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EVIDENCE OF 1,25-DIHYDROXYVITAMIN D_3-RECEPTORS IN HUMAN DIGESTIVE MUCOSA AND CARCINOMA TISSUE BIOPSIES TAKEN AT DIFFERENT LEVELS OF THE DIGESTIVE TRACT, IN 152 PATIENTS

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Summary—Epidemiological and experimental data suggest that dietary calcium and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are protective against colorectal cancers, while their activity on colon mucosa still remains unknown.

Since the presence of receptors is required for steroid action, specific 1,25-$(OH)_2D_3$ receptors were investigated in biopsies taken at different levels of the digestive tract from the oesophagus to the rectum and in pancreas. The total study involved biopsies from 152 patients. In 82% of the cases they were paired biopsies in adenocarcinoma tissue and in adjacent normal mucosa (NM). There were 120 operated on for colorectal adenocarcinoma (HCRA).

1,25-$(OH)_2D_3$ receptor was assayed in tissue extract by the dextran-coated charcoal (DCC) technique and also characterised by sucrose density gradient centrifugation.

Scatchard analyses showed a single class of specific high affinity–low capacity sites binding for 1,25-$(OH)_2D_3$ with a $K_d = 1.48 \pm 0.8 \cdot 10^{-10}$ M ($n = 119$). The sedimentation coefficient of the steroid receptor complex was approximately 3.2 S.

The incidence of 1,25-$(OH)_2D_3$ receptors was significantly higher in NM (82.5%) than in HCRA (34.5%). In HCRA this incidence decreased from right colon (64.7%) to left colon (27.7%) and rectum (15%). All positive HCRA in left colon and rectum (16/76) were histologically well differentiated.

The receptor content in NM and HCRA was in the same range: (median) 10–314 (58) and 13–175 (64) fmol/mg protein.

These data suggest that 1,25-$(OH)_2D_3$ may modulate calcium transport in colon, as in the intestine. Also, loss of receptivity to 1,25-$(OH)_2D_3$ is observed as associated with malignant transformation of the human colorectal mucosa.

INTRODUCTION

It is now well established that the active hormonal metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) acts via a mechanism similar to that of the other steroid hormones. In a target tissue, 1,25-$(OH)_2D_3$ binds to a specific nuclear cell receptor and forms a hormone–receptor complex [1, 2]. The complex binds to DNA specific sequences [3] and induces an increase in the activity of certain genes [4].

1,25-$(OH)_2D_3$ receptors were first detected in intestine [5–7], bone [1], kidney [8] and other organs from chicken and rat [9–13]. Such receptors have also been reported in malignant cells not only of digestive origin [14, 15]. The presence of these receptors in tissues which are not known as traditional targets for 1,25-$(OH)_2D_3$ suggests that the role of this hormone is more general than its effect on calcium homeostasis. 1,25-$(OH)_2D_3$ seems to be a modulator of growth and differentiation of various kinds of neoplastic cells [12–17]. Thus, the differentiation stages have been related to the receptor level [18] in the human cancer cell line HT29.

Cancer of the large bowel is clinically the second most widespread in developed countries, in terms of both incidence and mortality [19]. Epidemiologic evidence accumulated over the past few years suggests that dietary calcium and 1,25-$(OH)_2D_3$, from diet and solar exposure, are involved in protection against colorectal cancers [20–22].

We previously demonstrated, for the first time, the existence of 1,25-$(OH)_2D_3$ receptors in the human colon cancer cell line LOVO [23], and reported an inhibition of the growth associated with a more differentiated phenotype after five days of treatment.

In the present work, the status of 1,25-$(OH)_2D_3$ receptors in human colorectal tumors and in the corresponding normal mucosa have been investigated.

Table 1. Incidence of 1,25-dihydroxyvitamin D_3 receptor detectability in the digestive tract and in tumor biopsies of the same origin

	Vitamin D_3 receptors incidences			Fmol/mg protein Range (median)		
	Mucosa	Tumor	X^2P	Mucosa	Tumor	Mann–Whitney tests
Oesophagus	4/5	1/5	>0.05	73–188 (120)	91	—
Stomach	6/10	2/10	>0.05	15–54 (46)	31–34 (33)	—
Duodenum and ileum	6/6	0/2	<0.01	14–75 (59)	—	—
Right colon	29/35	22/34	>0.05	17–314 (62)	13–126 (62)	>0.05
Left colon	36/47	10/36	<0.001	10–292 (56)	40–175 (75)	>0.05
Rectum	34/38	6/40	>0.001	12–188 (58)	40–86 (64)	>0.05
Pancreas	9/11	0/9	<0.001	27–93 (54)	—	—

The biopsies were simultaneously taken in normal and tumor tissue in the same patients. The content in receptors is expressed as the range of the values and the median ( ) calculated in each class of tissue and analysed statistically by the Mann–Whitney tests. The incidences were tested by χ^2 tests.

