Virchows Arch (2000) 437:501–507
DOI 10.1007/s004280000275

ORIGINAL ARTICLE

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In situ mRNA hybridization analysis and immunolocalization of the vitamin D receptor in normal and carcinomatous human colonic mucosa: relation to epidermal growth factor receptor expression

Received: 27 September 1999 / Accepted: 6 June 2000 / Published online: 9 September 2000

Abstract There is evidence that vitamin D receptor (VDR)-mediated action of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) could limit colon cancer cell growth particularly when induced by activation of the epidermal growth factor receptor (EGFR). We therefore wanted to ascertain the relevance of this observation for human colon cancerogenesis. Utilizing in situ mRNA hybridization and immunocytochemical techniques, we analyzed cell-specific expression of VDR and EGFR in normal and malignant human colonic mucosa. In normal mucosa, VDR positivity is weak and observed only in a small number of luminal surface colonocytes. In contrast, EGFR expression at a relatively high level is also found in cells at the crypt base. The number of VDR-positive colonocytes increases remarkably during tumor progression. It reaches its maximum in low grade adenocarcinomas and returns to lower levels in highly malignant cancers. In both low- and high grade carcinomas, the great majority of tumor cells contain the EGFR message. The relative abundance of EGFR over VDR in normal mucosa and in high grade carcinomas would create a situation in which mitogenic effects from EGFR activation are only ineffectively counteracted by signaling from 1α,25-$(OH)_2D_3$/VDR. In contrast, in well to moderately differentiated tumors, upregulation of VDR could retard further tumor progression.

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Keywords Colon adenocarcinoma · Growth control · Differentiation · Steroid hormone receptors · 1,25-Dihydroxyvitamin D_3

Introduction

1α,25-Dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), the hormonally active metabolite of vitamin D_3, not only plays a major role in calcium and phosphate homeostasis, but also functions as a regulator of cell proliferation and differentiation of normal and malignant cells (for review see e.g., [13]). In particular, growth inhibition by 1α,25-$(OH)_2D_3$ has been demonstrated in various colon carcinoma cell lines such as Caco-2 [4, 8, 9, 10] or HT-29 [30] and in primary cultures of human colon adenoma and carcinoma [22]. The presence of the nuclear vitamin D receptor (VDR), which functions as a gene transcription factor [2] in all of these various types of colon cancer cells, is considered a requirement for their responsiveness to the anti-mitogenic action of the steroid hormone ([14, 21]).

Interestingly, colon cancer cells not only possess the VDR [21, 22], but as we have shown recently [5, 12], are also able to synthesize its ligand, 1α,25-$(OH)_2D_3$, from its endogenous precursor, 25-$(OH)D_3$. This suggests that proliferation of human colon carcinoma cells can be controlled in an autocrine fashion through their intrinsic 1α,25-$(OH)_2D_3$/VDR system. Its efficiency necessarily depends not only on the availability of 1α,25-$(OH)_2D_3$ from endogenous or exogenous sources, but also on the extent of VDR abundance. Consequently, the magnitude of VDR expression might be a key factor in controlling neoplastic cell proliferation at certain stages of colon cancer development.

Studies of VDR expression in colorectal carcinoma have so far yielded rather conflicting data. While some investigators reported that receptor density in tumor tissue is low [17], others observed higher VDR expression

in carcinomatous tissue than in normal colonic mucosa [11, 28]. In all of these studies, VDR expression was assessed using northern and/or western blot analysis of whole tissue homogenates. Because the VDR has been shown to be present also in fibroblasts, smooth muscle cells, endothelial cells, macrophages, and activated lymphocytes (e.g., [29]), it was not possible to determine the relative contribution of receptor expression in these cell types to the total VDR content as measured. To obtain unambiguous data on VDR density and distribution among epithelial versus non-epithelial cells during development of colorectal cancer, it was therefore mandatory to study changes in VDR expression in intact colonic tissue. In the present study, we used semiquantitative in situ hybridization (ISH) and immunohistochemical techniques to detect VDR mRNA transcripts and protein in epithelial and stromal cells of normal and cancerous human colonic mucosa.

