Sex Steroid and 1,25-Dihydroxyvitamin D₃ Receptors in Human Colorectal Adenocarcinoma and Normal Mucosa

Farid Meggouh, Patrice Lointier, and Simone Saez

ABSTRACT

In order to determine the potential role of sex steroid and 1,25-dihydroxyvitamin D₃ in the spreading of colorectal cancer, we previously hypothesized from epidemiological and experimental data, specific androgen (AR, n = 94), estrogen (ER, n = 60), progesterone (PGR, n = 50), and 1,25-dihydroxyvitamin D₃ receptors (VDR, n = 111) were investigated in human colorectal adenocarcinoma (AC) and compared with the normal adjacent mucosa (NM).

Scatchard analysis and competition studies of binding data did not reveal any difference between the biochemical behavior (affinity, specificity, and sedimentation coefficient) of the normal and tumoral tissue receptors. For ARs and ERs, high incidences were found (92 of 94 and 90 of 94 in NM versus 46 of 60 and 40 of 60 in AC, respectively) in both classes of tissues, while they were low for progesterone (7 of 50 and 5 of 50 in NM versus AC). While sex steroid receptors the incidences did not vary with sex and age of the patients or the location and histopathological grade of the tumor, the VDR incidence was lower in AC (35 of 111) than in NM (99 of 111) and decreased significantly from the right colon to the rectum in adenocarcinoma. Binding capacities were similar in NM and AC for ERs and VDRs, whereas AR levels in NM were significantly higher than in AC.

The expression of VDRs in some colorectal tumors suggests a possible clinical significance. No known function for sex steroid receptors is related to their presence in human colorectal tissues and their pattern in carcinoma does not support any hypothesis previously raised in the case of chemically induced colonic tumors in rodents.

INTRODUCTION

During the last few years there has been considerable interest in comparing epidemiological data concerning colorectal and breast cancer in humans. For both tumors, it has been suggested that dietary fat represents a potential associated risk. A positive correlation has been observed between the incidence of colon and breast cancers (1, 2). The incidence of large bowel cancer as a secondary neoplasm in women with previous breast or gynecological cancer is not rare (3). The protective effect of early age at the first pregnancy has been noted for both cancers (4, 5).

The digestive tract cannot be considered as a typical target for sex steroid hormones such as breast cancer (6), but several lines of evidence indicate a potential role for hormonal and reproductive factors in the risk of colorectal cancer (5, 7). Data are conflicting with regard to the role of sex steroids in experimental carcinogenesis (8).

However, the observation that breast glandular epithelium in women and the digestive mucosa are both targets for 1,25-

(R4)D3, 3 which can be considered as an authentic hormone, merits more attention. Indeed, a number of experiments on colorectal carcinogenesis and epidemiological observations are in favor of a protective role of 1,25-(OH)2D₃, which could be direct or indirect (10–12).

Since any hormone activity depends on the presence of the corresponding receptors, we have investigated the sex steroid and 1,25-(OH)2D₃ receptor status in the digestive tract of both normal mucosa and tumor biopsy specimens from human subjects. The existence of 1,25-(OH)2D₃ receptors in normal colonic mucosa had not been previously demonstrated and our aim was to investigate whether modifications of the status of this receptor was altered in tumor samples.

MATERIALS AND METHODS

Tissue and Collection. Samples of digestive carcinoma and corresponding normal mucosa (15 cm) were obtained from surgical specimens. None of the patients in this study had received chemo- or radiotherapy prior to surgical treatment. Colon biopsies from 111 patients (69 males and 42 females) were investigated. In 108 cases, paired specimens were obtained from normal mucosa and carcinoma in the same patients. The distribution of biopsy sites in the upper digestive tract was from esophagus to rectum as indicated in Table 4. In addition, 11 pancreas samples were included and 17 normal large bowel mucosa biopsy specimens were obtained from patients without neoplasms. Tumors were histologically confirmed as primary adenocarcinomas.

