Polyethylene glycol-mediated colorectal cancer chemoprevention: roles of epidermal growth factor receptor and Snail

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Abstract
Polyethylene glycol (PEG) is a clinically widely used agent with profound chemopreventive properties in experimental colon carcinogenesis. We reported previously that Snail/β-catenin signaling may mediate the suppression of epithelial proliferation by PEG, although the upstream events remain unclear. We report herein the role of epidermal growth factor receptor (EGFR), a known mediator of Snail and overexpressed in ~80% of human colorectal cancers, on PEG-mediated antiproliferative and hence antineoplastic effects in azoxymethane (AOM) rats and HT-29 colon cancer cells. AOM rats were randomized to either standard diet or one with 10% PEG-3350 and euthanized 8 weeks later. The colonic samples were subjected to immunohistochemical or Western blot analyses. PEG decreased mucosal EGFR by 60% (P < 0.001). Similar PEG effects were obtained in HT-29 cells. PEG suppressed EGFR protein via lysosomal degradation with no change in mRNA levels. To show that EGFR antagonism per se was responsible for the antiproliferative effect, we inhibited EGFR by either pretreating cells with gefitinib or stably transfecting with EGFR-short hairpin RNA and measured the effect of PEG on proliferation. In either case, PEG effect was blunted, suggesting a vital role of EGFR. Flow cytometric analysis revealed that EGFR-short hairpin RNA cells, besides having reduced membrane EGFR, also expressed low Snail levels (40%), corroborating a strong association. Furthermore, in EGFR silenced cells, PEG effect on EGFR or Snail was muted, similar to that on proliferation. In conclusion, we show that EGFR is the proximate membrane signaling molecule through which PEG initiates antiproliferative activity with Snail/β-catenin pathway playing the central intermediary function. [Mol Cancer Ther 2008;7(9):3103–11]

Introduction
Colorectal cancer is second most common cause of cancer-related deaths in the United States with 49,960 deaths estimated in 2008 (1). Because early-stage colorectal cancer (curable) is generally clinically silent, screening the entire at-risk (age >50) asymptomatic population has been advocated. However, the power of tests such as colonoscopy in reducing mortality and even incidence of colorectal cancer is juxtaposed with the reticence of the asymptomatic population to undergo invasive screening tests. From a patient preference perspective, chemoprevention represents an attractive approach for colorectal cancer prevention with numerous agents showing efficacy in epidemiologic and preclinical studies. However, the results of randomized controlled studies with several promising agents have been disappointing owing to suboptimal efficacy and/or higher toxicity of the agents used. For instance, although the evidence supporting aspirin-related colorectal cancer inhibition is convincing (2, 3), the U.S. Preventive Service Task Force concludes that the risk of toxicity with aspirin outweighs its chemopreventive benefit (4). This was the rationale for the employment of cyclooxygenase-2 inhibitors, which cause less gastrointestinal toxicity. However, the randomized controlled trial while showing efficacy also noted a 2- to 3-fold increase in cardiac toxicity (5, 6). Furthermore, although the epidemiologic evidence for a protective role of folate seems compelling, recent clinical data suggest that folic acid supplementation may enhance tumor progression (7). Likewise, dietary calcium supplementation by and large seems to be safe, but its chemopreventive efficacy is modest (10-15% risk reduction; ref. 8). Thus, safe and more effective chemopreventive agents are urgently needed.

Over the past several years, Corpet et al. have indicated that polyethylene glycol (PEG) has remarkable efficacy as a chemopreventive agent (9, 10). Indeed, the ability of this novel agent to suppress tumors or aberrant crypt foci in the azoxymethane (AOM)-treated rat model was >90%, generally outperforming reported efficacies of nonsteroidal anti-inflammatory drugs or that of other known chemopreventive agents (11). Our laboratory has both confirmed these findings (12) and extended it to the MIN mouse, another well-validated model of experimental colon...
cancer (13). Preliminary epidemiologic data support the potential of PEG to suppress adenomas in humans (14). This is coupled with the excellent safety data from the long-term use of PEG as a cathartic agent (15). Indeed, PEG is available over-the-counter and is widely clinically used.

