Carcinogenesis vol.25 no.5 pp.703–712, 2004
DOI: 10.1093/carcin/bgh079

Ascorbic acid suppresses drug-induced apoptosis in human colon cancer cells by scavenging mitochondrial superoxide anions

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Although a high alimentary intake of antioxidant vitamins such as ascorbic acid may play an important role in cancer prevention, a high level of antioxidants may have quite different effects at different stages of the transformation process. In cancer development, the resistance of cells to apoptosis is one of the most crucial steps. We have tested the effects of ascorbic acid on apoptosis in HT-29 human colon carcinoma cells when induced by two potent apoptosis inducers, the classical antitumor drug camptothecin or the flavonoid flavone. Apoptosis was assessed based on caspase-3-like activity, plasma membrane disintegration and finally nuclear fragmentation and chromatin condensation. Ascorbic acid dose-dependently inhibited the apoptotic response of cells to camptothecin and flavone. RT–PCR analysis and western blot analysis revealed that ascorbic acid specifically blocked the decrease of bcl-X_L by camptothecin or flavone. An increased generation of mitochondrial $O_2^{-\bullet}$ precedes the down-regulation of bcl-X_L by camptothecin and flavone and ascorbic acid at a concentration of 1 mM prevented the generation of this reactive oxygen species. In conclusion, ascorbic acid functions as a potent antioxidant in mitochondria of human colon cancer cells and thereby blocks drug-mediated apoptosis induction allowing cancer cells to become insensitive to chemotherapeutics.

Introduction

Dietary factors play an important role in the tumorigenesis of colonic tissue at both the level of initiation as well as progression (1–4). With regard to chemoprevention, dietary antioxidants are considered to act as protective in colon carcinogenesis (5,6). Amongst the antioxidants, ascorbic acid (vitamin C) plays a central role as it contributes to the regeneration of vitamin E and constitutes a strong line of defence in retarding free radical induced cellular damage (7). Ascorbic acid was shown to reduce the spontaneous mutation rate in mismatch-repair deficient human colon cancer cells and to reduce microsatellite instability (8). Moreover, the DNA of lymphocytes from colorectal carcinoma patients contains a higher level of 8-oxo-2′-deoxyguanosine, as a marker of base alterations by reactive oxygen species leading to mutations during replication of DNA, and that this was found to be associated with lowered plasma levels of antioxidant vitamins (9). It was suggested therefore, that the decreased concentration of antioxidant vitamins might cause a pro-oxidative environment in blood cells of colorectal carcinoma patients (9). With regard to later stages of the carcinogenesis process that are triggered, e.g. by mutations in cell–cycle regulating proteins in the initiation phase, it is important to note that supplementation of vitamins C, A and E in patients with colorectal adenomas is effective in reducing abnormalities in cell kinetics that may be indicative of a pre-cancerous condition (10). However, in a 3-year placebo-controlled endoscopic follow-up and intervention study against growth and recurrence of colonic polyps, supplementation of 150 mg ascorbic acid daily together with other antioxidant vitamins did not result in significant differences between the two groups (11). Apoptosis is a crucial parameter in the carcinogenesis process as genetically damaged or mutated cells can be eliminated by apoptosis (12). High vitamin C intake, however, was associated with reduced colorectal apoptosis rates among individuals with adenomas and it was suggested therefore, that vitamin C supplements might be contraindicated in patients with a history of adenomas (13). In contrast, in an *in vitro* solid tumor model ascorbic acid displayed cytotoxic activities towards tumor cells and was suggested as an adjuvant cancer treatment (14).

To assess whether ascorbic acid interferes with apoptotis execution in transformed cells, we investigated the impact of ascorbic acid on apoptosis when initiated by the classical antitumor drug camptothecin or the equally potent flavonoid flavone in HT-29 human colorectal cancer cells. By using a semi-quantitative RT–PCR approach and western blotting we determined mRNA and protein levels of apoptosis-relevant gene products and by confocal microscopy we determined the role of oxygen radicals in the apoptotic response of the cells.

Abbreviations: COX-2, cyclooxygenase-2; GAP-DH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcriptase–polymerase chain reaction.

Materials and methods

Cell culture

HT-29 cells (passage 106) were provided by American Type Culture Collection and used between passage 150 and 200. Cells were cultured and passaged in RPMI-1640 supplemented with 10% FCS and 2 mM glutamine. Antibiotics added to the media were 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged at pre-confluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA (all materials for cell culture were from Invitrogen, Karlsruhe, Germany).

Preparation of stock solutions

Stock solutions of 100 mM L-ascorbic acid (Sigma, Diesenhofen, Germany) and 1 M mannitol (Roth, Karlsruhe, Germany) were prepared fresh in a modified Krebs buffer, containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 0.3 mM KH_2PO_4, 10 mM glucose and 10 mM HEPES–Tris, pH 7.4. Dilutions of these stock solutions were made directly in cell culture media. Stock solutions of flavone (30 mM; Sigma), camptothecin (10 mM; Sigma) and *p*-benzoquinone (1 mM; Sigma) were prepared in DMSO. DMSO concentrations in the cell culture experiments did not exceed 1% and controls were always treated with the same amount of DMSO as used in the corresponding experiments.

Measurement of $O_2^{-\bullet}$ in mitochondria

$O_2^{-\bullet}$ in mitochondria of HT-29 cells were visualized by confocal laser scanning microscopy using a TCS SP2 microscope (Leica, Bensheim, Germany). For staining of mitochondria, cells were grown on glass slides placed into Quadriperm wells (Merck, Darmstadt, Germany) and loaded with 500 nM MitoTracker Red CMXRos (Bioprobes, Leiden, The Netherlands) for the last 30 min of incubation. For detection of $O_2^{-\bullet}$, cells were loaded with 50 μM proxylfluorescamine (Bioprobes) for the last 2 h of incubation. 200 μM cysteine were added to the incubation media to yield an increase in the emission of proxylfluorescamine fluorescence due to the reduction of the fluorophore nitroxide to its corresponding hydroxylamine in the presence of superoxide (15). $O_2^{-\bullet}$ was detected after excitation with the UV-laser at emissions of 440–480 nm and mitochondria were visualized after excitation at 543 nm at emissions of 590–650 nm, respectively. The fluorescence ratios of proxylfluorescamine over MitoTracker were determined exclusively for the mitochondrial areas using the Leica Confocal Software, Version 2.5.