EXPERIMENTAL

Chemicals

1,25-$(OH)_2$ [26,27-methyl^{3}H]cholecalciferol (176 Ci/mmol), with a radio purity of 98.4%, was obtained from Amersham, France. Inert 1,25-$(OH)_2D_3$ was a generous gift from F. Hoffmann, La Roche and Co. Ltd, Basel, Switzerland. It was prepared as pure ethanol solution (10^{-3} M) and stored under nitrogen in amber vials at −20°C.

Aprotinin and bovine serum albumin were from Sigma, France. All other reagents were purchased from Merck, Germany.

Biological specimens

Samples of digestive carcinoma and corresponding normal mucosa (15 cm) were obtained from surgical specimens taken in patients who had not undergone preoperative chemotherapy. The distribution of biopsy sites in the digestive tract was from oesophagus to rectum as indicated in Table 1, and involved, in addition, 9 pancreas samples. Colon biopsies from 120 patients (73 males and 47 females) were investigated. In 108 cases paired specimens were obtained from normal mucosa and carcinoma in the same patients. In addition, biopsies were obtained from non-neoplastic diseases.

Fresh samples were rinsed in isotonic buffer and immediately placed in liquid nitrogen until processing.

Cytosol preparation and receptor binding

The frozen tissues were pulverized into fine powder with a cryogrinder (Cryorivoire, Montpellier, France), and the cytosol was prepared by suspending the tissue powder with buffer: PEMDK (25 mM KH_2PO_4, 1.5 mM EDTA, 10 mM Na_2MoO_4, 1 mM DTT, 300 mM KCl and 20 μl of the protease inhibitor Aprotinin (0.6, TIU/ml homogenate)). Homogenization was done with 4–6 strokes of 5 s in a polytron PT-10 (Kinimatica, GMBM., Switzerland), and the mixture was maintained at 4°C in ice with frequent agitation for nuclear receptor extraction. Cytosols were then prepared by ultracentrifugation at 105,000 *g* at 4°C for 60 min. Clear supernatants were removed from beneath the fatty layer. Cytosol protein concentration was determined by the method of Lowry[24].

The cytosol was diluted to 0.1 M KCl with PEMD (PEMDK without KCl), before binding incubations.

1,25-$(OH)_2D_3$ receptors were determined [23] by incubating cytosol aliquots (80 μl) with [^{3}H]1,25-$(OH)_2D_3$ at increasing concentrations from 5×10^{-11} to 10^{-8} M. Non-specific binding was assessed by duplicate samples with a 100-fold excess of inert ligand. The samples were incubated at 4°C for 16 h. Unbound 1,25-$(OH)_2D_3$ was removed by dextran-coated charcoal (DCC) in PEMD (0.1% w/v) treatment at 4°C for 10 min under constant stirring. The DCC was pelleted by centrifugation at 2500 *g* for 10 min. The radioactivity in the supernatant was then counted in a scintillation counter. Receptors are expressed as bound 1,25-$(OH)_2D_3$ fmol/mg cytosol protein and were statistically analysed. The significance of the results (paired or unpaired) was assessed by the Wilcoxon analysis for nonparametric data.

Sucrose density gradient sedimentation

Cytosol aliquots were incubated with [^{3}H]1,25-$(OH)_2D_3$ at 10^{-9} M, with or without 100-fold excess of unlabeled ligand. Incubations and DCC treatment were as in binding assay. Aliquots were layered on the top of a linear 5–20% (w/v) sucrose density gradient prepared in PEMD containing 0.1 M KCl and sedimented at 300,000 *g* in a vertical rotor (Sorvall) for 3 h. BSA was used as density marker. Five drop fractions were collected from the bottom of the tube and their radioactive content determined.

RESULTS

Scatchard analysis of binding data obtained in the biopsies indicates the existence of a single class of high affinity binding sites with a K_d of $1.48 \pm 0.8 \times 10^{-10}$ M. The K_d mean values observed in the normal tissues and in tumor samples from other sites were $1.34 \pm 0.8 \times 10^{-10}$ and $1.62 \pm 0.7 \times 10^{-10}$ M respectively, (Fig. 1A, B).