One important issue, which has stymied the acceptance of PEG as a chemopreventive agent, is the lack of understanding of its mechanism of action. During early colon carcinogenesis, diffuse epithelial hyperproliferation along with suppression of apoptosis in the colonic mucosa is a key event that is often driven by alterations in the β-catenin signaling pathway (16). Down-regulation of β-catenin signaling, with a consequent suppression of proliferation, is a hallmark of a variety of chemopreventive agents including nonsteroidal anti-inflammatory drugs, green tea, etc. (17, 18). PEG has been shown previously to suppress colonic epithelial proliferation (12, 19). In both cell culture and AOM-treated rat model, our laboratory has further shown that the antiproliferative effect of PEG was accompanied by marked suppression of Snail, leading to induction of E-cadherin and subsequent sequestration of β-catenin away from the nucleus (12). This leads to decreased β-catenin-dependent transcriptional activity and cyclin D1 expression, an important regulator of cellular proliferation (12). The relevance of Snail in tumorigenesis is evident from our earlier studies showing that selective down-regulation of this transcriptional repressor, using antisense phosphorodiamidate morpholino oligomer, decreased epithelial proliferation in the normal histologic mucosa with a concomitant reduction in intestinal tumorigenesis in the MIN mouse model (20). Thus, there is compelling rationale to assume that inhibition of Snail signaling by PEG is critical for its chemopreventive effect.

PEG being a large molecule, the central issue is how is it accessible to the cell to regulate the expression of Snail, which is predominantly located in the nucleus. We reasoned that PEG may likely be influencing Snail-related signaling pathways by interacting with upstream targets at the cell membrane level. In this context, epidermal growth factor receptor (EGFR), a transmembrane glycoprotein, has been implicated in the control of intracellular Snail levels (21, 22). Moreover, EGFR is overexpressed in most colorectal cancers (23), making it an excellent candidate for a cell surface-based PEG target. In the present studies, we show that PEG down-regulates EGFR in both AOM-treated rat and colon cancer HT-29 cells. This down-regulation appears to be a result of increased endocytic lysosomal degradation. Moreover, using a short hairpin RNA (shRNA) approach, we show that EGFR down-regulation is central to PEG responsiveness with regards to its role in antiproliferation and Snail regulation.

Materials and Methods

**Experimental Animal Protocols**

All animal studies were done in accordance with the Institutional Animal Care and Use Committee of Evanston-Northwestern Healthcare. Twenty-four male Fisher 344 rats (100-120 g; Harlan) were maintained on defined (AIN-76A) diet for 2 weeks and then randomized into three equal groups. Group 1 was injected with saline (AOM vehicle) and groups 2 and 3 with AOM 20 mg/kg body weight/wk for 2 weeks (i.p.). Two weeks later, group 3 rats were switched to a PEG-3350-supplemented diet (10 g/100 g diet; Harlan Teklad) and continued until sacrifice 8 weeks after AOM. In another experiment, to study the short-term dose effect of PEG-3350 on EGFR expression, 16 additional AOM-treated rats were randomized to receive 5% or 10% gavages of PEG (w/v) for 1 week before euthanizing. Rats were provided water *ad libitum* and housed in polycarbonate cages in an environmentally controlled room (daily 12-h fluorescent light and dark cycles at 24°C and a relative humidity of 70%). All rats were euthanized in a nonfasted state and the colons were isolated and flushed with PBS (pH 7.4). Whereas small distal segments were removed and fixed in formalin for immunohistochemical studies, the remainder of the colon was opened longitudinally to expose the luminal mucosa. The colonic mucosa was collected by gentle scraping using glass slides, homogenized in Laemmli buffer, and subjected to Western blot analysis as described previously (24).

