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Original Paper

Effect of Anthraquinone-laxatives on the Proliferation and Urokinase Secretion of Normal, Premalignant and Malignant Colonic Epithelial Cells

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Even though 1,8-dihydroxyanthraquinone (DHA)-laxatives have been implicated in colon carcinogenesis, the available information is still inconclusive. The aim of this study was to demonstrate the effect of the DHA-laxatives, danthrone, rhein, aloe-emodin and sennidine, on colorectal tumour cells. In SW480 carcinoma cultures, dose-dependent induction of urokinase secretion into the medium was the predominant effect. Simultaneously, cell numbers were decreased by DHA-aglycones, but not by sennoside or the biphenylic laxative bisacodyl. DNA synthesis was not similarly reduced: 0.4–4 μM danthrone and sennidine even stimulated 5-bromo-2′-desoxyuridine (BrdU) uptake into DNA. When uptake was normalised to cell number, danthrone and sennidine doubled BrdU uptake/10^6 cells, 18 μM rhein and 0.7 μM aloe-emodin induced increases of 37 and 50%, respectively. This may at least partially be due to selective resistance of S-phase cells to DHA-caused cell loss. In VACO235 adenoma cells, sennidine and aloe-emodin did not affect urokinase secretion, but stimulated growth. Both cell numbers and DNA synthesis were increased. In contrast to SW480 carcinoma cells, VACO235 cells were also sensitive to sennoside and bisacodyl. No effects of DHA were observed in normal colorectal epithelial cells. The biological effects were preceeded by specific phosphorylation of cellular proteins with molecular weights of 110, 78, 63, 57 kDa, indicating the specific induction of a cellular signalling cascade by the laxatives. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

ANTHRAQUINONES AND dianthrones (1,8-dihydroxyanthraquinones (DHAs), Figure 1) are present in various plants as glycosides. After removal of the sugar moiety by intestinal bacteria, the DHA-aglycons have strong laxative effects. Both the purified compounds and teas prepared from DHA-containing plants are, therefore, used therapeutically. In contrast, the chronic use of laxatives has many adverse effects. Specifically, chronic use of DHAs might be involved in the development of colorectal cancer. However, data from epidemiological studies are contradictory: while no relationship between laxative use and colorectal cancer risk could be observed in the Melbourne Colorectal Cancer Study [1], other studies are more positive. Nusko and colleagues found an increase of adenoma risk with laxative intake of any kind, as well as specific effects of anthranoid drugs [2] and Siegers and associates observed significantly higher rates of melanosis coli—a condition brought about by anthraquinone abuse—in patients with adenomas and even higher in patients with carcinomas, and calculated a relative colon cancer risk of 3.04 for melanosis patients [3]. Mutagenic activity in bacteria, as well as in higher cells, has been shown for several DHAs in some, but not all, model systems [4–7]. Similarly, DHAs promoted cell growth in various cell lines [8], but neither carcinogenic nor tumour promoting activity in rodents have been observed [9]. Effects on the relevant target cells—the colonic mucosa—have not yet been investigated.

For investigations of this kind, we have previously made use of tumour cell lines, as well as primary cultures of colorectal

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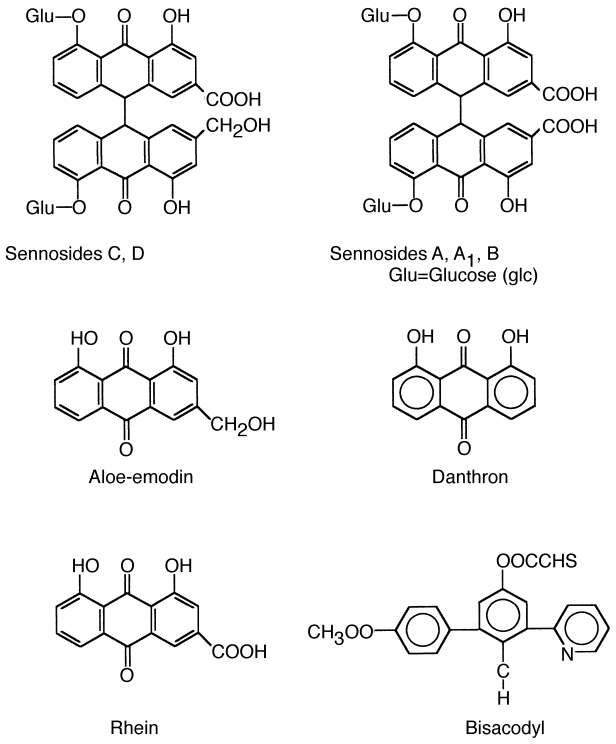

Figure 1. Structure of 1,8-dihydroxyanthraquinone (DHA) laxatives and bisacodyl.

epithelial cells. Several suspected tumour promoters in the colon cause selective growth stimulation in colonic epithelial cells. This has been shown for bile acids [10], phorbol esters [10, 11] and 1,2-diglycerides [12, 13]. In addition, phorbol esters and 1,2-diglycerides also stimulate secretion of urokinase, an enzyme that enables digestion of extracellular matrix and neighbouring cells and furthers cell spread and metastasis [11, 14–17]. These effects are highly tumour stage specific and reflect the successive changes in signal transduction pathways observed during colorectal carcinogenesis [10, 18–21]. They also provide a mechanistic basis for tumour promotion and progression induced by dietary factors. Any compound inducing similar effects on growth and urokinase secretion in the colorectal epithelium has to be regarded as a candidate tumour promoter in the colon. We have, therefore, measured the effects of DHAs on the growth and urokinase secretion of normal, premalignant and malignant colonic epithelial cells to elucidate further their role in colorectal tumour growth and spread.