Each tissue was washed with cold phosphate-buffered saline to remove blood cells. A glass slide was used, and the normal mucosa was removed by scraping the adjacent nonepithelial tissue which was then stored in liquid nitrogen (10–15 min, after resection) until processed. Multiple biopsy specimens were taken from different areas of the tumor in the operating room immediately following resection. Pathology reports were used to determine how far the lesion had penetrated the bowel wall and the presence or absence of lymph node metastases. Each lesion was staged by the Dukes' classification for carcinoma of the colon and rectum (13).

Steroids. The tritiated R2058 (1-ethyl,21-hydroxy,19nor-[6,7 n 3H]pregna-4-ene,3,20-dione; specific activity, 50.6 Ci/mmol) and DHT (specific activity, 51.6 Ci/mmol) were purchased from Amersham, France. Tritiated estradiol (2,4,6,7 n 3H) estradiol; specific activity, 100 Ci/mmol) was obtained from C.E.A., Gif sur Yvette, France.

Diethylstilbestrol, 5α-dihydrotestosterone, progesterone, dexamethasone, testosterone, and mibolerone (R1881) were obtained from Roussel (Romainville, France). Cyproterone acetate was purchased from Shering (France) and F2058 was purchased from Amersham.

1,25-(OH)2D3 [26,27-methyl-3H]hcholcalciferol (176 Ci/mmol), with a radiopurity of 98.4%, was obtained from Amersham. Inert 1,25-

(OM)2D3 was a generous gift from F. Hoffmann La Roche and Co., Ltd., Basel, Switzerland. It was prepared as a pure ethanol solution (1 mM) and stored under nitrogen in amber vials at −20°C.

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2 To whom requests for reprints should be addressed, at Laboratoire de Biologie Médicale, Centre Léon Bérard, Rue Laennec, 69373 Lyon, France.
All radiolabeled steroids were purified by paper chromatography, eluted with absolute ethanol, and stored under nitrogen in amber vials at -20°C until used.

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

Cytosol Preparation. The frozen tissues were pulverized into fine powder with a cryogrinder (Cryoviroire, Montpellier, France), and the cytosol was prepared by suspending the tissue powder in PEMD buffer and 300 mM KCl. Homogenization was done by 4-6 strokes for 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 et al. (14). The cytosol was diluted to 0.1 M KCl with PEMD before binding incubations.

Receptor Measurements. The supernatant (cytosol) was used immediately or stored at -80°C until assayed for receptor content.

The presence of specific ERs, PGRs, and ARs was determined by the dextran-coated charcoal assay, according to the recommendations of the EORTC (15, 16). For routine measurements, steroids were used at a final concentration of 10 nM and were displaced by 10-, 50-, 100-, and 200-fold excess of unlabeled sex steroids.

In order to assess the affinity of steroid receptors for their ligands, the Scatchard method was used. Equilibrium binding analysis was performed by measuring specific binding at concentrations of tritiated DHT or estradiol ranging from 0.025-5 nM. Nonspecific binding was calculated in a duplicate set of samples incubated with a 100-fold excess of unlabeled DHT or estradiol.

Finally, the specificity of ARs and ERs for their respective ligands DHT and estradiol was investigated by competition with several ligands for both receptors. The tritiated DHT and estradiol were used at a final concentration of 5 nM and were displaced by 10-, 20-, 50-, 100-, 200-, and 500-fold excess unlabeled steroids. For ARs, the following ligands were tested: DHT, testosterone, cyproterone acetate, mibolerone, estradiol, progesterone, and dexamethasone. Unlabeled estradiol, diethylstilbestrol, DHT, progesterone, and dexamethasone were used to compete with [3H]estradiol.

1.25-(OH)2D3 receptors were determined (17) by incubating cytosol aliquots (80 μl) with [3H]1,25-(OH)2D3 at increasing concentrations from 0.05-5 nM. Nonspecific binding was assessed by duplicate samples with a 1000-fold excess of inert ligand.

The final concentration of ethanol was <0.1%. All incubations were carried out at 0-4°C for 16-18 h. Unbound steroids were removed by DCC in PEMD (0.5% w/v) 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. Specific binding was calculated by subtracting nonspecific binding (in the presence of a large excess of unlabeled ligand) from total binding (in the absence of inert ligand). Receptors are expressed as bound steroid fmol/mg cytosol protein and they were statistically analyzed by the Wilcoxon analysis for nonparametric data.

