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Protein kinase C βII and TGFβRII in ω-3 fatty acid–mediated inhibition of colon carcinogenesis

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Increasing evidence demonstrates that protein kinase C βII (PKCβII) promotes colon carcinogenesis. We previously reported that colonic PKCβII is induced during colon carcinogenesis in rodents and humans, and that elevated expression of PKCβII in the colon of transgenic mice enhances colon carcinogenesis. Here, we demonstrate that PKCβII represses transforming growth factor β receptor type II (TGFβRII) expression and reduces sensitivity to TGF-β–mediated growth inhibition in intestinal epithelial cells. Transgenic PKCβII mice exhibit hyperproliferation, enhanced colon carcinogenesis, and marked repression of TGFβRII expression. Chemopreventive dietary ω-3 fatty acids inhibit colonic PKCβII activity in vivo and block PKCβII-mediated hyperproliferation, enhanced carcinogenesis, and repression of TGFβRII expression in the colonic epithelium of transgenic PKCβII mice. These data indicate that dietary ω-3 fatty acids prevent colon cancer, at least in part, through inhibition of colonic PKCβII signaling and restoration of TGF-β responsiveness.

Introduction

Epidemiological studies have shown a convincing link between dietary fat intake and colon cancer risk (for review see Bartsch et al., 1999). Consumption of fish oil, which contains a high level of ω-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid (c20:5, n-3) and docosahexaenoic acid (c22:6, n-3), is associated with reduced colon cancer incidence (Bang et al., 1976; Caygill and Hill, 1995; Caygill et al., 1996). Biochemical studies confirm that ω-3 fatty acids have potent chemopreventive effects on carcinogen-induced colon cancer in rodents (Deschner et al., 1990; Reddy et al., 1991; Chang et al., 1997). Despite the compelling epidemiological and biochemical evidence demonstrating the efficacy of ω-3 fatty acids in colon cancer chemoprevention (Rose and Connolly, 1999), little is known about how these dietary lipids mediate their protective effects. ω-3 fatty acids can inhibit the colonic epithelial hyperproliferation observed during colon carcinogenesis, suggesting that proliferative signaling is an important target of their chemopreventive effects (Anti et al., 1994, 1997; Latham et al., 1999).

Elevated colonic protein kinase C βII (PKCβII)* expression and activity correlate with colon carcinogenesis in both rodents and humans (Murray et al., 1999; Gokmen-Polar et al., 2001). Elevated PKCβII expression in the colonic epithelium of transgenic mice induces hyperproliferation and enhances susceptibility to azoxymethane (AOM)-induced colon carcinogenesis (Murray et al., 1999), demonstrating a direct role for PKCβII in colon carcinogenesis. Based on these data, we hypothesized that ω-3 fatty acids block colon carcinogenesis by interfering with proliferation via inhibition of PKCβII signaling. To test this hypothesis, we evaluated the effect of a diet high in ω-3 fatty acids on colonic PKCβII activity and signaling. Dietary ω-3 fatty acids inhibited PKCβII activity and suppressed PKCβII-mediated hyperproliferation and colon carcinogenesis in vivo. In addition, our data demonstrate that PKCβII represses transforming growth factor β receptor type II (TGFβRII) expression in vitro and in vivo, and that this repression is reversed by dietary ω-3 fatty acids.

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*Abbreviations used in this paper: ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, bromodeoxyuridine; PKCβII, protein kinase C βII; RIE, rat intestinal epithelial; TGFβRII, transforming growth factor β receptor type II.