MATERIALS AND METHODS

Cell lines and primary culture

SW480 colon carcinoma cells were obtained from the American Tissue Culture Collection (Rockville, Maryland, U.S.A.) and kept under standard tissue culture conditions using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. VACO235 adenoma cells were a gift from J.K.V. Willson, Ireland Cancer Center, Case Western University, Cleveland, Ohio, U.S.A. They were cultured on collagen I gels in HEPES-buffered DMEM containing 2% fetal calf serum, 2×10^{-10} M triiodo-L-thyronine, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 2 μg/ml transferrin, 5×10^{-9} M selenite (MEM2+ [18]).

Primary cultures of normal colonic epithelial cells were established from the colons of rat term embryos. After washing with phosphate buffered saline, minced colons were digested with 0.24 U/ml dispase grade II (Böhringer Mannheim, Germany) at 37°C until the tissue was dispersed into individual crypts and single cells. Crypts were separated from debris and single cells by fractionated centrifugation and plated into 1.8 cm^2 wells of a sonical seal slide (Nunc Inc. Naperville, Illinois, U.S.A.), coated with collagen type I and cultured in MEM2+ supplemented with 30 ng/ml epidermal growth factor.

Identification of epithelial cells

Cultures were washed with phosphate buffered saline, fixed with methanol/acetone (1:1) and stained using monoclonal anti-pan-cytokeratin antibodies obtained from Sigma (St Louis, Missouri, U.S.A.) at a concentration of 1 μg/ml. Control preparations received normal serum instead of the first antibody. Binding was visualised using second antibodies, coupled to biotin and avidin peroxidase reagents (Vectastain ABC reagents, Vector Laboratories, Burlingame, California, U.S.A.) and incubation with 3,3′-diaminobenzidine (Sigma) and H_2O_2.

DHA treatment

Rhein and aloe-emodin were obtained from Roth (Karlsruhe, Germany), danthrone from Aldrich (Beerse, Belgium) and sennidine A/B, sennoside and bisacodyl were a gift from Madaus AG, (Germany). Stock solutions were made in dimethylsulphoxide (DMSO) and stored at −80°C under nitrogen. The treatment media for carcinoma and adenoma cells contained DHAs in serum free medium supplemented with 1 mg/ml bovine serum albumin (Sigma), 10 μg/ml Tween 80 (Serva, Heidelberg), 1 mg/ml *N*-acetylcysteine (Sigma) and 0.5 μl/ml DMSO corresponding to the amount in the highest treatment group. For treatment of normal colonic epithelial cells, the treatment media also contained 2% fetal calf serum. DHA concentrations ranged from 0.2 to 5 μg/ml (0.4–20 μM depending on the compound).

Cells were plated at 5×10^4 cells/1.8 cm^2 well for experiments on growth and urokinase secretion and at 3×10^5 cells/78 cm^2 Petri dish for biochemical studies. Cells were left to attach for 48 h (SW480) or 72 h (VACO235) and then exposed to DHA-containing treatment media in a 90% N_2/5%O_2/5%CO_2 atmosphere. Control cultures received DMEM containing bovine serum albumin, Tween 80, *N*-acetylcysteine and the highest concentration of DMSO present in any treatment group.

Determination of cell number

Cell number was determined by neutral red uptake from serum-free DMEM, containing 50 μg/ml neutral red during a 2 h period. The dye is taken up into the lysosomes of viable cells from where it can be dissolved with 1% acetic acid in 70% ethanol.

Determination of DNA synthesis

Cell proliferation was determined by incorporation of 5-bromo-2′-desoxyuridine (BrdU) into the DNA using cell proliferation kits obtained from Amersham (Arlington Heights, Illinois, U.S.A.). BrdU was added to the medium of carcinoma cells for 2 h and to adenoma cells and normal colonic epithelial cells for the final 24 h of the incubation. At the end of the treatment period, cells were fixed with

methanol/acetic acid (3: 1) and BrdU uptake was determined by enzyme-linked immunosorbent assay (ELISA) (carcinoma and adenoma cells) or immunocytochemistry (adenoma cells and normal colonic epithelial cells) using the respective cell proliferation kit according to the instructions.

Alternatively, cells were incubated with 2.5 μCi/ml ^{3}H-thymidine (10 Ci/mmol; ICN Biomedicals, Costa Mesa, California, U.S.A.) for 2 h, lysed in 0.1 M NaOH. DNA was precipitated using 10% trichloroacetic acid and radioactivity incorporated in high molecular weight material counted in a Packard 1900 TR liquid scintillation counter

Urokinase assay

Urokinase secreted into conditioned medium was determined by incubation of 100 μl conditioned medium with the specific chromogenic substrate Spectrozyme urokinase (carbobenzoxy-L-c-glutamyl-a-t-butoxy]-glycyl-arginin-*p*-nitroanilide diacetate; American Diagnostics, Greenwich, Connecticut, U.S.A.) in the presence of aprotinin at 30°C. Under these conditions, digestion was dose-dependent and linear for 24 h. Purified urokinase (Serva Biochemicals, Heidelberg, Germany) was used as a standard.

Phosphorylation of endogenous substrates

Cultures were treated with laxatives as described above, but treatment was terminated after 1, 5, 30 or 60 min to assess short-term effects. Cultures were washed with phosphate buffered saline and lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM ethylene diamine tetraacetic acid (EDTA), 2 mM ethyleneglycol-aminoethyl tetra acetic acid (EGTA) 100 μM Na_3VO_4 and 1% Triton X100. Aliquots were used for the determination of protein content and 50 μg cellular protein was separated on 10% sodium dodecyl sulphate–polyacrylamide gels. Protein phosphorylation was determined after transfer to PVDF membranes by probing with a polyclonal antiphosphotyrosine antibody (Zymed Lab. Inc., San Francisco, California, U.S.A.) and a chemoluminescence detection system (Amersham).

