Anti-neoplastic effects of fucoxanthin and its deacetylated product, fucoxanthinol, on Burkitt's and Hodgkin's lymphoma cells

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Abstract. Fucoxanthin (FX) is a natural carotenoid with reported antitumorigenic activity. This study explored the effects of FX and its deacetylated product, fucoxanthinol (FXOH), on B-cell malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma and Epstein-Barr virus-immortalized B cells. Both FX and FXOH reduced the viability of these malignant B cells in a dose-dependent manner accompanied by the induction of cell cycle arrest during G_1 phase and caspase-dependent apoptosis. FXOH was approximately twice more potent than FX in these activities. In contrast, normal peripheral blood mononuclear cells were resistant to FX and FXOH. Strong and constitutive activation of nuclear factor-κB (NF-κB) is a common characteristic of many B-cell malignancies, and FXOH suppressed constitutive NF-κB activity. NF-κB inhibition was accompanied by downregulation of NF-κB-dependent anti-apoptotic and cell cycle regulator gene products, including Bcl-2, cIAP-2, XIAP, cyclin D1 and cyclin D2. The results indicated that FX and FXOH are potentially useful therapeutic agents in B-cell malignancies characterized by aberrant regulation of NF-κB.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human γ-herpesvirus and was the first human virus linked to cancer (1). EBV can infect and immortalize B-lymphocytes *in vitro*, giving rise to lymphoblastoid cell lines (LCLs). Consistent with this feature, persistent, latent EBV infection is present in several lymphoid malignancies including Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL) (2).

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BL represents a subtype of high-grade mature B-cell non-Hodgkin's lymphoma and is characterized by rapid cell proliferation and a generally aggressive clinical course. BL is typically seen in areas where malaria is endemic and can represent up to 50% of childhood cancers in some regions. In this endemic form of BL disease, EBV is present in all cases. BL also occurs sporadically at lower incidence throughout the world with associated lower levels of EBV positivity (2). The frequency of BL has increased in low-incidence countries since the 1980s, following the advent of human immunodeficiency virus/acquired immunodeficiency syndrome, and among these cases 30-40% of tumors are EBV-positive. Patients with human immunodeficiency virus-associated lymphoma pose additional therapeutic challenges, particularly the risk of overwhelming opportunistic infections (3).

HL is a B cell lymphoma generally comprising only a small proportion of the characteristic malignant Hodgkin/Reed-Sternberg (HRS) cells, but with abundant non-malignant immunocytes recruited by various cytokines secreted by HRS cells. EBV is present in the HRS cells in ~40% of classical HL cases (2). Although multidrug approaches have been very successful in the treatment of HL, unfortunately, latent toxicity of these agents appear after several years in the form of secondary malignancies and cardiovascular disease (4). In addition, some patients will suffer from refractory disease or experience a relapse. Therefore, the current goal in HL treatment is to find new therapies that specifically target the deregulated signaling cascades that cause HRS cell proliferation and resistance to apoptosis. In many cancers, nuclear factor-κB (NF-κB) is constitutively activated, protecting the tumor cells against apoptosis. Indeed, lesions from patients with EBV-associated B-cell lymphomas show activation of NF-κB, and thus inhibition of NF-κB is a much sought-after therapeutic target in a variety of cancers (5,6).

Carotenoids are a family of natural pigments that include fucoxanthin (FX), an abundant constituent of edible brown algae. FX has several reported biological functions including anti-oxidant, anti-inflammatory, anti-cancer, anti-obesity, anti-diabetic, anti-angiogenic and anti-malarial activities (7). FX is also an inhibitor of pivotal proinflammatory mediators including nitric oxide and cytokines, and affects signaling molecules involved in the inflammatory processes such as

NF-κB and mitogen-activated protein kinases (8). In mammals, dietary FX is deacetylated into fucoxanthinol (FXOH) in the intestinal tract by lipases and esterases from the pancreas or intestinal cells, and is then incorporated as FXOH into the blood circulation (7).