In parallel, specific immunostaining was used to study the cellular distribution of epidermal growth factor receptor (EGFR) in colonic mucosa in relation to tumor progression because EGFR expression levels rise in parallel to the metastatic potential of human colon carcinoma cells [19] and we found that $1\alpha,25\text{-}(OH)_2D_3$ is a specifically potent antagonist of EGF-induced growth of colonic tumor cells [10, 23, 25].

In the present study, we demonstrate that expression of both VDR and EGFR mRNA increases upon transition from normalcy to the malignant state. However, distinct dissimilarities in the receptor expression pattern at two critical stages of tumor development do exist. In normal mucosa, the relative abundance of EGFR over VDR is high and could therefore increase the risk of neoplastic transformation, whereas in high grade, i.e., poorly or undifferentiated adenocarcinomas, a similar abundance ratio could contribute to sustained malignant tumor cell growth.

Materials and methods

Surgical specimens

Tissue specimens of cancerous lesions and from histologically normal mucosa outside the tumor border were available from 24 patients who had to undergo surgery for primary colon adenocarcinoma at the Clinics of Surgery, Vienna General Hospital or at Krankenhaus Rudolfsstiftung, Vienna. For comparison, normal colon tissue was obtained from two diverticulitis patients after stoma reoperation (Krankenhaus Rudolfsstiftung). Permission from the Ethics Commission of the University of Vienna Medical School was granted prior to initiation of the study. Tumors (all of the adenocarcinoma type) were graded according to the World Health Organization (WHO) classification [15]. Twenty-one were of low grade, i. e., well to moderately differentiated, and three were of high grade, i. e., poorly differentiated or undifferentiated.

ISH techniques

All solutions used for this procedure were prepared in diethylpyrocarbonate-treated water. Tissue samples were embedded in Tissue-Tek OCT compound (Miles Inc, Elkhart, Ind.) and snap-frozen at –196°C in liquid nitrogen. Frozen blocks were stored at –80°C until used. Tissue sections (5 µm) were cut on a cryostat and adhered to silanized glass slides. Eighteen cryocuts from adenocarcinomas and adjacent mucosa and two cryocuts from normal mucosa from non-cancer patients were suitable for further processing. Cryocuts were fixed for 1–3 h in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). After washing three times in PBS, endogenous alkaline phosphatase was blocked with 0.2 N HCl at room temperature for 8 min. Proteinase K treatment was performed for 6–15 min at 37°C in preheated buffer [0.05 M Tris-HCl, pH 7.4, 0.005 M ethylene diamine tetraacetic acid (EDTA), 20 µg/µl proteinase K (Amresco Inc., Solon, Ohio]. After denaturation at 80 C for 5 min, probes were mixed with a hybridization solution [(50% formamide, 10% dextran sulphate, 1% Denhardt's solution, 2× sodium saline citrate (SSC)], applied to tissue samples and covered with parafilm. Slides were hybridized at 50 C overnight in a humidified chamber.

Subsequently, slides were incubated for 30 min at 37 C with RNase A (40 µg/ml) followed by washing in 2×SSC, 50% formamide for 5 min at 50 C and two washes in PBS. Goat serum (1.5%) in PBS for 30 min at room temperature was used for blocking. Anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Germany, concentration 1:500) was applied for 1 h at room temperature. The BCIP/NBT substrate Kit IV (Vector Labs, Burlingame, Calif.) was used to localize alkaline phosphatase activity. The development of a color reaction was monitored using light microscopy. Slides were counterstained with methyl green (Vector) or mounted directly with Histomount (Zymed Labs, South San Francisco, Calif.). Controls used were: (1) sense riboprobes, (2) RNase digestion of section before applying specific probes, and (3) no addition of a specific probe.