Stability of DHAs in the medium

DHAs were extracted from the medium with diisopropylether and separated by HPLC on a Merck cartridge 250×4 packed with LiChrospher 100 RP18, 5 μm using isocratic elution with water/methanol/acetonitrile/acetic acid (39/55/5/1). The DHAs were detected by ultraviolet absorption (254 nm) and quantified by integration of the peak areas.

RESULTS

Effects on SW480 colon carcinoma cells

The addition of DHAs to the medium of colonic carcinomas caused non-specific toxicity, damaging both cellular and nuclear membranes. This was ameliorated when reducing conditions were established in the medium by the addition of 1 μg/ml *N*-acetylcysteine and cultivation in a 90% N_2/5% O_2 atmosphere, mimicking the reducing conditions present within the colonic lumen. Under these conditions, concentrations of 5 μg/ml (≈20 μM) DHA caused between 50 and 70% cell loss (Figure 2a). DNA synthesis was not

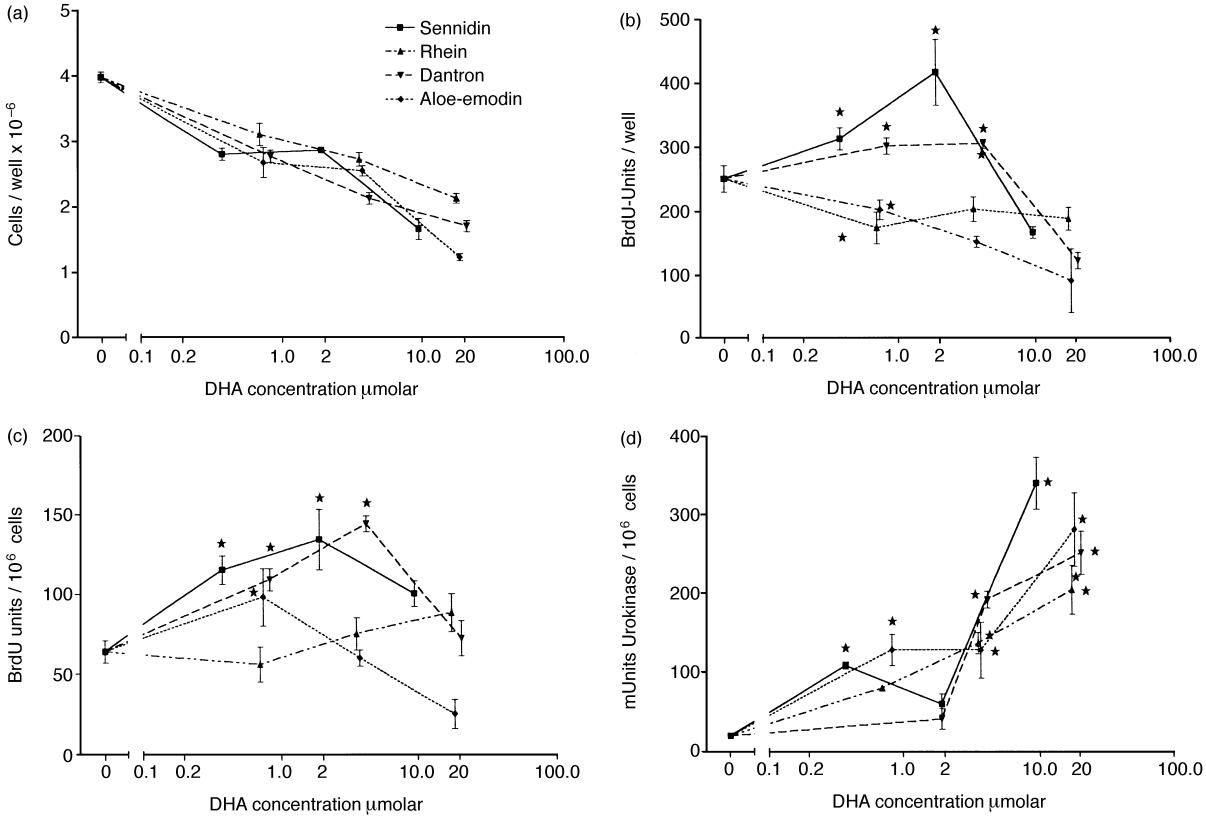

Figure 2. Effects of 1,8-dihydroxyanthraquinones (DHAs) on the growth and urokinase secretion of colonic carcinoma cells. (a) Cell number determined by neutral red uptake, (b) DNA synthesis, by total 5-bromo-2′-desoxyuridine (BrdU) incorporation or (c) BrdU incorporation/10^6 cells. (d) Urokinase secretion into the medium, measured by digestion of spectrozyme urokinase. Triplicates were analysed in three independent experiments and the results are presented as means ± standard deviation. *increased at $P < 0.05$.

similarly reduced—in some of the sennidine and danthrone treatment groups (0.4 and 4 μM) it was even increased (Figure 2b). Only the highest concentrations of these DHAs (10 μM sennidine and 20 μM danthrone) reduced total BrdU uptake per well. Rhein and aloe-emodin caused a reduction of BrdU incorporation at all concentrations. When the BrdU incorporation was normalised to cell numbers, increased incorporation (DNA synthesis) per 10^6 cells was seen for all DHAs at one or more concentrations (Figure 2c). 2 μM sennidine and 3.5 μM danthrone doubled BrdU uptake/10^6 cells. Rhein (18 μM) and aloe-emodin (0.7 μM) caused increases of 37 and 50%, respectively (significant at $P < 0.05$). Aloe-emodin (18 μM) is the only treatment group with a significantly decreased BrdU uptake/10^6 cells. In another series of experiments, DNA synthesis was measured by incorporation of 3H-thymidine and the same results were obtained.

