Pergamon

European Journal of Cancer, Vol. 34, No. 13, pp. 2119–2125, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00267-6

Original Paper

Growth Regulation of Human Colon Cancer Cells by Epidermal Growth Factor and 1,25-Dihydroxyvitamin D_3 is Mediated by Mutual Modulation of Receptor Expression

W.-M. Tong, E. Kállay, H. Hofer, W. Hulla, T. Manhardt, M. Peterlik and H.S. Cross

Department of General and Experimental Pathology, University of Vienna Medical School, Waehringer Guertel 18-20, A-1090 Vienna, Austria

The human colon adenocarcinoma-derived cell line Caco-2 was used as a model system to study the interaction of epidermal growth factor (EGF) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in control of colorectal cancer cell growth. The mitogenic stimulus of EGF was rapidly transduced via apical and basal membrane receptors alike into elevation of c-*myc* expression, causing a shift of Caco-2 cells from the G_0/G_1 into the S phase of the cell cycle. The stimulatory effect of EGF on cell division was effectively counteracted by $1,25(OH)_2D_3$: the presence of the steroid hormone prevents the negative effect of EGF on vitamin D receptor abundance and concurrently minimises ligand-occupied EGF receptor numbers on both sides of Caco-2 cell monolayers. Our data suggest that EGF and $1,25\text{-}(OH)_2D_3$ actions on mutual receptor levels represent a specific feature of the potent anti-mitogenic effect of the steroid hormone on colon cancer cells. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: colon adenocarcinoma, Caco-2 cells, vitamin D, epidermal growth factor receptor, vitamin D receptor, c-*myc* expression, cell cycle, apical/basal receptor distribution
Eur J Cancer, Vol. 34, No. 13, pp. 2119–2125, 1998

INTRODUCTION

EPIDERMAL GROWTH factor (EGF) is a 6000 kDa polypeptide which is produced predominantly in the gastrointestinal tract by salivary glands, Brunner's glands and Paneth cells. The peptide hormone is released into the digestive fluids as well as into the circulation and, hence, can locally as well as systemically regulate cell growth and function in a variety of tissues [1]. In the gastrointestinal tract, EGF inhibits acid secretion by gastric parietal cells [2] and, importantly, stimulates ornithine decarboxylase [3], DNA synthesis and, thus, proliferation of small and large intestinal epithelial cells [4]. There is also evidence that EGF plays a significant role in autocrine growth stimulation of colon tumour cells [5].

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) inhibits proliferation and induces differentiated cell functions in normal and, notably, also in malignant cells [6]. In this respect, vitamin D compounds have been shown, for example, to inhibit effectively cell growth and to support phenotypic redifferentiation of the human colon cancer cell line, Caco-2 [7–9]. The effects of $1,25(OH)_2D_3$ on cell growth and differentiation are mediated by binding to the nuclear vitamin D receptor (VDR) which then acts as a transactivation factor for various genes.

Regulation of receptor abundance may be an important mechanism for modulating the extent of target cell responsiveness to hormones and growth factors. In this respect, Krishnan and Feldman [10] showed that in mouse fibroblasts and human breast cancer cells, VDR gene expression can be upregulated by serum and growth factors. EGF has also been found to increase VDR in the small intestine of the neonatal rat [11]. Conversely, $1,25(OH)_2D_3$ has been reported to modulate EGF receptor (EGFR) expression, although in opposite directions depending on the cell type involved. In human T-47D breast cancer cells, growth inhibition induced by $1,25(OH)_2D_3$ is associated with a decrease in EGFR numbers [12], whereas Desprez and colleagues [13] reported an increase in EGFR expression as the result of $1,25(OH)_2D_3$ treatment in BT-20 breast cancer cells. Because no respective data are available for human colon cancer cells, we investigated

Correspondence to H.S. Cross.
Received 6 Mar. 1998; revised 26 May 1998; accepted 1 Jun. 1998.

the action and interaction of EGF and $1,25(OH)_2D_3$ in growth control and mutual receptor regulation using the human colon adenocarcinoma-derived cell line Caco-2 as a model system.

The cell line Caco-2, despite its malignant origin, has retained the potential for spontaneous redifferentiation in culture [14]. Caco-2 cells also display two distinct classes of EGFR, at the apical and basolateral plasma membrane, respectively [15, 16] and, importantly, express the VDR at the mRNA and protein level [17, 18].

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ was a gift from Hoffmann-LaRoche (Basle, Switzerland). EGF (tissue culture grade) was obtained from Sigma (Deisenhofen, Germany). An anti-c-myc monoclonal antibody was purchased from Oncogene Science (Cambridge, Massachusetts, U.S.A.). The anti-VDR monoclonal antibody was from Chemicon (Temecula, California, U.S.A.).

