1,25-dihydroxyvitamin D₃ and retinoid X receptor expression in human colorectal neoplasms

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Abstract

Epidemiological studies suggest that 1,25-dihydroxyvitamin D₃ (D₃) protects against colorectal carcinogenesis. Animal and in vitro studies show an antiproliferative effect of D₃ in a variety of tumours including those of large bowel origin. D₃ actions are mediated by D₃ receptors (VDR) alone or by VDR in conjunction with retinoid X receptors (RXRs) in all D₃ responsive tissues. The expression of mRNAs encoding VDR and RXRs in normal and malignant human colorectum was determined. Full length VDR (4.6 kB), RXR α (5.5 kB), and RXR γ (3.5 and 7 kB) mRNAs were expressed in all tissues, but RXR β mRNA was not expressed in any. VDR expression was reduced in 12 carcinomas relative to paired normal mucosa, and RXR α expression was reduced in nine. There was no correlation between VDR or RXR α expression and the site, grade of differentiation, or Dukes's staging of the tumour. The finding of persistent VDR and RXR coexpression in all colorectal tumours provides a rational basis for exploring a role for D₃ in the treatment of colorectal malignancy.

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The primary actions of 1,25-dihydroxyvitamin D₃ (D₃) in bone, kidney, and intestine in controlling Ca²⁺ and PO₄³⁻ homeostasis are well established but an important regulatory role in the control of cell differentiation has been recognised recently. D₃ is required for osteoblast differentiation during normal bone development and is a potent inducer of cell differentiation in several other tissues, including skin and bone marrow promyelocyte precursors.^{1,2} Together with its antiproliferative actions in mammalian models of breast cancer,^{1,3} these findings suggest a potential role for D₃ in the treatment of a wide variety of tumours.

Epidemiological studies suggest that D₃ confers significant protection against colorectal cancer when assessed by dietary intake⁴ or serum concentrations.⁵ D₃, in conjunction with dietary calcium supplementation, protects in animal models of colon cancer,^{6,7} and together with increased dietary calcium intake, reduces the incidence of K-ras mutations in 1,2-dimethylhydrazine-induced colonic tumours in rats.⁸ Colonic cancer cell xenografts regress after treatment of mice with hypercalcaemic doses of D₃⁹ and treatment of

the colonic cancer cell lines HT-29 and CACO-2 with supraphysiological doses of D₃ (10⁻⁶ to 10⁻⁸ M) induces differentiation and inhibits cell proliferation in vitro.^{10–13}

The actions of D₃ are mediated by its high affinity nuclear receptor (VDR) which is a member of the steroid/thyroid hormone receptor super-family.^{14,15} The VDR binds as a homodimer to specific target sequences of DNA, termed D₃ response elements, to function as a hormone inducible transcription factor that activates or represses transcription of D₃ responsive target genes.¹⁴ In addition, VDR can form functional heterodimers with retinoid receptors (RXR), the ligand for which is the vitamin A derivative 9-*cis* retinoic acid.^{16,17} There are three RXR genes, RXR α , β , and γ , encoding multiple receptor isoforms, which would allow modification of D₃ signalling pathways by 9-*cis* retinoic acid.^{16–19} Thus, D₃ can regulate gene expression by two pathways, one activated via VDR homodimers, and the other through VDR/RXR heterodimers.²⁰

If D₃ has a role in colorectal carcinogenesis, appropriate receptors must be present in both normal colon and cancers. Therefore, we have determined the expression of VDR and RXR mRNAs in tumour tissue from patients with colorectal cancers and from normal mucosa to investigate whether receptor mRNA expression persists in neoplastic tissue.

Methods

RNA PREPARATION AND NORTHERN ANALYSIS OF HUMAN TISSUE

Tissue samples were collected from surgical specimens immediately after resection, and within 15 minutes of excision were frozen in liquid nitrogen until use. Non-necrotic, colorectal tumour tissue was removed and paired normal mucosa from two sites (directly adjacent to and 5–10 cm distant from the tumour) was dissected off the muscularis propria.