Urokinase secretion was unequivocally increased by all DHAs tested in a dose-dependent manner (Figure 2d). The dose–response relationships were similar for all four compounds and stimulation by 20 μM concentrations ranged between 10- and 20-fold. Similar experiments were also performed with the DHA-glycoside sennoside and the diphenylic laxative bisacodyl. Concentrations up to 50 μM were used for sennoside and did not cause cytotoxicity or stimulation of DNA synthesis. We did, however, observe increased urokinase secretion at the highest concentration (Table 1). Bisacodyl was used at lower concentrations (0.2–5 μM), because of its much lower therapeutic doses. We observed no cytotoxic effects in bisacodyl-treated cultures. In contrast to DHA treatment, DNA synthesis and urokinase secretion were decreased (Table 1).

Effects on VACO235 adenoma cells and normal colonic epithelial cells

To measure the effects on VACO235 adenoma cells, sennidine and aloe-emodin were tested as typical examples for

Table 1. *Effects of sennoside and bisacodyl on growth and urokinase secretion in SW480 cells*

Compound	Concentration (μM)	Cell number (10^5 cells/well)	DNA synthesis (units/well)	Urokinase secretion (m units/well)
Sennoside	0	4.2 ± 0.1	152.8 ± 5.0	20.7 ± 4.5
	5	3.9 ± 0.03	153.4 ± 14.3	18.5 ± 2.1
	15	3.9 ± 0.2	94.0 ± 7.2	25.5 ± 4.9
	50	4.0 ± 0.8	110.0 ± 8.0	51.5 ± 3.5*
Bisacodyl	0	3.25 ± 0.4	125.0 ± 0.9	23.0 ± 4.9
	0.2	3.25 ± 0.3	124.6 ± 1.4	8.3 ± 1.2
	1	3.30 ± 0.1	97.1 ± 0.8	11.0 ± 3.0
	5	3.20 ± 0.4	98.8 ± 0.8	12.3 ± 2.5

*Increased above control $P < 0.05$.

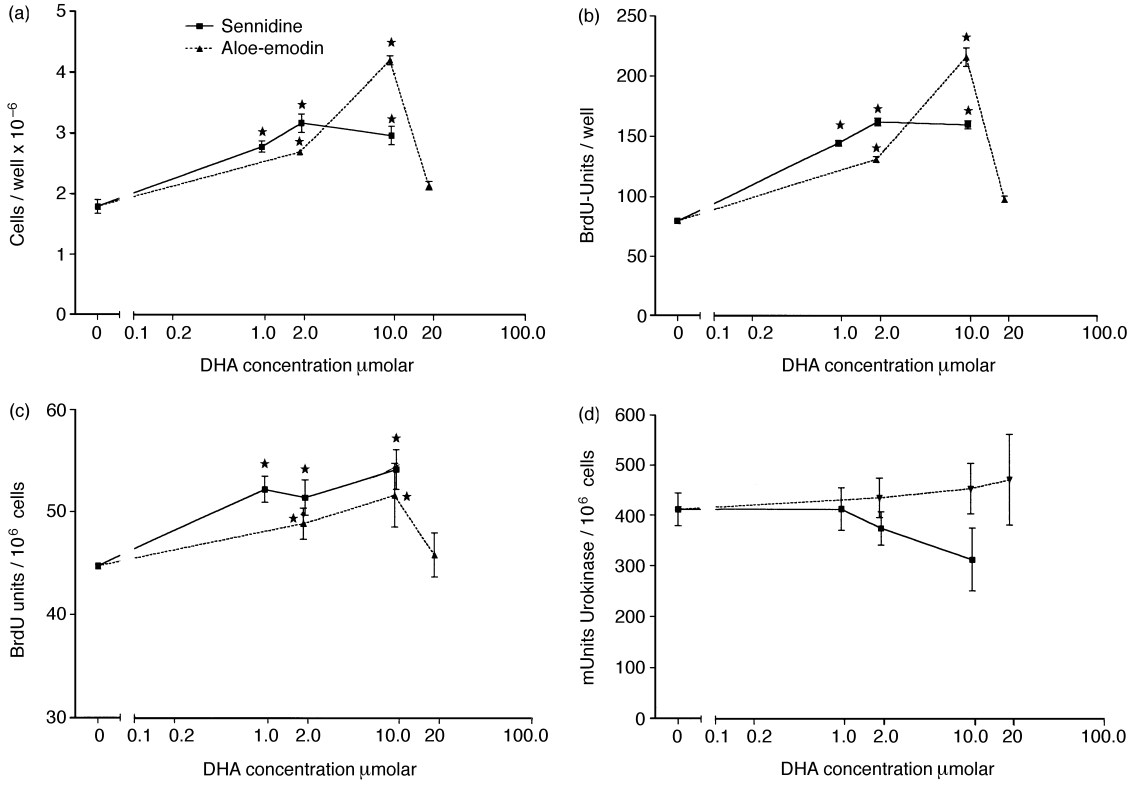

Figure 3. Effect of 1,8-dihydroxyanthraquinones (DHAs) on VACO235 adenoma cells. Cell numbers, DNA synthesis and urokinase secretion were determined as in Figure 2. (a) Cell number, (b) DNA synthesis per well, (c) DNA synthesis/10^6 cells, (d) urokinase secretion. Three representative experiments are presented as means ± standard deviation. *Increased at $P < 0.05$.

dianthrone- and anthraquinone-laxatives, respectively. Initial experiments had shown that their growth behaviour was highly dependent on cell density—dense cultures being less sensitive than sparse cultures. For optimal comparison with the results obtained with SW480 cells, cultures initiated with 5×10^4 cells/well were chosen for the final experiments shown in Figure 3. DHA treatment caused an approximate doubling of cell numbers and total BrdU incorporation by both compounds during the 48 h treatment period (Figure 3a, b). Dose–response relationships followed roughly the same kinetics for cell numbers and DNA synthesis: for sennidine it reached a plateau at 1 μM, while the effect of aloe-emodin had a maximum at 1 μM and then decreased again. The calculation of BrdU uptake/10^6 cells showed a slight increase at 10 μM for both DHAs (20 and 12%, respectively; significant at $P<0.05$). (Figure 3c). There was no effect of either substance on urokinase secretion (Figure 3d).