Riboprobes were designed complementary to two fragments [hormone-binding domain (280 bp) and hinge domain (580 bp)] of the human VDR and to a fragment of EGFR (400 bp) based on published reports of the complementary (c) DNA sequences [1, 26]. The specificity of the probes was checked by the Genetics Computer Group sequence analysis software package (GCG Inc., Madison, Wis.).

Total RNA was extracted from human colon adenocarcinoma-derived Caco-2 cells using a one-step method. cDNA was obtained using a standard reverse transcription reaction using random primers. The next specific primers were designed.The primers 5′-GTC AGT GAC GTG ACC AAA GCC-3′ and 3′-TAC TAG GTC TTC GAT CGG CTG-5′ were used for the hormone-binding domain of VDR, the primers 5′-GTG CAG AGG AAG CGG GAG ATG-3′ and 3′-TGG ACA CCG TTG GTT CTG ATG-5′ were used for the hinge domain of VDR, and the primers 5′-CAT TCA GGG GGA TGA AAG-3′ and 3′-GGA CAG ATA GTG AGT CGG-5′ were used for EGFR. Hot start PCR was done on a GeneAmp PCR System 9600 (Perkin Elmer, Foster City, Calif.). Amplification products were ligated to pCRII or pCR2.1 vectors (Invitrogen, San Diego, Calif.). Only the pCR2.1 vector was cut with *Eco*RI and subcloned into pBluescript II KS(+) vector (Stratagene, La Jolla, Calif.). Orientation of the inserts was checked by means of PCR using different combinations of external and internal primers and by digestion with the appropriate restriction enzymes.

Recombinant plasmid DNA was extracted using a Plasmid midi kit (Qiagen GmbH, Hilden, Germany) and linearized with *Hind*III, *Not*I or *BamH*I restriction enzymes (New England Biolabs Inc., Beverly, Mass.). The antisense and sense probes were synthesized in a transcription in vitro reaction with digoxigenin-11-UTP (Boehringer Mannheim, Germany) and SP6, T3, or T7 RNA polymerases (Promega, Madison, Wis.). Intact probes and the ones digested with alkaline hydrolysis were produced in order to validate results of hybridization. The molecular weight of riboprobes was checked on agarose gels. The concentration of probes was determined through serial dilution on dot-blots using nylon membranes (Amersham, Buckinghamshire, UK)

Evaluation of ISH signals

Percentage of positive cells and staining intensity were evaluated at a total magnification of 200× (Olympus AHBT microscope).

The number of positive cells was determined by counting the number of positive nuclei over a distance of 50 epithelial cells in three different areas of a tissue sample. The intensity of specific signals was scored as follows: 0, negative; 1, weak; 2, moderate; 3, high. Multiplying the percentage of positive cells with the staining intensity yielded a semiquantitative score according to Remmele and Stegner [20].

Immunohistochemistry

Sections of paraffin-embedded tissue (5 µm) on poly-L-lysine-coated slides were incubated 20 min at 60 C, deparaffinized, and rehydrated. After washing three times in PBS (pH 7.2), with the last washing containing 0.1% Tween-20, endogenous alkaline phosphatase was inactivated by incubation in 0.2 N HCl at room temperature for 8 min. Sections were washed in PBS and submerged in peroxidase quenching solution (3% H_2O_2 in absolute methanol) for 10 min. After washing in PBS, sections were permeabilized in 0.1% Tween-20 for 10 min. After washing in PBS, sections were boiled (three 5-min boils) in citrate buffer (pH 6.0) in a microwave oven for antigen retrieval.

