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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 $(OH)_2 D_3$ 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 $(OH)_2 D_3$ in concentrations of 1 μM–100 pM (10^{-6}–10^{-10} 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 D_2, 10 nM (10^{-8} M) reduced the CCPR from 4·74–2·74 ($p<0·05$) and calcipotriol from 4·86–2·38 ($p<0·05$). In familial adenomatous polyposis patients 1,25 $(OH)_2 D_3$ 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.

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There is a clear link between the intensity of cell proliferation and susceptibility to neoplasia.[1 2] In the large intestine, surgical or dietary manipulations that stimulate cell growth generally promote experimental colorectal carcinogenesis,[3-5] whereas mucosal hypoplasia (for example, by defunctioning colostomy) has a protective effect.[6] Patients with colonic adenoma or carcinoma show increased labelling indices in 'normal' biopsy specimens taken from multiple sites,[7] and those with ulcerative proctocolitis have increased crypt cell proliferation even in quiescent disease.[8] Thus, the cytokinetic status of the epithelium[9 10] could reflect a subject's susceptibility to colorectal neoplasia.

Calcium may protect against colorectal cancer by reducing epithelial cell turnover.[11 12] Vitamin D could be another chemopreventative agent[13] because of its ability to switch cellular activity (in various cancer cell lines) from proliferation to differentiation.[14-16] In particular, the human colon cancer cell lines HT-29[17 18] and LOVO[14] possess high affinity receptors for the active metabolite of vitamin D_3 (1,25 $(OH)_2 D_3$), which suppresses cell growth and induces changes indicative of differentiation. Receptors for 1,25 $(OH)_2 D_3$ have also been found in normal human colon.[19] Although the therapeutic use of 1,25 $(OH)_2 D_3$ and 1-alpha hydroxycholecalciferol is limited by their profound effects on calcium metabolism,[20] analogues such as calcipotriol (MC-903, a secosterol)[21-23] are now available which have limited hypercalcaemic and hypercalciuric effects.

We have used stathmokinetic and immunohistochemical techniques to study the effect of three agents – 1,25 $(OH)_2 D_3$, vitamin D_2, 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

RECTAL 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 $(OH)_2 D_3$ and ergocalciferol (vitamin D_2) were donated by Roche Products Ltd (Welwyn Garden City, UK). The secosterol calcipotriol

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(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 $(OH)_2 D_3$ at concentrations of 1 μM (10^{-6} M), 10 nM (10^{-8} M), or 100 pM (10^{-10} M); ergocalciferol (vitamin D_2) at 10 nM (10^{-8} M); and calcipotriol (MC-903) at 100 mM (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,[14-18] and the lowest dose of 1,25 $(OH)_2 D_3$ 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 $(OH)_2 D_3$ in the standard culture medium was 6 pM (6×10^{-12} M), as determined by batch testing the fetal calf serum. The organ culture dishes were sealed in an atmosphere of 95% O_2 and 5% CO_2 at a temperature of 37°C and were then gently rocked at 5 cycles per minute.[24] 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.[8 24] Explants were removed one, two, and three hours later, fixed in Carnoy's fluid, and stored in 70% alcohol. Biopsy 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,[25] 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.[11]

IMMUNOHISTOCHEMISTRY

After 18 hours' organ culture in medium with or without the addition of 1,25 $(OH)_2 D_3$ (100 pM), paired mucosal explants were mounted and then 3 μm cryostat sections were cut and air dried before blocking in H_2O_2 (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.[26 27] Slides were developed in diamino benzadine-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.[28] 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

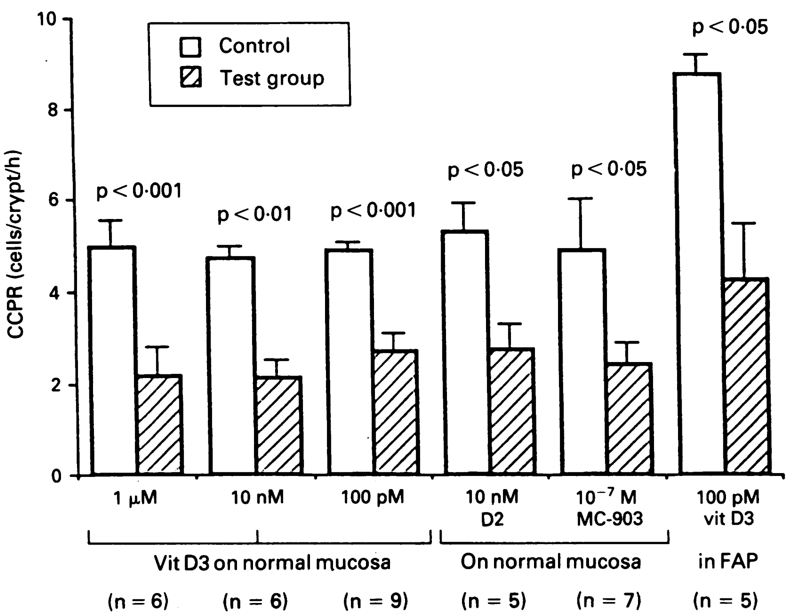
HT-29 cells were maintained as a monolayer of cells in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, High Wycombe, UK) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Serolabs, Crawley, UK). Cells were incubated at 37°C in 95% O_2 and 5% CO_2, and the medium was changed every 2 days. At 80–90% visual confluence, the cells were trypsinised with 0·25% trypsin and EDTA (Flow Laboratories). After washing in phosphate buffer saline (PBS), aliquots of 1×10^4 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^{-5} to 10^{-9} 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.[8 24] Explants showed excellent preservation of crypt architecture, with an infection rate and crypt necrosis rate of less than 1%. The active form of vitamin D_3 (1,25 $(OH)_2 D_3$) 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 $(OH)_2$ D_3 (57% *v* 45%: $p<0·05$). Ergocalciferol (vitamin D_2) 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