This study tested the hypothesis that FX and FXOH, through the inhibition of NF-κB, could be potentially useful therapeutic agents in the treatment of BL and HL.

Materials and methods

Extraction and isolation. FX was extracted from the brown seaweed *Cladosiphon okamuranus Tokida* using acetone as the solvent, and purified by column chromatography, liquid-liquid partition, and then recrystallization up to >95% purity. It was further purified by RP-HPLC up to >98% purity, in preparation for *in vitro* assays. FXOH was prepared by enzymatic hydrolysis of the purified FX using porcine pancreatic lipase. Briefly, 195 mg of FX, 2 g of sodium taurocholate and 2 g of porcine pancreatic lipase (Type II; Sigma-Aldrich, St. Louis, MO) were dissolved in 30 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction buffer was incubated at 37˚C for 3 h. FXOH was purified by ODS column chromatography, liquid-liquid partition and recrystallization. We prepared 142 mg of purified FXOH for this study (>95% purity, 72% yield). The FXOH was also further purified up to >98% purity by RP-HPLC, for *in vitro* assays. The identity and purity of the products were confirmed by comparison with reference FX (Wako Pure Chemical Industries, Osaka, Japan) and data in the literature.

Cells and cultures. Raji and Daudi are EBV-positive BL cell lines, whereas BJAB and Ramos are EBV-negative BL cell lines. B95-8/Ramos and B95-8/BJAB are Ramos and BJAB cells, respectively, infected with the B95-8 strain of EBV. LCL-Ka and LCL-Ku are EBV-immortalized human B-cell lines generated from peripheral blood mononuclear cells (PBMC) of healthy volunteers. PBMC were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden) and washed with phosphate-buffered saline. L428, KM-H2, HDLM-2 and L540 are HL cell lines. All cell lines were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10 or 20% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin.

Cell viability and assay of apoptosis. The effects of FX and FXOH on cell viability were assessed using water-soluble tetrazolium (WST)-8 (Wako Pure Chemical Industries). Briefly, $1x10^5$ cells/ml were incubated in a 96-well microculture plate in the absence or presence of various concentrations of FX or FXOH. After 24-h culture, WST-8 (5 μl) was added for the last 4 h of incubation and the absorbance at 450 nm was measured using an automated microplate reader. Mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provided a measure of cell proliferation. Apoptotic events in cells were detected by staining with phycoerythrin-conjugated Apo2.7 monoclonal antibody (Beckman-Coulter, Marseille, France) (9) and analyzed by flow cytometry (Epics XL, Beckman-Coulter, Fullerton, CA).

Cell cycle analysis. Cell cycle analysis was performed with the Cycletest™ Plus DNA DNA reagent kit (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Briefly, $1x10^6$ cells were washed with a buffer solution containing sodium citrate, sucrose and dimethyl sulfoxide, suspended in a solution containing RNase A, and then stained with 125 μg/ml propidium iodide for 10 min. Cell suspensions were analyzed on a Coulter EPICS XL using EXPO32 software. The population of cells in each cell cycle phase was determined with MultiCycle software.

In vitro measurement of caspase activity. Caspase activity was measured with the colorimetric caspase assay kits (MBL, Nagoya, Japan). Cell extracts were prepared using cell lysis buffer and assessed for caspases-3 and -9 activities using colorimetric probes. Colorimetric caspase assay kits are based on detection of the chromophore *p*-nitroanilide after cleavage from caspase-specific-labeled substrates. Colorimetric readings were performed in an automated microplate reader at an optical density of 405 nm.