Cell culture

Caco-2 cells (provided by A. Quaroni, Cornell University, Ithaca, New York, U.S.A.) were routinely cultured in Costar vented tissue culture flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) containing 4.0 mM glutamine, 10% fetal calf serum (heat-inactivated at 56°C for 30 min), 20 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin. The cultures were fed every 48 h and subcultured serially when approximately 80% confluent. They were used between passages 10 and 30.

For experimentation, 15 000 cells/ml were routinely seeded per well in 24 well Falcon plastic tissue culture dishes. For the investigation of selective stimulation of apical and basolateral EGFR, 60 000 cells/ml were seeded on to 25 mm Falcon polycarbonate filter units (0.4 μm pore size). Electron microscopy had been used previously to verify monolayer formation of confluent filter grown Caco-2 cells [15, 19].

The medium was changed and new treatments were added to Caco-2 cell cultures every 24 h, if not indicated otherwise. If appropriate, $1,25(OH)_2D_3$ was added to the cultures in ethanolic solution. The vehicle concentration in all cultures containing the steroid hormone was 0.01%.

Cell proliferation assay and cell counting

For the determination of [^{3}H]thymidine incorporation into cellular DNA, the cells were incubated for 4 h at 37°C in DMEM containing 4 μCi/ml of [^{3}H]thymidine (specific activity 70 Ci/mmol, American Radiolabeled Chemicals, St Louis, Missouri, U.S.A.). The cells were washed with phosphate buffered saline (PBS), fixed and extracted twice with 5% trichloroacetic acid. The cells were solubilised in 1 ml of 0.1 N NaOH and assayed for protein (BCA protein assay kit, Pierce, Rockford, Illinois, U.S.A.) and counted for radioactivity. Trypsinised cells were counted in a Coulter Counter (Model D, Coulter Electronics, Luton, U.K.).

Cell cycle analysis and c-myc protein determination

Cell nuclei were prepared as described elsewhere [20]. In brief, trypsinised cells were suspended in 2.0 ml citrate buffer, pH 7.6 (containing 0.1% Nonidet-P 40, 1.5 mM spermidine tetrahydrochloride (STC), 0.5 mM Tris/STC buffer and 0.03 mg/ml trypsin (all from Sigma). After incubation for 15 min at room temperature, 2.0 ml of STC buffer with 0.5 mg/ml trypsin inhibitor (Sigma) and 0.6 mg/ml RNase were added and the cells were incubated for 15 min at room temperature. The nuclei were collected by centrifugation at 1,000 *g* for 20 min after addition of 20 ml of STC buffer containing additional STC (1.6 mg/ml final concentration).

Nuclear c-myc protein was assessed using a monoclonal anti-c-myc antibody (final concentration 25 μg/ml), which was detected with a fluorescein isothiocyanate-labelled second antibody (25 μg/ml final concentration).

For cell cycle analysis, the nuclei were incubated with 50 μl of antibody solution on ice, washed twice with STC buffer and resuspended in 300 μl STC buffer containing 0.42 mg/ml propidium iodide (Sigma). The stained nuclei were analysed in a flow cytometer (FACStar; Becton Dickinson, Mountain View, California, U.S.A.) using linear amplification for propidium iodide fluorescence and logarithmic amplification of fluorescein isothiocyanate fluorescence. Cell cycle distribution was analysed using CELLFIT software (Becton Dickinson), fitting G_0/G_1 and G_2/M in normal distributions and S phase cells outside 3 standard deviations (S.D.) of the mean for G_0/G_1 and G_2/M.

EGFR radioligand assay

Binding of iodinated EGF to apical or basolateral membranes of Caco-2 cells grown on filter units was determined essentially as described by Cross and Quaroni [15]. Briefly, 1.0 ng/ml mouse ^{125}I-EGF (specific activity 100 μCi/μg, Amersham International, Buckinghamshire, U.K.) in 2 ml binding medium (DMEM with 0.1% bovine serum albumin (BSA)) was applied selectively to the apical or basal filter compartment for 3 h at 4°C. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled EGF. After incubation, the monolayers were washed, trypsinised and counted for radioactivity in an automated gamma counter (1277 GammaMaster, LKB).

Western blotting

The cells were homogenised on ice with a Polytron (Brinkmann Instruments, Westbury, New York, U.S.A.) in 10 mM Tris–HCl (pH 7.2) buffer containing a protease inhibitor mix: 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 μg/ml leupeptin, 50 μg/ml antipain, 0.1 mg/ml aprotinin (Boehringer Mannheim, Vienna, Austria). The homogenate was centrifuged at 4°C for 10 min at 1280 rpm. The supernatant was centrifuged for 60 min at 106 000 *g* and stored at − 80°C.