Parallel wells from control cultures and the 10 μM treatment groups were incubated with BrdU for the final 24 h of the treatment period, fixed and BrdU uptake determined by immunocytochemistry as described in Materials and Methods. The incidence of BrdU-positive cells and mitotic cells was determined by counting 2000 cells per well. The BrdU labelling index was elevated from 29 ± 2% in the controls to 35 ± 3% and 34 ± 2.5%, respectively, for sennidine- and aloe-emodin-treated cultures. Even though this stimulation was only slight, it correlated well with the increase in BrdU uptake/10^6 cells. The mitotic index was increased from 0.085% in controls to 0.35% and 0.63% for sennidine and aloe-emodin, respectively.

As sennoside and bisacodyl did not have any effects on SW480 carcinoma cells, VACO235 adenoma cells were exposed to high concentrations of both compounds. Surprisingly, both laxatives induced DNA synthesis in the adenoma cell line (Table 2). Sennoside (50 μM) did not affect the cell number per well, but increased BrdU uptake into the DNA 5-fold. The effect of bisacodyl was similar to the DHA effects on SW480 cells: using 5μM concentrations, the cell number was reduced to 25% of the control, while BrdU uptake increased 4-fold.

The effects of DHA-laxatives on normal colonic epithelial cells were tested using primary cultures of rat colonic epithelial cells. Cultures were established from rat term embryos and grew as co-cultures of fibroblasts and epithelial cells expressing cytokeratin (Figure 4a). They were treated at day 2 after seeding and only cytokeratin-positive cells were analysed. As an earlier study using human normal colonic epithelial cells had shown that they did not secrete significant amounts of urokinase [11, 13], only the effects on proliferation were measured. Normal colonic epithelial cells proliferate very slowly and BrdU uptake could not be measured by ELISA, only by immunocytochemistry, as described above. Untreated cells cultivated with MEM without EGF contained between 5 and 10% BrdU-positive S-phase cells (Figure 4b). This fraction was not significantly increased by sennidine or aloe-emodin at concentrations of up to 10 and 20 μM, respectively (Figure 4c).

Table 2. Effects of sennoside and bisacodyl on growth of VACO235 cells

Compound	Cell number (10^5 cells/well)	DNA synthesis (units/well)
Control	1.28 ± 0.08	92 ± 2
Sennoside (50 μM)	1.25 ± 0.03	339 ± 26
Bisacodyl (5 μM)	0.3 ± 0.02	275 ± 30

Short-term effects on protein phosphorylation

To determine whether the effects of DHAs on colon tumour cell lines are a secondary consequence of cytotoxic effects or are caused by specific actions of the laxatives, we determined their short-term effect on protein phosphorylation. Parallel cultures were treated with 5 μg/ml aloe-emodin and lysed after the times indicated in Figure 5(a). Fifty micrograms of protein per lane were analysed by electrophoresis and Western blot using an antibody against phosphotyrosine. The blot shows increased phosphate levels in proteins with molecular weights of 110, 78, 63, 57 and 37 kDa. Phosphorylation increased with time from 1 to 30 min after which a plateau level was reached. Aloe-emodin increased phosphorylation levels in a dose-dependent way in SW480 cells, while sennoside (50 μM) and bisacodyl (5 μM) did not have a significant effect (Figure 5b). In VACO235 adenoma cells, the same proteins were affected and

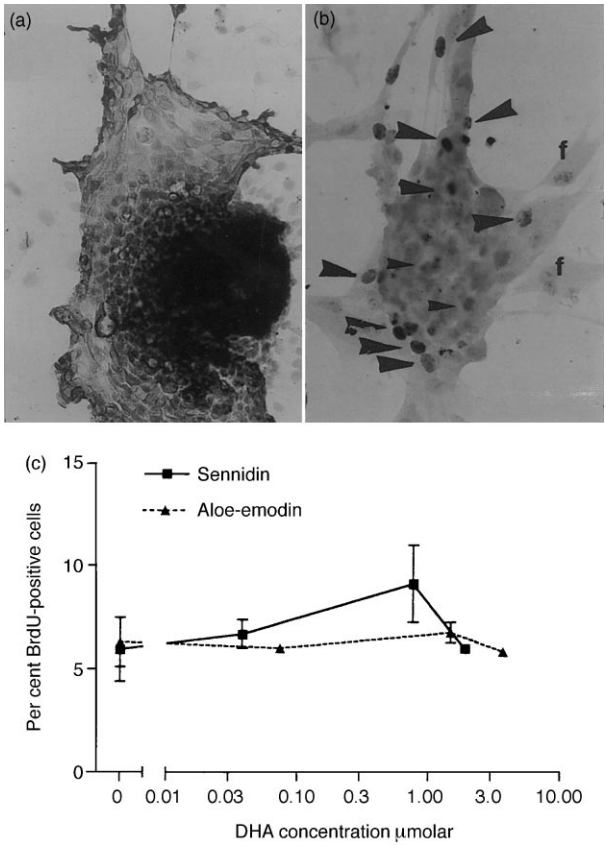

Figure 4. 1,8-Dihydroxyanthraquinones (DHAs) do not affect growth of normal colonic epithelial cells. Primary cultures of rat embryonic colonic epithelial cells were established as described in Materials and Methods and epithelial cells identified by their polygonal morphology and by staining with antikeratin antibodies (a). The fraction of S-phase cells in the culture was determined by 5-bromo-2′-desoxyuridine (BrdU) incorporation and immunocytochemistry (b). BrdU was incorporated in both epithelial cells (arrows) and fibroblasts (f). The fraction of epithelial cells in S-phase was determined by counting 1,000 cells in triplicate wells of control cultures and cultures after DHA treatment ((c) two experiments).