Results and discussion

PKCβII is a lipid-dependent protein kinase whose activity can be modulated in vitro by ω-3 fatty acids (Holian and Nelson, 1992; Davidson et al., 2000; Seung Kim et al., 2001). Therefore, we assessed the relationship between colonic PKCβII activity and dietary ω-3 fatty acids during AOM-induced colon carcinogenesis in vivo. Cytosolic and membrane extracts from colonic epithelia of male Sprague-Dawley rats fed an ω-6 fatty acid diet (15% maize oil by weight) or ω-3 fatty acid diet (3.5% maize oil, 11.5% fish oil) were subjected to immunoblot analysis for PKCβII (Fig. 1). A significant decrease in the level of membrane-associated PKCβII was observed in the colonic epithelium of animals fed ω-3 fatty acids, whereas cytosolic PKCβII levels were similar in both diet groups. The membrane association of conventional PKC isozymes, including PKCβII, is widely accepted as an indirect measure of PKC enzyme activation in vivo, because it is not technically feasible to directly determine the activity of the enzyme in situ. Our results are consistent with the direct inhibition of PKCβII activity by ω-3 fatty acids in vitro (Holian and Nelson, 1992).

We previously characterized transgenic mice expressing elevated levels of PKCβII in the colonic epithelium similar to those observed in mouse colon tumors (Murray et al., 1999; Gokmen-Polar et al., 2001). Transgenic PKCβII mice exhibit a hyperproliferative phenotype and enhanced colon carcinogenesis (Murray et al., 1999). Therefore, we assessed the effect of dietary ω-3 fatty acids on AOM-mediated carcinogenesis in transgenic PKCβII mice. Transgenic PKCβII mice fed a diet rich in ω-6 fatty acids exhibited enhanced colon carcinogenesis, as evidenced by increased numbers of preneoplastic lesions, aberrant crypt foci (ACF) (Fig. 2 A). In contrast, a diet rich in ω-3 fatty acids inhibited the enhanced colon carcinogenesis characteristic of transgenic PKCβII mice. Thus, dietary ω-3 fatty acids block PKCβII activation and attenuate the procarcinogenic effects of PKCβII in the colon in vivo.

Transgenic PKCβII mice exhibit hyperproliferation of the colonic epithelium (Murray et al., 1999), whereas dietary ω-3 fatty acids suppress carcinogen-induced hyperproliferation (Latham et al., 1999). Therefore, we assessed the effect of dietary ω-3 fatty acids on the hyperproliferative phenotype of transgenic PKCβII mice (Fig. 2 B). Whereas transgenic PKCβII mice exhibited hyperproliferation when fed an ω-6 fatty acid diet, an ω-3 fatty acid diet selectively suppressed this hyperproliferative phenotype (Fig. 2 B). These data demonstrate that dietary ω-3 fatty acids suppress the hyperproliferative phenotype observed in transgenic PKCβII mice and provide a plausible mechanism for the chemopreventive effects of dietary ω-3 fatty acids on PKCβII-mediated colon carcinogenesis.

To gain insight into how PKCβII enhances colon carcinogenesis, we established rat intestinal epithelial (RIE)-1 cells that stably express PKCβII (Fig. 3). Parental RIE-1 cells, which, like most cells of the intestinal epithelium, express little or no PKCβII, were infected with a retrovirus containing the full-length human PKCβII cDNA (Fig. 3 A). Comparative gene microarray analysis of RIE-1 and RIE/PKCβII cells was used to identify potential targets for PKCβII. Among the genes identified through this analysis, TGFβRII expression was found to be markedly repressed

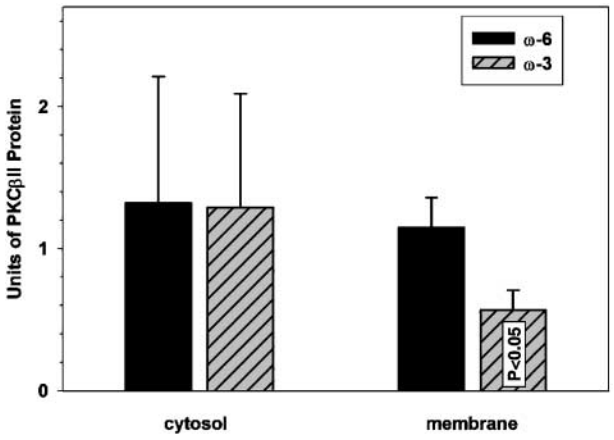

Figure 1. **Dietary ω-3 fatty acids block AOM-induced PKCβII activity.** PKCβII expression was assessed in membrane and cytosolic fractions from colonic epithelial cell extracts from the distal colon of AOM-treated Sprague-Dawley rats fed either an ω-6 or an ω-3 fatty acid diet. Values represent means ± SEM, $n = 5$.