Detection of apoptosis

Caspase-3-like activity was measured as described previously (16), based on the method of Nicholson *et al*. (17). In brief, HT-29 cells were seeded at a density of 5×10^5/well onto 6-well plates (Renner, Dannstadt, Germany) and allowed to adhere for 24 h. Cells were then exposed for 24 h to the test compounds. Subsequently to the exposure to the test compounds, cells were trypsinized, cell numbers were determined and then the cells were centrifuged at 2500 *g* for 10 min. Cytosolic extracts were prepared by adding 750 μl of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM dithiotreitol (DTT), 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin and 10 mM HEPES–KOH, pH 7.4, to each pellet and homogenizing by 10 strokes. The homogenate was centrifugated at 100 000 *g* at 4°C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC (Calbiochem, Bad Soden, Germany) at a final concentration of 20 μM. Cleavage of the apopain substrate was followed by determination of emission at 460 nm after excitation at 390 nm using a fluorescence microtiter plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany).

Changes in membrane permeability, as another early apoptosis marker, were assessed by incubating 3×10^4 HT-29 cells on 24-well plates (Renner) with the test compounds or cell culture medium alone (control) for 24 h. Cells were stained with 1 μg/ml Hoechst 33342 (Sigma) and rate of accumulation of the dye in early apoptotic cells (18) was detected using an inverted fluorescence microscope (Leica DMIRBE) equipped with a bandpass excitation filter of 340–380 nm and a longpass emission filter of 425 nm. Photographs were made from at least three independent cell batches and the number of apoptotic cells was determined as the percentage of the total cell count.

Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33258 (Sigma). HT-29 cells (3×10^4) were incubated with the test compounds for the times indicated. Thereafter, cells were washed with PBS, allowed to air-dry for 30 min and then fixed with 2% paraformaldehyde prior to staining with 1 μg/ml Hoechst 33258 and visualization under the inverted fluorescence microscope. Photographs were made from at least three independent cell batches and apoptotic cells were determined according to the number of cells displaying chromatin condensation and nuclear fragmentation in comparison with total cell counts.

Semi-quantitative RT–PCR

RNA from HT-29 cells was isolated at the times indicated according to the method described by Chomczynski and Sacchi (19) with slight modifications (16). Reverse transcription was performed with 5 μg of isolated RNA. First strand cDNA synthesis was accomplished with an oligo-$(dT)_{15}$ primer (MBI Fermentas). Amplification of sequence-specific fragments (*Taq* polymerase was from Sigma) was performed with 30 cycles (95°C denaturation for 1 min, 55°C hybridization for 2 min, 72°C extensions for 2 min). RT–PCR products were separated on a 1% agarose gel and visualized by ethidium bromide. The amount of first strand used to amplify specific sequences was derived from the linear range of amplification. The amplified glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) sequence was used as a constitutively expressed control. The amplified products were photographed and the intensity of the bands was analyzed using the SigmaGel software. No products were obtained for all genes without reverse transcription indicating the specificity of mRNA determination. A λ-DNA/Eco*RI* + Hin*dIII* marker (MBI Fermentas) was used in all PCR experiments as a size control of the amplified products. Primers (Eurogentech) used were: GAP-DH (5′GACCACAGTCCATGCCATCACT3′ and 5′TCCACCACCCTGTTGCTGTAG3′), bax (5′ACCAGCTCTGAGCAGATCATG3′ and 5′CTTCTTCCAGATGGTGAGCGA3′), bak (5′TTACCGCCATCAGCAGGAACA3′ and 5′ATGGGACCATTGCCCAAG TTC3′), p21 (5′GGGATGTCCGTCAGAACCCAT3′ and 5′TCTTGGAGAAGATCAGCCGGC3′), cyclooxygenase-2 (COX-2) (5′TAGGAATGTTCCACCCGCAGT3′ and 5′GATATCATCTAGTCCGGAGCG3′), NF-κB (5′CTGAAGATGTGAAGCTGCAGC3′ and 5′TCTGAGCACCTTTGGATGCAC3′), c-myc (5′AGCTTGTACCTGCAGGATCTG3′ and 5′CAACTGTTCTCGTCGTTTCCG3′) and bcl-X_L (5′ACTGAATCGGAGATGGAGACC3′ and 5′AGGCTCTAGGTGGTCATTCAG3′).

Immunoblotting

HT-29 cells were incubated in 6-well cell culture plates (Renner) with or without effectors for 24 h and scraped off in Laemmli equilibration buffer containing 50 mM Tris, 100 mM DTT, 10% glycerin, 2% SDS and 0.1% bromphenol blue. Samples were centrifuged at 2500 *g* for 5 min and protein content in the supernatant was determined by the Bradford reaction (Bio-Rad, Munich, Germany). The samples were resolved by SDS–PAGE according to the method described by Schagger and von Jagow (20) and were electroblotted onto PVDF membranes (Roth). Control of protein transfer and identification of the molecular weight marker proteins was achieved by Ponceau Red staining. Thereafter, the blotting membranes were blocked for 1 h with TBST and then incubated with the primary antibody (anti-bcl-X_L, anti-actin, sc-1615; Santa Cruz, Heidelberg, Germany) for 1 h in a 1:1000 dilution in TBST. Bound antibodies were detected after 1 h incubation with HRP-conjugated secondary reagents (sc-2020 for anti-actin and sc-2004 for bcl-X_L; Santa Cruz) and 3-amino-9-ethylcarbazole and H_2O_2 as substrates. The stained bands were analyzed using the SigmaGel sofware with the area under the curve of density serving as the measure of protein quantity.

Calculations and statistics

Variance analysis between groups was performed by one-way ANOVA and significance of differences between control and treated cells were determined by a Bonferroni's multiple comparison test (GraphPadPrism). For each variable at least three independent experiments were carried out. Data are given as the mean ± SEM.

Results

Ascorbic acid blocks camptothecin- and flavone-induced apoptosis

Camptothecin and flavone were shown previously to stimulate caspase-3-like activity in HT-29 cells 6- and 7-fold, respectively, with a maximum of activation of ~24 h (16). These effects are achieved at concentrations of camptothecin and flavone of 50 and 150 μM, respectively, which represent the highest concentrations not causing any cytotoxic side effects (16). When ascorbic acid was provided it reduced the caspase-3-like activity when increased by camptothecin or flavone in a dose-dependent manner with EC_{50}-values for half maximal inhibition of 250.8 ± 11.4 μM and 189.4 ± 11.8 μM, respectively (Figure 1).

