour development, and it has been proposed that alteration of one or more factors involved in cell cycle checkpoint regulation is a prerequisite for cancer (19). Increased proliferative activity has been described in the colon of animals treated with carcinogens and of patients at increased risk of colon cancer and has been proposed as a biomarker of increased susceptibility to colonic neoplasia (20). On the one hand we investigated expression of proliferating cell nuclear antigen (PCNA), a polymerase δ-associated protein synthesized in early G1 and S phases of the cell cycle. On the other hand we monitored level and distribution of cyclin D1, the major cyclin involved in transition from G1/S phase. This protein is known to be frequently overexpressed in, among others, early colorectal tumours (21) though no cyclin D1 gene amplification is found. Evidence for overexpression in precursor lesions may be a result of regulation of cyclin D1 expression by the APC/β-catenin pathway (22). In addition, we wanted to monitor oxidative stress under hyperproliferative conditions by evaluating 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation. Oxidative stress caused by reactive oxygen species (ROS) may play a role in a variety of tumour-associated cellular events. 8-OHdG is one of the most abundant oxidatively modified lesions in DNA. Kondo et al. (23) demonstrated that increased 8-OHdG levels in colorectal carcinoma may be attributable, at least in part, to increased formation during enhanced proliferation.

By using these three markers for early colonic pathogenesis we were able to show that with loss of VDR protein expression, PCNA and 8-OHdG positivity rises in parallel to that of cyclin D1 in colon descendents. In colon descendents, however, loss of VDR leads only to non-significant enhancement of cyclin D1 and PCNA expression, which does not lead to enhanced 8-OHdG positivity.

**Materials and methods**

**Animals**

VDR null mutant mice, generated as described previously (16) were housed in the Centre for Laboratory Animal Care at the University of Vienna in a contained environment. Animals for this study were generated by breeding heterozygotes to produce offspring with all three genotypes. Mice were weaned at 2–3 weeks of age, and then fed ad libitum on a standard diet (rat and mouse No. 3 breeding diet, Special Diets Services, Witham, UK). Drinking water was supplemented with calcium lactate (5 mM). When mice (male and female) were 2–3 weeks old, ears were notched for identification and tail samples were obtained for PCR analysis. Mice used for experimentation were always 12–15 weeks old, except when indicated otherwise. The study protocols were reviewed and approved by the Institutional Committee of Animal Experimentation of the University of Vienna Medical School and by the Austrian Ministry of Science and Education.

**DNA preparation**

Genomic DNA was extracted from mouse tail tissue using standard proteinase K-SDS digestion. Briefly, 3 mm of the mouse tail was suspended in Tail buffer (50 mM Tris–HCl, pH 8.0, containing 0.1 M EDTA, 100 mM NaCl, 1% SDS, 20 μg/ml RNase DNase-free, 0.5 mg/ml protease K). Samples were incubated for 3 h at 55°C with agitation. Proteins were precipitated by adding 170 μl saturated NaCl. Supernatant was removed to a fresh tube and an equal volume of isopropanol was added to precipitate DNA. DNA washed once in 70% ethanol was allowed to air-dry for 10–15 min and was dissolved in a 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA buffer.

**PCR**

PCR was performed at a final concentration of 1× TaqPlus Precision buffer (Stratagene, La Jolla, CA, USA), 10 mM of each dNTP, 10 pmol of each primer (for wild-type (WT) or VDR-KO sequence) and 2.5 U of TaqPlus precision polymerase (Taq and Pfu together). Conditions were: 35 cycles at 94°C for 15 s, 66°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 10 min. The WT primers amplify a 140 bp product (located between bases 109 and 249 of the mouse vitamin D receptor complete coding sequence, accession number: D31969) that codes for amino acids 1–46 (DNA-binding domain and first zinc finger). The KO primers amplify a 455 bp product from the inserted targeting vector (16). The primer sets were:

- WT forward: ATG GAG GCA ATG GCA GCC AGC ACC TTC
- WT reverse: GAA ACC TGG GCA GCC TGC ACA GGT CA
- KO forward: GCC TCC TTT CGG AAT ATC ATG GTG GA
- KO reverse: AGC CAG GTG AGT TTA CCT ACC ACT TCC

The PCR products were visualized with ethidium bromide on a 2% agarose gel.