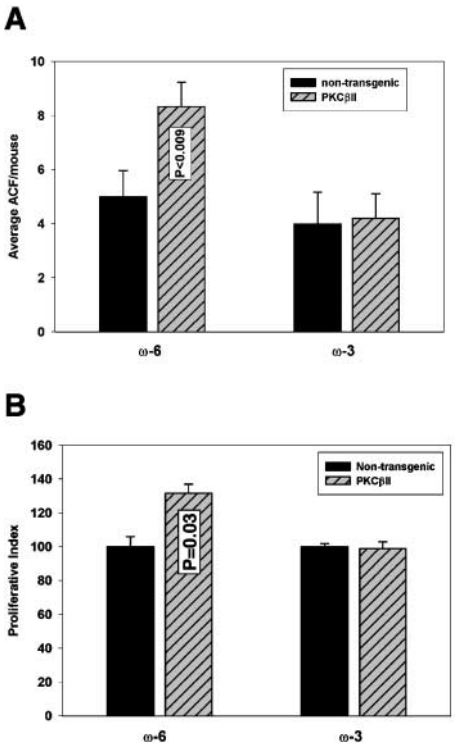

Figure 2. **Dietary ω-3 fatty acids block PKCβII-enhanced colon carcinogenesis and colonic hyperproliferation.** (A) Effect of dietary fat intake on AOM-induced ACF formation in transgenic PKCβII mice. Mice were terminated 12 wk after the final AOM injection and the colons analyzed for ACF formation as described previously (McLellan et al., 1991; Murray et al., 1999). Values represent means ± SEM, $n = 6–18$ animals/experimental group. (B) Mice were fed an ω-6 or ω-3 diet for 18 d. 1 h before sacrifice, mice were injected with 50 mg/kg BrdU and distal colon was isolated, fixed in 4% paraformaldehyde, and analyzed for proliferation as determined by BrdU labeling (Chang et al., 1997). Results respresent proliferative index relative to nontransgenic controls. Values represent means ± SEM, $n = 4–5$ animals/experimental group.