Double immunostaining was performed using a Histostaint-DS Kit (Zymed Laboratories, Inc., South San Franscisco, Calif.) according to the manufacturer's instructions. Briefly, after washing with PBS/0.1% Tween-20 and incubation with a blocking solution, sections were incubated overnight at 4 C with the monoclonal rat anti-VDR antibody (Chemicon, Temecula, Calif.; dilution 1:20). After washing in PBS/0.1% Tween-20, the biotinylated second antibody was applied for 20 min, followed by streptavidin-alkaline phosphatase (10 min). The VDR was localized by adding the alkaline phosphatase substrate, which produced a blue stain. Then the sections were washed with PBS. To detect the second antigen, the monoclonal mouse anti-EGFR antibody (Ylem, Rome, Italy, dilution 1:20) was applied for 1 h at room temperature. After incubation with the biotinylated second antibody (10 min) and with streptavidin-peroxidase (10 min), the EGFR was visualized by adding the peroxidase substrate, which produced a red stain. Finally, sections were mounted in EUKITT (Kindler, Freiburg, Germany).

No specific staining for VDR or EGFR was detected when negative controls were produced by substituting the respective primary antibody with PBS or pre-immune serum. To control for a possible cross-reaction between the streptavidin used for EGFR detection and unoccupied biotin residues on the second antibody decorating the anti-VDR antibody, the anti-EGFR antibody was substituted by pre-immune immunoglobulin (Ig)G_1 before the biotinylated second antibody, and the streptavidin-peroxidase complex were applied. No false positive signals were detected.

Results

ISH of normal and carcinomatous colonic mucosa was achieved with digoxigenin-labeled antisense VDR mRNA probes, which recognized either a 280-bp fragment of the hormone-binding domain or a 580-bp fragment of the hinge domain. In all cases, both probes yielded identical results. Representative samples of individual patient tissue are shown in Fig. 1. In normal mucosa outside the tumor border, only a few colonocytes at the luminal surface and in the upper part of the crypts exhibited a weak hybridization signal (Fig. 1A). An identical pattern with respect to cellular localization and intensity of hybridization signals from VDR mRNA was observed in healthy mucosa from diverticulitis patients (not shown).

In the 18 adenocarcinomas investigated, ISH reactivity for VDR mRNA was most prominent in tumor cells. Intensity of hybridization signals ranged between moderate and strong. Figure 1B shows a low grade adenocarci-