The sedimentation coefficient of the 1,25-$(OH)_2D_3$–receptor complex prepared in hypertonic buffer (PEMD, 0.3 M KCl) was approximately 3.3 S. The radioactivity was associated with a single peak

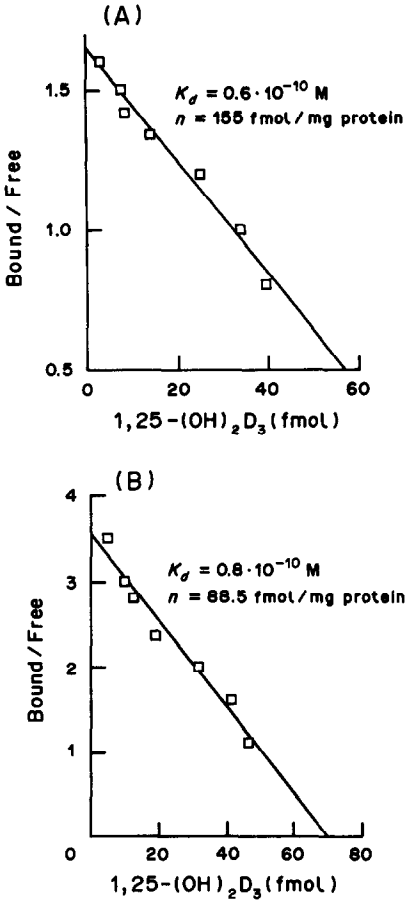

Fig. 1. Scatchard analysis of specific [^{3}H]1,25-$(OH)_2D_3$ binding to cytosols from normal colon mucosa (A) and the corresponding tumor (B). Cytosols were incubated as indicated in methods. Each point represents the mean of three determinations.

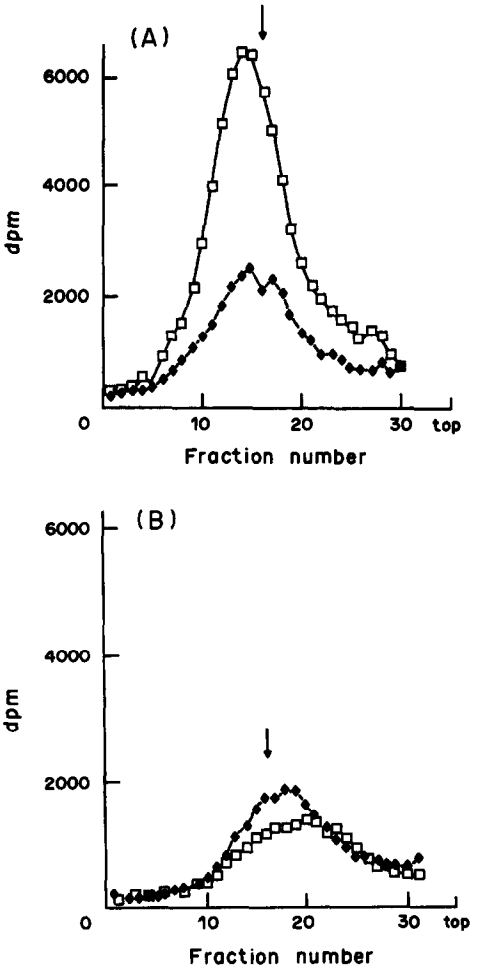

Fig. 2. Sucrose density gradient sedimentation (5–20%) of [^{3}H]1,25-$(OH)_2D_3$ binding to cytosols from normal colon mucosa (A) and the corresponding tumor (B). 0.2 ml aliquots of high salt cytosol were incubated with 1 nM [^{3}H]1,25-$(OH)_2D_3$ for 16 h at 4°C in the absence (□) or in the presence of 100-fold excess of unlabeled 1,25-$(OH)_2D_3$ (◆), and then layered on the top of 5–20% sucrose density gradient and sedimented at 300,000 *g*, 4 h at 4°C in a vertical rotor (Dupont). Arrow: sedimentation of BSA.

(Fig. 2A), which was totally quenched in samples incubated in the presence of an excess of inert ligand. No radioactive peak was observed in tumor samples which did not display specific binding by Scatchard analysis (Fig. 2B). Findings were similar when using samples from different localization.

Evidence of specific 1,25-$(OH)_2D_3$ binding was found in the 6 samples of small bowel mucosa which were tested.