Western blotting. Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the specific antibodies. The bands were visualized by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Antibodies to cyclin D2, cIAP-2, IκBα and NF-κB subunits p50, p65, c-Rel, p52 and RelB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Bax, Bcl-2 and actin were purchased from NeoMarkers (Fremont, CA). Antibodies to XIAP and cyclin D1 were obtained from Medical & Biological Laboratories (MBL, Nagoya, Japan). Antibodies to survivin, caspase-3, -9, Bcl-x_L and phospho-IκBα (Ser32 and Ser36) were purchased from Cell Signaling Technology (Beverly, MA). The antibody to poly(ADP-ribose) polymerase (PARP) was purchased from BD Transduction Laboratories (San Jose, CA).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Cells were cultured and examined for inhibition of NF-κB after exposure to FXOH for 24 h. Nuclear proteins were extracted as described by Antalis and Godbolt (10) with modifications, and NF-κB binding activity to the NF-κB element was examined by EMSA. Briefly, 5 μg of nuclear extracts were preincubated in a binding buffer containing 1 μg poly-deoxy-inosinic-deoxycytidylic acid (GE Healthcare Biosciences), followed by the addition of ^{32}P-labeled oligonucleotide probes containing the NF-κB element. The mixtures were incubated for 15 min at room temperature. The DNA protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography. The probes and competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: a typical NF-κB element from the IL-2 receptor α chain (IL-2Rα) gene (5'-gatcCGGCAGGGGAATCTCCCTCTC-3') and an AP-1 element of the IL-8 gene (5'-gatcGTGATGACTCAGGTT-3'). The above underlined sequences represent the NF-κB and AP-1 binding sites, respectively.

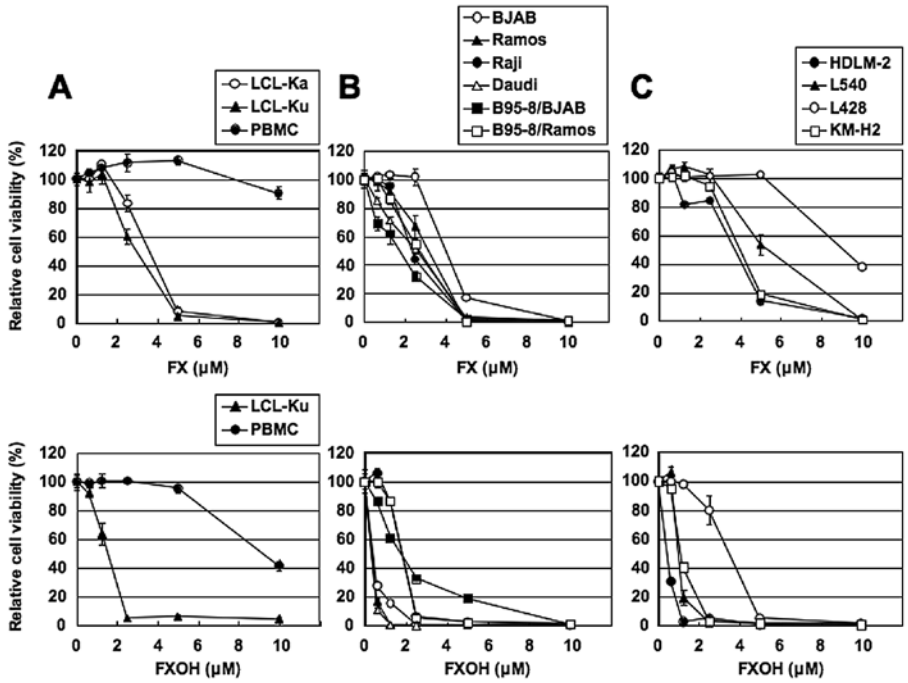

Figure 1. FX and FXOH reduce cell viability of EBV-immortalized human B (A), BL (B) and HL cell lines (C). Cell lines and PBMC from a healthy control were incubated in the presence of various concentrations of FX or FXOH for 24 h. The viability of the cultured cells was determined by WST-8 assay. Relative viability of the cultured cells is presented as the mean ± SD determined for cells from triplicate cultures.