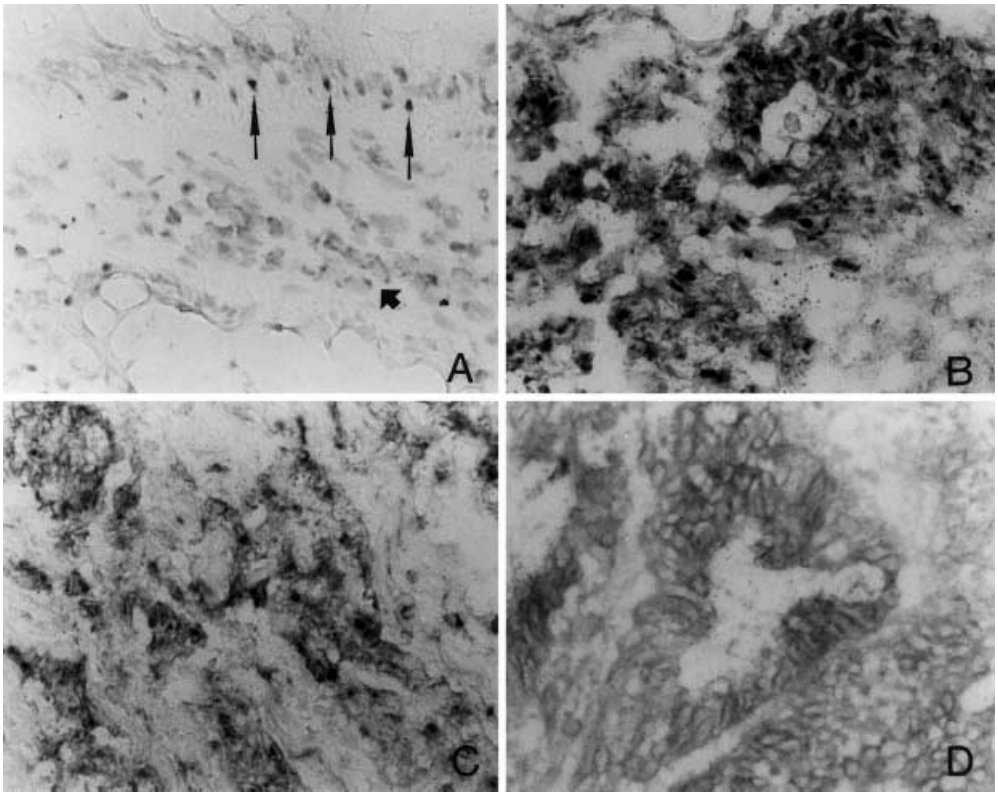

Fig. 1 In situ hybridization for vitamin D receptor (VDR) mRNA in human colonic mucosa with digoxigenin-labelled antisense RNA probes. Magnification ×100. **A** Normal mucosa: weakly positive reaction in colonocytes within the luminal and crypt epithelium (*arrows*) and in non-epithelial cells in the lamina propria (*arrow heads*). **B** Adenocarcinoma (low grade): high abundance of VDR mRNA in tumor cells. **C** Adenocarcinoma (high grade): lower abundance of VDR mRNA. **D** Negative control (sense VDR riboprobe, low grade adenocarcinoma)

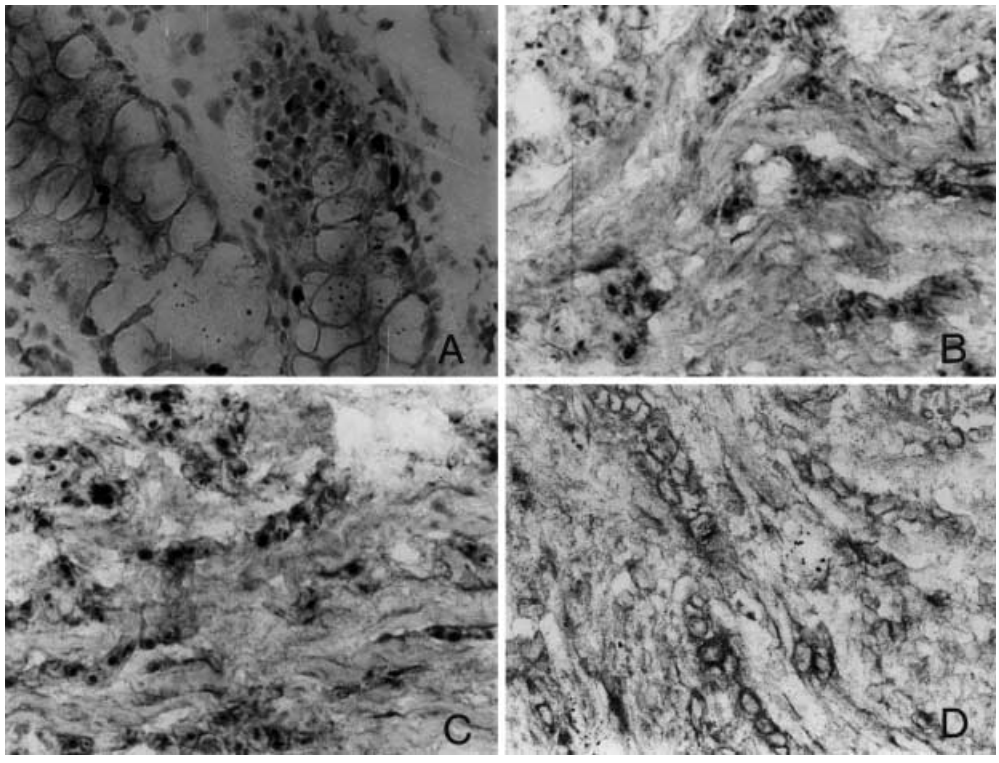

Fig. 2 In situ mRNA hybridization analysis of epidermal growth factor receptor (EGFR) in human colonic mucosa. Magnification ×100. **A** Normal mucosa: EGFR mRNA-positive colonocytes in crypt epithelium. **B** Adenocarcinoma, low grade and (C) adenocarcinoma, high grade: strong positive reaction in tumor cells. **D** negative control with a sense probe (adenocarcinoma, low grade)