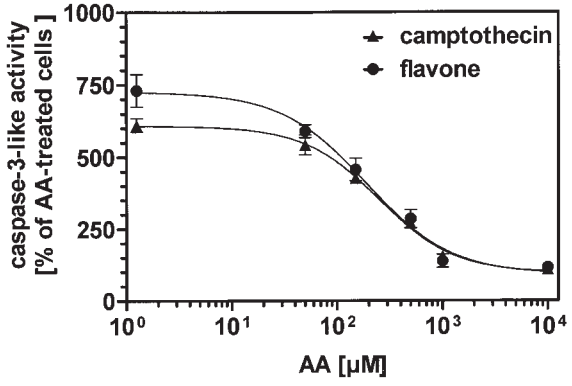

Fig. 1. Effects of ascorbic acid (AA) on camptothecin- and flavone-induced activation of caspase-3-like activity. HT-29 cells were incubated for 24 h in the presence of 50 μM camptothecin or 150 μM flavone and increasing concentrations of ascorbic acid. The caspase-3-like activity of cells treated with ascorbic acid alone was set as 100%. Caspase-3-like activity was assessed according to the cleavage of Ac-DEVD-AMC.

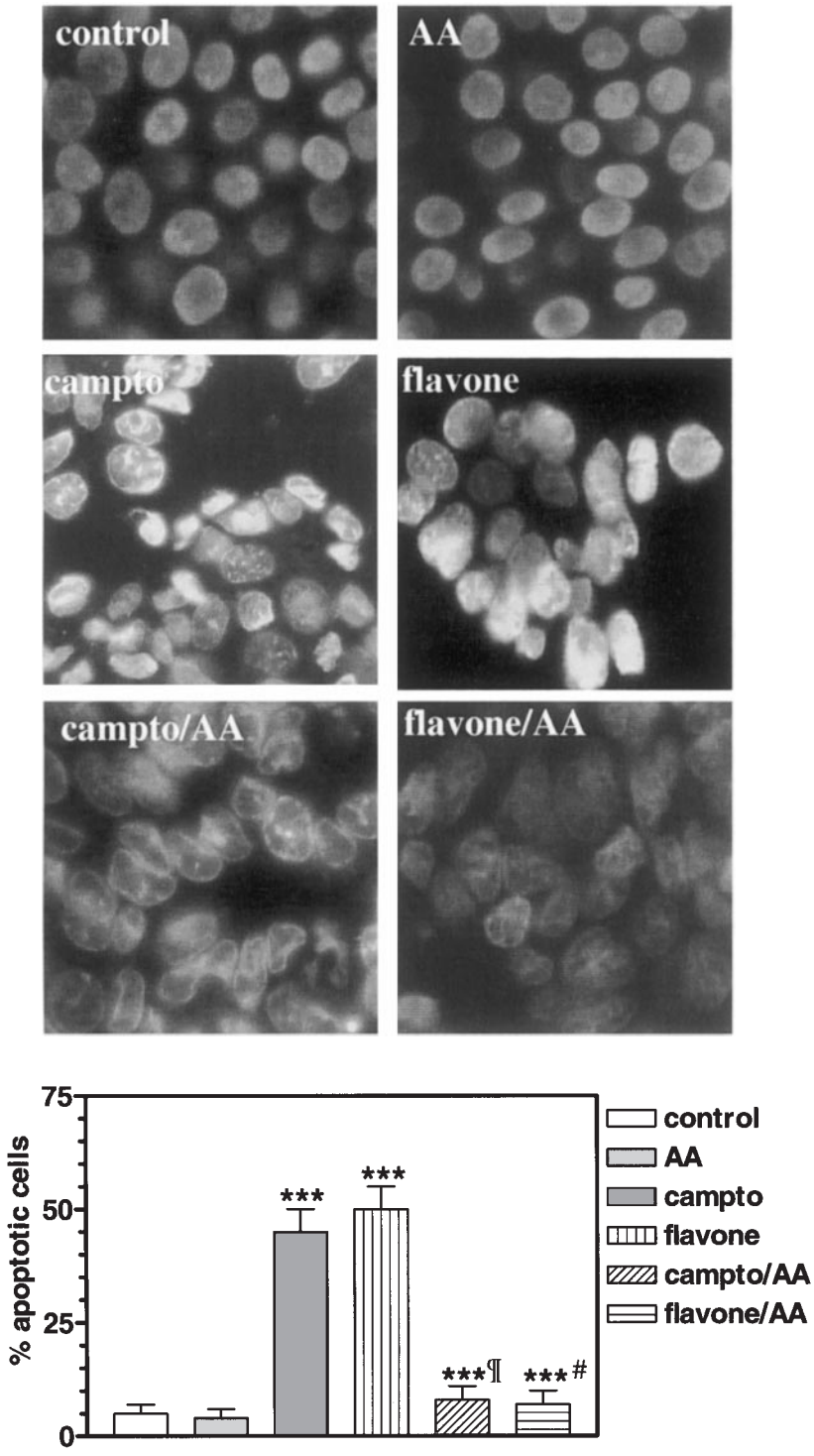

Fig. 2. Camptothecin- and flavone-mediated plasma membrane disintegration is reduced by ascorbic acid. Membrane desintegration in cells treated with medium alone (control) or with 1 mM ascorbic acid, or with 50 μM camptothecin (campto) or 150 μM flavone in the absence or presence of 1 mM ascorbic acid (AA) was assessed by uptake of Hoechst 33342 after 24 h. The percentage of apoptotic cells at 24 h is given in the lower panel. $^{***}P < 0.001$ versus control cells, or versus cells treated with ¶ camptothecin or # flavone.

Caspase-3-like activation by camptothecin and flavone was associated with the disintegration of the plasma membrane at 24 h of incubation (Figure 2) and was followed by nuclear fragmentation after 48 h (Figure 3). Both processes were strongly inhibited by 1 mM ascorbic acid (Figures 2 and 3). Ascorbic acid did not influence any of the apoptotic parameters measured when applied alone (Figures 2 and 3).

Ascorbic acid scavenges mitochondrial $O_2^{-\bullet}$

We have shown previously that nitric oxide (NO) scavenges mitochondrial $O_2^{-\bullet}$ and thereby prevents the down-regulation of bcl-X_L that is crucial for the occurrence of apoptosis in HT-29 cells (21). To investigate whether ascorbic acid blocks apoptosis also by its antioxidative capacity, we measured its influence on the camptothecin- and flavone-induced