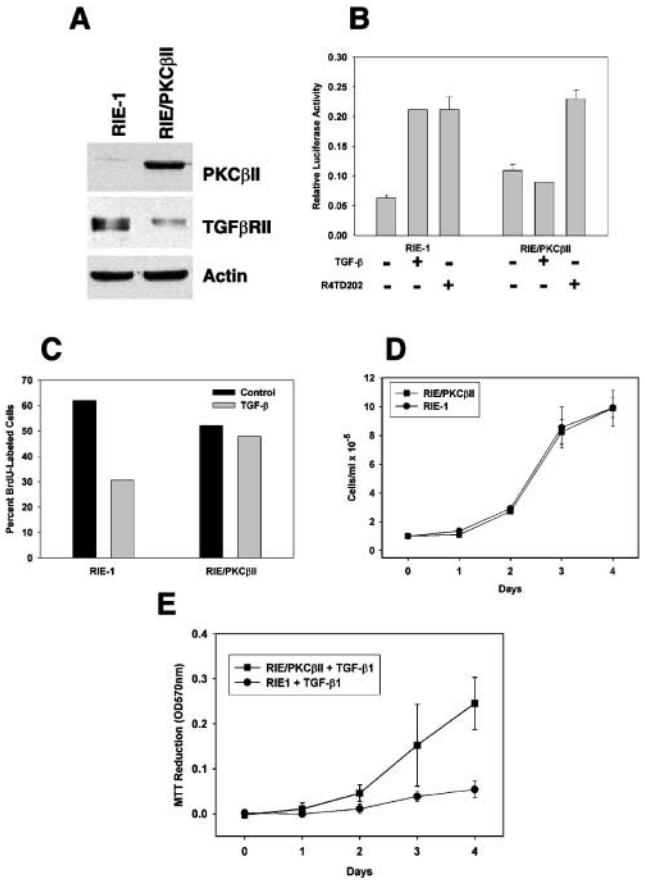

Figure 3. **Characterization of TGF-β signaling in RIE/PKCβII cells.** (A) Expression of PKCβII, TGFβRII, and actin in RIE-1 and RIE/PKCβII cells. RIE-1 cells were infected with pBABEpuro containing full-length human PKCβII or empty pBABEpuro vector. Puromycin-resistant cell populations were isolated and screened for expression of PKCβII, TGFβRII, and actin by immunoblotting. (B) Effect of PKCβII expression on TGF-β–induced transcription in RIE-1 cells. RIE-1 and RIE/PKCβII cells were transiently transfected with 0.4 μg of 3TP/luc TGF-β reporter plasmid plus 1 μg of TK/renilla as an internal control for transfection efficiency. TGF-β1 (3 ng/ml) was added to cells as indicated. Cellular extracts were prepared after 24 h of TGF-β exposure and assayed for firefly and renilla luciferase activity. In addition, some cells were cotransfected with an expression vector encoding constitutively active TGF-β chimeric receptor R4TD202. Data represent the mean ± SD from three independent experiments measuring firefly luciferase activity normalized to renilla luciferase activity. (C) Effect of PKCβII expression on TGF-β–mediated inhibition of BrdU incorporation in RIE-1 cells. TGF-β responsiveness of RIE-1 and RIE/PKCβII cells was assayed by measuring inhibition of BrdU incorporation. (D) Effect of PKCβII expression on RIE-1 cell proliferation. Growth of RIE-1 and RIE/PKCβII cells in growth medium in the absence of TGF-β was measured by hemocytometer count on a daily basis. Data represent the mean ± SD from three dishes. (E) Effect of PKCβII expression on TGF-β–mediated inhibition of RIE-1 cell proliferation. Cultures of RIE-1 and RIE/PKCβII cells were treated with 3 ng/ml TGF-β1, and cell growth was monitored by measuring OD_{570} after reduction of MTT. Data represent the mean ± SD from three wells.

in RIE/PKCβII cells, when compared with RIE-1 cells. These results were confirmed by Western blotting using a specific antibody to TGFβRII (Fig. 3 A). Given the importance of TGF-β signaling in colonic epithelial cell proliferation and differentiation, and the frequent loss of TGF-β responsiveness during colon carcinogenesis (Markowitz and Roberts, 1996), we assessed whether this change in TGFβRII expression leads to an alteration in the well-documented response of the RIE-1 cells to TGF-β (Ko et al., 1995). RIE-1 and RIE/PKCβII cells were transfected with a reporter plasmid containing a TGF-β–responsive element from the tissue plasminogen activator promoter linked to luciferase (Wrana et al., 1992). RIE-1 cells respond to TGF-β by activation of this TGF-β–dependent reporter construct (Fig. 3 B). However, RIE/PKCβII cells showed a diminished transcriptional response to TGF-β consistent with repression of TGFβRII expression. Cotransfection of the TGF-β reporter plasmid with a plasmid containing R4TD202, a constitutively active mutant of TGFβRI (Feng and Derynck, 1996), resulted in comparable increases in transcriptional activity in both RIE-1 and RIE/PKCβII cells (Fig. 3 B), indicating that signaling downstream of the TGF-β receptor is intact in both RIE-1 and RIE/PKCβII cells. Consistent with the transcriptional response to TGF-β, RIE-1 cell proliferation was inhibited by TGF-β as determined by a reduction in the number of bromodeoxyuridine (BrdU)-labeled cells after addition of TGF-β1 to mid-log phase cultures (Fig. 3 C). RIE/PKCβII cells, however, were relatively insensitive to TGF-β inhibition of BrdU labeling (Fig. 3 C). Although there was no detectable growth phenotype associated with expression of PKCβII in the absence of added TGF-β (Fig. 3 D), RIE/PKCβII cells were much less sensitive to TGF-β–mediated inhibition of proliferation (Fig. 3 E). Thus, expression of PKCβII leads to repression of TGFβRII and loss of TGF-β responsiveness in RIE-1 cells.

