Dietary-induced ERβ upregulation counteracts intestinal neoplasia development in intact male ApcMin/+ mice

Michele Barone, Sabina Tanzi, Katia Lofano, Maria Principia Sava, Mario Pricci, Lucia Demarinis, Samanta Papagni, Raffaella Guido, Eugenio Maiorano1, Giuseppe Ingravallo1, Maria Cristina Comelli2, Antonio Francavilla and Alfredo Di Leo1

Section of Gastroenterology, Department of Emergency and Organ Transplantation and 1Department of Pathological Anatomy, University of Bari, Ospedale Policlinico, Piazza Giallo. Cesare 11, 70124 Bari, Italy and 2CM&D Pharma Limited, London, SW1W9TR, UK

*To whom correspondence should be addressed. Tel: +39 080 5593514; Fax: +39 080 5593251; Email: a.dileo@gastro.uniba.it

Most sporadic colorectal cancers (CRCs) develop through the adenoma–carcinoma sequence pathway and are initiated by adenomatous polyposis coli (APC) gene mutations. Estrogen receptor beta (ERβ) is recognized to progressively reduce its expression in adenomatous and carcinomatous tissues in humans. Moreover, ERβ deficiency enhances small intestinal tumorigenesis in rodents. In the ApcMin/+ mouse model, we evaluated intestinal polydysplasia and ERβ expression plus other biological parameters influencing tumor growth (epithelial cell proliferation, apoptosis and migration) following the addition of a combination of the ERβ-selective agonist silymarin (SIL) and/or lignin (LIG) to a high-fat/low-fiber diet. Forty-five ApcMin/+ mice were divided in four groups: animals fed on the tumorigenic high-fat/low-fiber diet, the tumorigenic diet supplemented with SIL (0.02%) or purified LIG (6.24%) or SIL (0.005%) + LIG (6.24%). In these animals, we assessed polydysplasia, epithelial cell proliferation, migration and apoptosis. The latter group of parameters was evaluated in normal and adenomatous mucosa and the results compared with those found in wild-type (WT) mice fed on the control diet. The addition of SIL or LIG to the diet and even more the specific combination of the two significantly counteracted intestinal tumorigenesis and increased ERβ mRNA and protein levels. Cell proliferation and apoptosis were re-balanced and cell migration accelerated, restoring values similar to those observed in WT animals. Our results further support a protective effect of ERβ in CRC suggesting the use of the combination of SIL-LIG as a potential approach against CRC development.

Introduction

Colorectal cancer (CRC) is the final outcome of a multi-step process that, in most cases, goes through the adenoma–carcinoma sequence pathway (1). Some familial forms of and ~80% of the sporadic CRCs are associated with mutations of the adenomatous polyposis coli (APC) tumor suppressor gene (2). For these reasons, the ApcMin/+ mice are considered one of the most suitable models for CRC studies (3). In humans, APC mutation provides the genetic background to tumor initiation, making intestinal cells susceptible to tumor progression and promotion through accumulation of further mutations by epigenetic phenomena, mostly influenced by environmental factors (4). Since the discovery of the estrogen receptors (ERs) in the tumoral colonic tissue (5,6), several epidemiological and clinical studies supported estrogens as protective hormones against the pathogenesis of colorectal neoplastic lesions, suggesting their potential use in the prevention of CRC (7–11). Of the two known ERs, ERα and ERβ, ERβ seems to mostly drive the estrogen-mediated proliferative activity, and ERβ is thought to be the antiproliferative form (12,13). ERβ is the prevalent form in the gut (14), supporting ERβ as the most probable mediator of estrogenic antiproliferative effects in the colon. ERβ deficiency enhances small intestinal tumorigenesis in ApcMin/+ mice (15). As a proof of concept, the administration of estrogens or the β-selective agonist coumestrol (16,17) similarly abolished increased intestinal neoplasia development in ovariec-tomized ApcMin/+ mice. So far as CRC development in humans is concerned, a markedly reduced ERβ expression is associated with worsening of CRC stage and grade (18–20). It is worth noting that an inverse correlation between ERβ expression and intestinal epithelial cell turnover has been described in human adenomatous sporadic polyps (21). Promotion and progression of carcinogenesis are susceptible to nutritional interventions aimed at counteracting cancer development (4). Traditionally supplemented as an antioxidant and antiinflammatory agent in chronic liver diseases (22), the milk thistle extract silymarin (SIL) exerts selective ERβ agonism (23). SIL has been documented as an effective chemopreventive against intestinal tumor promotion and progression (24–26). Lignin (LIG), a non-starch insoluble dietary fiber (27) has been similarly reported as effective in CRC chemoprevention (28), most probably due to its capability to absorb potential carcinogens into the intestinal lumen (29,30). Moreover, LIG has been recently reported to be intestine converted to enterolignans and particularly to enterolactone (31). Enterolignans are phytooestrogens with rather weak binding affinities to ERs (32) and chemopreventive properties against CRC (33) and breast cancer (34).