noma with many tumor cells expressing VDR mRNA. No signal was detected when the antisense mRNA probe was substituted by the respective sense probe for control of specificity of hybridization (Fig. 1D). A somewhat different staining pattern was observed in high grade adenocarcinomas (Fig. 1C). The number of positive tumor cells seemed to be much lower than in more differentiated tumors but still higher when compared with normal musosal tissue (Fig. 1A, B)

Results from semiquantitative evaluation of ISH reactivity (Fig. 3) confirmed the notion that VDR mRNA expression is highest in low grade adenocarcinomas and considerably less in high grade cancerous lesions. In accordance with previous reports from other laboratories, we were able to detect the VDR mRNA signal also in stromal and, interestingly, in infiltrating cells (e.g., [29]).

ISH of EGFR mRNA revealed that in normal colonic mucosa, predominantly colonocytes in the lower part of the crypts contained the message for the EGFR, as indicated by moderate to strong hybridization signals (Fig. 2A). Evidence for expression of EGFR mRNA was obtained in all adenocarcinomas inspected. Regardless of whether in high grade or low grade tumors, virtually all cancer cells exhibited a moderate to strong signal for EGFR (Fig. 2B, C), which was not detected when the sense instead of the anti-sense probe was used in the hybridization assay (Fig. 2D). The respective semiquantitative ISH reactivity scores are shown in Fig. 3.

When normal colonic mucosa was probed for the presence of VDR and EGFR protein by means of double immunostaining, a distribution pattern identical to that from ISH analysis was obtained for both receptors

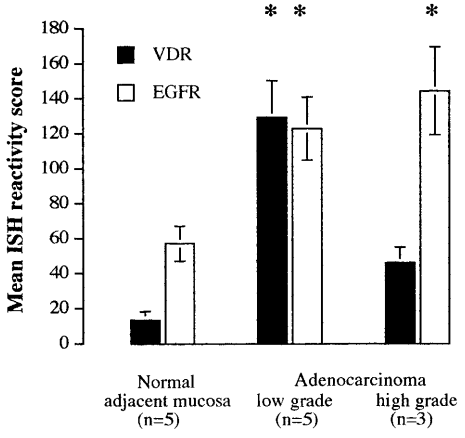

Fig. 3 Semi-quantitative evaluation of vitamin D receptor (VDR) and epidermal growth factor receptor (EGFR) mRNA expression in epithelial cells. In situ hybridization (ISH) reactivity scores were calculated by multiplying the percentage of receptor-positive cells by the average signal intensity (see Methods section). Data are means ±SEM from *n* samples per group. *Asterisk* indicates statistically significant difference from respective ISH score in normal adjacent mucosa at $P<0.01$ level (using Student's unpaired *t*-test)

(Fig. 4). With respect to the VDR, specific nuclear staining was apparent only in colonocytes within the surface epithelium and in the upper part of the crypts (Fig. 4A). In contrast, membrane and cytoplasmic staining specific for the EGFR was detected throughout the entire mucosal epithelium but predominantly in the lower part of the crypts (Fig. 4A).