To directly demonstrate that PKCβII activity is responsible for repression of TGFβRII protein expression in RIE/PKCβII cells, we determined the effect of the PKCβ-selective inhibitor LY379196 on TGFβRII expression. Treatment of RIE/PKCβII cells with increasing concentrations of LY379196 led to a dose-dependent increase in TGFβRII protein expression (Fig. 4 A). Quantitative analysis of these expression data (Fig. 4 B) indicate an apparent ED_{50} for re-expression of TGFβRII of ~40 nM LY379196, consistent with the reported IC_{50} of LY379196 for PKCβII of 30 nM (Jirousek et al., 1996). It should also be noted that re-expression of TGFβRII is accompanied by reduced electrophoretic mobility of TGFβRII upon SDS-PAGE. Because TGFβRII is highly glycosylated, it is possible that this shift reflects changes in posttranslational processing of the protein. The significance of this observation to TGF-β–mediated signaling is unclear. Nevertheless, these data provide direct evidence that PKCβII activity is responsible for repression of TGFβRII in these cells.

TGF-β is a potent growth inhibitor in epithelial cells (Lamprecht et al., 1989) and the loss of TGF-β responsiveness is a common feature of many human colon cancers, particularly those exhibiting the microsatellite instability phenotype (Markowitz et al., 1995). Loss of TGF-β responsiveness in hereditary nonpolyposis colon cancer syndrome is due to inactivating mutations within TGFβRII (Markowitz et al., 1995; Kim et al., 2000). Recent studies indicate that transcriptional repression of TGFβRII expression is a common mechanism for loss of TGF-β responsiveness in

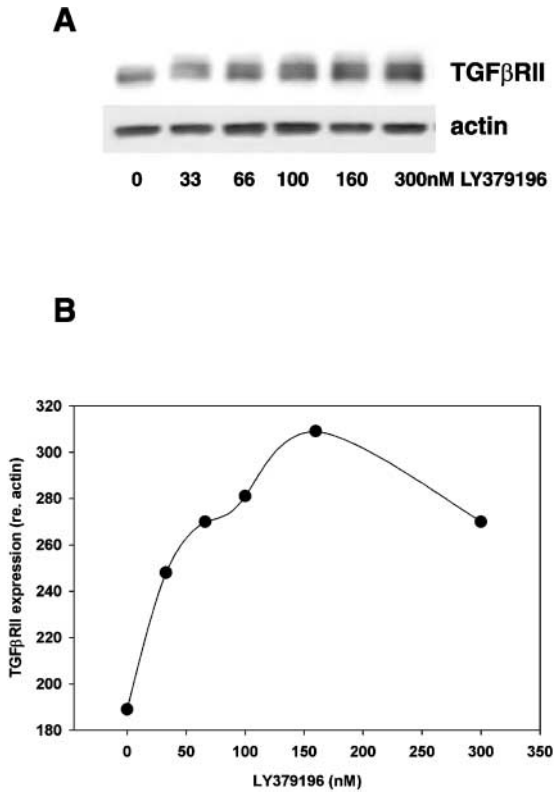

Figure 4. **The selective PKCβ inhibitor LY379196 restores TGFβRII expression in RIE/PKCβII cells.** (A) RIE/PKCβII cells were treated with the indicated concentration of LY379196 for 48 h, and total cellular protein extracts were subjected to immunoblot analysis for TGFβRII expression. (B) Quantitative analysis of the immunoblot data shown in A.