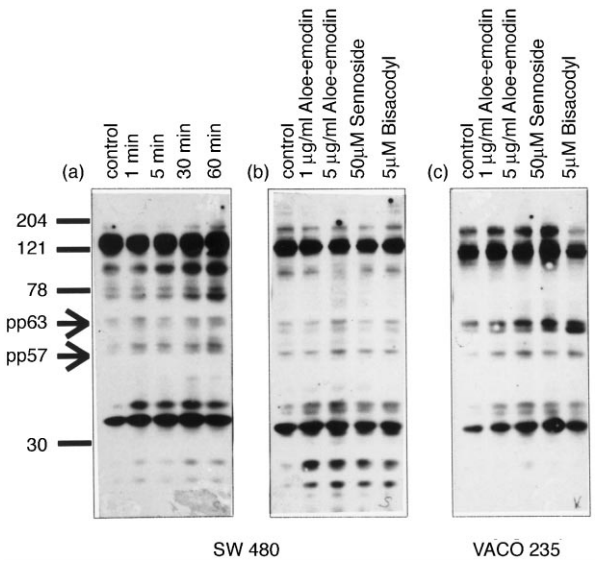

Figure 5. Laxative induced protein phosphorylation. Cultures were treated with aloe-emodin as described previously and lysed after 1, 5, 30 and 60 min. Proteins were analysed by sodium dodecyl sulphate–gel electrophoresis and Western blot using an anti-phosphotyrosine antibody. Phosphoproteins with molecular weights of 110, 78, 63, 57 and 37 kDa appeared after 1 min and increased in intensity with time (a) and dose (b, c) in both SW480 and VACO235 cells. Sennoside and bisacodyl did not have this effect in SW480, only in VACO235 (b, c).

phosphorylation was increased by aloe-emodin, as well as sennoside and bisacodyl (Figure 5c).

Stability of DHA in solution

To ensure that the DHAs rather than the reaction products created under culture conditions were responsible for the effects, we investigated the stability of the anthraquinone aloe-emodin and the dianthrone sennidine in the medium. The DHAs were extracted from the medium immediately after preparation and after 20 and 45 min of incubation. Analysis was performed by 'reverse phase' HPLC, as described in Materials and Methods. DHA concentrations were quantified relative to the concentration at time 0. Aloe-emodin levels remained completely stable for 45 min. Sennidine levels in the medium also did not decrease under reducing conditions and no peaks indicating the appearance of oxidation products (aloe-emodin or rhein) were observed for at least 45 min.

DISCUSSION

Epidemiological observations have suggested an association between laxative abuse and cancer risk, but the data are still contradictory [1–3]. Furthermore, DHAs—especially aloe-emodin—have been shown to induce mutations in various *in vitro* assays [4–7] and tumour promoting effects have also been shown in tissue culture models [8]. However, among the cell systems tested, there were no target cells for colon carcinogenesis. As tumour promotion is a highly species and tissue specific phenomenon, this fact makes interpretation of the data extremely difficult. Tumour promoting activity *in vivo* could not be demonstrated in the single study performed so far [9].

Our results demonstrate for the first time specific biological effects of DHA-laxatives on colonic epithelial cells. Performing such assays has the advantage that target cells for colorectal carcinogenesis can be investigated and tissue specific differences in growth control and sensitivity do not complicate the analysis. In addition, the premalignant and malignant cells used were of human origin. Normal colonic epithelial cells have only been obtained from rats, because sufficient human material has not been available. The use of rat colonic epithelial cells is valid as we have shown in a previous paper that they follow the same growth mechanisms as human colonic epithelial cells [22].

While this tissue culture model cannot mimic *in vivo* conditions, it does permit the study of relevant growth mechanisms, and it has been postulated that the changing growth behaviour of colonic epithelial cells *in vitro* mirrors tumour progression *in vivo*. These models have been used previously to investigate possible tumour enhancing or inhibiting compounds [11–14, 20]. Using phorbol esters, 1,2-diglycerides and bile acid, it has been established that these compounds induce stage specific effects in normal, premalignant and malignant colorectal epithelial cells *in vitro*. The effects reflect tumour stage specific regulatory mechanisms and are consistent with a stimulation of tumour growth and spread [10–14]. The present study has shown that the DHA-aglycones likewise induce growth and urokinase secretion in this model system in a stage specific way.

In carcinoma cells, the secretion of urokinase is the predominant effect of all DHAs. This protease facilitates metastasis by activating matrix degradation and digesting normal cells [15]. It has been shown to dissolve adenoma cells in coculture with malignant cells, but also causes rounding up of the tumour cells themselves and their subsequent death [11]. Simultaneously, cells were lost from the cultures. In this situation, it is difficult to ascertain the cause–effect relationship: liberation of urokinase into the medium might be an active effect causing cell loss or it could be shed into the medium as a consequence of cellular damage and lysis. The demonstration of specific signalling events prior to urokinase secretion argues for the former possibility. In addition, we have observed cell loss after addition of urokinase to the medium, especially under the serum free conditions used in our experiments (data not shown). We, therefore, assume that urokinase secretion is the primary factor causing cell loss.

DNA synthesis in DHA-treated cells was found to be stimulated in the remaining population both by BrdU uptake and by ^{3}H-thymidine incorporation. Unfortunately, cell loss makes any growth effects difficult to assess, because it counteracts stimulation of DNA synthesis or proliferation. At least some of the increase of DNA synthesis relative to cell number might be caused by preferential loss of resting cells.