For immunoblotting, the samples were run on 12% sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (total protein per lane 120 μg) and were blotted to a Hybond ECL nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) with a Hoefer Transblot™ Unit (Pharmacia Biotech., San Francisco, California, U.S.A.). The gels were checked for equal protein loading by staining with Coomassie Blue. Non-specific binding was blocked in 3% BSA in PBS/0.1% Tween for 2 h at room temperature. The membranes were incubated with rat anti-VDR antibody (IgG_{2b}, 1:1000, Chemicon International, Temecula, California, U.S.A.) in PBS/Tween with 1% BSA at 4°C overnight. Incubation of the membrane with a horseradish peroxidase-conjugated antirat IgG (Amersham, 1:10 000 in PBS/0.1% Tween) was performed for 2 h at room temperature, with subsequent detection by the SuperSignal TM CL-HRP substrate system (Pierce, Rockford, Illinois, U.S.A.).

RNA isolation and Northern blot hybridisation analysis

The cells were lysed in 3.0 ml guanidinium thiocyanate buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5 N-lauroylsarcosine, 0.1 M 2-mercaptoethanol, pH 7) and total RNA was isolated. RNA was quantified by absorbance at 260 nm. The intactness of the RNAs was visualised on a non-denaturing, ethidium bromide stained 1% agarose gel. Fifteen micrograms of total RNA were then run on a denaturing (3% formaldehyde), 0.8% agarose gel and blotted with 20×SSC (3.0 M sodium chloride, 0.3 M sodium citrate) on to Nylon (Immobilon-N, Millipore, Bedford, Massachusetts, U.S.A.) or nitrocellulose (Schleicher & Schuell) membranes. A 358 bp Cla1-Msp1 fragment from c-*myc* exon 3, a cDNA probe for the human VDR, as well as β-actin were ^{32}P-labelled by random priming (Promega, Madison, Wisconsin, U.S.A.) and used for hybridisation. After baking for 2 h at 80°C, the membranes were prehybridised in a solution of 25 mM phosphate buffer (pH 7.4), 5×SSC, 5× Denhardt's solution, 50% formamide and 100 μg/ml salmon sperm DNA at 42°C, and then hybridised for 12–24 h in the same buffer containing the radiolabelled probe. The membranes were washed twice with 2×SSC/0.1% SDS for 5 min at room temperature, then twice with 0.2×SSC/0.1% SDS under the same conditions, and twice at 42°C with 0.2×SSC/0.1% SDS for 15 min and then exposed to a Kodak X-OMAT AR film at −70°C for 1–5 days. To normalise the signals for gel loading variations, the membranes were stripped by boiling in 0.1% SDS and rehybridised using a radiolabelled probe for β-actin. Autoradiographs were evaluated using a laser densitometer (LKB UltroScan XL and GelScan XL software).

The c-*myc* probe was obtained from an original American Type Culture Collection clone. VDR cDNA was kindly provided by J. W. Pike (Ligand Pharmaceuticals, San Diego, California. U.S.A.). The β-actin probe was reverse transcription–polymerase chain reaction (RT–PCR)-generated from Caco-2 RNA using commercially available primers from Clontech (Palo Alto, California, U.S.A.).