In this study, intact male ApcMin/+ mice were selected to test a putative dietary-induced ERβ upregulation against intestinal tumorigenesis, in the presence of physiologically stable estrogen levels. To this end, SIL, purified LIG and the association of the two were supplemented to a high-fat/low-fiber tumor-promoting diet. SIL and LIG were found to be synergistic in reducing intestinal neoplasia development in that a reduction of the number and degree of dysplasia of polyps and the volume of colonic polyps were observed. ERβ-reduced expression was confirmed as the underlying condition for decreased apoptosis in non-adenomatous mucosa and increased proliferation in the adenomatous ApcMin/+ mucosa. In ApcMin/+ dietary-managed animals, ERβ upregulation occurred with no changes in ERα expression. Interestingly, the non-adenomatous mucosa recovered ERβ expression to levels comparable with the healthy intestinal mucosa of the syngeneic wild-type (WT) mice, suggesting a normalization of epithelial turnover behavior.

Abbreviations: APC, adenomatous polyposis coli; BrdU, bromodeoxyuridine; CC-3, cleaved caspase-3; CRC, colorectal cancer; DSI, distal small intestine; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; FAP, familial adenomatous polyposis; LIG, lignin; mRNA, messenger RNA; PCR, polymerase chain reaction; SIL, silymarin; WT, wild-type.

Materials and methods

Animals

Five-week-old C57BL/6J syngeneic WT and C57BL/6J mice with an heterozygote mutation for the APC gene (ApcMin+) were obtained from Charles River (Calco, CO, Italy). They were kept in temperature, air- and light-controlled (light on from 7 a.m. to 7 p.m.) conditions and received food and water ad libitum. Animals did not receive any surgical or hormonal manipulation but were kept anatomically and physiologically intact. All animals received care in compliance to the 'Guide for the Care and Use of Laboratory Animals'.

Dietary treatments and study design

Forty-five intact male ApcMin/+ mice were fed on a rodent chow diet for 10 weeks, as follows: (i) control group, n = 15 mice on a high-fat/low-fiber diet (35) (5K20: 18.5% protein, 10.5% fat, 4.5% soluble and insoluble fibers, 7%...
ashes and 60% non-nitrogenous compounds; Mucedola Srl, Settimo Milanese, Italy), from now on referred to as the ‘control diet’ [according to dietary fiber definitions and amounts set up in the Codex Alimentarius (CL/07/3-NSFDU), a total fiber amount of 3 g/100 g is defined as ‘source of fiber’ in the diet, whereas an amount >6 g/100 g defines the ‘high-fiber’ diet]; (ii) SIL group, n = 15 mice on a control diet supplemented with 0.02% SIL (purchased from Mucedola Srl) [this amount of LIG increased fiber content in the diet to ~30%; silibinin by high-performance liquid chromatography analysis, kindly offered by Madaus Srl, Padova, Italy] [as the assessment of the dose of SIL concerned, in a previous phase of this experiment, using WT mice, the optimal dose was defined as the one producing the highest ERβ induction (supplementary data, available at Carcinogenesis Online)]; (iii) LIG group, n = 9 mice on the control diet supplemented with 6.24% purified LIG (purchased from Mucedola Srl) [this amount of LIG increased fiber content in the diet to ~10%, that is the quantity of fibers previously used in other experimental studies for intestinal protection from carcinogenic stimuli (36)] and (iv) SIL + LIG group, n = 6 mice on the control diet supplemented with 0.005% SIL and 6.24% purified LIG [considering the daily allowance of food and the concentration of SIL in the diet, this group our mice assumed a dose per day of SIL similar to that used for clinical purposes in humans]. Any diet was provided in pellets and dietary intakes were monitored throughout the study period (3 ± 0.1 g/day was the average intake per animal). Body weights were monitored at study entry and at sacrifice.