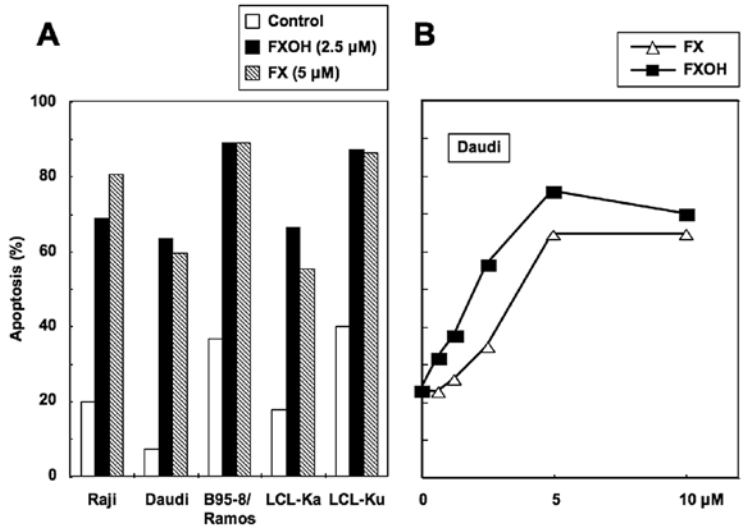

Figure 2. FX and FXOH induce apoptosis. (A) Cell lines were incubated with or without FX (5 μM) or FXOH (2.5 μM) for 24 h. (B) Daudi cells were treated with various concentrations of FX or FXOH for 24 h. Cells were harvested, then stained with the Apo2.7 monoclonal antibody and analyzed by flow cytometry. Data are percentages of apoptotic cells.

Results

FX and FXOH reduce cell viability of EBV-immortalized human B-cell lines, BL and HL cell lines. We first examined the effects of FX and FXOH on the cell viability of EBV-immortalized human B-cell lines. Cells cultured in the presence of various concentrations of FX or FXOH for 24 h showed dose-dependent decrease in cell viability in two EBV-immortalized human B-cell lines, as assessed by WST-8 assay (Fig. 1A). PBMC from healthy volunteers were less susceptible to FX and FXOH than the human B-cell lines. Treatment of EBV-positive and -negative BL cell lines (Fig. 1B) and HL cell lines (Fig. 1C) with FX or FXOH also resulted in lower cell viability. The FXOH-induced suppression of cell viability was more pronounced than that induced by FX.

FX and FXOH induce apoptosis. We next examined whether induction of apoptosis accounted for the reduced cell viability observed in these cell lines. Cells were treated with 5 μM of FX or 2.5 μM of FXOH and then probed with the Apo2.7 monoclonal antibody. FX (5 μM) and FXOH (2.5 μM) increased the proportion of apoptotic cells in EBV-immortalized human B-cell lines and BL cell lines by similar levels (Fig. 2A). Exposure of Daudi cells to FX and FXOH also induced apoptosis in a dose-depen-