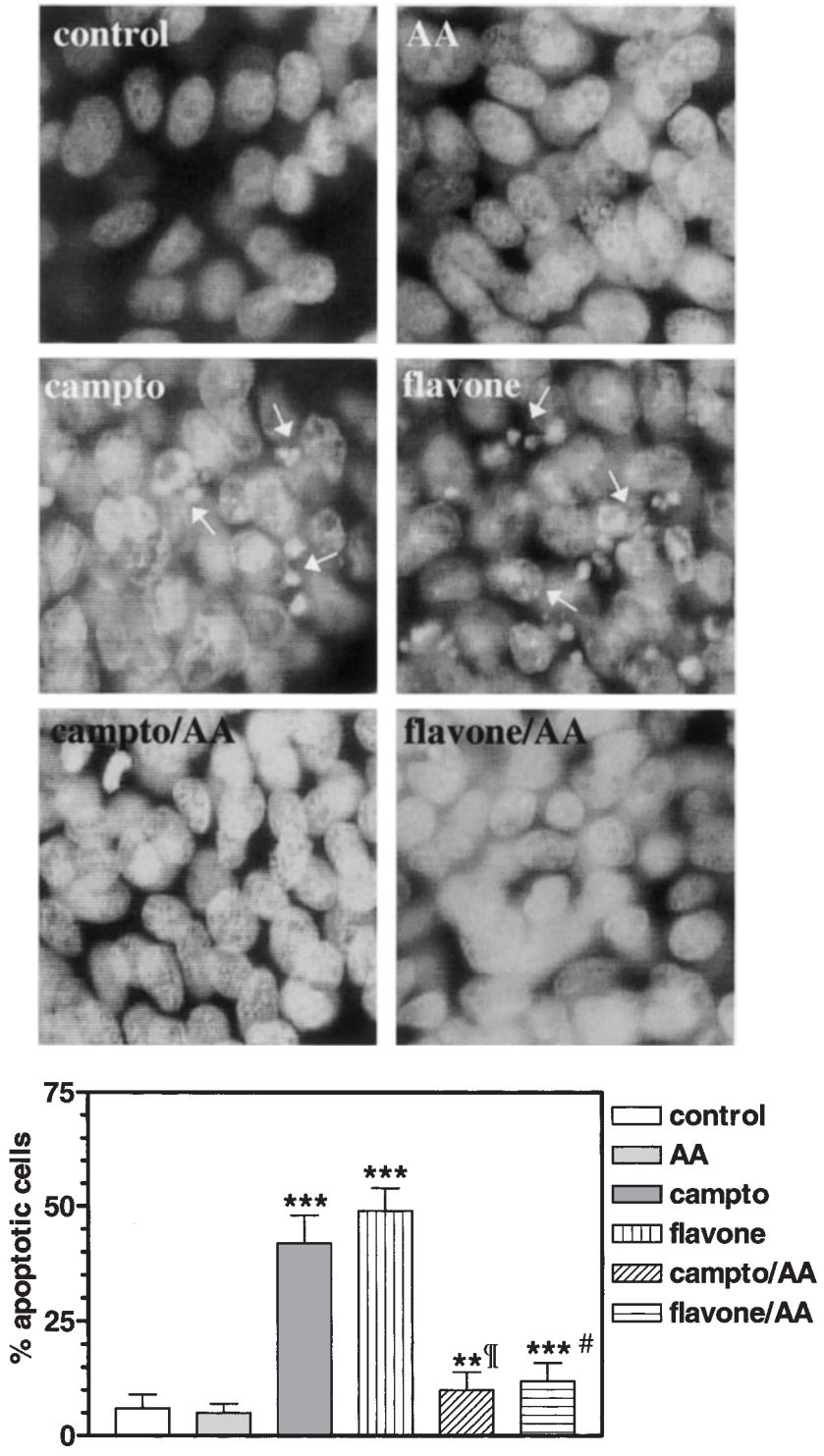

Fig. 3. Influence of ascorbic acid on nuclear fragmentation as induced by camptothecin or flavone in HT-29 cells. Nuclear fragmentation (arrows) was assessed after 48 h by Hoechst 33258 staining in HT-29 cells treated with medium alone (control) or with 1 mM ascorbic acid, or with 50 μM camptothecin (campto) or 150 μM flavone in the absence or presence of 1 mM ascorbic acid (AA) using an inverted fluorescence microscope. The percentage of cells displaying signs of chromatin condensation and DNA fragmentation is given in the lower panel at 48 h of incubation. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control, or ¶ versus cells treated with camptothecin, or # versus flavone-treated cells.

generation of $O_2^{-\bullet}$. Whereas camptothecin and flavone significantly increased the level of $O_2^{-\bullet}$ in mitochondria, ascorbic acid at a concentration of 1 mM potently reduced the amount of $O_2^{-\bullet}$ to a level observed in control cells (Figure 4). Levels of $O_2^{-\bullet}$ in cells treated with ascorbic acid alone did not differ from those in control cells (Figure 4).

Ascorbic acid prevents the down-regulation of bcl-X_L

The increased levels of $O_2^{-\bullet}$ in mitochondria of camptothecin- and flavone-treated cells were accompanied by a decrease of bcl-X_L at the mRNA-level (Figure 5A) as well as at the protein level (Figure 5B). Whereas ascorbic acid did not influence the level of bcl-X_L when applied alone, it clearly blocked the

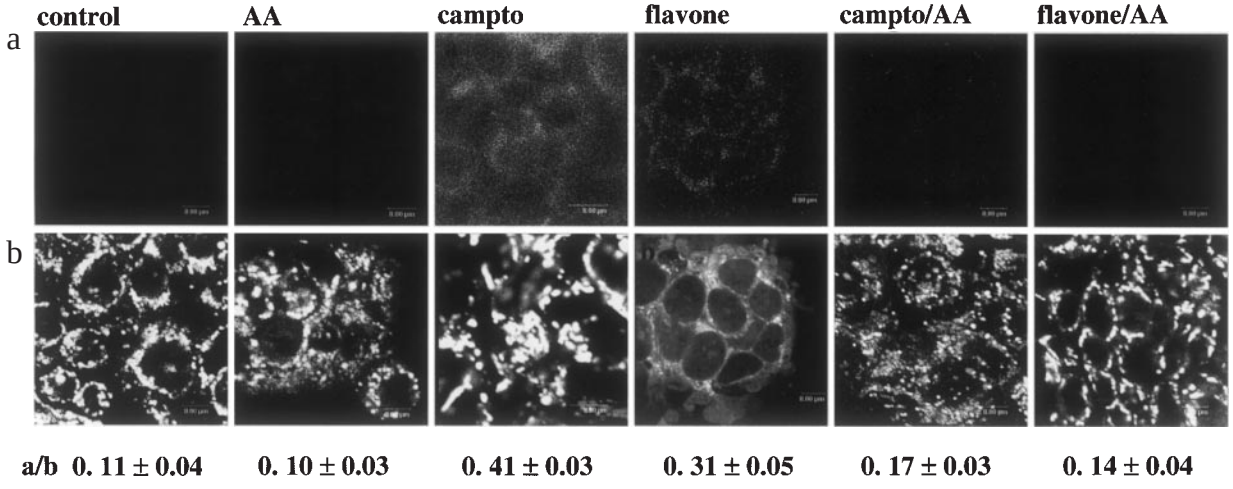

Fig. 4. Ascorbic acid prevents the appearance of mitochondrial $O_2^{-\bullet}$. Cells were incubated with medium alone (control), or with 1 mM ascorbic acid (AA), or with 50 μM camptothecin (campto) or 150 μM flavone in the absence or presence of 1 mM ascorbic acid for 6 h. During the last period of incubation cells were loaded with proxylfluorescamine for the detection of $O_2^{-\bullet}$ (a) in combination with MitoTracker for the visualization of mitochondria (b). The fluorescence ratios of a over b were determined for the mitochondrial areas only.