In adenoma cultures, no urokinase secretion or cell loss was observed. As a nonspecific cytotoxic effect should not discriminate between cells from different tumour stages, this observation also supports the assumption that urokinase secretion in the carcinoma cell line was a primary effect in its own right. In the absence of cell loss, growth stimulation was more easily observed. Both cell numbers/well and BrdU uptake were increased in all six experiments. Parallel assessment of BrdU uptake/10^{6} cells and the fraction of S-phase cells in the culture showed that both are increased to a similar extent, indicating that the stimulation of proliferation is caused by a slight but significant increase in growth fraction. In normal colonic epithelial cells, the growth fraction was not

increased by DHA treatment. These effects could well enhance tumour growth and metastasis [11].

The DHA-glycoside sennoside did not stimulate DNA synthesis in the SW480 carcinoma cell line, but it did affect urokinase secretion, albeit at 5-fold higher concentrations than the aglycone. Bisacodyl—a diphenylic laxative—stimulated neither growth nor urokinase production, indicating that in SW480 the effect is specific for DHA-laxatives. In the adenoma cell line VACO235, sennoside and bisacodyl not only stimulated DNA synthesis, they were even more effective than the DHA-aglycons tested.

The phosphorylation patterns observed after laxative treatment resembled those identified after phorbol 1,2-diglyceride treatment [23] and after addition of various growth factors to the medium ([24] and data not shown). Specific phosphorylation of at least five different proteins including a pp63 and a pp57 band was seen, which has been described in previous publications [23, 24]. Phosphorylation is both time- and dose-dependent and it occurs under such conditions that cause biological effects subsequently.

An important problem with the study of DHAs—sennidine in particular—is their chemical instability. Sennidines are dianthrones that spontaneously disintegrate into two anthrone moities both in the colonic lumen and in various buffers. The anthrones are then oxidised to anthraquinones via a radical mechanism [25, 26]. As a certain amount of decomposition cannot be prevented, we tried to mimic conditions in the gut by providing reducing conditions and a low O_2 atmosphere, so that the reaction should be similar under both conditions. This minimised non-specific toxicity and membrane damage to the cells. Analysis of the treatment media has shown that both aloe-emodin and sennidine remained stable for at least 45 min under our culture conditions. As most of the DHA is rapidly excreted with the resulting diarrhoea [26], exposure times *in vivo* may be even shorter. This need not preclude effective exposure—1,2-diglycerides and phorbol ester [23] laxatives induce signalling events within minutes after exposure. It has to be assumed that DHAs remain stable for a sufficient amount of time to induce mitogenic signals.

In the colonic lumen, concentrations may be much higher: effective therapeutic doses of 0.15–0.3 g DHA represent approximately 0.5–1 mmol and could yield effective concentrations well in the dose range causing necrotic cell damage in carcinoma cell cultures. This is the effect that has also been observed *in vivo*—DHA treatment *in vivo* did not induce proliferation [27] but cell damage in rodents [28]. Such cell damage can induce regenerative proliferation and enhance tumour promotion *in vivo* [29]. However, colonic epithelium *in vivo* is protected from exogenous substances by a mucus barrier and it is difficult to judge the amount of DHA that actually reaches the colonic epithelium.

Metabolic studies have shown that DHAs are produced from their glycosides and reduced to anthrones by intestinal bacteria in the lower parts of the gastrointestinal tract [25]. The anthrone is supposed to be the active drug, but could not be studied because it cannot be obtained in a stable form. However, as it reacts to form sennidine and rhein in aqueous solution their effects on colonic epithelial cells are probably more relevant than that of the anthrone [30]. It is scarcely resorbed, but the oxidation products, namely the anthraquinones, are resorbed and undergo enterohepatic circulation [25, 26, 31]. While aloe-emodin has not been detected in the plasma or tissues, rhein plasma levels of 150–160 ng/ml have been measured between 3 and 11 h after a therapeutic dose of DHA-laxatives. This concentration stimulated urokinase secretion, but not DNA synthesis of carcinoma cells in our study.

In summary, out data demonstrate that laxatives and especially DHAs have distinct stage specific biological effects on colorectal tumour cells. DHA stimulates urokinase secretion of colorectal carcinoma cells and growth in the premalignant adenoma cells. Bisacodyl was more effective on premalignant cells. Growth effects on normal epithelium could not be observed but might well be caused by indirect mechanisms *in vivo*. Together these effects could facilitate tumour growth and spread.