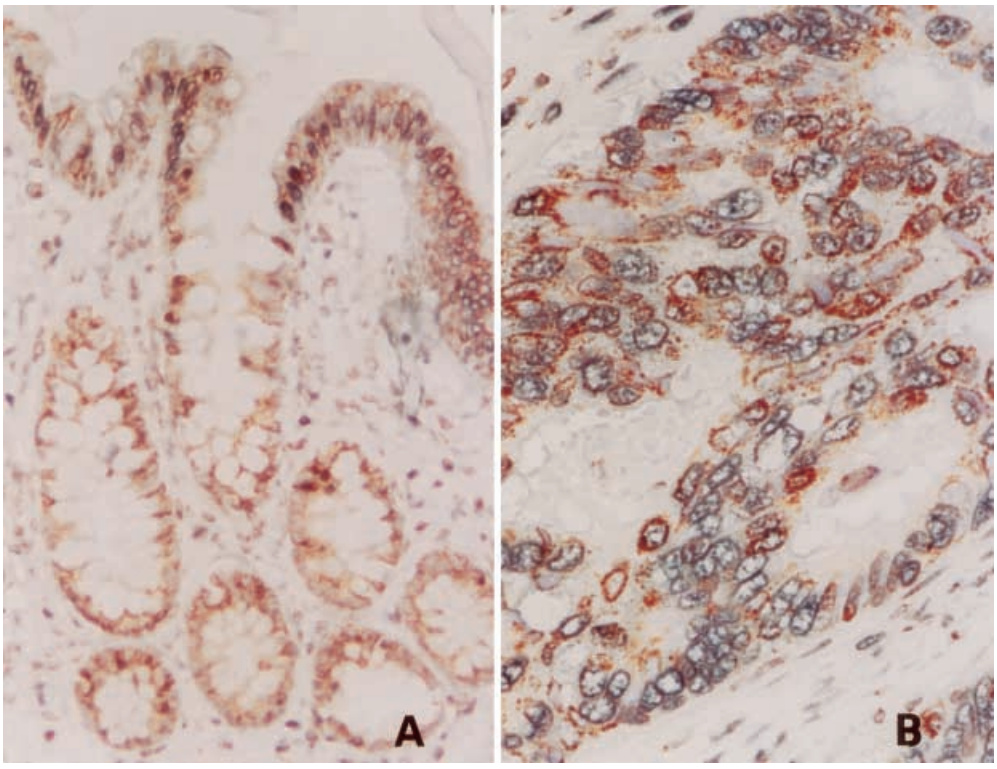

Fig. 4 Expression of vitamin D receptor (VDR) and epidermal growth factor receptor (EGFR) in normal adjacent mucosa (**A**) and cancerous lesion (**B**) visualized by means of double immunohistochemical staining. VDR: *blue* nuclear staining; EGFR: *red* membrane and cytoplasmic staining. Negative controls (not shown) were done as detailed in methods. Magnification: **A** ×175, **B** ×350

In all adenocarcinomas expressing the VDR message, also the protein could be detected by means of specific immunostaining. As a representative example, Fig. 4B shows that many of the tumor cells in a low grade adenocarcinoma exhibit positive immunoreactivity for the VDR in the nuclear region. Generally, in these cells, coexpression of EGFR was observed. In addition, it is apparent that VDR-negative cells stain positively for the EGFR so that EGFR is abundantly present in virtually all carcinoma cells.

Discussion

The notion that vitamin D protects against the risk of colorectal cancer was substantiated by several in vitro studies showing that vitamin D compounds in fact reduce proliferation and promote differentiation in colon carcinoma cells [4, 9]. Consequently, the feasibility of vitamin D treatment of colorectal cancer is presently under intensive investigation. Since the mechanism of antimitogenic action of $1\alpha,25(OH)_2D_3$ and of its functionally related synthetic analogs involves gene activation by the $1\alpha,25(OH)_2D_3$/VDR complex, evaluation of VDR expression could be of clinical importance with respect to prevention or treatment of the disease.