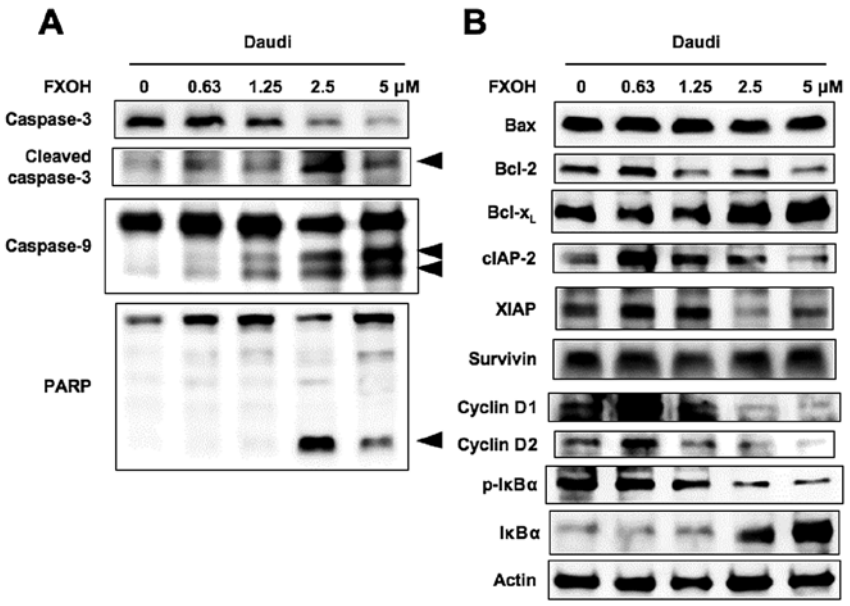

Figure 3. Western blot analysis. (A) Induction of caspase activity by FXOH. Daudi cells were incubated with the indicated concentrations of FXOH for 24 h. Cellular proteins were resolved by SDS/polyacrylamide gel electrophoresis, and caspase activity was detected by cleavage of PARP, caspase-3 and -9 using immunoblot analysis. Arrowheads indicate the cleaved forms of PARP, caspase-3 and -9. (B) FXOH inhibits the expression of NF-κB-regulated proteins involved in driving cell proliferation and survival, as well as upstream events in the NF-κB pathway. Daudi cells were incubated with the indicated concentrations of FXOH for 24 h. The content of different apoptosis and cell cycle regulatory proteins as well as phosphorylated and total IκBα was evaluated by western blotting. Actin was used as a protein-loading control.

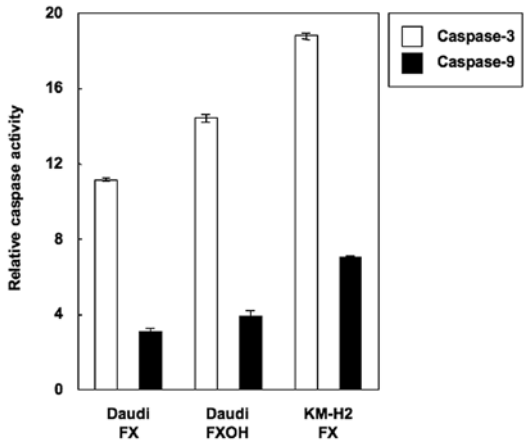

Figure 4. FX and FXOH induce caspase activities. Daudi and KM-H2 cells were treated with or without FX (10 μM) or FXOH (5 μM). After 6 h, cell lysates were prepared and incubated with the labeled caspase substrates, and caspase activity was measured using an automated microplate reader. Caspase activity is expressed relative to untreated cells, which were assigned a value of 1. Values are mean ± SD (n=3).

dent manner (Fig. 2B). The FXOH-induced apoptosis was more pronounced than that induced by FX. These results indicate that the inhibitory effects of FX and FXOH on the viability of cell lines reflect the agents' pro-apoptotic properties.

FX- and FXOH-induced apoptosis is caspase-dependent. We next investigated whether the observed apoptosis was due to caspase activation. Cell extracts were obtained after various treatments and processed for immunoblot analysis. As shown in Fig. 3A, immunoblot analysis demonstrated the cleaved products of PARP, caspases-3 and -9 induced by FXOH in a dose-dependent manner. The immunoblotting allowed us to examine the processing of caspases, but did not indicate whether the cleavage products were enzymatically active. Therefore, caspases-3 and -9 activities were determined by cleavage of caspase-specific-labeled substrates in colorimetric assays. Both FX and FXOH activated caspase-3 and -9 in Daudi and KM-H2 cells (Fig. 4). These results indicated that caspase activation plays a role in the FX- and FXOH-induced apoptosis observed in these cell lines.