Total cellular RNA (approximately 200 μ g) was extracted and the mRNA fraction enriched by oligo (dT) column chromatography ([dT] cellulose type 7, Pharmacia-LKB).²¹ Poly-A⁺ RNA was loaded into a denaturing 1.2% formaldehyde agarose gel and resolved by electrophoresis (100 V, 4 hours).²¹ RNA was transferred overnight to Hybond N⁺ membranes (Amersham International, Buckinghamshire, UK) and subsequently bound to filters by UV irradiation (245 nm, 120 mJ). Filters were prehybridised (8 hours) and hybridised (16 hours) at 65°C to ³²P-labelled cDNA or cRNA probes in phosphate (0.77 M NaH₂PO₄/Na₂HPO₄ pH

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TABLE 1 Details of patients with colorectal cancer

Site of cancer	Sex		Histological grade of differentiation			Dukes's staging		
	M	F	Well	Moderate	Poor	A	B	C
Caecum+ascending colon	2	3	3	1	1	0	4	1
Transverse+descending colon	0	2	0	2	0	0	0	2
Sigmoid colon	5*	3	2*	5	1	2	0	6*
Rectum	6†	1	2	5†	0	2	3†	2

* and † refer to two patients with coincidental adenomatous polyps.

7.2, 5 mM EDTA, 7% sodium dodecyl sulphate (SDS), 100 µg/ml sonicated salmon sperm DNA) or formamide (50% deionised formamide, 5x SSPE, 0.15 M Tris pH 8, 1% SDS, 5x Denhardt's solution, 100 µg/ml sonicated salmon sperm DNA) buffers, respectively. Filters were washed to high stringency (0.1x SSC, 0.1% SDS at 65°C for one hour (cDNA probe) or 75°C for one hour (cRNA probes)) before autoradiography for between six hours and 28 days, depending on the probe used. Autoradiographs were quantified by laser densitometry and receptor mRNA expression standardised relative to β-actin.

PROBES

A full length mouse β-actin cDNA was radiolabelled with α-(³²P)dCTP (Amersham International, Buckinghamshire, UK) by nick translation (Amersham).²¹ cRNA probes were labelled with ³²P UTP (Amersham) by in vitro transcription (Riboprobe system, Promega, Southampton UK) using T7 or T3 bacteriophage RNA polymerase.²¹ cRNAs were synthesised from a full length human VDR cDNA²² subcloned into pBSKS⁺ (Stratagene, Cambridge, UK) and linearised with Hind III and from full length human RXRα²³ and mouse RXRβ and γ cDNAs²⁴ cloned into pBSSK⁺ (Stratagene) and linearised with Xba I, Xba I or Xho I, respectively.

Results

Specimens were analysed from 22 patients undergoing elective colorectal resections for malignancy, two of whom had synchronous adenomatous polyps (Table I), and all of whom had normal renal function, serum alkaline phosphatase (bone isoenzyme), and corrected calcium levels. Colonic mucosa from two patients undergoing a total colectomy for intractable constipation was also analysed to assess gene expression throughout non-neoplastic colon.

Colonic tissue from each site (normal adjacent mucosa, normal distant mucosa, benign adenomatous polyp, and cancer) in all the patients studied expressed a single 4.6 kB mRNA encoding VDR and a single 5.5 kB mRNA encoding RXRα (Fig 1). No RXRβ mRNAs were expressed in any tissue samples examined from any patient (data not shown). 7 kB and 3.5 kB RXRγ specific mRNA transcripts, expressed at very low levels in all tissue samples, were detected only after three weeks' autoradiography. VDR, RXRα, and RXRγ mRNA transcripts, as described above, were present in non-neoplastic colon

excised from the patients who underwent total colectomy for constipation. There was no detectable expression of RXRβ mRNA, but expression of VDR, RXRα, and RXRγ mRNAs was seen throughout the non-neoplastic large bowel in all of the samples tested from these two patients (Fig 2). There was no obvious regional variation in receptor mRNA levels along the colon of these two patients but we cannot conclude that this is generally the case as the numbers were so limited.