many tumor types, including colon cancers (Kim et al., 2000). Our data demonstrate that PKCβII represses TGF-βRII expression in RIE-1 cells, and indicate that PKCβII-mediated loss of TGF-β responsiveness is involved in the hyperproliferative and procarcinogenic effects of PKCβII in the colonic epithelium. Therefore, we assessed whether TGFβRII is a target for colonic PKCβII in vivo (Fig. 5). Colonic epithelial cell extracts from transgenic PKCβII and nontransgenic mice fed an ω-6 fatty acid diet were subjected to immunoblot (Fig. 5 A) and immunofluorescence analysis (Fig. 5 B, 1 and 4) using a specific TGFβRII antibody. Extracts from nontransgenic mice contained little PKCβII, but abundant TGFβRII, whereas those from transgenic PKCβII mice contained elevated PKCβII and a reduced level of TGFβRII (Fig. 5 A). Immunofluorescence analysis for TGFβRII confirmed these immunoblotting results (Fig. 5 B). Colonic crypts from nontransgenic mice fed an ω-6 fatty acid diet (Fig. 5 B, 4) exhibited abundant staining for TGF-βRII. Staining was strongest in the upper third and along the luminal surfaces of the colonic crypts, consistent with the reported distribution of TGFβRII in the colonic epithelium (Guda et al., 2001). In contrast, colonic crypts from transgenic PKCβII mice fed an ω-6 fatty acid diet (Fig. 5 B, 1) expressed much lower levels of TGFβRII, confirming that TGFβRII expression was repressed by PKCβII in the colonic epithelium in vivo.

Because PKCβII mediates repression of TGFβRII expression and dietary ω-3 fatty acids inhibit PKCβII signaling, we predicted that dietary ω-3 fatty acids would restore TGFβRII expression in transgenic PKCβII mice. Indeed, transgenic PKCβII mice fed an ω-3 fatty acid diet showed levels of TGFβRII comparable to those seen in nontransgenic mice (Fig. 5 B, 2). Therefore, TGFβRII is a critical gene target of PKCβII-mediated repression in RIE-1 cells in vitro and in the colonic epithelium in vivo. Our data are consistent with the conclusion that PKCβII down-regulates TGFβRII expression, thereby inducing a TGF-β–insensitive state. Because TGF-β inhibits proliferation and promotes differentiation of intestinal epithelial cells (Lamprecht et al., 1989), PKCβII-mediated repression of TGF-βRII imposes a hyperproliferative state that increases sensitivity to carcinogens, such as AOM. Dietary ω-3 fatty acids inhibit PKCβII activity and restore TGFβRII expression in the colonic epithelium of transgenic PKCβII mice, resulting in reversal of the hyperproliferative phenotype and attenuation of the enhanced colon carcinogenesis characteristic of transgenic PKCβII mice.