**Immunohistochemical Staining**

Formalin-fixed colonic tissue sections were examined by immunostaining to assess changes in the expression levels of Snail and EGFR. Briefly, paraffin-embedded colonic segments were sliced (4 μm thick) along the vertical axis of the crypts and the sections were mounted on Vectabond-coated Superfrost + slides. These slides were then baked for 1 h at 60°C to 70°C, deparaffinized in xylene, and rehydrated by graded ethanol washes. The antigen epitope retrieval for Snail and EGFR were achieved by pressure microwaving (NordicWare) in antigen unmasking solution for 9 min × 2 cycles (Vector Laboratories). Endogenous peroxidase activity was quenched by treating with 3% H2O2 for 5 min and nonspecific binding was blocked by 5% horse serum for 2 h. Sections were then incubated overnight (at 4°C) with appropriate primary antibodies [anti-Snail (SNA11; T-18, 1:100) and anti-EGFR (1:150; Santa Cruz Biotechnology)] followed by incubation with suitable biotinylated secondary antibodies (1:2,000). After washing, the samples were incubated with avidin-biotin peroxidase complex using Vectastain Elite ABC kit (Vector Laboratories) and the stain developed with DAB (Vector Laboratories). Only complete longitudinal crypts, extending from the muscularis mucosa to colonic lumen, were included for immunohistochemical analysis (8 crypts per colon and 6 rats in each group). Staining intensity was quantified on a five-point scale by a gastrointestinal pathologist blinded to the treatment group.

**Cell Culture and PEG Treatment**

The human colon cancer cell line HT-29 (American Type Culture Collection) was cultured in McCoy’s 5A medium with 10% fetal bovine serum. The cells were seeded in 100 mm Petri dishes (10⁵ cells/mL), washed twice with PBS, and serum starved (0.5% fetal bovine serum) for 72 h before treating with PEG for the indicated time. Cells

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were then harvested and subjected to flow cytometric analysis, Western blotting, and reverse transcription-PCR.

**Flow Cytometry Analyses**

This technique was employed to measure the surface expression of EGFR as well as levels of intracellular Snail in HT-29 cells treated with PEG. Cells (70% confluent) were treated with PEG as described above and then fixed in 4% buffered paraformaldehyde for 30 min. For EGFR surface staining, the cells were washed twice in a PBS (pH 7.4) containing 2% fetal bovine serum, 0.2% bovine serum albumin, and 0.02% sodium azide; for intracellular staining of Snail, 0.2% saponin was also included in this buffer. Cells were later incubated in their respective PBS buffers with either anti-EGFR (1:200; kindly provided by Dr. H. Band) or anti-Snail (T-18; 1:200) antibodies at room temperature for 1 h. After washing three times with their respective buffers, cells were incubated with secondary antibody Alexa 488 Green FITC-labeled anti-goat (for Snail) or anti-mouse (for EGFR, Invitrogen) for 40 min. Cells were subsequently washed three times with their respective buffers and subjected to flow cytometric analysis (Becton Dickinson Labware).

**Western Blot Analysis**

Western blotting was done using standard techniques as described previously (24). Briefly, samples were prepared for SDS-PAGE analysis by adding Laemmli sample buffer to clear whole-cell lysates and then heated for 5 min at 95°C before loading. An equal amount (40 μg) of the protein samples from rat colonic mucosa or HT-29 cells were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), blocked with 5% nonfat milk, and probed with appropriate antibodies [anti-EGFR, 1:200; anti-proliferating cell nuclear antigen (PCNA), 1:250] using standard techniques. Xero-grams were developed with enhanced chemiluminescence (Santa Cruz Biotechnology) and image analysis was done using image acquisition analysis software (Labworks, 4.6; UVP). Expression levels were normalized to the levels of β-actin or α-tubulin as control after stripping and reprobing with anti-β-actin (1:300) or anti-α-tubulin (1:200) antibody.