Sucrose Density Gradient Sedimentation. In order to characterize ARs and VDRs, by assessing their sedimentation coefficient, cytosol aliquots were incubated with [3H]1,25-(OH)2D3, or [3H]DHT at 1 nM, with or without a 100-fold excess of unlabeled ligands. Incubations and DCC treatment were the same as in the binding assay.

Labeled cytosol aliquots (200 μl) were layered on the top of a linear 5-20% (w/v) sucrose density gradient prepared in PEMD containing 0.1 M KCl for VDRs and 0.3 M KCl for ARs and sedimented at 300,000 × g in a vertical rotor (Sorvall) for 3 h. Bottom serum albumin was used as a density marker. Five-drop fractions were collected from the bottom of the tube and their radioactive content was determined.

RESULTS

The four types of steroid receptor (VDR, AR, ER, and PGR) measurements were performed in relation to the available volume of each pair of samples. ARs were determined in 94, ERs in 60, and PGRs in 50 biopsy pairs. VDRs were determined in 111 colonic biopsy, 5 esophageal, 10 gastric, 6 small bowel, and 11 pancreatic samples.

The threshold of receptor positivity was 10 fmol/mg protein. The clinical characteristics of the patients studied are shown in Table 1.

Sex Steroid Receptors. Most frequently ARs and ERs could be detected. The incidences were 96 and 98% for ARs and 67 and 77 for ERs, respectively, in carcinoma versus the corresponding adjacent normal mucosa. The distribution of the two receptor species did not differ along the three colorectal localizations (Table 2).

The incidences of ARs and ERs were not influenced by age, sex, and site of biopsy. Also, the histological grade of tumors and their local extension, according to the Dukes' classification, did not seem to have an affect on these incidences. The number of PGR-positive biopsy specimens was small (5 of 50 in tumors and 7 of 50 in normal mucosa) and for this reason not further analyzed.

Scatchard analysis of binding data obtained for ARs and ERs indicated the existence of one single class of high affinity-binding sites. The Kd values for AR and ER binding were 1.7 ± 0.6 (SD) and 0.7 ± 0.3 nm, respectively, as calculated from 10 samples for each. No difference was found between the Kd of the receptors in tumors and that of normal tissues for both ARs and ERs. We also assessed the specificity of ARs and ERs by competition analysis with several steroids (Figs. 1 and 2). The ARs and ERs measured in this study were highly specific for their respective ligands (DHT and estradiol), and no difference was observed between tumors and normal mucosa. ARs were also characterized by sucrose density gradient sedimentation in hypertonie buffer (0.3 M KCl), which allows extraction of nuclear bound receptors (Fig. 3). The sedimentation coefficient was about 4.5 S, similar to that of the classical ARs in prostatic tissue. This analysis was not done for ERs because of their low concentration in the studied samples. Generally the binding of...
Fig. 1. Ligand specificity of androgen-binding sites. The specificity was studied by incubating 80 μl of cytosol of colon mucosa (A) and adenocarcinomas (B) taken from the same patient with radiolabeled DHT (5 nM) for 16 h at 4°C, without or with 10-, 20-, 50-, 100-, 200-, and 500-fold molar excess of unlabeled ligand. 0, 5α-dihydrotestosterone; □, testosterone; O, cyproterone acetate; ●, mibolerone; ▲, estradiol; ▼, diethylstilbestrol; ▼, progesterone. Treatment is described in “Materials and Methods.” Each value was determined in triplicate. Points, mean values. For clarity, SDs were omitted since they were <3%.

Fig. 2. Ligand specificity of estrogen-binding sites. The specificity was studied by incubating 80 μl of cytosol of colon mucosa (A) and adenocarcinomas (B) taken from the same patient with radiolabeled estradiol (5 nM) for 16 h at 4°C, without or with 10-, 20-, 50-, 100-, 200-, and 500-fold molar excess of unlabeled estradiol. *, estradiol; □, diethylstilbestrol; ▼, progesterone; O, dexamethasone; ▲, 5α-dihydrotestosterone. Treatment of labeled cytosols was as described in “Materials and Methods.” Each value was determined in triplicate. Points, mean values. For clarity, SDs were omitted since they were <3%.

capacity was higher for DHT than for estradiol, while it was very low for progesterone. The binding capacities of ARs and ERs in right colon, left colon, and rectum are shown in Table 3. Receptors for progesterone were omitted since the incidences of PGR-positive biopsy specimens of carcinoma and normal mucosa were very small (10 and 14%, respectively). The range of PGR content was 3–22 fmol/mg of cytosol protein. For ERs, no significant difference was found between the receptor content of the normal and the tumoral tissue (P > 0.05), whatever the localization. The AR level was significantly higher in normal mucosa than in carcinoma (P < 0.001) as assessed by the Wilcoxon test for paired data.