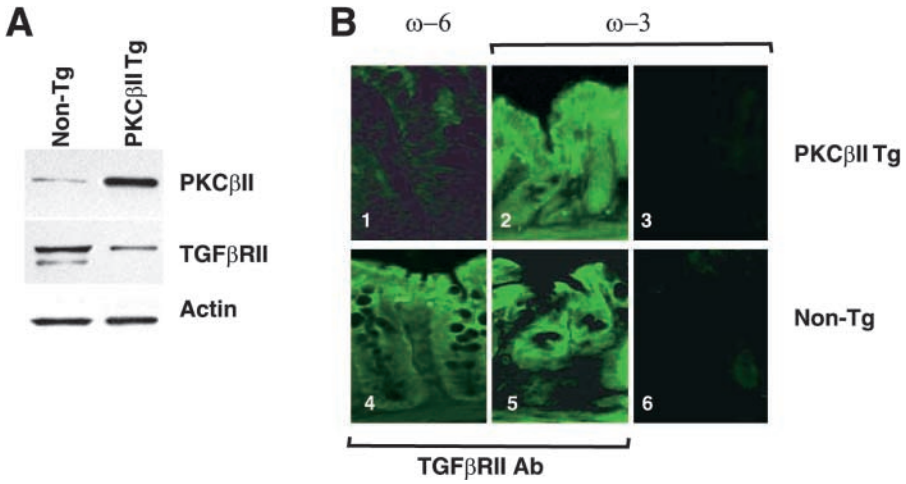

Figure 5. **ω-3 fatty acids restore colonic TGFβRII expression in PKCβII mice.** (A) Immunoblot analysis of TGFβRII expression. PKCβII transgenic mice and nontransgenic littermates were killed, the colonic epithelia were scraped, and 150 μg of the resulting protein extract was subjected to immunoblot analysis using antibody specific for TGFβRII, PKCβII, or actin (Santa Cruz Biotechnology, Inc.). (B) Immunofluorescence analysis of TGF-βRII expression. PKCβII transgenic mice (1, 2, and 3) and nontransgenic littermates (4, 5, and 6) were fed (ad libitum) either an ω-3– (2, 3, 5, and 6) or ω-6– (1 and 4) containing diet beginning at 6 wk of age. After 18 d, mice were killed and distal colons were subjected to immunofluorescence analysis using a specific antibody to TGFβRII (1,2, 4, and 5) or secondary antibody only (3 and 6). All panels are at 400× magnification.

Our results provide a plausible link between chemopreventive dietary ω-3 fatty acids, colonic PKCβII activity, TGF-β signaling, cellular proliferation, and susceptibility to colon cancer. Therefore colonic PKCβII, which is induced early during colon carcinogenesis, represents a novel and potentially highly effective target for chemopreventive therapy in colon cancer. Given the strong association of dietary ω-3 fatty acids with the chemoprevention of other epithelial cancers, including breast and prostate cancer (Rose and Connolly, 1999), as well as neurological conditions such as bipolar disorder (Seung Kim et al., 2001), it is possible that selective PKCβII inhibition could prove to be an important therapeutic modality in the treatment and chemoprevention of multiple epithelial cancers and central nervous system disorders. Likewise, because repression of TGFβRII expression has been documented in many cancer cell types, including gastric cancer, colon cancer, small cell lung cancer, esophageal cancer, hepatocellular carcinoma, squamous cell carcinoma, breast cancer, endometrial cancer, bladder cancer, and osteosarcoma (for review see Kim et al., 2000), it is possible that PKCβII inhibition, either through dietary modulation or pharmacological intervention, may be of therapeutic value for a broad range of major cancer types by restoring TGF-β responsiveness in these tumors.

Materials and methods

Production and maintenance of transgenic mice

Transgenic PKCβII mice were generated and maintained essentially as described previously (Murray et al., 1999), except that the transgene construct used a modified version of the rat liver fatty acid binding protein promoter containing four copies of a heptad repeat enhancer region that directs enhanced and more sustained expression in the entire colonic epithelium (Simon et al., 1997).

Cell fractionation and PKCβII immunoblot analysis

3-wk-old Sprague-Dawley rats fed either an ω-6 fatty acid diet (15% maize oil by weight) or an ω-3 fatty acid diet (3.5% maize oil, 11.5% fish oil, by weight; Chang et al., 1997). After 1 wk on experimental diets, rats were injected with AOM (Chang et al., 1997). Animals were terminated at 16 wk after AOM injection. Colonic epithelial cell extracts from the distal colon were fractionated into membrane and cytosolic fractions and subjected to immunoblot analysis for PKCβII expression (Davidson et al., 2000). Band intensity was quantitated using the Bio-Rad Laboratories Fluor-s imaging system.