FX and FXOH cause G_1 cell cycle arrest. We next examined the effects of FX and FXOH on cell cycle-regulatory mechanisms using Daudi, KM-H2 and L540 cells. Cultivation with 2.5 μM of FX or 1.25 μM of FXOH for 24 h increased the population of cells in the G_1 phase, with reduced numbers of cells in the S phase, relative to untreated cells (Fig. 5). Thus, FX and FXOH suppressed the proliferation of BL and HL cell lines by arresting the cells in the G_1 phase of the cell cycle.

Effects of FXOH on the expression of apoptosis and cell cycle regulatory proteins. To clarify the molecular mechanisms of FX- and FXOH-induced inhibition of cell growth and apoptosis, we used western blot analysis to investigate the mechanism of FXOH-induced changes in the expression of several intracellular regulators of cell cycle and apoptosis. As shown in Fig. 3B, FXOH did not alter the expression levels of anti-apoptotic proteins Bcl-x_L and survivin, or pro-apoptotic protein Bax. In contrast, FXOH downregulated the expression levels of anti-apoptotic proteins Bcl-2, cIAP-2 and XIAP in a dose-dependent manner. Furthermore, FXOH downregulated the expression levels of cell cycle regulatory proteins cyclins D1 and D2 dose-dependently. The results demonstrated that FXOH-mediated growth inhibition and apoptosis was associated with reduced expression of Bcl-2, cIAP-2, XIAP, cyclin D1 and cyclin D2 in the Daudi cells.

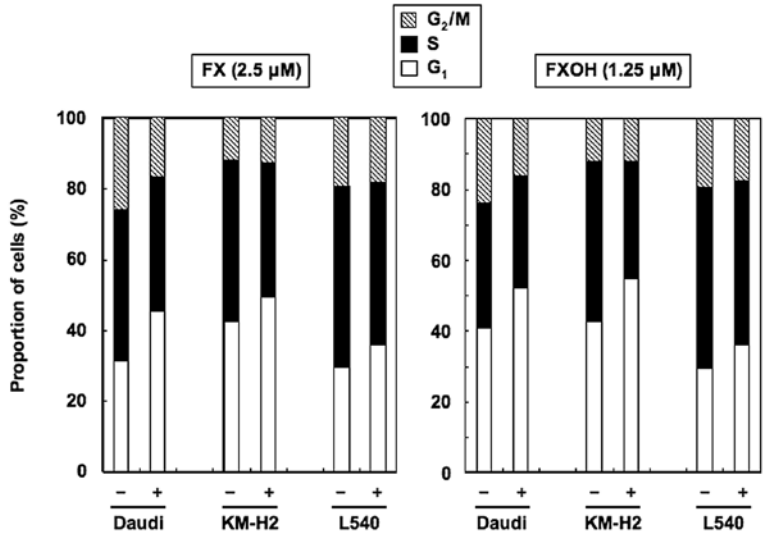

Figure 5. FX and FXOH induce G_1 cell cycle arrest in BL and HL cell lines. Daudi, KM-H2 and L540 cells were incubated in the absence or presence of FX (2.5 μM) or FXOH (1.25 μM) for 24 h. Then, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry.