Effect of vitamin D on the crypt cell production rate (CCPR) in human rectal tissue. Values are mean (SEM).

the CCPR in seven patients from 4·86–2·38 cells/crypt/hour.

In patients with familial adenomatous polyposis, the baseline CCPR seemed to be higher than the overall control value (8·75 *v* 4·74 cells/crypt/hour) but the difference did not reach statistical significance. Again, 1,25 $(OH)_2$ D_3 halved the CCPR from 8·75–4.22 cells/crypt/hour (Figure).

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The active metabolite, 1,25 $(OH)_2$ D_3 reduced the crypt labelling index (measured using Ki-67) from a control value of 7·28 (0·68) (mean (SEM)) to 3·74 (0·64) (n=4, $p<0·01$, paired Student's *t* test)

CELL CULTURE

HT-29 cells in control media showed a rapid log phase growth in the first 14 days of culture. Thereafter cells continued to proliferate but at a diminished rate (possibly because of cell to cell contact). After 21 days' culture there were 8·29 (0·74)$\times 10^6$ cells in control cultures (mean (SEM)). Calcipotriol produced a clearcut dose dependent inhibition of proliferation. After 21 days' culture calcipotriol reduced the number of cells to 0·06% of the control value at 10^{-5} M ($p<0·01$), to 0·11% at 10^{-6} M ($p<0·01$), to 8·45% at 10^{-7} M ($p<0·001$), and to 94·10% at 10^{-8} M (see Table).

Discussion

We have shown for the first time an inhibition of 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 D_3 receptors in normal and malignant colorectal tissue,[19] together with the increased colonic absorption of calcium after small bowel resection,[29] had previously suggested that the colon could be a target organ for 1,25 $(OH)_2$ D_3. We have now shown an in vitro physiological response in colorectal tissue to the active metabolite of vitamin D_3 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 $(OH)_2$ D_3.[17 18] Calcipotriol has limited effects on calcium metabolism, while it retains potent cell regulatory properties.[20–23] Clinical trials using calcipotriol suggest that it is a safe and effective topical treatment for psoriasis vulgaris.[23] Our data suggest that it also has an effect on colorectal tissue.

There is now substantial evidence to show that 1,25 $(OH)_2$ D_3 acts as a differentiating agent[15–18 30 31] and that some of these actions are associated with a modulation of receptor concentrations.[31 32] Differentiating cells have in some cases shown a reduction in vitamin D receptor expression (for example HT-29 cells),[7] and the modulation of receptor expression may be dependent on the state of differentiation.[33] Although our observations could reflect a receptor associated genomic effect, to establish the fact would require correlation with changes in a measurable gene product.[14] The almost equal inhibition of CCPR by ergocalciferol compared with the active metabolite (1,25 $(OH)_2$ D_3) argues against a receptor mediated effect, since non-hydroxylated vitamin D_2 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)[34] or to a post-receptor binding effect.[20] In support of this, both verapamil and glucocorticoids (which influence calcium transport) affect the morphological changes induced by 1,25 $(OH)_2$ D_3 in LOVO cells.[14]

Irrespective of the mode of action, vitamin D, 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 effect of calcipotriol on HT-29 cell growth. Values are total cell numbers (mean (SEM))

Concentration of calcipotriol (M)	*Day 7*	*Day 14*	*Day 21*
Control	12·84 (0·88)$\times 10^5$	64·75 (3·97)$\times 10^5$	82·92 (1·45)$\times 10^5$
1×10^{-5}	3·34 (1·09)$\times 10^4$**	4·40 (0·53)$\times 10^3$**	4·70 (1·37)$\times 10^3$**
1×10^{-6}	6·40 (1·00)$\times 10^4$**	10·90 (3·48)$\times 10^4$**	9·00 (1·50)$\times 10^3$**
1×10^{-7}	1·97 (0·73)$\times 10^5$***	4·44 (1·80)$\times 10^5$***	7·01 (2·92)$\times 10^5$***
1×10^{-8}	4·50 (0·23)$\times 10^5$*	48·70 (1·44)$\times 10^5$*	78·00 (2·49)$\times 10^5$
1×10^{-9}	8·00 (0·41)$\times 10^5$*	45.00 (0·81)$\times 10^5$*	110·00 (0·01)$\times 10^5$

Significance *v* control (analysis of variance): *$p<0·05$; **$p<0·01$; ***$p<0·0001$.

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