ACF analysis

6–8-wk-old transgenic PKCβII mice and nontransgenic littermates were maintained on either an ω-6 or ω-3 fatty acid diet (ad libitum) until termination of the experiment. 1 wk after introduction of the diet, mice received intraperitoneal injections of 10 mg/kg AOM 1×/wk for 4 wk. Mice were terminated 12 wk after the final AOM injection and the colons were analyzed for ACF formation (McLellan et al., 1991; Murray et al., 1999).

Colonic epithelial cell proliferation analysis

6–8-wk-old transgenic PKCβII mice and nontransgenic littermates were maintained on an ω-6 or ω-3 fatty acid diet for 18 d. 1 h before sacrifice, mice were injected with 50 mg/kg BrdU. Distal colon was isolated, fixed in 4% paraformaldehyde, and analyzed for proliferation as determined by BrdU labeling (Chang et al., 1997).

TGF-β transcriptional response assay

RIE-1 and RIE/PKCβII cells were transiently transfected with 0.4 μg of 3TP/luc TGF-β reporter plasmid (Wrana et al., 1992) plus 1 μg of vector expressing renilla luciferase under control of the thymidine kinase promoter as an internal control for transfection efficiency. TGF-β1 (3 ng/ml) was added to cells. After 24 h of TGF-β exposure, cellular extracts were prepared and assayed for firefly and renilla luciferase activity. Data are presented as firefly luciferase corrected for the level of renilla luciferase. Some cells were cotransfected with 0.1 μg of an expression vector encoding constitutively active TGF-β chimeric receptor R4TD202 (Feng and Derynck, 1996) and assayed for TGF-β reporter activity as described above.

Effect of PKCβII on RIE-1 cell proliferation and TGF-β responsiveness

TGF-β responsiveness of RIE-1 and RIE/PKCβII cells was assayed by measuring inhibition of BrdU incorporation. Midlog phase cells were treated with 3 ng/ml TGF-β for 24 h followed by 1 mM BrdU for 30 min. Cells were then permeabilized and stained with anti-BrdU antibody using the BD PharMingen BrdU FlowKit®, according to the manufacturer's recommendations. Approximately 15,000 cells from each culture were analyzed using a Becton Dickinson flow cytometer. RIE-1 and RIE/PKCβII cells were plated at $\sim 10^5$ cells per 6-cm dish. At 24-h intervals, three dishes from each line were harvested by trypsinization and the cells were counted using a hemocytometer. RIE-1 and RIE/PKCβII cells were plated in 96-well plates at 2×10^4 cells in 0.2 ml of medium ± 3 ng/ml TGF-β1. At 24-h intervals, cell growth was assayed in triplicate wells by measuring OD_{570} after reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Analysis of TGF-β RII expression

Equal amounts of cell lysates from RIE-1 and RIE/PKCβII cells were subjected to immunoblot analysis using antibody specific for TGFβRII, PKCβII, or actin (Santa Cruz Biotechnology, Inc.). PKCβII transgenic mice and nontransgenic littermates were killed, the colonic epithelia were scraped, and 150 μg of the resulting protein extract was subjected to immunoblot analysis using the same antibodies listed above. Immunoblot images were captured using a Stratagene Eagle Eye imager. To assess the effect of PKCβII inhibition on TGFβRII protein expression, confluent cultures of RIE/PKCβII cells were placed in medium containing 0.2% FBS plus the concentrations of LY379196 indicated in the figure legend. All cultures contained 0.1% DMSO as a diluent control. After 2 d, total cellular protein was extracted and 50 μg of protein per lane was subjected to immunoblot analysis for TGFβRII expression. Chemiluminescent exposure was adjusted to conform to a linear response range, and data were quantified using a Lynx digital image processor.

Immunofluorescence analysis of TGFβRII expression

6-wk-old PKCβII transgenic mice and nontransgenic littermates were fed either an ω-3– or ω-6–containing diet ad libitum. After 18 d on the defined diets, the mice were killed. Distal colon was isolated, fixed in 4% paraformaldehyde, sectioned, and subjected to immunofluorescence analysis for TGFβRII expression. Images were captured using a CCD color digital camera and digital image capture software.

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