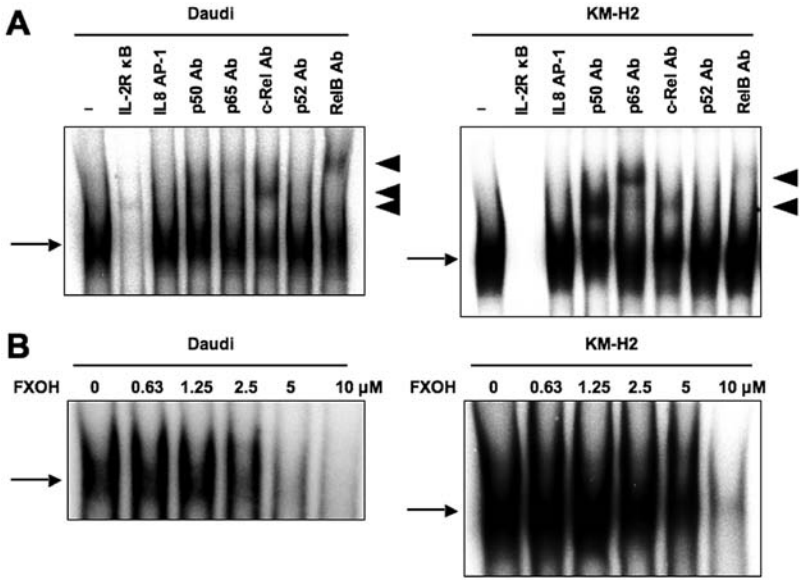

Figure 6. FXOH inhibits NF-κB-DNA binding. (A) Abundant constitutive NF-κB-DNA binding activity in Daudi and KM-H2 cells. EMSA using untreated Daudi and KM-H2 nuclear extracts and radiolabeled NF-κB probe generated DNA-protein complexes, which were eliminated by 100-fold molar excess of self-competitors, but not by the same molar excess of the irrespective oligonucleotides. Supershift assays using the radiolabeled NF-κB probe, untreated nuclear extracts and the indicated antibodies (Ab) to NF-κB components showed that the NF-κB bands consisted of p50, p65, c-Rel and RelB subunits. Arrows, the specific complexes; arrowheads, the DNA binding complexes supershifted by antibodies. (B) Effect of FXOH on NF-κB-DNA binding activity. Daudi and KM-H2 cells were treated with the indicated concentrations of FXOH for 24 h. Nuclear extracts were examined for NF-κB-DNA binding activity by EMSA.

Inhibitory effects of FXOH on NF-κB activity. Mammalian NF-κB characterizes a family of five transcription factors: RelA/p65, c-Rel, RelB, NF-κB1 (p50) and NF-κB2 (11). After activation, the NF-κB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes (12). Because Bcl-2, cIAP-2, XIAP, cyclin D1 and cyclin D2 are NF-κB target genes (12-18), we examined whether FXOH directly inhibits the NF-κB pathway. To study the DNA-binding activity of NF-κB, we performed EMSA with radiolabeled double-stranded NF-κB oligonucleotides and nuclear extracts from Daudi and KM-H2 cells, and confirmed the constitutive activation of NF-κB in these cells. Supershift analysis showed that the NF-κB bands were composed of p50, p65, c-Rel and RelB subunits (Fig. 6A). We next examined the effects of FXOH on Daudi and KM-H2 cells in this context by EMSA and found reduced DNA binding to NF-κB in a dose-dependent manner (Fig. 6B), suggesting that FXOH could inhibit the DNA-binding activity of NF-κB.

NF-κB is inactive in the cytosol where it is bound to IκB, and only becomes active after IκB has been phosphorylated and subsequently degraded (11). Immunoblotting showed that in the absence of FXOH, the levels of phosphorylated IκBα steadily increased in Daudi cells (Fig. 3B). FXOH thus reduced the phosphorylation and degradation of IκBα in a dose-dependent manner. These results indicated that FXOH inhibits NF-κB

activation by preventing the degradation of phosphorylated IκBα.

Discussion

The main issue addressed in this study was whether FX and FXOH have inhibitory activity against human B-cell malignancies including BL and HL, and the possible mechanisms underlying such activities. Because of its central role in cell proliferation and survival, the NF-κB transcription factor has become an important molecular marker of the malignant transformation of cells, especially in hematopoietic malignancies (5,19), and particularly in the pathogenesis of BL and HL (19-21). The present study therefore characterized this transcription factor as a good target for the treatment of BL and HL (19,20). FX is one of the most biologically active and abundantly found carotenoids (7). Our findings strongly suggested that FX and its deacetylated product, FXOH, inhibit constitutive NF-κB activation via the IκBα phosphorylation mechanism, resulting in less cell proliferation and increased cell death by apoptosis. Such results encourage the search for novel carotenoids for the treatment of cancers characterized by aberrant regulation of NF-κB.