**Western blot analysis**

Tissue samples were obtained from ascending and descending colon of 10–15-week-old mice. For one set of experiments 6-month-old mice were used. For the proximal part, mucosa from the caecum to the middle of the colon transversum, for the distal part the subsequent tissue, excluding the rectum, was taken. Mucosal cells were scraped into 10 mM Tris buffer, pH 7.4, with protease inhibitors and 1% SDS. After homogenization with a Polytron (PT1200, Kinematica AG, Switzerland) and subsequent boiling for 5 min, homogenate was centrifuged for 10 min at 1500 r.p.m. and supernatant was frozen at −70°C.

Equal amounts of protein samples per lane were separated by 12% SDS–PAGE and subsequently blotted to a nitrocellulose membrane. Unspecific binding was blocked with 3% BSA in PBS containing 0.1% Tween 20 (T-PBS) for 2 h at room temperature. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-cyclin D1 antibody (NeoMarkers, Fremont, CA, USA), or mouse monoclonal anti-PCNA antibody (DAKO, Copenhagen, Denmark) in T-PBS with 1% BSA. After washing three times in T-PBS membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2 h at room temperature with subsequent detection with the SuperSignal CL-HPR Substrate system (Pierce, Rockford, IL, USA). Bands were quantified with the E.A.S.Y Win 32 software (Henzol, Wiesloch, Germany). Specific staining of the VDR band was abolished by pre-incubating the antigen with the peptide to which the antibody was raised. Cyclin D1 or PCNA proteins (NeoMarkers, Fremont, CA, USA) were used as positive controls on relevant blots.

**Immunohistochemistry**

Mouse tissue from the proximal part of colon descendents and the distal part of colon descendents was formalin-, respectively, Bouin’s-fixed and paraffin-embedded.

For immunostaining of PCNA, 5-μm paraffin serial sections on poly-L-lysine-coated slides were incubated for 20 min at 60°C, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by treatment with 3% H2O2 in methanol. For antigen retrieval slides were boiled in 0.01 M Tris buffer, pH 6.0, in a microwave oven at 600 W. A mouse monoclonal anti-PCNA antibody (DAKO, Copenhagen, Denmark) was used according to the HistoMouse-SIP kit instructions (Zymed Laboratories Inc., San Francisco, CA, USA). For negative control sections were incubated with non-immune mouse IgG (Amersham Pharmacia Biotech, Uppsala, Sweden). Immunostaining for 8-OHdG was performed as previously described (24,25). The monoclonal antibody N45.1 specific for 8-OHdG evaluation was used on Bouin’s fixed colon sections, which were derived from a total of 15 mice (five per genotype). For quantification three 35 mm photo slides were taken randomly from each sample. Slides were digitized using a Leitz Diaplan (Wetzlar, Germany), with a 100× objective using a cooled charge-coupled device camera (DS-F1, Nikon, Tokyo, Japan) in connection with Quadra 800 (Apple Computer Japan, Tokyo, Japan) and were analysed by NIH Image freeware (version 1.62).
Biochemical analyses of mineral homeostasis

**Results**

**Vitamin D receptor DNA evaluation in WT, HT and KO mice**

Mouse litters were routinely characterized by PCR analysis of genomic DNA extracted from tail clippings. Figure 1 demonstrates DNA analysis of littermates at 4 weeks of age. WT animals were identified by a predicted single VDR band of 140 bp, and KO animals by a single VDR band consisting of 455 bp. HT animals were identified by VDR bands consisting of both 140 and 455 bp. One-fourth of newborn litters presented with the KO genotype, which started to manifest by 10 weeks after weaning.

**Biochemical analyses of mineral homeostasis**

A total of 38 animals were evaluated. Table I demonstrates serum levels of calcium, phosphate, 25-D$_3$, and 1,25-D$_3$. All biochemical parameters of mineral homeostasis determined were similar in the WT and HT animals. In KO mice, however, serum calcium levels were significantly lower when compared with both WT and HT animals. Concerning serum phosphate levels, no statistically significant differences were found among the three genotype groups; nevertheless, phosphate tended to be lowest in KO mice. As expected, serum 1,25-D$_3$ levels were significantly higher by at least 50-fold in the KO mice than in the other two groups. Interestingly, 25-D$_3$ levels were significantly lower in KO animals than in both the WT and the HT group possibly indicating exhaustion of precursor supply for 1α-hydroxylation.