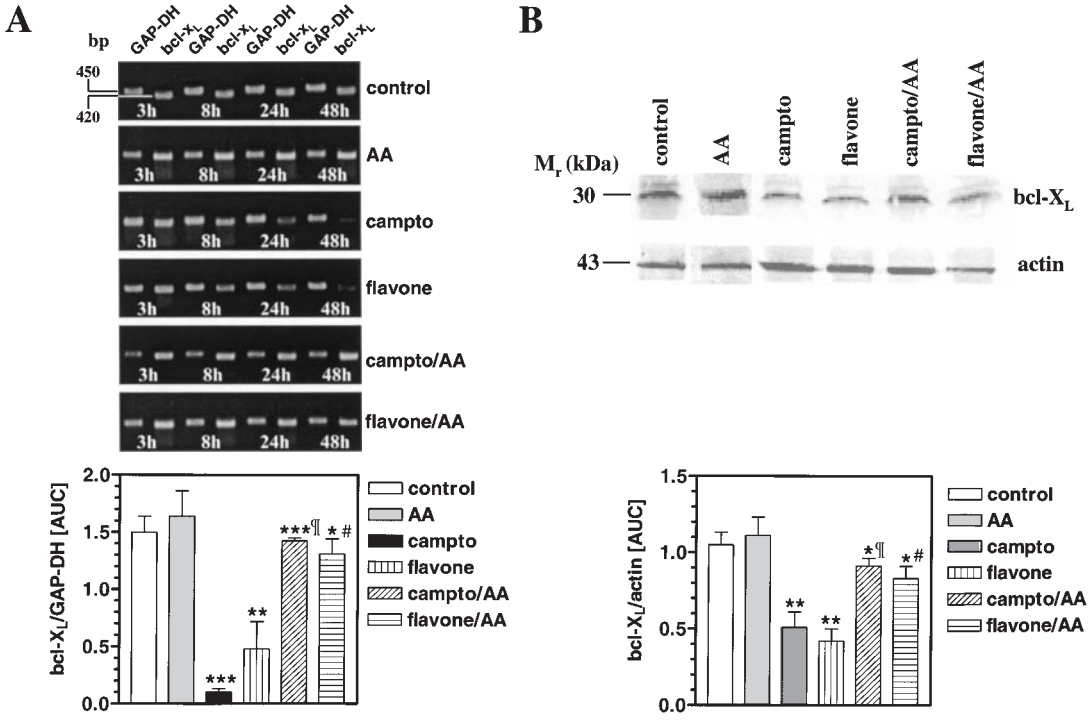

Fig. 5. Camptothecin- and flavone-mediated decreases of bcl-X_L are inhibited by ascorbic acid at the mRNA and protein level. (**A**) Semi-quantitative determination of bcl-X_L mRNA-levels in HT-29 cells by use of RT–PCR. Cells were incubated for the indicated time points either with medium alone (control), with 1 mM ascorbic acid (AA), with 50 μM camptothecin (campto), with 150 μM flavone or with flavone or camptothecin in combination with 1 mM ascorbic acid. Subsequently RNA was isolated, reverse transcribed and cDNA sequences of bcl-X_L and GAP-DH (as a constitutively expressed standard gene) were amplified by specific primers. The upper panels show the amplified cDNA products (representative experiment), the lower panels the ratio of target gene to standard gene at 24 h of incubation. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.01$ versus the control, or ¶ versus cells treated with camptothecin, or # versus flavone-treated cells ($n = 4$). (**B**) Determination of bcl-X_L by western blotting. Cells were incubated for 24 h as described under (A). Immunochemically detected bcl-X_L and actin (used as a constitutively expressed control) in the homogenate is shown in the upper panel, the ratio of bcl-X_L to actin is given in the lower panel. $^{*}P < 0.05$; $^{**}P < 0.01$ versus the control, or ¶ versus cells treated with camptothecin, or # versus flavone-treated cells ($n = 3$).

reductions caused by camptothecin or flavone (Figure 5). Moreover, this blockade of bcl-X_L down-regulation by ascorbic acid was specific, since the levels of other apoptosis-relevant genes, such as *Bax*, *Bak*, *p21* or *COX-2* were altered by camptothecin and flavone but those alterations remained unaffected by ascorbic acid (Table I). The transcript levels of *NF-κB* were reduced by camptothecin and flavone and this reduction was inhibited by the addition of ascorbic acid, although the inhibition did not reach the level of significance here (Table I). Changes in c-myc mRNA levels, however, were completely prevented in cells exposed to camptothecin and flavone when ascorbic acid was present