1,25-(OH)2D3 Receptors. Scatchard analysis of binding data obtained in the biopsies indicates the existence of a single class of high affinity-binding sites with a Kd of 0.15 ± 0.08 nM (n = 222). The Kd mean ± SD values observed in the normal tissues and in tumor samples from other sites were 0.13 ± 0.05 (n = 25) and 0.16 ± 0.07 nM (n = 25), respectively.

The sedimentation coefficient of the 1,25-(OH)2D3 receptor complex prepared in hypertonic buffer (PEMD and 0.3 M KCl) was approximately 3.3 S. The radioactivity was associated with a single peak whatever the nature of the tissue (malignant or not; Fig. 4), which was totally quenched in samples incubated in the presence of a 200-fold excess of inert ligand. No radioactive peak was observed in tumor samples which did not display specific binding by Scatchard analysis (data not shown). Findings were similar when using samples from different localizations along the digestive mucosa.

Evidence of specific 1,25-(OH)2D3 binding was found in the upper digestive tract (esophagus and stomach) in the 6 samples of small bowel mucosa tested, as well as in the colon (Table 4). The VDRs were expressed also in normal mucosa taken from patients undergoing surgery for nonneoplastic diseases (Table 5). However, they were not present at the same rate along the digestive tract. 1,25-(OH)2D3 receptors were not detected in all of the normal mucosa biopsy specimens of the upper tract (esophagus and stomach), while they were present in the normal
colonie specimens taken in cancerous and in non-tumor-bearing cytosols from normal colon mucosa (A) and the corresponding tumor (B). Aliquots (200 μl) of high salt cytosol (0.3 M KCl) were incubated with 1 nM [3H]5α-DHT for 16 h at 4°C in the absence (C) or presence of 200-fold excess of unlabeled DHT (D) and then layered on the top of a 5–20% sucrose density gradient and sedimented at 3000,000 x g for 4 h at 4°C in a vertical rotor (Dupont). Arrows, sedimentation of bovine serum albumin.

DISCUSSION

In this study, the status of sex steroid and vitamin D3 receptors in the digestive tract was investigated. Sex steroid receptors (AR and ER) were shown to be present in malignant tumors and adjacent normal mucosa from the colon and rectum. Estradiol- and progesterone-binding sites have already been demonstrated in these tumors (18, 19) and sometimes at high levels (20, 21). Here, we found a low concentration of estrogen receptors (between 2 and 66 fmol/mg protein) in the majority of biopsy specimens.

The number of positive progesterone receptor specimens and their mean receptor concentrations were low. The discrepancy with other previously published data could be related to the use of a very specific ligand in our experiments (19–24).

ARs were present in all the samples at a high frequency (98 and 96%, normal mucosa versus adenocarcinomas) and at a significant high level (21–279 fmol/mg protein). The age and sex of the patients, the site, and histological grade of the tumors did not appear to influence steroid receptor content. These data are in agreement with the findings of others (7, 19–24). Specificity and affinity of ARs and ERs studied by competition of different ligands and with Scatchard analysis did not display any difference in these properties between tumoral and normal tissue receptors.

The higher incidence of colon adenocarcinomas in females and of rectal cancer in males (5, 25) and the reported high frequency of this cancer in patients with a history of gynecological or breast tumors suggest that the development of the colorectal cancers may be influenced by steroid hormones. Our study seems to point to a minor role of estrogens and progestins in colorectal tumor behavior. In contrast, androgen receptors are present with a high incidence at a significant level in colorectal tumors, and they are present in all samples of normal colorectal mucosa at a higher level than in carcinomas. They are also detected in colonic mucosa from healthy patients and patients without neoplastic diseases (Table 5).