The entire small bowel and colon were excised to assess polypl number and volume. For the calculation of polypl volume, we considered them as hemispheres (v = 4/3πr³). To this end, the small intestine and the colon were cut along the mesenteric insertion, placed on a paper strip at 0–4°C and analyzed trough a stereomicroscope at ×3 magnification by two independent observers (S.T. and K.L.) (for the macroscopic appearance of polyps see supplementary Figure I, available at Carcinogenesis Online). To assess ERs expression and apoptotic activity, the small intestine was further divided into proximal, medial, and distal segments. The proximal segment underwent scraping of the mucosa, which in part was immediately put into RNA later and stored at -20°C, and in part in liquid nitrogen, in order to run real-time polymerase chain reaction (PCR) and western blotting, respectively. Medial and distal small intestine (DSI), as well as colon specimens were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin in a ‘Swiss roll’ fashion, enabling the full intestinal tract to be microscopically examined on 4μm thick slices by two independent observers (E.M and G.I.). Number and degree of dysplasia of intestinal polypos were assessed on hematoxylin–eosin preparations, by two pathologists in a blinded fashion (E.M and G.I.). Severity of dysplasia was graded on a three-tiered system (mild, moderate, and severe) according to the occurrence of disorganized architecture, cellular atypia and abnormal differentiation as described in the ‘Histological studies’ section. WT syngeneic mice (n = 10) were similarly fed the control diet for 10 weeks and killed for immunohistochemistry, PCR and western blot studies, as described hereafter.

Real-time PCR

To evaluate ERβ and ERα mRNA expression, real-time PCR was performed on an ABI Prism 7900 PCR cycler (Applied Biosystems, Foster City, CA). Murine ERβ (Mm00599819-m1), ERα (Mm00433149-m1) and 18S ribosomal RNA (Assay ID: 4331182, endogenous control) validated PCR primers and TaqMan MGB probes (Applied BioSystems, Warrington, UK) were used. PCR mix was prepared according to the manufacturer’s instructions. Thermal cycler conditions were: 1 × 2 min 50°C, 1 × 10 min 95°C, 40 cycles denaturation (15 s, 95°C) and combined annealing and extension (1 min, 60°C). Relative ERβ and ERα relative expression were calculated using the ΔACT method (2-DDCt) and calibrated to WT mouse intestine as reference.

Western blotting

Frozen intestinal mucosa was homogenized in lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail from Roche Diagnostic, Germany). Lysates were centrifuged at 14 000 r.p.m. for 20 min at 4°C. The Bradford method was applied for protein evaluation. Aliquots containing 50 μg of protein were resolved on a 10% polyacrylamide gel and electrophoresis and successively transferred onto nitrocellulose membranes. The molecular weight markers were run adjacent to test samples to serve as size standards. ERβ and ERα expression was detected by 1:200 diluted H-150 and MC-20 rabbit polyclonal antibody, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Cleaved caspase-3 (CC-3) and β-actin were detected by 1:1000 diluted Asp175 rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) and a 1:500 diluted sc-81178 mouse monoclonal antibody (Santa Cruz, Biotechnology), respectively. After overnight incubation at 4°C, antigen–antibody complexes were visualized by horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G followed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) detection. Computer-assisted densitometry provided with level of expression in arbitrary units, after β-actin normalization.

Histological studies

To evaluate the grade of dysplasia, hematoxylin–eosin stained sections were examined in a blinded fashion by two pathologists (E.M and G.I.). Dysplasia was defined as the occurrence of disorganized glandular architecture, depletion of mucin-producing cells and goblet cells, nuclear atypia and increased mitotic activity. Similarly to the technique commonly applied in human intestinal biopsies, a three-tiered system (mild, moderate and severe) was used for grading dysplasia. This system can be schematically illustrated as the following.