Table I. Transcript levels of apoptosis-relevant genes

Gene	Treatment	Time of treatment			
		3 h	8 h	24 h	48 h
Bax	Control	1.15 ± 0.06	1.13 ± 0.07	1.18 ± 0.05	1.27 ± 0.07
	Ascorbic acid	1.18 ± 0.18	0.94 ± 0.12	1.01 ± 0.11	1.09 ± 0.22
	Campto	1.22 ± 0.18	0.91 ± 0.19	0.66 ± 0.16[a]	0.63 ± 0.22[a]
	Flavone	1.12 ± 0.09	1.21 ± 0.13	1.15 ± 0.09	0.96 ± 0.16
	Campto/ascorbic acid	1.21 ± 0.06	1.15 ± 0.07	0.75 ± 0.09	0.83 ± 0.13
	Flavone/ascorbic acid	1.22 ± 0.11	1.18 ± 0.19	1.10 ± 0.14	1.19 ± 0.08
Bak	Control	0.80 ± 0.10	0.69 ± 0.08	0.75 ± 0.08	0.72 ± 0.07
	Ascorbic acid	0.91 ± 0.09	0.85 ± 0.11	0.69 ± 0.15	1.08 ± 0.18
	Campto	0.66 ± 0.10	0.39 ± 0.13	0.31 ± 0.05[b]	0.14 ± 0.05[c]
	Flavone	0.60 ± 0.04	0.68 ± 0.14	0.87 ± 0.12	2.02 ± 0.43[a]
	Campto/ascorbic acid	0.70 ± 0.10	0.43 ± 0.10	0.31 ± 0.11	0.22 ± 0.10
	Flavone/ascorbic acid	0.73 ± 0.11	0.73 ± 0.11	0.82 ± 0.08	1.16 ± 0.22
p21	Control	0.71 ± 0.07	0.57 ± 0.12	0.50 ± 0.22	0.65 ± 0.21
	Ascorbic acid	0.75 ± 0.23	0.82 ± 0.27	0.96 ± 0.24	1.11 ± 0.31
	Campto	0.57 ± 0.09	0.49 ± 0.11	0.65 ± 0.09	1.80 ± 0.12[b]
	Flavone	0.83 ± 0.11	0.90 ± 0.11	1.32 ± 0.09[a]	3.32 ± 0.60[b]
	Campto/ascorbic acid	0.53 ± 0.21	0.50 ± 0.09	0.52 ± 0.11	1.75 ± 0.32
	Flavone/ascorbic acid	0.83 ± 0.09	0.99 ± 0.12	1.27 ± 0.12	2.66 ± 0.21
COX-2	Control	0.70 ± 0.11	0.81 ± 0.12	0.80 ± 0.06	0.85 ± 0.10
	Ascorbic acid	0.85 ± 0.18	0.78 ± 0.21	0.81 ± 0.21	0.85 ± 0.04
	Campto	0.62 ± 0.12	0.38 ± 0.17	0.31 ± 0.09[b]	0.12 ± 0.07[c]
	Flavone	0.68 ± 0.20	0.41 ± 0.15	0.24 ± 0.16[a]	0.12 ± 0.08[b]
	Campto/ascorbic acid	0.71 ± 0.22	0.40 ± 0.10	0.29 ± 0.11	0.15 ± 0.08
	Flavone/ascorbic acid	0.62 ± 0.15	0.47 ± 0.17	0.23 ± 0.13	0.23 ± 0.12
NF-κB	Control	1.25 ± 0.17	1.43 ± 0.16	1.28 ± 0.13	1.31 ± 0.15
	Ascorbic acid	1.30 ± 0.26	1.40 ± 0.28	1.43 ± 0.26	1.36 ± 0.25
	Campto	1.18 ± 0.16	1.30 ± 0.23	0.26 ± 0.15[b]	0.24 ± 0.03[c]
	Flavone	1.20 ± 0.18	1.31 ± 0.17	0.55 ± 0.17[a]	0.30 ± 0.09[b]
	Campto/ascorbic acid	1.23 ± 0.23	1.18 ± 0.21	0.58 ± 0.11	0.51 ± 0.17
	Flavone/ascorbic acid	1.09 ± 0.23	1.05 ± 0.23	0.71 ± 0.18	0.65 ± 0.16
C-myc	Control	2.44 ± 0.25	2.18 ± 0.23	2.12 ± 0.31	2.31 ± 0.13
	Ascorbic acid	2.52 ± 0.26	2.69 ± 0.31	2.54 ± 0.20	1.82 ± 0.22
	Campto	2.31 ± 0.28	2.05 ± 0.31	1.13 ± 0.09[a]	1.02 ± 0.15[c]
	Flavone	2.07 ± 0.37	2.12 ± 0.16	2.16 ± 0.15	0.35 ± 0.06[c]
	Campto/ascorbic acid	2.22 ± 0.14	1.92 ± 0.22	1.66 ± 0.13[a]	1.60 ± 0.12[a]
	Flavone/ascorbic acid	2.43 ± 0.22	2.25 ± 0.25	1.961 ± 0.23	1.23 ± 0.23[a]

Transcript levels are given as the ratios of band intensities (AUC) of amplified cDNAs from target genes versus GAP-DH (used as a constitutively expressed control). Prior to semi-quantitative RT–PCR cells were treated with medium alone (control), with 1 mM ascorbic acid or with 150 μM flavone, 50 μM camptothecin (campto), 150 μM flavone and 1 mM ascorbic acid (flavone/ascorbic acid) or 50 μM camptothecin and 1 mM ascorbic acid (campto/ascorbic acid) for the indicated times.
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$ ($n = 4$). Comparisons were made for ascorbic acid, campto and flavone versus the control and for campto/ascorbic acid and flavone/ascorbic acid treated cells versus campto and flavone treated cells, respectively.

(Table I). Since flavone-induced apoptosis is observed already at 24 h of incubation (Figures 2 and 3) whereas c-myc mRNA levels are reduced no earlier than 48 h, we conclude that c-myc most likely does not contribute to apoptosis initiation by flavone or its inhibition by ascorbic acid.

Mitochondrial $O_2^{-\bullet}$ but not $OH^{-\bullet}$ are responsible for submitting HT-29 cells to apoptosis

To assess whether $O_2^{-\bullet}$ radicals or other $O_2^{-\bullet}$-derived reactive oxygen species such as hydroxyl radicals ($OH^{\bullet}$) are responsible for the induction of apoptosis we determined apoptosis rates induced by camptothecin or flavone in the presence of *p*-benzoquinone, a scavenger of $O_2^{-\bullet}$ (22), or of mannitol serving as a $OH^{\bullet}$ scavenger (23).

p-Benzoquinone prevented the generation of proxylfluorescamine fluorescence in mitochondria of both, camptothecin- or flavone-treated HT-29 cells, whereas mannitol was without effect (Figure 6A). Moreover, *p*-benzoquinone but not mannitol also prevented the disappearance of the bcl-X_L protein (Figure 6B) that was observed when cells were exposed to camptothecin or flavone alone (Figure 5B). The data strongly suggest that $O_2^{-\bullet}$ but not $OH^{\bullet}$ are the reactive oxygen species that regulate the expression of bcl-X_L. Finally, *p*-benzoquinone blocked camptothecin- or flavone-induced apoptosis as potently as ascorbic acid, whereas mannitol was without any effect (Figure 7).