**Reverse Transcription-PCR**

HT-29 cells were treated with 10% PEG-3350 for 24 h and RNA was extracted with TRI Reagent (Sigma) as described previously (25). The cDNA was synthesized using 5 μg RNA and Superscript RT (Invitrogen Life Technologies). Amplification of Snail mRNA was done using nested PCR protocols (26). Cyclophilin was used as a control for RNA loading (25).

**Cell Proliferation Assay**

To show that EGFR modulation by PEG is important in its antiproliferative activity, we pretreated HT-29 cells with a known EGFR inhibitor to study if it blunts the
antiproliferative activity of PEG. For this experiment, HT-29 cells were pretreated with EGFR inhibitor gefitinib (25 μmol/L; Iressa; AstraZeneca) for 2 h at 37°C before treating with 10% PEG-3350 for 24 h. For control, cells were pretreated with equal volume of DMSO (gefitinib vehicle) and then with PEG. For these assays, cells were seeded in 96-well plates. At the end of the incubation, the medium was replaced with fresh medium (100 μL) containing 5 μL 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) reagent (Roche Diagnostics). After 5 to 15 min incubation, the absorbance was read at 440 nm in a Spectramax Plus Spectrophotometer plate reader (Molecular Devices).

**EGFR Knockdown Assay**

To further explore the importance of EGFR in PEG-mediated antiproliferation, we studied the effect of PEG on cellular proliferation in EGFR-deficient HT-29 cells. We knocked down EGFR gene expression in these cells using shRNA (Origene). The EGFR-shRNA and control vectors were transfected in HT-29 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were incubated at 37°C in a humidified 5% CO2 incubator for 72 h. Transfectants were selected by puromycin (0.5 μg/mL). The cells were then incubated with 10% PEG for 24 h before assaying expression of EGFR and PCNA.

**Statistical Methods**

Values were expressed as mean ± SE as indicated. Quantitative densitometry values were compared by unpaired Student’s t test. Differences with P < 0.05 were considered statistically significant.

**Results**

**PEG Down-regulates Colonic Epithelial EGFR in AOM-Induced Rats**

PEG supplementation was well tolerated by the rats with no apparent toxicity as reflected by normal body weight gain as reported previously (12). Because cellular hyperproliferation has been found to be associated with overexpression of EGFR in the premalignant colonic mucosa of humans as well as AOM rat model of colon cancer (27, 28), we reasoned if the ability of PEG to inhibit proliferation (12) is mediated via modulation of the mucosal EGFR expression.

**Figure 2.** Mechanism of EGFR down-regulation in HT29 cells treated with PEG-3350. The human colon cancer cells, HT29, were seeded in 100 mm Petri dishes (10^5/mL) using McCoy’s 5A medium containing 10% fetal bovine serum. After 24 h, the cells were washed off the medium with PBS before further incubation in serum-starved medium (0.5% fetal bovine serum) for 72 h and then treated with 5% or 10% PEG for 6 or 24 h. A, as shown in Western blot analysis, both 5% and 10% PEG treatment for 6 or 24 h inhibited EGFR expression; however, the effect was statistically significant at 10% dose for both short (P < 0.05) and long (P < 0.001) exposure times. B, similar effects on membrane EGFR inhibition (55%) could be ascertained by flow cytometric analysis. C, further as depicted in the representative reverse transcription-PCR blot, PEG exposure did not cause any significant differences in the EGFR mRNA levels. To further study if PEG may induce EGFR down-regulation by inducing lysosomal degradation, cells were pretreated with 250 nmol/L bafilomycin A1 (BFL; specific lysosomal inhibitor) for 2 h before treating with 10% PEG for 24 h. D, block in lysosomal degradation almost completely prevented PEG to inhibit EGFR.
expression. For these experiments, we used the AOM rat model, and as shown in Fig. 1A (representative Western Blot), there was a significant induction of EGFR expression ($P < 0.001$) at premalignant time point (8 weeks post-AOM treatment) compared with saline-treated controls. In contrast, PEG supplementation in AOM-treated rats (10 g PEG-3350/100 g diet) significantly decreased EGFR expression by 60% ($P < 0.001$) almost to the control levels of saline-treated rats. In another AOM rat experiment (Fig. 1B) to study if relatively shorter PEG treatments could also down-regulate EGFR, 7 weeks post-AOM-treated rats were gavaged with vehicle or 5% or 10% (w/v) PEG-3350 for 1 week before sacrifice. As shown, only a week of oral PEG gavages caused significant decline in EGFR expression. Whereas 5% PEG showed a trend, 10% PEG caused a highly significant ($P < 0.001$) decrease in EGFR levels.