- Mild dysplasia: presence of hyperplastic glands with minimal architectural distortion, no mucin-depleted cells and minimal decrease of goblet cells, slight increase of nuclear:cytoplasmatic ratio, preservation of nuclear polarity, negligible stratification of nuclei, undetectable mitotic figures.
- Moderate dysplasia: presence of crowded hyperplastic glands with stratiﬁed, pimple-shaped nuclei limited to the basal portion of the cell cytoplasm, abundant mucin-depleted cells and consistent decrease of goblet cells moderate increase of nuclear:cytoplasmatic ratio, slight alterations of nuclear polarity with evidence of scattered elongated (cigar-shaped) nuclei scattered mitotic ﬁgures with typical features.
- Severe dysplasia: back-to-back architectural glandular arrangement and presence of cribriform glands, depletion of goblet cells, markedly increased nuclear:cytoplasmatic ratio with evidence of pleomorphic nuclei, sometimes showing amphophilic nucleoli, loss of nuclear polarity and evidence of (pseudo-)immaturation of nuclei, frequent and atypical mitotic ﬁgures.

Inflammation was minimal or absent in polyphs showing true dysplasia (37).

Immunohistochemical analysis

After the 10 weeks of dietary management, all animals received an intraperitoneal injection of bromodeoxyuridine (BrdU, 30 mg/kg) and were killed under metathane anesthesia at 24, 48 and 72 h after injection (animals on diet A: n = 5; diet B: n = 5; diet C: n = 3 and diet D: n = 2). BrdU immunostaining was performed using the BrdU Labeling and Detection kit II (Roche Diagnostics) following the manufacturer’s instructions. Immunohistochemical analysis was performed in the DSI since ApcMin/+ mice develop the majority of tumors in the small intestine (14,15). Ten well-oriented crypt-villi from the normal and non-adenomatous mucosa from WT and ApcMin/+ mice, respectively, and 10 randomly selected fields from the adenomatous mucosa of ApcMin/+ mice were assessed at 48, 72 h after BrdU injection for cell proliferation and migration. The labeling index, i.e. the percentage of BrdU immunolabeled cells over the total number of counted cells, was used to quantify epithelial cell proliferation. The highest labeled cell within the height of the crypt-villus axis, reported as percentage of covered crypt-villus axis (16), was used to estimate cell migration.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay

Apoptic cells were detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay, accordingly to manufacturer’s instructions (In situ Cell Death Detection Kit, Roche). Briefly, deparaffinized slides were immersed in 0.1 M citrate buffer (pH 6.0) and microwave-irradiated at 350 W for 10 min. After phosphate-buffered saline rinse, slides were incubated with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling reaction mix at 37°C for 60 min and then counterstained with TO-PRO-3 (Invitrogen-Molecular Probes, Carlsbad, CA). All sections were screened Leica TCS SP2 (Leica, Wetzlar, Germany) confocal laser scanning microscope at ×20 magnification for screening. At least 10 randomly selected fields were examined. Confocal images were recorded and stored as TIFF files in Adobe Photoshop software (Adobe Systems, San Jose, CA).

Statistical analysis

Either t-test for independent samples or one-way analysis of variance and the multiple comparisons for independent proportions among groups were applied, using a SigmaStat3.1 system. When the one-way analysis rejected the hypothesis of the mean equality among groups, the Holm–Sidak method was applied for comparisons with control group.

Results

Intestinal tumor data

Although the dietary intake was similar in all animal groups (3 ± 0.1 g/day), body weight was significantly increased in mice receiving either SIL, LIG or SIL + LIG as compared with mice fed the control diet (23.9 ± 2.1, 25.0 ± 2.0 and 26.8 ± 2.1, respectively, versus
21.2 ± 3.5 in Apc\textsuperscript{Min+/+} control group, \(P < 0.001\) one-way analysis of variance), supporting the gross observation of a generalized well-being and reduced cachexia in the dietary-managed animals, when compared with the mice fed the control diet only.

As summarized in Table I, the SIL and the SIL + LIG diet protected against intestinal neoplasia development, as per the reduced total polyp number along the entire intestinal tract (−27 and −40\% versus control diet, respectively, \(P = 0.029\)). However, only SIL + LIG gave the most significant reduction in the DSI (\(P = 0.028\)). All the dietary-managed treated groups presented with a significantly reduced number of mice bearing colon polyps, being such reduction particularly marked in the SIL + LIG group (\(P < 0.001\)). Polyp volume was reduced along the entire intestine in all the dietary-managed groups and particularly at the DSI and colon (Table II).

Low, moderate and highly dysplastic adenomatous polyps were observed in all animals. Although the degree of dysplasia significantly decreased in all dietary-managed groups as compared with control, paralleling the observed reduction in polyp number and volume (Table I), the SIL supplemented group presented with the lowest number of mice bearing high-grade dysplastic polyps (\(P < 0.001\)).