1,25-$(OH)_2D_3$-receptors were not detected in all the biopsies of the upper tract (oesophagus, stomach) (Table 1), while they were present in the complete series of normal or non-neoplastic diseases of the colon (Table 1). The mean incidence in HCRA was lower (34.5%) and decreased from the right colon (64.7%) to the left (27.7%) colon and rectum (15%). All the positive HCRA in these two former localizations (16/76) were histologically well differentiated (data not shown).

The number of receptors was very similar in the positive biopsies, whatever their nature (malignant or not) and their localization (Table 1, median values). The median values in NM and HCRA were 58 and 64 fmol/mg protein and the corresponding ranges were 10–314 and 13–175 respectively.

We also detected 1,25-$(OH)_2D_3$-receptors in the upper digestive tract (oesophagus and stomach) as in the colon (see Table 1).

There was no correlation with age and sex. Assays in different parts of the same tumor revealed no heterogeneity of the receptor distribution. No correlation was found with the degree of local extension (Dukes stage).

DISCUSSION

Until recently, most of the studies which have led to our current knowledge of the mechanism of action of 1,25-$(OH)_2D_3$ were carried out using animal model systems. Only in the last few years have the technical

difficulties associated with the study of mammalian 1,25-$(OH)_2D_3$-receptors been substantially overcome. It has been shown that the active metabolite of vitamin D3 (1,25-$(OH)_2D_3$) is involved in growth and differentiation of many cancer cells [15, 25]. The 1,25-$(OH)_2D_3$ receptor is found throughout a wide range of species suggesting that it is a highly conserved molecule [28] and a high degree of homology exists between receptor species.

The role of human colon in calcium absorption has been reported as clinically significant and related to 1,25-$(OH)_2D_3$ activity [26, 27], but no demonstration of the biochemical mechanisms by which these effects are mediated was given.

The present study provides a complementary set of data indicating that 1,25-$(OH)_2D_3$ is involved in the physiology of the colon. The results show that the normal colorectal epithelium contains a high affinity 1,25-$(OH)_2D_3$-binding protein, specially the right colon. In contrast, only a few tumors are positive. The high affinity binding protein appears to be similar to the 1,25-$(OH)_2D_3$-receptor present in classical vitamin D target tissues [6–9] and in some tumor cell lines [14–16], with respect to their affinity for 1,25-$(OH)_2D_3$ (Fig. 1) and sedimentation coefficient (Fig. 2). These similarities lead us to assume that human colorectal cells possess 1,25-$(OH)_2D_3$ receptors, which are similar in normal and malignant cells. No contamination with the 6S macromolecule which preferentially binds 25-$(OH)_2D_3$ was observed. The value of the K_d (10^{-10} M) is the most appropriate for physiological active receptor. This is the first report of 1,25-$(OH)_2D_3$ receptors in biopsies from different sites of the digestive tract, and also in the pancreatic annex gland.

While in HCRA 1,25-$(OH)_2D_3$ receptors were found to be present only in well differentiated biopsies, other authors [18] reported a loss of 1,25-$(OH)_2D_3$ receptors after differentiation of human colon carcinoma cell line HT29. Moreover, the high frequency of 1,25-$(OH)_2D_3$ receptors in colon carcinoma cell lines [14, 23] is discordant with the low frequency in primary colon adenocarcinoma. This suggests a special selection of 1,25-$(OH)_2D_3$ receptor positive cells among various colon carcinomata which have been subjected to culturing attempts. In addition the modulation of 1,25-$(OH)_2D_3$ binding capacity might be dependent on the stage of differentiation [18] and probably limited to specific functional stages in the physiology of the normal and malignant cells.

It is not possible at this time to correlate the relative amount of 1,25-$(OH)_2D_3$ receptors present in human colorectal epithelium with calcium absorption. However, the colon mucosa may play a significant role in calcium absorption [26], and 1,25-$(OH)_2D_3$ seems to increase this ion transport [27]. From this point of view, 1,25-$(OH)_2D_3$ could play a role in cell proliferation. Our findings provide strong support for this hypothesis. Whether the active metabolite of vitamin D or analogues may influence colon carcinoma cells *in vivo*, and the prognostic implication for patients, remains to be seen. However, this demonstration of the presence of 1,25-$(OH)_2D_3$ receptors in some HCRA should encourage further investigations on the effect of 1,25-$(OH)_2D_3$ on colorectal carcinogenesis to be undertaken.

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