FX was hydrolyzed to FXOH during uptake by Caco-2 cells, a tissue culture model for studying the absorption of dietary compounds by human intestinal epithelium (22). Dietary FX is also thought to be hydrolyzed to FXOH in the mammalian gastrointestinal tract by digestive enzymes such as lipase and cholesterol esterase before being absorbed into intestinal cells (22). It was also reported that FXOH is a major metabolite of dietary FX in humans, implying that the major compound in the circulation after FX intake is FXOH (23). Therefore, the bioavailability of FXOH is higher than that of FX in the body. In addition, our study found that FXOH was more potent in inducing apoptosis than FX in the BL and HL cell lines. Taken together, it seems likely that most dietary FX is converted to FXOH, which could then exert a suppressive effect on cells at concentrations lower than the effective concentrations of FX used in the present study.

A dose-dependent suppression of NF-κB constitutive activity by FXOH was also observed in the BL and HL cell lines studied here. In Daudi and KM-H2 cells, FXOH inhibited NF-κB-DNA binding by preventing IκBα phosphorylation, which is a crucial prerequisite to NF-κB-DNA binding. The capacity of FXOH to inhibit nuclear NF-κB-DNA binding was associated with the inhibition of NF-κB-regulated gene expression. In this regard, FXOH reduced the expression of genes involved in cell proliferation (cyclins D1 and D2) and anti-apoptosis (Bcl-2, cIAP-2 and XIAP). The decrease in cell viability could be associated with a decreased cell proliferation and/or an increase in apoptotic cell death, and both D-type cyclins play a key role in cell proliferation through the activation of cyclin-dependent kinases (24). Furthermore, both cyclins are required for the progression of cells from the G_1 phase to the S phase of the cell cycle (24). This scenario could therefore explain our findings that FXOH induces G_1/S cell cycle arrest and thus inhibits cell proliferation.

We found that both FX and FXOH induced apoptosis of cells, accompanied by activation of caspases-3 and -9. A possible mechanism underlying the induction of apoptosis by FXOH could be its capacity to inhibit NF-κB-regulated anti-apoptotic proteins. Bcl-2 prevents the process of mitochondrial release of pro-apoptotic factors, such as cytochrome c (25), and treatment of Daudi cells with FXOH in this study caused a reduction in Bcl-2 expression as well as other NF-κB-dependent anti-apoptotic proteins, cIAP-2 and XIAP (14,15). These IAP proteins are known to inhibit both extrinsic (i.e., death receptor) and intrinsic (i.e., mitochondrial) pathways of apoptosis; cIAP-2 and XIAP directly bind and inhibit effector caspases, acting downstream of the initiator caspases. Our results demonstrated that FXOH induces apoptosis through downregulation of NF-κB-dependent gene products, Bcl-2, cIAP-2 and XIAP.

Notably, FX and FXOH up to 10 and 5 μM, respectively, did not inhibit cell viability of PBMC from healthy volunteers that presented no NF-κB activity. These results together indicated that inhibition of NF-κB activity and of NF-κB-dependent expression of cell survival proteins plays a major role in the pro-apoptotic activity of FX and FXOH in B cell malignancies.

In conclusion, this study demonstrated that FX and FXOH can inhibit NF-κB constitutive activation in BL and HL cell lines. The ability of both carotenoids to reduce cell viability reflects their capacity to decrease cell proliferation by causing G_1 cell cycle arrest and to induce apoptotic cell death. The observed effects combined with the well-established pharmacological safety of both carotenoids (7) provide strong rationale for the potential use of FX and FXOH as new therapeutic agents for patients with BL and HL.

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