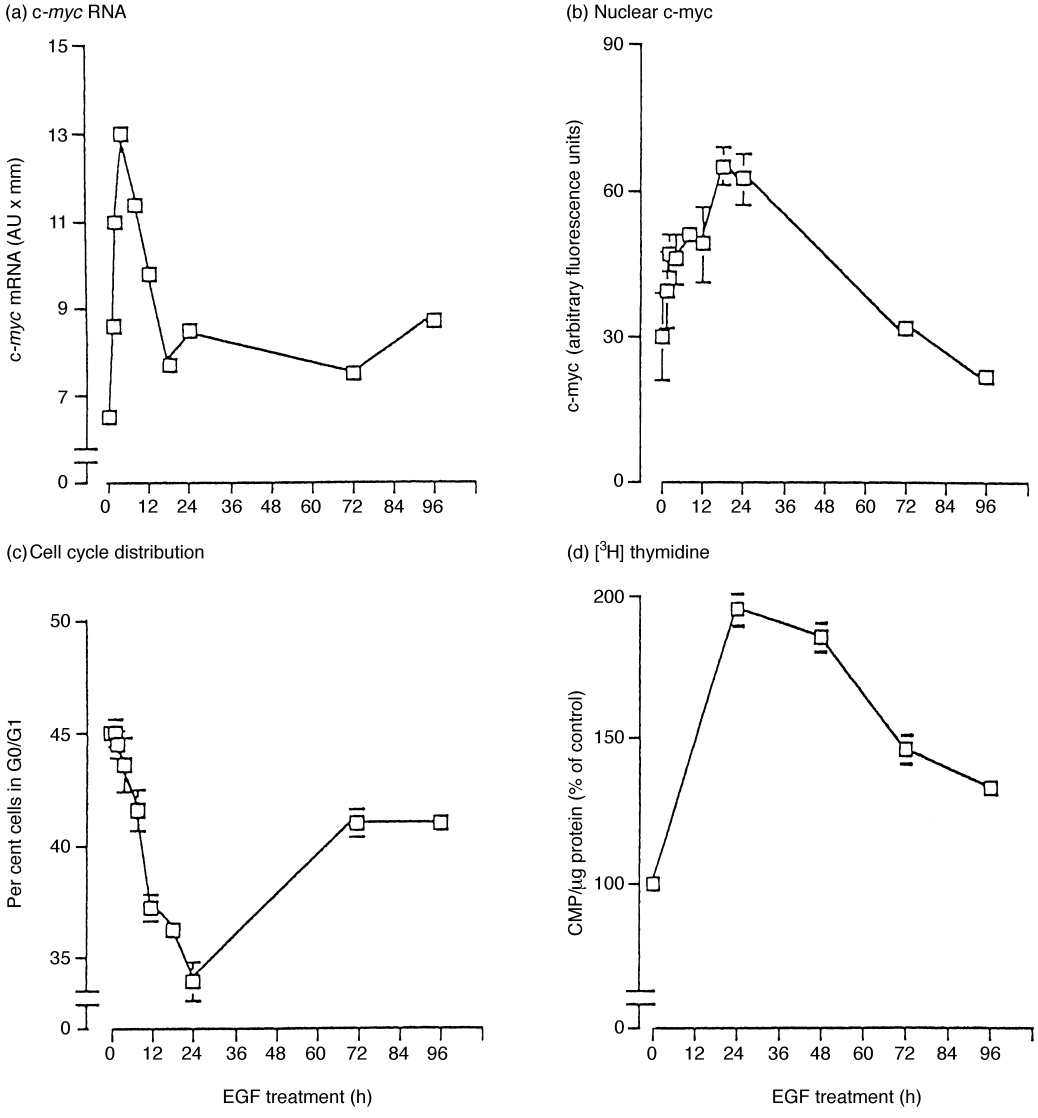

Figure 1. Effect of epidermal growth factor (EGF; 25 ng/ml) on c-*myc* mRNA (a), nuclear c-myc (b) cell cycle distribution (c) and [^{3}H]thymidine incorporation (d) of confluent Caco-2 cells. One representative experiment (out of three) is shown. (b–d) data are means ± standard error of the mean (SEM) from four measurements.

Data presentation and statistical analyses

Data are presented as means ± standard error of the mean (SEM), if appropriate. One-way analysis of variance was used for statistical evaluations. Groups versus control group were compared by Bonferroni's method.

RESULTS

*EGF affects proliferation and c-*myc *expression*

The time-course of the mitogenic effect of EGF on Caco-2 cells is illustrated in Figure 1, showing the relationship between [^{3}H]thymidine incorporation into DNA, shifts in cell cycle distribution and expression of c-*myc* mRNA and of nuclear c-myc protein. Within 6 h after the addition of EGF, c-*myc* mRNA was increased 2-fold and then sharply declined to a level still above that of untreated controls (Figure 1a). Nuclear c-myc protein also showed a transient change inasmuch as its initial value doubled, reaching a maximum after 24 h and then steadily declined to control values at 96 h (Figure 1b). This initial increase in nuclear association of the c-myc protein is exactly mirrored by a shift of Caco-2 cells out of the resting, i.e. G_0/G_1, phase of the cell cycle and a corresponding maximum of DNA synthetic activity (Figure 1c, d).

In order to investigate from which cell side the EGFR was stimulated, we added EGF to either the apical or basal cell aspect of filter grown Caco-2 cell monolayers. This resulted in a significant rise in c-*myc* mRNA which became apparent after 2 h, reached a peak level 4–5 times above control at 4 h and the rise was similar regardless of the direction of the stimulation (Figure 2). Thereafter, c-*myc* mRNA expression declined to a steady state level which was still approximately twice as high as in zero time controls (cf. Figure 1a).

Combined stimulation of apical and basolateral EGFR populations had no additional effect on c-*myc* mRNA (not shown).

EGF-induced proliferation: attenuation by $1,25(OH)_2D_3$

Figure 3 shows that growth of Caco-2 cells exposed to EGF was significantly reduced by $1,25(OH)_2D_3$ at a concentration as low as 10^{-10} M. In contrast, in the absence of EGF, the steroid hormone inhibited cell growth only at a 10-fold higher concentration. In order to explain the increased sensitivity of Caco-2 cells to $1,25(OH)_2D_3$ in the presence of EGF, we investigated the mutual effects of the growth factor and of $1,25(OH)_2D_3$ on expression levels of their respective receptors.

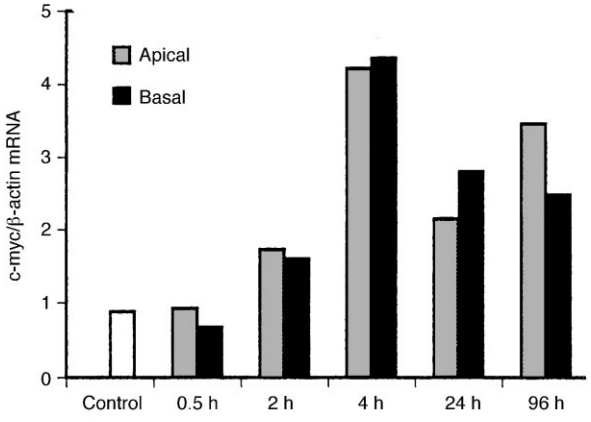

Figure 2. Selective stimulation of apical or basolateral epidermal growth factor receptor (EGFR) and c-*myc* mRNA expression. EGF (25 ng/ml) was added on day 2 after confluence. Data are from one representative experiment (out of three).