**PEG Inhibits EGFR Expression in HT-29 Cells**

Because we showed previously that PEG inhibits proliferation of human colon cell line HT-29, we investigated if PEG down-regulated EGFR expression in these cells. As depicted by a representative Western blot in Fig. 2A, there was a dose- and time-dependent inhibition of EGFR protein expression in these cells. Both 5% and 10% PEG treatments for 6 or 24 h inhibited EGFR expression; however, the upper dose (shown previously to be antiproliferative in these cells; ref. 12) caused a significantly greater effect ($P < 0.05$ at 6 h and $P < 0.001$ at 24 h). Analysis of EGFR expression by flow cytometric analysis confirmed these results (Fig. 2B). We further investigated if the effect of PEG occurred at the translational or transcriptional level. As can be seen in a representative reverse transcription-PCR blot (Fig. 2C), PEG (10% for 24 h) treatment did not cause any significant difference in the mRNA levels in these cells. This suggests that the down-regulation of EGFR by PEG may be a result of the post-transcriptional modulation of the receptor.

**PEG Down-regulates EGFR via Lysosomal Degradation in HT-29 Cells**

EGFR is recognized to be down-regulated by receptor internalization from the plasma membrane into endosomes and other cytoplasmic vesicles where it is degraded by lysosomal/proteosomal processes and may undergo ubiquitination (29). To study the role of lysosomal degradation in PEG induced decrease in EGFR expression, HT-29 cells were pretreated with specific lysosomal inhibitor, bafilomycin A1 (Calbiochem) before treatment with PEG for 24 h. The levels of EGFR expression was analyzed by Western blotting. As shown in Fig. 2D, PEG in the absence of the inhibitor resulted in expected decrease in EGFR expression. When the cells were pretreated with bafilomycin for 2 h at 37°C, there was a small stabilizing effect on the EGFR; however, under these conditions, the ability of PEG to down-regulate EGFR was almost completely lost. There is some evidence to suggest that EGFR degradation could also be blocked by proteosomal inhibitors (29). In our studies, when we used lactacystin (Calbiochem) to inhibit proteosomal degradation, there was only a nonsignificant partial block (by 17%) in EGFR degradation by PEG. Taken together, these studies suggest that EGFR down-regulation by PEG may be a consequence of lysosomal degradation of EGFR and only partially facilitated by proteosomal degradation.

**EGFR Antagonist (Gefitinib) Blocks PEG-Induced Antiproliferative Activity**

To establish the relevance of EGFR as the primary membrane target of PEG-mediated antiproliferative effects, we investigated if gefitinib, a known EGFR antagonist, could blunt the effect of PEG on HT-29 cell proliferation. The cells were pretreated either with gefitinib or with 25 μmol/L gefitinib for 24 h and then assayed for proliferation using standard WST-1 assay. As shown in Fig. 3A, whereas in DMSO-treated cells PEG caused more than 50% decrease in proliferation rate, this effect was abolished in cells pretreated with EGFR inhibitor gefitinib. In our studies, when we used lactacystin (Calbiochem) to inhibit proteosomal degradation, there was only a nonsignificant partial block (by 17%) in EGFR degradation by PEG. Taken together, these studies suggest that EGFR down-regulation by PEG may be a consequence of lysosomal degradation of EGFR and only partially facilitated by proteosomal degradation.
in gefitinib-treated cells, the antiproliferative effect of PEG was lost. These studies strongly suggest that the antiproliferative effects of PEG may indeed be mediated by its effect on the EGFR down-regulation.

