Vitamin D and its metabolites inhibit cell proliferation in human rectal mucosa and a colon cancer cell line

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Abstract
Like calcium, vitamin D may protect against colorectal neoplasia as it reduces epithelial cell proliferation and induces differentiation. Although its therapeutic use is limited by its effects on calcium metabolism, analogues such as calcipotriol produce little hypercalcaemia. Stathmokinetic and immunohistochemical techniques were used to study the effect of 1,25(OH)2D3 and its analogues on cell proliferation in human rectal mucosa and a colon cancer cell line. Paired sigmoidoscopic biopsy specimens were obtained from 17 control patients and five patients with familial adenomatous polyposis. Explants were established in organ culture, with or without the addition of vitamin D. Proliferation was assessed using (1) metaphase arrest to determine the crypt cell production rate (CCPR) and (2) Ki-67 monoclonal antibody directed against an antigen present in proliferating cells. 1,25(OH)2D3 in concentrations of 1 μM–100 μM (10–10–10–6 M) reduced the CCPR (cells/crypt/hour) from 4.74 to 2.15–2.67 (p<0.001), and the Ki-67 labelling index from 7.28–3.74 (p<0.01). Likewise, vitamin D3, 10 nM (10–8 M) reduced the CCPR from 4.72–2.74 (p<0.05) and calcipotriol from 4.86–2.38 (p<0.05). In familial adenomatous polyposis patients 1,25(OH)2D3, 100 pM (10–10 M) halved the CCPR from 8.75–4.22. Calcipotriol (10–5 M to 10–9 M) produced a clearcut dose response inhibition of HT-29 cell growth. Thus, vitamin D and its metabolites inhibit proliferation in normal and premalignant rectal epithelium and suppress growth in a colorectal cancer cell line.

(Gut 1992; 33: 1660–1663)

There is a clear link between the intensity of cell proliferation and susceptibility to neoplasia. In the large intestine, surgical or dietary manipulations that stimulate cell growth generally promote experimental colorectal carcinogenesis, whereas mucosal hypoplasia (for example, by defunctioning colostomy) has a protective effect. Patients with colonic adenoma or carcinoma show increased labelling indices in 'normal' biopsy specimens taken from multiple sites, and those with ulcerative proctocolitis have increased crypt cell proliferation even in quiescent disease. Thus, the cytokinetic status of the epithelium could reflect a subject's susceptibility to colorectal neoplasia.

Calcium may protect against colorectal cancer by reducing epithelial cell turnover. Vitamin D could be another chemopreventative agent because of its ability to switch cellular activity (in various cancer cell lines) from proliferation to differentiation. In particular, the human colon cancer cell lines HT-29 and LOVO possess high affinity receptors for the active metabolite of vitamin D3 (1,25(OH)2D3), which suppresses cell growth and induces changes indicative of differentiation. Receptors for 1,25(OH)2D3 have also been found in normal human colon. Although the therapeutic use of 1,25(OH)2D3 and 1-alpha hydroxycholecalciferol is limited by their profound effects on calcium metabolism, analogues such as calcipotriol (MC-903, a secoester) are now available which have limited hypercalcaemic and hypercalcifer effects.

We have used stathmokinetic and immunohistochemical techniques to study the effect of three agents – 1,25(OH)2D3, vitamin D3, and calcipotriol – on cell proliferation in human rectal epithelium. We have studied both macroscopically normal rectal tissue in control patients and those with familial adenomatous polyposis (at increased risk of neoplasia) and HT-29 cells in culture.

Methods

RECTORAL BIOPSY SPECIMENS

Paired rectal biopsy specimens were taken from 17 patients (mean age 60 years, range 37–80 years) with macroscopically normal rectal mucosa who were attending the outpatient clinic with incidental anal conditions. Biopsy specimens were also obtained from five patients (mean age 38 years, range 35–40 years) with familial adenomatous polyposis who had previously undergone total abdominal colectomy with ileorectal anastomosis and were attending regularly for follow up. One specimen from each patient was examined histologically to exclude mucosal disease, notably microadenoma in familial adenomatous polyposis. The other biopsy specimen was maintained in organ culture with or without the addition of vitamin D metabolites. In four control patients, tissue was frozen to –80°C (after organ culture) for subsequent immunohistochemistry.

Local ethical committee approval was obtained, and all patients had given fully informed consent to the procedures.