Differential expression of apical and basolateral EGFR: effects of EGF and $1,25(OH)_2D_3$

Because Caco-2 cells display an unequal distribution of EGFR on their apical and basolateral plasma membrane [15], which might also reflect functional differences [16], the site-specific expression and function of EGFR was studied on filter grown Caco-2 cells. Analysis of selective ^{125}I-EGF binding to either the apical or basolateral surface of Caco-2 monolayers revealed a particularly high density of basolateral membrane EGFR in untreated Caco-2 cells, whereas only approximately one-fifth of the total number was found at the apical side (Table 1). Treatment with 25 ng/ml EGF, either at the apical or basolateral side of the filter chamber, initiated a decrease of EGFR numbers within 30 mins. Homologous downregulation was very efficient on the basolateral side, where receptor density was reduced to approximately 20% of its initial level, whereas reduction of EGFR numbers was less conspicuous on the apical side (Table 1). Steady state EGFR levels were achieved after little more than 2 h of EGF treatment and were practically in the same order of magnitude on the apical and the basolateral side (Table 1).

Treatment of Caco-2 cells with 10^{-8} M $1,25(OH)_2D_3$ reduced EGFR numbers in the steady state on the apical side by approximately 50% and on the basolateral side by around 25%. When combined with EGF, the steroid hormone induced a significant further decrease in EGFR numbers, leading to extremely low receptor density on both sides of Caco-2 cells (Table 1).

VDR mRNA and protein expression: effect of EGF and $1,25(OH)_2D_3$

Selective stimulation of apical or basolateral EGFR by addition of 25 ng/ml EGF to one of the respective cell compartments on the filter units suppressed VDR mRNA

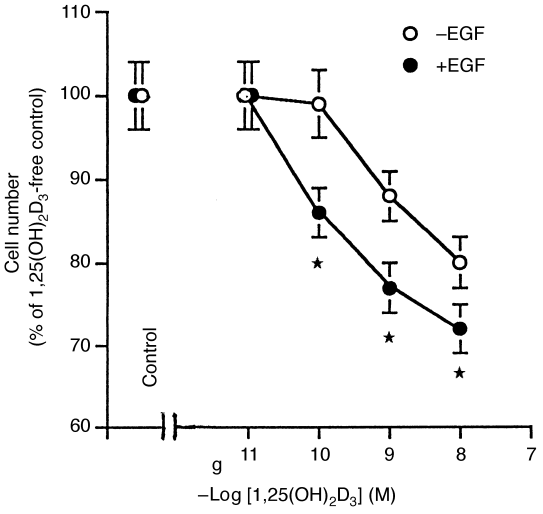

Figure 3. Antimitotic effect of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on confluent Caco-2 cells in the absence (○) or presence of epidermal growth factor (EGF; 25 ng/ml) (●). $1,25(OH)_2D_3$-free vehicle controls contained 0.01% ethanol. Cell counts in triplicate after 4 days treatment. *Statistically significant difference from respective EGF-free group (at least $P < 0.05$).

Table 1. *Homologous and heterologous regulation of epidermal growth factor receptor (EGFR) density on luminal and basolateral (Caco-2 cell membranes)*

	EGFR density (fmol/mg protein)					
	Apical (luminal) Additions to luminal side			Basolateral Additions to basolateral side		
Time (h)	EGF	$1,25(OH)_2D_3$	EGF plus $1,25(OH)_2D_3$	EGF	$1,25(OH)_2D_3$	EGF plus $1,25(OH)_2D_3$
0	847 ± 12	847 ± 12	847 ± 12	2,433 ± 57	2,433 ± 57	2,433 ± 57
0.5	392 ± 31*	N.D.	N.D.	897 ± 57*	N.D.	N.D.
2.0	256 ± 28*	N.D.	N.D.	567 ± 20*	N.D.	N.D.
48	214 ± 48*	N.D.	N.D.	386 ± 49*	N.D.	N.D.
168	314 ± 20*	413 ± 86*	81 ± 7*,†	435 ± 17*	1,776 ± 203*	51 ± 13*†

EGF (25 ng/ml) was added to either the luminal or basolateral compartment of filter grown Caco-2 cells. $1,25(OH)_2D_3$ (10^{-8} M) was added on both sides. Control cultures contained 0.01% ethanol as the vehicle. EGFR was quantified by duplicate radioligand assays. Data are means ± standard error of the mean (SEM) from two separate experiments. *Statistically significant difference (at least $P \leq 0.05$) from control. †Statistically significant difference (at least $P \leq 0.05$) from EGF treated group at the same time point. N.D., not determined; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

abundance within 4 h to 40% of initial values. Further exposure to EGF for up to 96 h maintained VDR mRNA expression at a similarly low level (Figure 4). Combined stimulation of apical and basolateral EGFR had no additional effect on VDR expression (data not shown).