VDR mRNA levels were reduced by more than 50% in 12 cancers but were approximately equal in a further 10 malignancies when compared with the paired normal adjacent mucosa. VDR mRNA levels were unchanged in two adenomatous polyps when compared with normal adjacent mucosa. RXRα mRNA levels were reduced in nine cancers but unchanged in 13 others when compared with paired normal mucosa. RXRα mRNA levels were no different in both adenomatous polyps relative to paired normal mucosa. There was no clear correlation between levels of VDR and RXRα expression; the nine cancers with low RXRα mRNA levels did not comprise a subset of the 12 cancers in which VDR mRNA was reduced. Levels of RXRγ mRNA were low in all specimens examined, precluding accurate

Figure 1: Northern blot analyses of vitamin D₃ receptor (VDR), retinoid X receptor (RXR)α, RXRγ, and β-actin mRNAs from normal and neoplastic colorectal tissue from three patients; 1=caecal carcinoma, 2=splenic flexure carcinoma, 3=rectal carcinoma with synchronous benign, adenomatous rectal polyp. Transcripts encoding VDR (4.6 kB), RXRα (5.5 kB), and RXRγ (3.5 kB and 7 kB) are shown in each tissue; N₁=normal mucosa 5–10 cm distant from the tumour, N₂=normal mucosa directly adjacent to the tumour, C=neoplasm, P=adenomatous polyp. The RXRγ cRNA probe cross hybridised to the 5.5 kB RXRα mRNA (▶). Sizes of RNA standard markers are indicated on the right of the autoradiograph. The filter was exposed to x ray film with intensifying screens at -70°C for the following times: β-actin=six hours; VDR=48 hours; RXRα=six days; RXRγ=three weeks.

Figure 2: Northern blot analyses of vitamin D₃ receptor (VDR), retinoid X receptor (RXR) α , RXR γ and β -actin mRNAs from non-neoplastic large bowel in two patients with intractable constipation. Lanes 1–4 are from a 48 year old man and lanes 5–9 are from a 33 year old woman. Lanes 5, 6 and 7–9 are non-contiguous lanes from the same gel. Sample numbers represent region of sample; 5=caecum, 1 and 6=ascending colon, 2 and 7=transverse colon, 3 and 8=sigmoid colon, 4 and 9=rectum. mRNA transcripts are of identical size to, and exposure times the same as, those described in Figure 1.

quantification, but no clear difference between tumours and normal mucosa was observed. There was no clear relationship between levels of VDR, RXR α , and RXR γ mRNA expression and site within the large bowel, histological grading, or Dukes's staging of each tumour. Group sizes were too small to allow valid statistical analysis of these data which are presented in full in Table II.

TABLE II Summary of results

	Site of tumour in colon			Histological grade of differentiation			Dukes's staging		
	Right	Left	Rectum	Well	Moderate	Poor	A	B	C
Total	7	8	7	7	13	2	4	7	11
VDR>50%	3	5	2	5	4	1	2	3	5
VDR<50%	4	3	5	2	9	1	2	4	6
RXR>50%	4	6	3	6	6	1	3	3	6
RXR<50%	3	2	4	1	7	1	1	4	5
{ VDR>50% RXR>50% }	3	5	1	4	4	1	1	3	5
{ VDR>50% RXR<50% }	0	0	1	1	0	0	1	0	0
{ VDR<50% RXR>50% }	1	1	2	2	2	0	2	1	1
{ VDR<50% RXR<50% }	3	2	3	0	7	1	0	3	5

<50%=the relative mRNA expression in the tumour is less than 50% of that in the paired normal mucosa.
>50%=the relative mRNA expression in the tumour is more than 50% of that in the paired normal mucosa.
VDR=vitamin D₃ receptor; RXR=retinoid X receptor.