Discussion

To understand why tumor cells resist apoptosis in response to the death signals has become a central theme in the understanding of tumor development (24) and one of the major goals

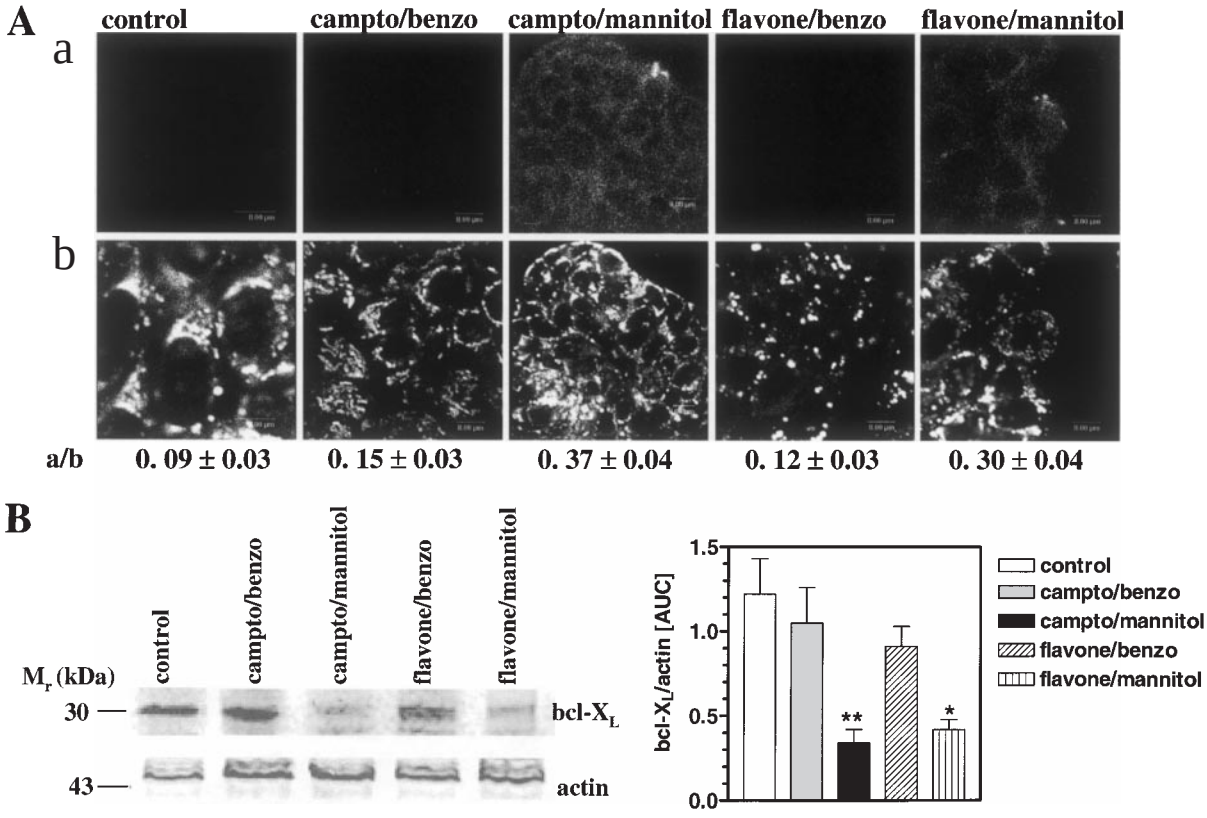

Fig. 6. Superoxide anion radicals as produced in mitochondria reduce the amount of bcl-X_L in HT-29 cells. Cells were incubated with medium alone (control), or with 50 μM camptothecin (campto) or 150 μM flavone either in the presence of 10 mM mannitol or 10 μM *p*-benzoquinone (benzo) for 6 h. (**A**) Fluorescence of $O_2^{-\bullet}$-sensitive proxylfluorescamine (a) was determined over mitochondrial areas as visualized by MitoTracker (b). (**B**) Levels of bcl-X_L protein were determined after 24 h of incubation by western blotting in relation to the actin control. The bcl-X_L to actin ratios are given in the right panel. $^{*}P < 0.05$; $^{**}P < 0.01$ versus the control.

in cancer therapy is to restore the sensitivity of transformed cells towards apoptotic signals and to allow the execution of apoptotic cell death (25,26). Whereas antioxidants are in particular regarded as effective chemopreventive agents due to their potent scavenging activities of reactive oxygen species (9), their role in later stages of the transformation process is not that clear. Reactive oxygen species may be essential as activators of apoptosis to remove cells that have accumulated mutations (27–29) and it was demonstrated that depletion of antioxidants is able to inhibit tumor growth in a transgenic mouse brain tumor model (30). Moreover, anti-apoptotic proteins that act as antioxidants, such as certain members of the bcl-2 family, are usually up regulated in cancer cells as a mechanism to escape apoptosis (31–33).

In the present study we have investigated the impact of ascorbic acid as a prototype antioxidant and essential micronutrient in human nutrition on apoptosis in HT-29 human colorectal carcinoma cells when initiated either by the classical antitumor drug camptothecin or by the flavonoid flavone. HT-29 cells in this regard serve here as a model for a colonic cancer tissue carrying all the genetic alterations acquired during the early stages of colorectal tumorigenesis (34) with expression of the truncated APC protein (35), as well as all alterations occurring in later stages with loss-of-function mutations in the tumor suppressor gene *p53* (36). We show here that ascorbic acid reduced the activity of the apoptosis effector caspase-3 dose-dependently with half maximal inhibitor concentrations of ~250 μM for camptothecin- and 190 μM for flavone-induced caspase-3 activation. In addition to caspase-3 activation, two further indicators of apoptosis occurrence, membrane disintegration and nuclear fragmentation, were highly increased by camptothecin and flavone and were effectively inhibited when 1 mM ascorbic acid was provided additionally. The concentrations at which ascorbic acid exerted its pronounced effects are found in normal human cells, such as brain cells (37) or circulating blood cells (38). We have shown previously that nitric oxide also prevents apoptosis in HT-29 cells by scavenging mitochondrial $O_2^{-\bullet}$ and thereby inhibits the flavone-mediated reduction in the levels of anti-apoptotic bcl-X_L and the subsequent depolarization of the mitochondrial membrane potential with the release of cytochrome c from mitochondria and finally the activation of caspase-3 (21). These results suggested that ascorbic acid by its radical scavenging activity could act via a similar or even identical mechanism to block apoptosis in HT-29 cells. Indeed ascorbic acid was able to potently reduce $O_2^{-\bullet}$ in mitochondria of cells treated with camptothecin or flavone. We did not see such a potent reduction of $O_2^{-\bullet}$ in mitochondria of camptothecin-treated cells by nitric oxide in our previous studies (21), suggesting that ascorbic acid possesses a higher antioxidative potential. Moreover, the ability of camptothecin (39) but not of flavone (40) to release reactive oxygen species, independent on mitochondrial $O_2^{-\bullet}$ generation, might explain the higher EC_{50} values found for inhibition of camptothecin-induced caspase-3 activation versus flavone-induced increase in caspase-3-like activity.