Heterologous downregulation of the VDR by EGF was also observed at the protein level (Figure 5). $1,25(OH)_2D_3$ has a positive effect on receptor abundance, even in the presence of EGF (Figure 5).

DISCUSSION

The present study strongly suggests that, regardless of whether the stimulatory effect of EGF on the growth of human colon adenocarcinoma-derived Caco-2 cells is elicited via luminal or basolateral EGFR activation, it eventually involves upregulation of the cell cycle regulatory protein c-myc. This would facilitate Caco-2 cells to enter into and progress through the G_1 phase of the cell cycle in response to EGF.

Until now the mechanism underlying the antimitogenic action of $1,25(OH)_2D_3$ in Caco-2 cells has remained an enigma, since, unlike in other tumour cells, c-*myc* expression in Caco-2 cells is resistant to modulation by $1,25(OH)_2D_3$ [18]. It had to be assumed, therefore, that the antimitotic effect of the steroid hormone results from VDR-mediated modulation of a regulatory site downstream or independent of c-myc action. This could probably involve an appropriate effect of vitamin D on the expression of other cycle regulators, such as cyclins, cyclin-dependent kinases or their inhibitors, as observed by Kawa and colleagues [21] in pancreatic cancer cells or by Wang and associates [22] in human leukaemia HL-60 cells.

The observation in the present study that EGF-stimulated Caco-2 cells responded particularly well to growth inhibition by $1,25(OH)_2D_3$ (cf. Figure 3), could be explained, at least in part, if one assumes that, apart from cell cycle regulatory events, changes in receptor abundance may also be an important mechanism for modulating the extent of target cell

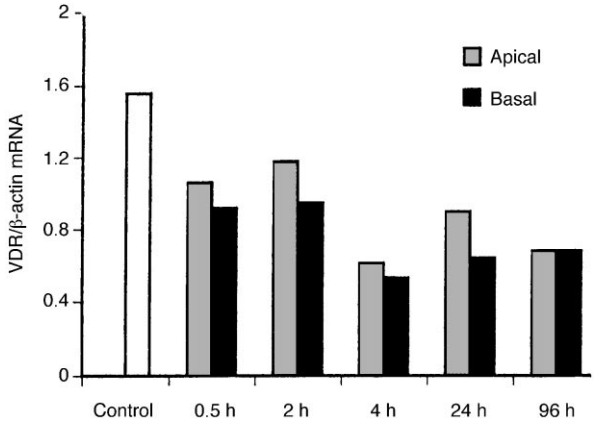

Figure 4. Selective stimulation of apical or basal epidermal growth factor receptor (EGFR): effect on vitamin D receptor (VDR) mRNA expression. Treatment with 25 ng/ml EGF apically or basally for the time period indicated. Data shown are from one representative experiment (out of three).

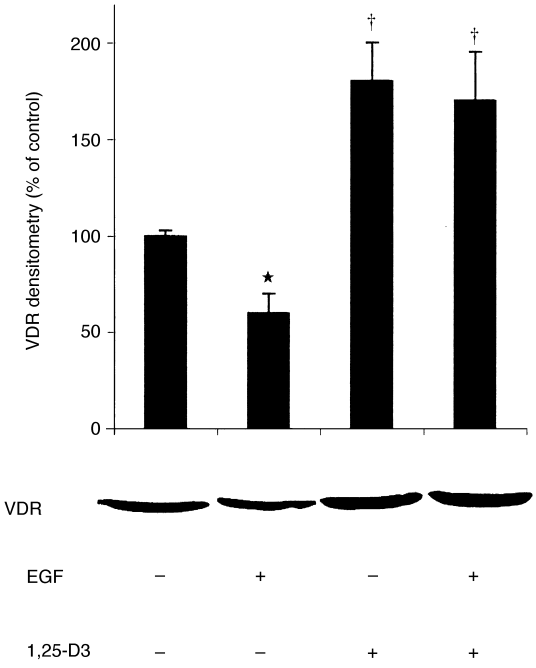

Figure 5. Western blot analysis of vitamin D receptor (VDR) density in Caco-2 cells. Epidermal growth factor (EGF; 25 ng/ml) or/and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; 10^{-8} M) were added 2 days after confluence for 96 h. Untreated controls contained 0.01% ethanol as vehicle. Data from one representative experiment (out of three) are shown. Means ± standard error of the mean (SEM). Statistically significant difference from the controls. *$P < 0.05$; †$P < 0.01$.