VITAMIN D PREPARATIONS

1,25(OH)2D3 and ergocalciferol (vitamin D2) were donated by Roche Products Ltd (Welwyn Garden City, UK). The secoester calcipotriol...
(MC-903) was supplied by Leo Pharmaceutical Products Ltd (Ballerup, Denmark).

Stock solutions of test compounds were prepared in absolute alcohol and stored at -20°C until use. Control medium was prepared with a similar dilution of alcohol and in a pilot study had no obvious effect on proliferation.

ORGAN CULTURE
Rectal biopsy specimens were divided into tiny explants and orientated mucosal surface uppermost on a metal grid within an organ culture dish (Lux Laboratories). Explants were cultured as paired samples in standard culture medium (CMRL 1066, Gibco, Paisley, UK) or in standard culture medium to which vitamin D metabolites had been added: 1,25 (OH)₂ D₃ at concentrations of 1 nM (10⁻⁸ M), 10 nM (10⁻⁷ M), or 100 µM (10⁻⁴ M); ergocalciferol (vitamin D₂) at 10 nM (10⁻⁸ M); and calcipotriol (MC-903) at 100 µM (10⁻⁷ M). Thus each biopsy specimen acted as its own control. The concentrations of vitamin D chosen were in a similar range to previous studies,³-six and the lowest dose of 1,25 (OH)₂ D₃ is probably within the human physiological range. In total, 34-biopsy specimens were obtained from controls and 10 from familial adenomatous polyposis patients, and each biopsy was divided into several explants (between six and 15).

The concentration of 1,25 (OH)₂ D₃ in the standard culture medium was 6 µM (6 x 10⁻¹² M), as determined by batch testing the fetal calf serum. The organ culture dishes were sealed in an atmosphere of 95% O₂ and 5% CO₂ at a temperature of 37°C and were then gently rocked at 5 cycles per minute.³-six After 15 hours, vincristine 0·6 µg/ml (Oncovin, Eli Lilly, Basingstoke, UK) was added to the culture medium to induce metaphase arrest within the colonic crypts.³-six Explants were removed one, two, and three hours later, fixed in Carnoy’s fluid, and stored in 70% alcohol. Biozy tissues were rehydrated later in successive solutions of 50%, 25%, and 10% alcohol. After acid hydrolysis in 1 M HCl at 60°C for 6 minutes, explants were stained with Schiff’s reagent. At least 20 crypts were microdissected from each specimen,³-six and the number of arrested metaphases per crypt was plotted against time from vincristine administration. The slope of this line (determined by least squares linear regression analysis) gave a value for the crypt cell production rate in cells/crypt/hour.³-six

IMMUNOHISTOCHEMISTRY
After 18 hours’ organ culture in medium with or without the addition of 1,25 (OH)₂ D₃ (100 µM), paired mucosal explants were mounted and then 3 µm cryostat sections were cut and air dried before blocking in H₂O₂ (0·22%) and methanol for 5 minutes. After washing in tap water and then Tris buffer (pH 7·3) for 5 minutes (×3), sections were stained using a three stage peroxidase procedure in which Ki-67 monoclonal antibody (1·50 in Tris buffer), biotinylated rabbit anti-mouse (1·300 in Tris buffer), and avidin-biotin complex (Dakopatts) were applied. The Ki-67 antibody is directed against an antigen expressed in proliferating cells.³-six Slides were developed in diamino benzidine-hydrogen peroxide (DAB) in Tris buffer for 5 minutes, then washed in tap water. Haematoxylin was used as a counterstain.

To assess rectal epithelial proliferation, the labelling index was determined in at least 15 crypts per section.³-six The labelling index was calculated as the ratio of Ki-67 positive to negative cells per crypt. The mean values of these counts were compared using a paired Student’s t test (each case acting as its own control).

CELL CULTURE
HT-29 cells were maintained as a monolayer of cells in Dulbecco’s modified Eagle’s medium (DMEM, Flow Laboratories, High Wycombe, UK) with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Serolabs, Crawley, UK). Cells were incubated at 37°C in 5% O₂ and 5% CO₂, and the medium was changed every 2 days. At 80–90% visual confluence, the cells were trypsinised with 0·25% trypsin-EDTA (Flow Laboratories). After washing in phosphate buffered saline (PBS), aliquots of 1 x 10⁶ cells were added to a six well tissue culture plate (Gibco). The cells were cultured in DMEM, with or without the addition of calcipotriol (MC-903) at a final concentration of 10⁻¹² to 10⁻⁸ M. All plates were set up in quadruplicate.