**EGFR Gene Knockdown in HT-29 Cells Blunts the Antiproliferative Activity of PEG**

To further establish a direct causal relationship between EGFR down-regulation and antiproliferative activity of PEG, we determined if specific EGFR knockdown could blunt the effect of PEG on the expression of PCNA in HT-29 cells. The expression of PCNA is known to be stimulated by EGFR (27) and is widely used as a marker of cell proliferation (30, 31). As shown in Fig. 3B, transfection of EGFR shRNA into HT-29 cells caused 50% reduction in the expression of PCNA compared with vector-transfected cells as control. Whereas in control cells PEG (10% for 24 h) caused 60% inhibition in the PCNA expression (Fig. 3C), in EGFR knockdown cells it produced only 25% inhibition. Taken together, these studies provide evidence that EGFR down-regulation may be an important mediator of the antiproliferative effects of PEG in colon cancer cells.

**EGFR, an Upstream Effecter of PEG-Induced Inhibition of Transcriptional Repressor Snail**

Our laboratory has shown previously that Snail is activated in colon cancer (32) and PEG treatment inhibits it (12). This down-regulation of Snail leads to increased plasma membrane E-cadherin, which in turn limits $\beta$-catenin transcriptional activity resulting in cyclin D1 inhibition and hence decreased proliferation (12). Because EGFR is also known to induce Snail (21) and increase cyclin D1 (29), we tested if this membrane receptor may be modulated by PEG and mediate its antiproliferative effects downstream of the Snail/$\beta$-catenin pathway. As depicted in Fig. 4A (representative immunohistochemical images of colonic sections from AOM-treated rats with or without PEG-3350 supplementation), PEG clearly reduced the expression of Snail as well as that of EGFR. To show a causal relationship between PEG-induced EGFR and Snail expression, we used the EGFR shRNA knockdown approach to study the effect of PEG on both EGFR and Snail expressions in HT-29 cells. Both control and EGFR-shRNA-transfected HT-29 cells were treated with 10% PEG-3350 for 24 h. Cells were subjected to flow cytometric analysis to measure EGFR and Snail levels as described in “Materials and Methods.” As shown in Fig. 4B, PEG significantly decreased both EGFR and Snail levels ($P < 0.001$) in the control cells. However, the effect of PEG was blunted for both EGFR and Snail in shRNA-EGFR-transfected cells. These studies provide strong evidence that the effect of PEG on Snail and related antiproliferative signaling may be initiated from its upstream modulation of membrane EGFR.

**Discussion**

We extend our previous finding that PEG suppresses epithelial proliferation via modulation of Snail/$\beta$-catenin signaling by identifying upstream regulators of this process. In the present study, we show, for the first time, an essential role of EGFR down-regulation in the PEG-mediated antiproliferative and hence chemopreventive effects. The present study provides evidence that PEG down-regulates membrane EGFR possibly via lysosomal degradation and this event may control the Snail/$\beta$-catenin

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**Figure 4.** A causal relationship between PEG-mediated reduced expression of EGFR and Snail. Because EGFR is known to induce Snail, which we have reported previously to be inhibited by PEG, we tested if this receptor may be the upstream membrane target for PEG to interact and initiate antiproliferative signaling via Snail. **A,** representative immunohistochemical images of colonic sections from AOM treated rats with or without PEG-3350 supplementation. PEG treatment clearly reduced the expression of both Snail and EGFR. To show a relationship between EGFR and Snail expression, we transfected HT-29 cells with EGFR shRNA and studied the effect of PEG on both EGFR and Snail expressions. Both parental and EGFR-shRNA HT-29 cells were treated with 10% PEG-3350 for 24 h. Cells were subjected to flow cytometric analysis to measure EGFR and Snail levels as described in Materials and Methods. **B,** PEG caused a significant decrease in both EGFR and Snail levels ($P < 0.001$) in the parental cells. However, reductions by PEG were blunted for both EGFR and Snail in shRNA-EGFR cells.