responsiveness to growth factors and hormones. Bishop and Wen [16] had suggested that Caco-2 cell proliferation is driven exclusively by ligand-activated basolateral membrane EGFR because they could observe an appropriate increase in intrinsic EGFR tyrosine kinase activity and [^{3}H]thymidine uptake only when Caco-2 cells were exposed to 1–5 ng/ml EGF at their basolateral but not at their apical aspect. However, the present study shows that by raising the EGF concentration to 25 ng/ml signalling via apical EGFR can be effectively transduced (cf. Figures 2 and 4). Since Cross and Quaroni [15] found no difference in the K_D or ligand binding between apical and basolateral EGFR, it remains to be seen whether the lack of sensitivity of apical membrane EGFR to low ligand concentrations can be attributed to any structural difference outside the ligand binding domain.

There exists, however, a significant difference between the two populations of EGFR with respect to modulation of their density. Whereas expression of apical membrane EGFR remains relatively unchanged in the presence of the ligand, their basolateral counterparts undergo rapid and efficient reduction in numbers upon ligand binding (Table 1). In view of the preponderance of EGFR on the basolateral membrane, effective homologous downregulation can be seen as a mechanism of self-attenuation of the response to EGF, which could protect colonocytes from mitogenic overstimulation.

Our data indicate a pattern of EGF and $1,25(OH)_2D_3$ interaction at the level of receptor expression that seems to be characteristic for human colon carcinoma cells. Unlike, for instance, in normal enterocytes [11], in Caco-2 cells expression of the VDR can be substantially suppressed by EGF (Figures 4 and 5). A similar effect of EGF on VDR levels has only been observed in osteoblast-like cells [23]. Importantly, in the presence of $1,25(OH)_2D_3$, Caco-2 cells are partially resistant to the inhibitory effect of EGF on VDR expression (cf. [24] and Figure 5). In addition, in Caco-2 cells, $1,25(OH)_2D_3$ decreases the abundance of EGFR, especially in the presence of its ligand (Table 1), whereas in breast cancer cells the steroid hormone induces a 3-fold rise in EGFR numbers [25].

The present study points at a pivotal role of the steroid hormone $1,25(OH)_2D_3$ in counteracting mitogenic stimulation by EGF in Caco-2 cells and, thus, lends experimental support for the notion derived from epidemiological data that vitamin D insufficiency is a contributing factor to the development of colorectal carcinomas in humans [26]. We suggest that this is a condition which would facilitate downregulation of the VDR by EGF (cf. Figures 4 and 5) which, in turn, would further diminish the antimitotic potential of any residual $1,25(OH)_2D_3$ and at the same time alleviate VDR-mediated control of EGFR levels (Table 1) favouring the transduction of mitogenic signals via the EGF/EGFR pathway. In contrast, if vitamin D is also available, for example, from endogenous production by colonocytes [27] in sufficient amounts to protect VDR expression from downregulation by EGF (cf. [24] and Figure 5), the steroid hormone could then effectively attenuate the proliferative impulse from EGF signalling and thereby reduce the risk of neoplastic transformation of colonocytes.