However, according to our results, the involvement of ARs in colorectal carcinogenesis remains intriguing. It is at variance with data reported in experimental colon carcinogenesis (26,

<table>
<thead>
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<th>Tumor localization</th>
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<td>Mucosa</td>
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<td>Right colon</td>
<td>59–303 (143)</td>
<td>21–285 (93)*</td>
<td>10–65 (29)</td>
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<td>Left colon</td>
<td>27–279 (131)</td>
<td>25–153 (84)*</td>
<td>13–60 (17)</td>
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<td>Rectum</td>
<td>42–442 (94)</td>
<td>27–279 (68)*</td>
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* P < 0.001.
results show a higher content of ARs in normal mucosa than in carcinomas of the human colon and this seems to be in agreement with the experimental results obtained by Izbicki et al. (32). However, the presence of steroid receptors does not systematically imply a hormonal dependence of tumors.

In rat colon mucosa, the early appearance of steroid receptors during its exposure to chemical carcinogens (28) suggested that their presence in human histologically normal adjacent mucosa might accompany or play a role in a biological transformation of the colon epithelium. Our results did not support this hypothesis since we found ARs in normal colorectal mucosa taken from patients without cancer.

We have also studied vitamin D3 receptors. The results show that the normal colorectal epithelium contains a high affinity 1,25-(OH)2D3-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)2D3 receptor present in classical vitamin D target tissues (17, 35, 36) and in some tumor cell lines (37-39) with respect to their affinity for 1,25-(OH)2D3 and their sedimentation coefficient. These similarities lead us to assume that human colorectal cells possess 1,25-(OH)2D3 receptors which are similar in normal and malignant cells. The value of the Ka (10^-10 M) is the most appropriate for physiologically active receptors. This is the first report of 1,25-(OH)2D3 receptors in biopsy specimens from different sites of the human digestive tract and also in the pancreatic annex gland.

While in adenocarcinomas 1,25-(OH)2D3 receptors were found to be present only in well-differentiated specimens, other authors (40) reported a loss of 1,25-(OH)2D3 receptors after differentiation of human colon carcinoma cell line HT29. Moreover, the high frequency of 1,25-(OH)2D3 receptors in colon carcinoma cell lines (40-42) is discordant with their low frequency in primary colon adenocarcinoma. This suggests a special selection of 1,25-(OH)2D3 receptor-positive cells among various colon carcinoma cells which have been subjected to culturing attempts. In addition, the modulation of 1,25-(OH)2D3-binding capacity might be dependent on the stage of differentiation (40, 41) and probably limited to specific functional stages in the physiology of the normal and malignant cells.

It has been shown that the active metabolite of vitamin D3
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(1,25-(OH)2D3) is involved in growth and differentiation of many cancer cells lines (12, 42, 43). In addition, 1,25-(OH)2D3 suppresses growth of human cancer solid tumor xenografts in nude mice (44) and inhibits dietary fat-promoted colon carcinogenesis in rodents (45). The 1,25-(OH)2D3 receptor is found throughout a wide range of species suggesting that it is a highly conserved molecule (46) and that a high degree of homology exists between receptor species.

While for sex steroid hormones no specific function has been identified along the human digestive mucosa, it is well known that the digestive tract is one of the main targets for 1,25-(OH)2D3. The role of the colon in calcium absorption in humans has been reported as clinically significant and related to 1,25-(OH)2D3 activity (47, 48), but no demonstration of the biochemical mechanisms by which these effects are mediated was given.

Whether the active metabolite of vitamin D or analogues may influence colon carcinoma cells in vivo, and the prognostic implications for patients, remains to be seen. However, this demonstration of the presence of 1,25-(OH)2D3 receptors in some colorectal tumors should encourage further investigations into the effect of 1,25-(OH)2D3 on colorectal carcinogenesis. The answers to the issues raised here are likely to come from an understanding of the function of sex steroid and 1,25-(OH)2D3 receptor protein products in regulating cellular growth at the molecular level in the colon and other tissues. In addition, it will be important that we extend our series of patients to determine whether expression of the VDR gene has prognostic significance in colorectal cancer.

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