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pathway. The significance of EGFR in PEG chemoprevention is further highlighted by our results indicating that not only is EGFR down-regulated by PEG in the pre-malignant mucosa, but by blocking EGFR in cell culture there is a marked decrease in the antiproliferative efficacy of PEG.

Previous studies have suggested that PEG is a remarkably potent chemopreventive agent with effects seen throughout the spectrum of carcinogenesis. Specifically, PEG has been shown to cause regression of established lesions such as aberrant crypt foci (33) and also inhibit the earliest stages of colon carcinogenesis including at the pre-dysplastic mucosa (12). It is well established that there is an increased epithelial proliferation in the histologically normal mucosa of the AOM-treated rats before development of neoplasia. Importantly, in humans, analysis of the proliferation in the histologically normal mucosa can predict which patients harbor neoplasia elsewhere in the colon (34). The proliferation rate has been used as a biomarker in several chemopreventive studies.

Proliferation in the colonic mucosa is governed by a numerous genetic and epigenetic events. Arguably, the most important is dysregulation of the β-catenin signaling cascade, which, by transactivation of Tcf-1/Lef-1, induces transcription of myriad of genes responsible for proliferation (cyclin D1, c-myc, etc.). β-Catenin signaling can be augmented through increased protein stability (via mutations in adenomatous polyposis coli or CTNNB1) or its altered cellular distribution (35, 36). The latter occurs through loss of E-cadherin, a membrane protein that avidly binds β-catenin to the cell membrane and hence away from the nucleus (37). The importance of E-cadherin is underscored by the observation that its loss triggers increased tumor initiation in the adenomatous polyposis coli-driven murine model of intestinal tumorigenesis (38). Moreover, several reports have indicated that E-cadherin induction may be an important mechanism for chemoprevention with agents such as cyclooxygenase-2 inhibitors, nonsteroidal anti-inflammatory drugs, and ursodeoxycholic acid (18, 24). Thus, corroborating our earlier findings that PEG induced E-cadherin was biologically plausible as a means of chemoprevention (12).

We have shown recently that the induction of E-cadherin by PEG is related to down-regulation of the transcriptional repressor Snail (39). We and others have shown that Snail was overexpressed in colorectal cancers (32, 40). Importantly, we showed that using antisense oligonucleotides to Snail induced E-cadherin expression in the uninvolved MIN mouse mucosa lead to decreased proliferation and tumorigenesis (20). Based on those studies, we presented a paradigm that, in the uninvolved mucosa, PEG leads to

\[\text{Snail} \rightarrow \text{E-cadherin} \rightarrow \beta\text{-catenin transcriptional activity} \rightarrow \text{cell proliferation} \rightarrow \text{tumorigenesis (Fig. 5).}\]

One challenge of this model is linking the bulky PEG molecule with Snail, generally found in the nucleus. Intuitively, given the size of PEG, it would appear more probable that PEG is controlling Snail levels via membrane receptor events rather than directly accessing the nucleus.

The candidate cell surface target of PEG would need to meet several criteria such as (a) should be able to regulate Snail expression, (b) should be overexpressed early in colon carcinogenesis (that is when PEG begins to show effectiveness), and (c) its inhibition must lead to a profound decrease in tumorigenesis (that is consonant with the effect of PEG). In this context, EGFR seems to meet all these requisite conditions. For instance, Lu et al. published a seminal report linking EGFR to Snail regulation in breast cancer cells (21). Our data from the EGFR knockdowns confirmed this by indicating an increase in basal Snail protein. Moreover, Mann et al. showed that inhibition of EGFR signaling blocked Snail and hence Snail-induced degradation of the procarcinogenic prostaglandin E2 (22). Similarly, reduced radiotherapy-induced apoptosis in tumors and cancer cell lines expressing high levels of EGFR has been recently linked to altered Snail/slug expression (41).