1. Carpenter G. Epidermal growth factor. *Handb Exp Pharmacol* 1981, **57**, 90–126.
2. Bower JM, Camble R, Gregory H, Gerring EL, Willshire IR. The inhibition of gastric acid secretion by epidermal growth factor. *Experimentia (Basel)* 1975, **31**, 825–826.
3. Feldman EJ, Aures D, Grossman MI. Epidermal growth factor stimulates ornithine decarboxylase activity in the digestive tract of the mouse. *Proc Soc Exp Biol Med* 1978, **159**, 400–402.
4. Scheving LA, Yeh YC, Tsai TH, Scheving LE. Circadian phase-dependent stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the duodenum, jejunum, ileum, caecum, colon and rectum of the adult male mouse. *Endocrinology* 1980, **106**, 1498–1503.
5. Gross ME, Zorbas MA, Daniels YJ, *et al.* Cellular growth response to epidermal growth factor in colon carcinoma cells with an amplified epidermal growth factor receptor derived from a familial adenomatous polyposis patient. *Cancer Res* 1991, **51**, 1452–1459.
6. Frampton RJ, Omond SA, Eisman JA. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D3 metabolites. *Cancer Res* 1983, **43**, 4443–4447.
7. Cross HS, Huber C, Peterlik M. Antiproliferative effect of 1,25-dihydroxyvitamin D3 and its analogs on human colon adenocarcinoma cells (Caco-2): influence of extracellular calcium. *Biochem Biophys Res Comm* 1991, **179**, 57–62.
8. Cross HS, Pavelka M, Slavik J, Peterlik M. Growth control of human cancer cells by vitamin D and calcium *in vitro*. *J Natl Cancer Inst* 1992, **84**, 1355–1357.
9. Cross HS, Farsoudi KH, Peterlik M. Growth inhibition of human colon adenocarcinoma-derived Caco-2 cells by 1,25-dihydroxyvitamin D3 and two synthetic analogs: relation to *in vitro* hypercalcemic potential. *Arch Pharmacol* 1993, **347**, 105–110.
10. Krishnan AV, Feldman D. Stimulation of 1,25-dihydroxyvitamin D3 receptor gene expression in cultured cells by serum and growth factors. *J Bone Mineral Res* 1991, **6**, 1099–1107.
11. Bruns DE, Krishnan AV, Feldman D, *et al.* Epidermal growth factor increases intestinal calbindin-D9k and 1,25-dihydroxyvitamin D receptors in neonatal rats. *Endocrinology* 1989, **125**, 478–485.
12. Koga M, Eisman JA, Sutherland RL. Regulation of epidermal growth factor receptor levels by 1,25-dihydroxyvitamin D3 in human breast cancer cells. *Cancer Res* 1988, **48**, 2734–2739.
13. Desprez PY, Poujol D, Falette N, Lefebvre MF, Saez S. 1,25-dihydroxyvitamin D3 increase epidermal growth factor receptor gene expression in BT-20 breast carcinoma cells. *Biochem Biophys Res Commun* 1991, **176**, 1–6.
14. Pinto M, Robine-Leon S, Appay MD, *et al.* Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 1983, **47**, 323–330.
15. Cross HS, Quaroni A. Inhibition of sucrase-isomaltase expression by EGF in the human colon adenocarcinoma cells Caco-2. *Am J Physiol* 1991, **261**, C1173–C1183.
16. Bishop WP, Wen JT. Regulation of Caco-2 cell proliferation by basolateral membrane epidermal growth factor receptors. *Am J Physiol* 1994, **267**, G892–G900.
17. Giuliano AR, Franceschi RT, Wood RJ. Characterization of the vitamin D receptor from the Caco-2 human colon carcinoma cell line: effect of cellular differentiation. *Arch Biochem Biophys* 1991, **285**, 261–269.
18. Hulla W, Kállay E, Krugluger W, Peterlik M, Cross HS. Growth control of human colon adenocarcinoma-derived Caco-2 cells by vitamin D compounds and extracellular calcium: relation to c-*myc* proto-oncogene and vitamin D receptor expression. *Int J Cancer* 1995, **62**, 711–716.
19. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 1989, **96**, 736–749.
20. Pedrazolli P, Bains MA, Watson R, Risher J, Hoy TG, Jacobs A. c-myc and c-myb oncoproteins during induced maturation of myeloid and erythroid human leukemic cell lines. *Cancer Res* 1989, **49**, 6911–6916.
21. Kawa S, Nikaido T, Aoki Y, *et al.* Vitamin D analogues up-regulate p21 and p27 during growth inhibition of pancreatic cancer cell lines. *Br J Cancer* 1997, **76**, 884–889.
22. Wang QM, Jones JB, Studzinski GP. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. *Cancer Res* 1996, **56**, 264–267.

23. Leeuwen JPTM van, Pols HAP, Schilte JP, Visser TJ, Birkenhäger JC. Modulation by epidermal growth factor of the basal $1,25(OH)_2D_3$ receptor level and the heterologous up-regulation of the $1,25(OH)_2D_3$ receptor in clonal osteoblast-like cells. *Calcif Tissue Int* 1991, **49**, 35–42.
24. Cross HS, Hulla W, Tong W-M, Peterlik M. Growth regulation of human colon adenocarcinoma-derived cells by calcium, vitamin D and epidermal growth factor. *J Nutr* 1995, **125**, 2004S–2008S.
25. Falette N, Frappart L, Lefebvre MF, Saez S. Increased epidermal growth factor receptor level in breast cancer cells treated by 1,25-dihydroxyvitamin D3. *Mol Cell Endocrinol* 1989, **63**, 189–198.
26. Garland C, Shekelle RB, Barrett-Connor E, Criqui MH, Rossof AH, Paul O. Dietary vitamin D and calcium and risk of colorectal cancer: a 19-year prospective study in men. *Lancet* 1985, **1**, 307–309.
27. Cross HS, Peterlik M, Reddy GS, Schuster I. Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: expression of 25-hydroxyvitamin D3-1α-hydroxylase activity and regulation of side-chain metabolism. *J Steroid Biochem Mol Biol* 1997, **62**, 21–28.

Acknowledgements—These investigations were supported by grants from the Austrian Science Foundation (Project nos. P 09917-MED and P 10133-MED). from the Herzfelder Foundation, from the Oncology Research Fund and from the Anton Dreher Memorial Fund (Project no. 225/93) of the University of Vienna Medical School. Drs Wolfgang Hulla and Wei-Min Tong are presently affiliated with the International Agency for Research on Cancer, 150 cours Albert Thomas, 69008 Lyon, France. Dr W. Hulla acknowledges gratefully personal support from the Hans Moser Stiftung, Austria. The authors thank Mrs Erika Bajna for skilful technical assistance.