After viability assessment using trypan blue exclusion, the total cell number was determined at 7, 14, and 21 days by counting at least three samples from each concentration using a haemocytometer. Results were analysed using one way analysis of variance and the Mann-Whitney U test.

Results
ORGAN CULTURE
The overall mean (SEM) crypt cell production rate (CCPR) in 17 normal patients (Figure) was 4·74 (0·25) cells/crypt/hour, with a range of 2·85–7·07. This value is similar to but slightly lower than our previously reported results.³-six Explants showed excellent preservation of crypt architecture, with an infection rate and crypt necrosis rate of less than 1%. The active form of vitamin D₃ (1,25 (OH)₂ D₃) consistently halved the overall CCPR in normal tissue irrespective of the dose used (analysis of variance). Thus, at 1 µM the CCPR was reduced from 4·96–2·15 cells/crypt/hour, at 10 nM it was reduced from 4·71–2·10 cells/crypt/hour, and at 100 pM it was reduced from 4·86–2·67 cells/crypt/hour. The data showed a dose-response trend when individual results were compared with their own paired controls (as opposed to the overall control values), percentage reductions being 57%, 55%, and 45% with diminishing doses of 1,25 (OH)₂ D₃ (57%, 45%, 45%; p<0·05). Ergocalciferol (vitamin D₂) at a dose of 10 nM reduced CCPR in the normal rectal tissue from 5·27–2·74 cells/crypt/hour. Calcipotriol (10⁻⁷ M) also reduced

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in vitro CCPR in normal and premalignant human rectal epithelium by vitamin D and its metabolites. The data have been verified immunohistochemically using the monoclonal antibody Ki-67 to show a reduction in the labelling index. The presence of vitamin D₃ receptors in normal and malignant colorectal tissue, together with the increased colonic absorption of calcium after small bowel resection, had previously suggested that the colon could be a target organ for 1,25 (OH)₂ D₃. We have now shown an in vitro physiological response in colorectal tissue to the active metabolite of vitamin D₃ and related compounds.

The dose dependent inhibition of HT-29 cell growth by calcipotriol (MC-903) correlates well with reports of the responsiveness of this cell line to 1,25 (OH)₂ D₃. Calcipotriol has limited effects on calcium metabolism, while it retains potent cell regulatory properties. Clinical trials using calcipotriol suggest that it is a safe and effective topical treatment for psoriasis vulgaris. Our data suggest that it also has an effect on colorectal tissue.

There is now substantial evidence to show that 1,25 (OH)₂ D₃ acts as a differentiating agent and that some of these actions are associated with a modulation of receptor concentrations. Differentiating cells have in some cases shown a reduction in vitamin D receptor expression (for example HT-29 cells), and the modulation of receptor expression may be dependent on the state of differentiation. Although our observations could reflect a receptor associated genomic effect, to establish the fact would require correlation with changes in a measurable gene product. The almost equal inhibition of CCPR by ergocalciferol compared with the active metabolite (1,25 (OH)₂ D₃) argues against a receptor mediated effect, since non-hydroxylated vitamin D₃ binds poorly to the vitamin D receptor.

An alternative hypothesis is that our observed effect on proliferation may be a non-genomic effect, possibly related to calcium ion transport (as suggested by the presence of cytosolic calcium binding proteins in the colonic mucosa of short bowel syndrome) or to a post-receptor binding effect. In support of this, both verapamil and glucocorticoids (which influence calcium transport) affect the morphological changes induced by 1,25 (OH)₂ D₃ in LOVO cells.

Irrespective of the mode of action, vitamin D₃, its metabolites and analogues inhibit colonic epithelial proliferation, at least in vitro. In premalignant conditions associated with an accelerated epithelial cell proliferation, a reduction in CCPR might be beneficial. Further studies to evaluate the mode of action and possible therapeutic use of vitamin D metabolites and analogues are obviously required.

The authors thank Dr Lise Bindenerp from Leo Pharmaceuticals, who provided us with the secosterol Calcipotriol (MC 903). Financial assistance for this work was provided by the Nutritional Consultative Panel.

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Gut 1992 33: 1660-1663
doi: 10.1136/gut.33.12.1660

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