**ERα mRNA and protein**

When compared with the healthy intestinal mucosa from WT mice fed the high-fat/low-fiber control diet, the intestinal mucosa of Apc\textsuperscript{Min+/+} mice fed the same diet confirmed previous report (16) of ER\(\beta\) mRNA and protein reduced expression. Figure 1A compares ER\(\beta\):ER\(\alpha\) mRNA ratio in the small intestine of WT and Apc\textsuperscript{Min+/+} differently fed groups. Whereas a 2-fold decrease in ER\(\beta\):ER\(\alpha\) mRNA was observed in Apc\textsuperscript{Min+} control compared with the WT mice (\(P < 0.001\)), SIL, LIG and SIL + LIG Apc\textsuperscript{Min+/+} groups almost fully recovered ER\(\beta\):ER\(\alpha\) to WT values. ER\(\alpha\) expression resulted substantially unmotivated among WT and Apc\textsuperscript{Min+/+} groups, whereas ER\(\beta\) upregulation was further confirmed in western blot analysis (Figure 1B).

**Cell migration**

Cell migration along the crypt-villus axis (Figure 2A–D) revealed a time-related progression of labeled cells, as per Figure 2A–C showing labeled cells position at 24 (A), 48 (B) and 72 (C) h after BrdU injection. All the dietary-managed Apc\textsuperscript{Min+/+} groups presented with a similarly accelerated cell migration, when compared with Apc\textsuperscript{Min+/+} control group (\(P < 0.05\), Figure 2D), suggesting a higher rate of epithelial renewal.

**Cell proliferation and apoptosis**

Figure 3 refers to the percentage of BrdU labeled cells in the intestinal mucosa from WT and Apc\textsuperscript{Min+/+} groups, as a measure of epithelial cell proliferation. A similar proliferative activity was encountered in the healthy WT and the non-adenomatous Apc\textsuperscript{Min+/+} mucosa from all animal groups. Whereas the adenomatous mucosa of control Apc\textsuperscript{Min+/+} mice fed the control diet presented with a 5-fold increase in cell proliferation, the Apc\textsuperscript{Min+/+} dietary-managed groups presented with a significant reduction in this parameter (\(P < 0.001\)). Among dietary-managed mice, 0.02% SIL supplemented group showed a higher trend to WT-like proliferative behavior. Figure 4 refers to cell apoptosis in healthy mucosa from WT mice, and in non-adenomatous and adenomatous mucosa from Apc\textsuperscript{Min+/+} mice, as assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay and CC-3 expression.

When compared with WT, mice fed on control diet presented with a 3-fold and 5-fold decreased number of apoptotic cells in non-adenomatous and adenomatous tissues, respectively. Either the SIL or SIL + LIG Apc\textsuperscript{Min+/+} presented with a fully restored apoptotic activity in the non-adenomatous tissues, but only the SIL + LIG group presented with a fully restored apoptotic activity in the adenomatous tissue, i.e. the mucosa maintained its pro-apoptotic activity.

A further confirmation comes from the results shown in Figure 4D, referring to CC-3 protein. Our findings clearly show that CC-3 markedly decreased in the Apc\textsuperscript{Min+/+} mice fed on the control diet (line 2) as compared with the mice fed the SIL + LIG diet (line 5).

### Table I. Number of polyps in all the intestinal tract and in the DSI, number of mice presenting more than one colonic polyp and number of mice with high-grade dysplastic polyps

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>No. of polyps</th>
<th>No. of mice with more than one colonic polyp (%)</th>
<th>No. of mice with high-grade dysplasia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>in all intestinal tracts(^a)</td>
<td>in DSI(^b)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>56.5 ± 21.0</td>
<td>23.5 ± 10.5</td>
<td>13 (87)%</td>
</tr>
<tr>
<td>SIL</td>
<td>15</td>
<td>40.9 ± 16.3(^f)</td>
<td>17.6 ± 7.9</td>
<td>8 (53)</td>
</tr>
<tr>
<td>LIG</td>
<td>9</td>
<td>46.8 ± 14.0</td>
<td>16.4 ± 6.9</td>
<td>6 (60)</td>
</tr>
<tr>
<td>SIL + LIG</td>
<td>6</td>
<td>34.0 ± 10.7(^f)</td>
<td>11.5 ± 5.1</td>
<td>1 (17)</td>
</tr>
</tbody>
</table>

\(^a\)The number of polyps represents the mean ± SD obtained using all mice in each group.