**Site-specific distribution of VDR protein**

In order to relate activity of the VDR to possible relevance for prevention of pre-malignant changes in certain areas of the colon, the site-specific presence of the protein was evaluated by immunoblotting of total protein preparations from proximal and distal colon mucosa. For statistical purposes five animals were investigated in each genotype group and cumulative data were presented in a histogram (Figure 2). A VDR band as predicted at ~52 kDa is present in WT colon ascendens, and, to a slightly less extent, in WT colon descendens. In HT colon ascendens, significantly less VDR was found when compared with WT mice. Interestingly, the amount of VDR was even further reduced in colon descendens. No trace of the 52 kDa band was found in KO mice.

As control, the VDR antibody was pre-incubated with the peptide against which it was raised. No band at 52 kDa was found in any genotype when using this antibody on an immunoblot (not shown).
Fig. 3. Representative site-specific western analysis of proliferation markers in WT, HT and KO colon of 12–15-week-old animals. (A) Total protein (100 µg) extracted from colon ascendens (Ca) and colon descendens (Cd) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with a polyclonal antibody against cyclin D1. (B) Total protein (50 µg) from Ca and Cd were separated by SDS–PAGE. A monoclonal PCNA antibody recognized a specific band at 36 kDa.

Marker evaluation by immunoblotting

Cyclin D1 expression

Figure 3A demonstrates a typical immunoblot of cyclin D1 expression in different colon segments of the three mouse genotypes. Cyclin D1 showed initially a much higher expression level in colon ascendens than in descendens, but the increment during VDR loss was much more pronounced in colon descendens.

PCNA expression

Immunoblotting for PCNA demonstrated a similar pattern (Figure 3B): again, there was higher expression of this protein in colon ascendens. PCNA was increased during VDR loss in HT mouse proximal and distal colon, but its highest level was reached in colon descendens of KO mice. Statistical evaluation of PCNA immunoblot band density from five animals per genotype was used for Figure 6.

When PCNA in the proximal and distal colon of aged mice of all three genotypes was evaluated by immunoblotting and compared with expression in 6-month-old mice no differences were found (not shown).

Immunohistochemistry

PCNA expression

Immunohistochemical analysis revealed a totally different pattern of proliferating cells in the proximal part of colon ascendens when compared with the distal part of colon descendens (Figure 4). In colon ascendens (Figure 4A and B) PCNA-positive cells were much more dispersed, not only located at the crypt base, but they were also present in the upper half of crypts. Though there was some increase in proliferating cells in this segment during loss of VDR, the increment did not prove to be statistically significant and was less than that found in colon descendens (see also Figure 3B). In contrast, in the sigmoid colon proliferating cells were found without exception only at the base of the crypt. In KO animals, this proliferative compartment was enlarged towards the upper part of the crypt, and staining became very dense (Figure 4C and D).

8-OHdG evaluation

DNA oxidation was evaluated by immunohistochemistry in the proximal part of colon ascendens [(A) WT; (B) KO] and in the distal part of colon descendens [(C) WT; (D) KO] in 12–15-week-old mice. Bar = 50 µm.
Data were derived from 8-OHdG was rising with loss of VDR. Complete loss resulted in colon descendens not only PCNA, but also positivity for 8-OHdG positivity were not altered statistically.

Quantitative evaluation of PCNA and 8-OHdG positivity were not altered statistically when comparing the WT genotype (Figures 5C,D and 6B).

Quantification of PCNA and 8-OHdG positivity

Data were derived from five mice per genotype by immunoblotting for PCNA and by immunohistochemistry for 8-OHdG. Positivity for the two markers was quantified as described under Materials and methods and was expressed in percent of WT control. Data are presented as mean ± SD. Student’s t-test was used for statistical group analysis: **P < 0.01; *P < 0.05.

Discussion

A recent case control-study nested in the prospective Nurses’ Health Study demonstrated that low levels of circulating 1,25-D₃ enhanced risk of distal colorectal adenomas (26). In an animal model for human familial adenomatous polyposis it was demonstrated that a diet low in calcium and vitamin D resulted in increased incidence of several tumour end points (27). This suggested to us that a mouse model with complete, or partial inactivation of the steroid hormone receptor could be an excellent model to study the relevance of an intact vitamin D system for pre-malignant changes in the colon.

We, therefore, characterized VDR expression in HT and KO mice in comparison with their WT age-matched littermates by immunoblotting. As expected, expression of the VDR was highest in WT mice, and non-existent in the KO genotype. In the HT colon, however, significantly less VDR was found compared with WT. This, however, did not influence serum parameters for vitamin D metabolites and calcium (see Table I). Although in the WT genotype quantitative evaluation of proximal/distal VDR expression showed similar distribution, this was different in HT mice: there was a definite gradient of decreasing VDR levels towards the distal colon.