1. Kune GA. Laxative use not a risk for colorectal cancer: data from the Melbourne Colorectal Cancer Study. *Z Gastroenterol* 1993, **31**, 140–143.
2. Nusko G, Schneider B, Muller G, Kusche J, Hahn EG. Retrospective study on laxative use and melanosis coli as risk factors for colorectal neoplasma. *Pharmacology* 1993, **47**, 234–241.
3. Siegers CP, Hertzberg-Lettin E von, Otte M, Schneider B. Anthranoid laxative abuse—a risk for colorectal cancer? *Gut* 1993, **34**, 1099–1101.
4. Westendorf J, Marquardt H, Poginsky B, Dominiak M, Schmidt J, Marquardt H. Genotoxicity of naturally occurring hydroxyanthraquinones. *Mutat Res* 1990, **240**, 1–12.
5. Bosch R, Friedrich U, Lutz WK, Brocker E, Bachmann M, Schlatter C. Investigations on DNA binding in rat liver and in Salmonella and on mutagenicity in the Ames test by emodin, a natural anthraquinone. *Mutat Res* 1987, **188**, 161–168.
6. Kodama M, Kamioka Y, Nakayama T, Nagata C, Morooka N, Ueno Y. Generation of free radical and hydrogen peroxide from 2-hydroxyemodin, a direct-acting mutagen, and DNA strand breaks by active oxygen. *Toxicol Lett* 1987, **37**, 149–156.
7. Bruggeman IM, Hoeven JC van der. Lack of activity of the bacterial mutagen emodin in HGPRT and SCE assay with V79 Chinese hamster cells. *Mutat Res* 1984, **138**, 219–224.
8. Wolfle D, Schmutte C, Westendorf J, Marquardt H. Hydroxyanthraquinones as tumor promoters: enhancement of malignant transformation of C3H mouse fibroblasts and growth stimulation of primary rat hepatocytes. *Cancer Res* 1990, **50**, 6540–6544.
9. Siegers CP, Siemers J, Baretton G. Sennosides and aloin do not promote dimethylhydrazine-induced colorectal tumors in mice. *Pharmacology* 1993, **47**, 205–208.
10. Friedman EA. Differential response of premalignant epithelial cell classes to phorbol ester tumor promoters and to deoxycholic acid. *Cancer Res* 1981, **41**, 4589–4599.
11. Friedman E, Urmacher C, Winawer S. A model for human colon carcinoma evolution based on the differential responses of cultured preneoplastic, premalignant and malignant cells to 12-*o*-tetradecanoylphorbol-13-acetate. *Cancer Res* 1984, **44**, 1568–1578.
12. Friedman E, Isaksson P, Rafter J, Marian B, Winawer S, Newmark H. Fecal diglycerides as selective mitogens for premalignant and malignant human colonic epithelial cells. *Cancer Res* 1989, **49**, 544–548.
13. Kahl-Rainer P, Marian B. Retinoids inhibit protein kinase C-dependent transduction of 1,2-diglyceride signals in human colonic epithelial cells. *Nutr Cancer* 1994, **21**, 157–168.
14. Marian B, Harvey S, Infante D, Markus G, Winawer S, Friedman E. Urokinase secretion from human colon carcinomas induced by endogenous diglycerides. *Cancer Res* 1990, **50**, 2245–2250.
15. Harvey SR, Lawrence DD, Madeja JM, Abbey SJ, Markus G. Secretion of plasminogen activators by human colorectal and gastric tumor explants. *Clin Exp Metastasis* 1988, **6**, 431–450.
16. Kohga S, Harvey SR, Weaver RM, Markus G. Localization of plasminogen activators in human colon cancer by immunoperoxidase staining. *Cancer Res* 1985, **45**, 1787–1796.
17. Wang H, Skibber J, Juarez J, Boyd D. Transcriptional activation of the urokinase receptor gene in invasive colon cancer. *Int J Cancer* 1994, **58**, 650–657.

18. Willson JKV, Bittner G, Oberley T, Meisner L, Weese JL. Cell culture of human colon adenomas and carcinomas. *Cancer Res* 1987, **47**, 2704–2713.
19. Weinstein IB. Nonmutagenic mechanisms in carcinogenesis: role of proteinkinase C in signal transduction and growth control. *Environ Health Perspect* 1991, **93**, 175–179.
20. Kahl-Rainer P, Karner-Hanusch J, Weiss W, Marian B. Five of six PKC-isoenzymes present in normal epithelial cells are lost during tumor development in the human colon. *Carcinogenesis* 1994, **15**, 779–782.
21. Kahl-Rainer P, Sedivy R, Marian B. Protein kinase C tissue localization in human colonic tumours suggests a role for adenoma growth control. *Gastroenterology* 1996, **110**, 1753–1759.
22. Schörkhuber M, Karner-Hanusch J, Sedivy R, *et al.* Survival of normal colonic epithelial cells from both rats and humans is prolonged by coculture with rat embryo colonic fibroblasts. *Cell Biol Toxicol* (in press).
23. Marian B, Winawer S, Friedman E. Tyrosine phosphorylation of a Mr 63,000 protein induced by an endogenous mitogen in human colon carcinoma cells, but not in normal colonocytes. *Cancer Res* 1989, **49**, 4231–4236.
24. Lee R, Ghose-Dastidaar J, Winawer S, Friedman E. Signal transduction through extracellular signal-regulated kinase-like pp57 blocked in differentiated cells having low protein kinase Cβ activity. *J Biol Chem* 1993, **268**, 5255–5263.
25. deWitte P. Metabolism and pharmacokinetics of anthranoids. *Pharmacology* 1993, **47**, 86–97.
26. deWitt P, Lemli L. The metabolism of anthranoid laxatives. *Hepatogastroenterol* 1990, **37**, 601–605.
27. Geboes K. Laxatives and intestinal epithelial cells: a morphological study of epithelial cell damage and proliferation. *Verh K Acad Geneeskd Belg* 1995, **57**, 51–74.
28. Mengs U, Rudolph RL. Light and electron-microscopic changes in the colon of the guinea pig after treatment with anthranoid and non-anthranoid laxatives. *Pharmacology* 1993, **47**, 172–177.
29. Marks F, Berry DL, Bertsch S, Fürstenberger G, Richter H. On the relationship between epidermal hyperproliferation and skin tumor promotion. In Hecker E, Fusenig NE, Kunz W, Marks F, Thielmann HW, eds. *Carcinogenesis* Vol. 7, *Cocarcinogenesis and Biological Effects of Tumour Promotors*. New York, Raven Press, 1982, 331–346.
30. deWitt P, Cuveele J, Lemli J. *In vitro* deterioration of rhein anthraquinone in cecal content of rats. *Pharm Acta Helv* 1992, **67**, 198–203.
31. Krumbegel G, Schulz HU. Rhein and aloe-emodin kinetics from Senna laxatives in man. *Pharmacology* 1993, **47**, 120–124.

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