Secondly, emerging evidence indicates that increased expression of EGFR is not only relevant in established colorectal cancers but also plays an important role early on at the premalignant stage of colon carcinogenesis (42). For instance, increased expression of EGFR has been reported to be involved in the development of large human aberrant crypt foci (27) and formation of microadenoma in an animal model of colon cancer (28). Our present study clearly shows EGFR up-regulation in the histologically normal mucosa of the AOM-treated rat at a pre-dysplastic time point as a marker of field carcinogenesis. The relevance to human disease is supported by the observation that EGFR activation in the histologically normal-appearing rectal mucosa is a marker of neoplasia in the proximal colon (43). Thus, EGFR clearly meets the relevance/timing criteria as a molecular target of PEG.

Thirdly, biological importance of EGFR in colorectal cancer is underscored by the demonstration that direct targeting of EGFR, achieved with either monoclonal antibodies or pharmacologic inhibitors (44, 45), is an effective strategy in treating colorectal cancer. With regards to prevention of neoplasia by the EGFR inhibitors, EKB-569
caused a profound reduction in the polyp number in the APC(Min) mouse model (45). Similarly, EGFR inhibitor gefitinib reduced tumorigenesis in the AOM-treated rat model by 69% with a concomitant reduction in aberrant crypt foci (46). It is therefore clear that EGFR is a plausible candidate PEG target during colorectal cancer chemoprevention. Our data show that PEG treatment decreases EGFR expression in vivo (AOM-treated rat) and in vitro (HT-29 cells). Furthermore, we show that EGFR is critical in PEG chemopreventive effect by showing that a modest decrease of EGFR using shRNA approach in HT-29 markedly blunted the responsiveness of these cells to PEG as assessed by either the antiproliferative effect or the ability to decrease Snail.

The mechanism of EGFR down-regulation by PEG remains largely unexplored. Our studies rule out regulation at the transcriptional level but suggest post-translational modulation. EGFR protein levels are regulated through a complex process of endocytosis, targeting the vesicles for either destruction via the lysosomes or recycled back to the membrane. The lysosomal inhibitor experiments suggest that PEG induces endocytosis and hence degradation as has been reported previously for sulindac (47). The mechanism by which PEG induces EGFR endocytosis is not clear. EGFR may involve clathrin-independent pathway that includes early and late endosomes (48), or clathrin-independent pathways that involve membrane invaginations (caveole; ref. 49). The latter pathway has been implicated in the proto-oncogene decorin regulation of EGFR (49). In addition, mechanisms involving noncaveolar lipid rafts (50) and formation of transient, circular dorsal ruffles or “waves” are some other recently recognized processes that have been shown to internalize EGFR. More studies are planned to elucidate the mechanisms through which PEG treatment results in internalization and degradation of EGFR.

In summary, we have described for the first time that down-regulation EGFR is an important mechanism for the chemopreventive activity of PEG. EGFR down-regulation leads to decrease in Snail expression with a consequent decrease in β-catenin signaling (as we described previously) and hence suppression of proliferation (Fig. 5). This study provides fundamental insights into the mechanism of action of PEG and will bolster further clinical studies. In that context, we are embarking on a phase II trial of PEG in human colon carcinogenesis. Although it is possible that the required chemopreventive dose may lead to increased bowel movement, this would still allow PEG to be used in the 20% to 30% of the population with chronic constipation (a putative colorectal cancer risk factor). Thus, given the safety and efficacy data, PEG has the potential of being an outstanding chemopreventive agent. Moreover, this novel mechanism of action may allow development of inexpensive, nontoxic topical agents that could target EGFR-dependent malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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