Discussion

We have shown the expression of VDR, RXR α , and RXR γ mRNAs and the absence of RXR β mRNA expression in all normal, adenomatous, and malignant human colonic mucosa specimens examined (Figs 1 and 2). Both benign tumours expressed similar levels of identical VDR and RXR specific mRNAs similar to those in paired normal mucosa whereas VDR mRNA was reduced in 12 malignant tumours and RXR α mRNA was lower in nine. Functional VDR signalling in the normal large bowel is indicated by studies which show antiproliferative actions of D₃ and its analogue MC 903 in human rectal mucosa,²⁵ and VDR expression has been shown previously by immunocytochemistry in normal human colonic mucosa,²⁶ but there are no data concerning neoplastic tissue. One small study has recently suggested a reduction of VDR mRNA expression in colorectal tumours¹³ and others have shown VDR protein by relatively insensitive radioligand binding assay in only 32% of tumours.²⁷ It has been suggested, therefore, that the level of VDR expression may be a useful marker to predict clinical outcome. In contrast, we have shown that VDR mRNA is expressed in normal, premalignant, and malignant mucosa and that no consistent changes in receptor expression could be detected in relation to the degree of tumour cell differentiation or the Dukes's staging. Our data, therefore, question the usefulness of changes in VDR expression as predictors of clinical outcome but provide a rational basis to explore the potential for therapeutic intervention with D₃ and its derivatives at all stages of colorectal tumourigenesis. Despite the pivotal role of RXRs in D₃ action, the role of retinoids, in particular of 9-*cis* retinoic acid, in the development of large bowel cancer has not been established. There are no epidemiological data concerning vitamin A status and colorectal tumours, although a protective role has been suggested for vitamin A against oesophageal cancers.²⁸ The expression of RXR α and γ mRNAs in human large bowel has not been reported before. A 5.5 kB RXR α transcript has been shown previously in human skin²⁴ but has not been studied in other human tissues. RXR γ mRNA expression has not been reported before in man, but several RXR γ mRNAs have been detected in a variety of murine and rat tissues.^{24 29} The presence of full length VDR and RXR mRNAs in normal and malignant colonic tissue, indicates that no mRNA splicing variations, receptor gene deletions, or major rearrangements are likely to be implicated in colonic tumourigenesis. The presence of normal sized mRNA transcripts does not, however, exclude abnormalities of VDR and RXR protein translation as factors in colorectal tumourigenesis. Factors which may alter receptor translation and function include changes in mRNA stability, point mutations in the VDR gene, or abnormal post-translational processing of mature proteins. Finally, other cofactors that interact with D₃ signalling could contribute to colorectal carcinogenesis.

Radioligand binding assay data indicating that only a third of colorectal tumours are VDR positive might suggest that receptor protein function is compromised in large bowel cancer. However, Scatchard analysis is relatively insensitive and recent evidence indicates that some breast cancers which are oestrogen receptor negative by Scatchard analysis do indeed possess functional oestrogen receptor when analysed by more sensitive techniques.³⁰ In addition, VDR protein has been shown to have a relatively short half life of between two and six hours (depending on the absence or presence of D₃)³¹ which could reduce the sensitivity of the radioligand binding assay in human tissues.

Functional heterodimerisation between VDR and RXR may play a central role in controlling the specificity of D₃ signalling in a variety of tissues,^{16-19 32 33} which could include the colon. The recent in vitro demonstration of two distinct D₃ signalling pathways, dependent on VDR homodimers or VDR/RXR heterodimers,²⁰ indicates that physiological specificity of D₃ signalling requires coexpression of VDR and RXR in the same cell. We report the full functional repertoire of receptors required for D₃ signalling in both normal and malignant colorectum. If these receptors are functional, they represent a means whereby disease occurrence could be reduced or established disease behaviour might be modified.

The therapeutic potential of D₃ as a systemic antiproliferative agent has been limited by its toxic calcaemic effects but synthetic D₃ analogues, with low calcaemic activity relative to differentiation potency, have been synthesised and possess similar or more potent antiproliferative and differentiating properties to D₃ in vitro but have equal binding affinity for VDR.^{1 34 35} Our studies suggest that these compounds may be rational antiproliferative treatments for colorectal cancer and warrant further detailed study.

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