Topical distribution of human colon cancer is unequal. For instance, in white male Americans colon cancer is more frequent in the distal, in African male Americans in the proximal colon (28). Reasons for this unequal distribution are unknown, except that there are some indications for a nutrition-related aetiology for tumours of the sigma and of the rectum (29,30). It is also becoming increasingly apparent that many genetic changes in colorectal carcinomas show marked difference in frequency of occurrence when the site of tumour formation is analysed: for instance in proximal tumours the karyotype is more stable and cells are diploid with few allelic deletions, whereas distal tumours often have an unstable karyotype with aneuploid DNA content, c-myc oncogene deregulation, and different c-ki-ras activation (31,32).

There have been considerable efforts in the last years to establish relevance of certain early markers for human colorectal pathogenesis. Especially relating proliferative activity to the risk of malignant transformation is of some controversy. Recently, this aspect was investigated definitively by human colon crypt microdissection and mitosis counting by Mills et al. (33). They provided evidence that (i) proliferation is independent of age and it is decreasing from the proximal to the distal gut, (ii) proliferation is increased in patients with familial adenomatous polyposis and sporadic carcinomas, and (iii) morphologically normal mucosa in close proximity to a tumour also shows elevated mitosis. Still, they suggest that the latter may be a consequence rather than an aetiologcal factor in development of neoplasia, such as production of eicosanoids because of a switch-on of COX-2 in tumours.

There are strong similarities to the above observations found in our murine model: there was no age-related increase of proliferation either. Quantitative evaluation of PCNA and cyclin D1 positivity by immunoblotting also indicated a decreasing gradient from the proximal to the distal WT mouse gut (Figure 3B), which cannot be related to differential distribution of the VDR. Enhanced proliferation with decreases...
ing VDR expression was independent of serum calcium changes, as it occurred already in the HT mouse gut. With loss of VDR however, proliferation rose more in colon descendens than in ascendens, and this was paralleled by a proportionally much greater loss of VDR in the distal colon of HT mice. Thus, in KO animals PCNA positivity was equally high in colon descendens as in colon ascendens though cyclin D1 expression only reached 50% of that found in colon ascendens. Why this marker of DNA synthesis was elevated so much in response to loss of growth control by 1,25-D3 in contrast to cyclin D1 is presently under investigation by us.

Toyokuni et al. (34) suggested that there is a correlation between the level of oxidative stress and cellular proliferation. This concurs with our data demonstrating much higher levels of 8-OHdG in parallel to high PCNA positivity in colon ascendens in WT mice. When VDR loss led to enhanced PCNA positivity in colon descendens, it was accompanied by an extremely high (6-fold) increase of 8-OHdG positivity.

1,25-D3 is known to control normal villus and crypt development by regulating proliferation and differentiation of intestinal cells moving up the villus axis (35). Apparently, when this growth control is reduced or completely abrogated, enhanced proliferation and highly increased radical formation occurs, however only in the distal colon. We suggest that, because of normally enhanced proliferative activity in colonocytes of the proximal colon, antioxidant enzymes and/or repair mechanisms for 8-OHdG cleavage may already be upregulated at this site. The distal colon, however, was initially a site of lower proliferation. Highly increased proliferation, respectively, DNA synthesis only occurred with loss of VDR, and subsequent loss of growth control by 1,25-D3. In this case, repair mechanisms against DNA damage may not have been sufficiently activated. Such a lack of a localized protective system was also suggested by the observation that in tumours of the distal colon DNA changes are much more prevalent than in tumours of the proximal colon (31). It is known that diets rich in fat and poor in dietary fibres enhance risk for human colorectal cancer and appear to increase the potential for hydroxyl radical formation in the faeces (36), which in turn could damage genomic integrity of distal colonocytes. In this respect it is interesting that dietary changes can reduce distal tumour formation, but not in the proximal part of the rat colon (37).

We, therefore, suggest that loss of VDR may provide a link to the complex course of multi-step carcinogenesis by causing direct DNA damage when 1,25-D3-mediated growth control is diminished and 8-OHdG expression rises. In addition, the distal part of the colon of our VDR-KO mouse model fulfills many requirements of an animal model for studies of human colorectal pre-malignancy. Induction of early lesions and/or their prevention by nutritional means is presently under investigation in our laboratory.

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References


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