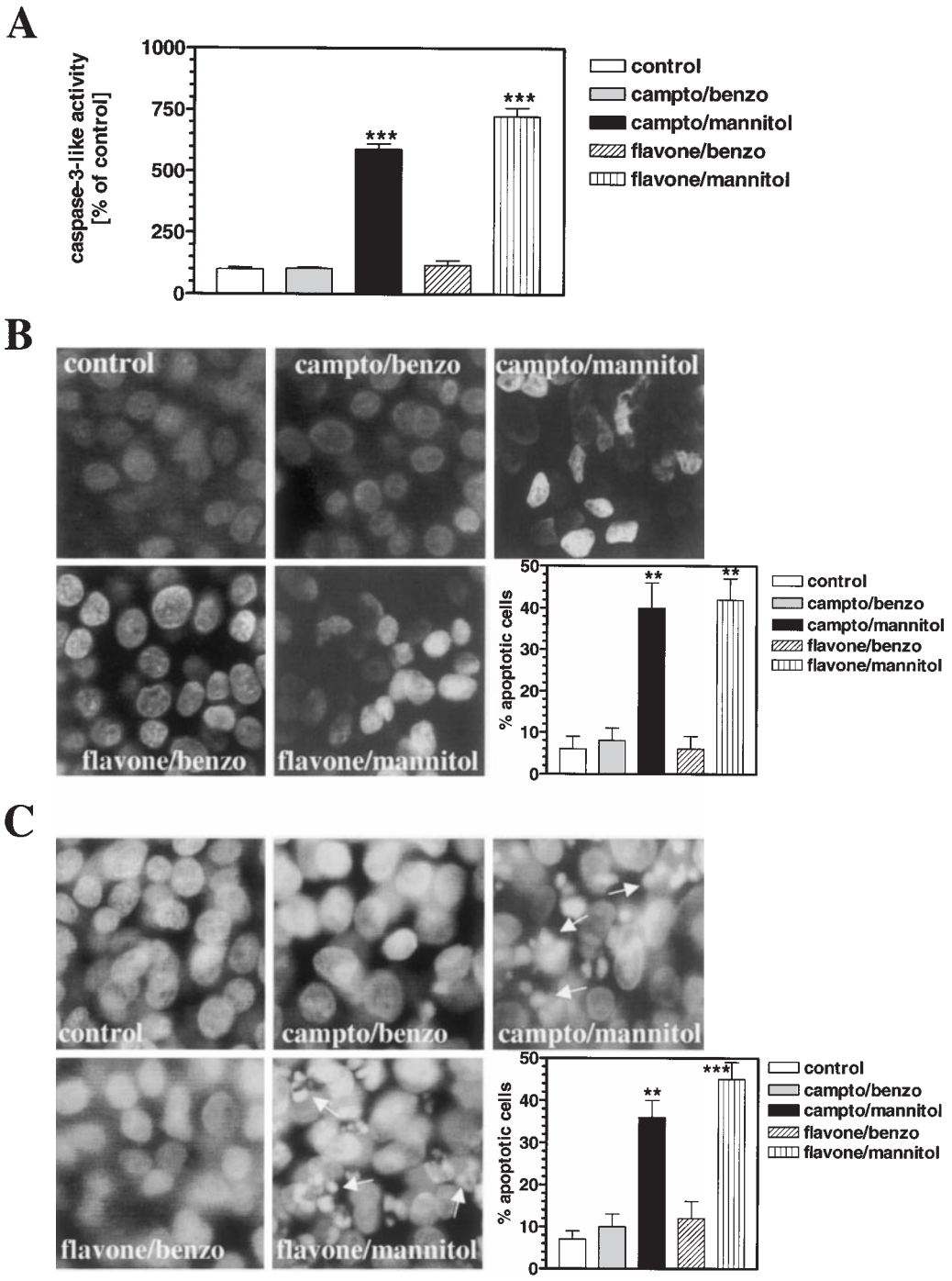

Fig. 7. Mitochondrial superoxide anion radicals induce apoptosis in HT-29 cells. (**A**) Caspase-3-like activities were assessed in HT-29 cells subsequently to their incubation for 24 h in medium (control), or in 50 μM camptothecin (campto) or 150 μM flavone either in the presence of 10 mM mannitol or 10 μM *p*-benzoquinone (benzo). $^{***}P < 0.001$ versus the control. (**B**) Uptake of Hoechst 33342 as an early apoptosis indicator was determined after 24 h incubation. The percentage of apoptotic cells at that time point is given in the lower right panel. $^{**}P < 0.01$ versus control cells. (**C**) Chromatin condensation (arrows) served as a late apoptosis marker and was estimated at 48 h by Hoechst 33258 staining. The percentage of cells displaying signs of condensed chromatin is shown in the lower right panel. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control cells.

The expression of the mitochondrial anti-apoptotic protein bcl-X_L was shown to be sensitive to reactive oxygen species (41) and we have shown previously that its down-regulation is crucial for the occurrence of apoptosis in transformed and non-transformed human colonocytes (21,42). Here we show that ascorbic acid prevents the decrease in bcl-X_L levels upon camptothecin and flavone treatment at the mRNA level as well as at the protein level. The effects of ascorbic acid on bcl-X_L expression and on apoptosis were mimicked by *p*-benzoquinone, a $O_2^{-\bullet}$ scavenger, whereas the $OH^{\bullet}$ scavenger mannitol did not alter bcl-X_L expression nor apoptosis. The data suggest strongly that $O_2^{-\bullet}$ are the reactive oxygen species that regulate bcl-X_L expression and apoptosis in HT-29 cells. Other apoptosis-relevant genes such as *Bax*, *Bak*, *p21*, *COX-2* and *NF-κB* also displayed altered transcript levels after camptothecin and flavone treatment of cells but ascorbic acid

failed to affect their changes. The observed down-regulation of *NF-κB* by camptothecin and flavone was also inhibited by ascorbic acid although this did not reach statistical significance. In this context, it is interesting to add that nitric oxide was shown to reduce mitochondrial $O_2^{-\bullet}$ generation in flavone-treated HT-29 cells and to block the flavone-mediated reduction in *NF-κB* mRNA levels (21). Our data presented here consequently provide additional evidence that *NF-κB* transcript levels are regulated by $O_2^{-\bullet}$. However, a clear difference was observed between the effects of NO and ascorbic acid on gene expression of *Bak*. In the presence of nitric oxide, bak mRNA levels declined in flavone-treated cells below that in control cells (21), whereas ascorbic acid reduced the flavone-mediated increase in bak transcript-levels at 48 h of incubation only modestly. This indicates, that besides their common action of scavenging reactive oxygen species, NO and ascorbic acid also alter gene expression selectively.

In conclusion, our studies provide evidence that ascorbic acid by its antioxidative capacity reduces drastically the production of reactive oxygen species in mitochondria that are required for the execution of drug-induced apoptosis. The data consequently raise the question of whether a high intake of ascorbic acid during chemotherapy of tumors is beneficial.

Acknowledgements

The authors greatly acknowledge the expert technical assistance of Mrs Beate Rauscher and Mrs Margot Siebler.

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Received October 6, 2003; revised December 19, 2003; accepted January 6, 2004