Possibly because of inherent methodical ambiguities, studies on changes in VDR density in colorectal carcinomas during tumor progression have yielded conflicting data so far. For this reason, we extended our previous investigation of VDR expression in human colon tumors based on immunoblotting [11] to ISH and immunohistochemistry. In order to further validate our results, we used two riboprobes for VDR mRNA hybridization that recognize either the hinge or the hormone binding domain of the VDR gene. In essence, our data demonstrate unequivocally that VDR mRNA increases remarkably from low expression in a few normal colonocytes and stromal cells to high levels in the majority of carcinoma cells. This increase in VDR expression as reflected by a conspicuous difference of the respective ISH reactivity scores (Fig. 3) is a composite of the increase in numbers of receptor-positive cells but also of mRNA copies per cell as indicated by intensified staining (Fig. 1A, B). The only exception appears to be high grade cancers. Although only tissue specimens from three patients with a high grade adenocarcinoma were available for the present study, we consider the observation that in high grade cancers VDR is less abundant than in more differentiated carcinomas (Fig. 3) significant, especially since it corresponds well with our previous immunoblotting data [11].

The remarkable rise in EGFR mRNA and protein expression in colonic adenocarcinomas (Fig. 4) is consistent with reports from other laboratories as well [19]. In addition, in a recent study, we calculated the average number of specific EGF-binding sites in primary cultured cells derived from low grade adenocarcinomas to be at least three times as high as in the human colon cell line Caco-2 undergoing re-differentiation in culture [24]. It should also be noted that the rise in receptor numbers is accompanied by the appearance of a typical oncofetal pattern of EGFR polarity inasmuch as colon cancer cells exhibit the majority of EGFR on their apical and not, as normal colonocytes, on their basolateral plasma mem-

brane [24]. Although not all of the apical EGF-binding sites might represent functional receptors, the high levels of EGFR mRNA expression in colonic tumor cells, as observed in the present study, nevertheless might reflect increased ability to respond to EGFR activation with a rise in the rate of cell division.

It should be noted that in normal mucosa, the location of cells expressing the EGFR and the VDR are frequently dissimilar (Fig. 1, Fig. 2 and Fig. 4). VDR positivity is mainly observed in the luminal surface epithelium and in the upper part of crypts, whereas EGFR-expressing cells are frequently found in the proliferative compartment at the crypt base. This implies that the latter cell type is particularly responsive to EGFR activation, because EGF-induced cell growth in this case cannot be checked via the antagonistic $1,25(OH)_2D_3$/VDR system. Likewise, in poorly differentiated adenocarcinomas, as a consequence of reduced VDR expression (Fig. 1; [11]), EGFR-mediated proliferation cannot be effectively counteracted by the intrinsic $1,25(OH)_2D_3$/VDR system.

The obvious coexistence of the EGFR and the VDR at high expression levels in low grade adenocarcinoma cells (Fig. 3 and Fig. 4) merits to be discussed. In vitro data have indicated that VDR levels can be up-regulated by EGF, e.g., in keratinocytes, enterocytes, and osteoblast-like cells [6, 7, 27]. In addition, transfection of mouse-skin fibroblasts with the c-*myc* oncogene, a cell cycle controlling gene, specifically induces expression of the VDR [18]. This, we believe, could imply that in the human colon under autocrine/paracrine growth stimulation and hyperproliferation, VDR expression is increased and therefore the vitamin D growth regulatory system can function at enhanced efficiency during availability of the steroid ligand.

High levels of VDR protein have been demonstrated by immunohistochemical methods in lung and breast cancer and in skin basal cell carcinomas [3, 16, 31]. Thus, VDR protein up-regulation may be a common feature of epithelial tumors. We therefore suggest that activation of the vitamin D system, which in the case of colon cancerogenesis would comprise enhancement of steroid hormone synthesis and also of receptor expression, may constitute a physiological defense system against epithelial tumor progression.

Acknowledgements This work was supported by grants from ASTA Medica GmbH, Vienna, Austria (to M.P. and H.S.C.), from the Austrian Federal Ministry of Science and the Arts (to M.P.), and from the Austrian Science Foundation (project no. P09917-MED to H.S.C.). We thank Ms. Teresa Manhardt for preparation of cryostat sections and Dr. Giovanna Bises for her invaluable help with the double immunostaining technique. Parts of this work were presented at the Tenth Workshop on Vitamin D, Strasbourg, France, May 1997.

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