\(^b\)One-way analysis of variance demonstrated a significant difference among groups (\(P = 0.029\)).

\(^c\)Significantly reduced as compared with the value found control group, that is 13 (87%), by multiple comparisons for independent proportions.

\(^d\)Significantly reduced versus control group by multiple comparisons for independent proportions.

\(^e\)Among these 13 animals, only three showed high-dysplastic colonic polyps, whereas no dysplastic colonic polyp was observed in dietary-managed groups.

\(^f\)Significantly reduced as compared with control group by Holm–Sidak method.

### Table II. Effect of different dietary interventions on polyp volume (mm\(^3\)) calculated in all intestinal tracts, in the DSI and in the colon

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>All intestinal tract(^a)</th>
<th>DSI(^b)</th>
<th>Colon(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>248.7 ± 95.7</td>
<td>77.6 ± 55.3</td>
<td>42.8 ± 36.1</td>
</tr>
<tr>
<td>0.02% SIL</td>
<td>15</td>
<td>133.3 ± 79.8(^d)</td>
<td>44.1 ± 31.5</td>
<td>16.1 ± 19.2</td>
</tr>
<tr>
<td>6.24% LIG</td>
<td>9</td>
<td>93.1 ± 62.5(^d)</td>
<td>25.7 ± 20.2</td>
<td>10.5 ± 22.0</td>
</tr>
<tr>
<td>0.005% SIL + LIG</td>
<td>6</td>
<td>99.0 ± 53.1(^d)</td>
<td>25.9 ± 20.3</td>
<td>7.9 ± 19.4</td>
</tr>
</tbody>
</table>

\(^a\)The values relative to polyp volume represent the mean ± SD.

\(^b\)One-way analysis of variance demonstrated a significant difference among groups in all intestinal tracts (\(P < 0.001\)).

\(^c\)One-way analysis of variance demonstrated a significant difference among groups in DSI (\(P = 0.006\)).

\(^d\)One-way analysis of variance demonstrated a significant difference among groups in Colon (\(P = 0.008\)).

\(^e\)Significantly reduced as compared with control group by Holm–Sidak methods.
compared with WT mice (line 1), whereas almost returned to the same level observed in WT mice in the dietary-managed groups (lines 3–5).

Discussion

The higher prevalence of CRC in males as compared with females, even in colon cancer models (38), stimulated an interest on the role of estrogens and ERs in the colon tumorigenic process. Our experimental conditions support a decreased ERβ expression as a cell proliferation signaling and the impaired apoptotic activity as the basal requirement for polyp development on the Apc mutated mucosa.

**Fig. 1.** ERβ:ERα mRNA ratio and ERβ and ERα protein expression in WT and ApcMin/+ mice. (A) The values reported represent the mean ± SD deviation of at least five animals from WT and ApcMin/+ groups. One-way analysis of variance demonstrated a significant difference among ApcMin/+ groups (P = 0.005). Asterisk represents significantly higher versus control group by Holm–Sidak method. (B) Western blot for ERβ, ERα and β-actin in WT mice (line 1), control (line 2), SIL (line 3), LIG (line 4) and SIL + LIG (line 5) animals. Blots are representative of three runs; each performed using three different tissue samples, 50 μg protein per lane.

Compared with WT mice (line 1), whereas almost returned to the same level observed in WT mice in the dietary-managed groups (lines 3–5).
Dependent kinase inhibitors, thus driving growth arrest at the G1 and induce apoptosis involving increased tumor suppressor p53 expression in the intestinal cells with Apc gene mutation. SIL has been shown to restore proliferation:apoptosis ratio in the non-adenomatous tissue and a net reduction in cell proliferation (P < 0.001) in the adenomatous mucosa. SIL, LIG, and noteworthy the combination of SIL + LIG, acted as selective ERβ inducers in ApcMin/+ mice, whereas ERα remained substantially unchanged.

In several cancer cell lines, including the human D-lactate dehydrogenase-1 colon cancer cells, specific ERβ ligands induced ERβ upregulation, and this effect was related to antiproliferative effects (39). Our dietary management similarly provided with a significant antiproliferative protection. Taking into account the increased development of intestinal neoplasia in ERβ-deficient ApcMin/+ mice and the ERβ deficiency observed in human adenomas and more markedly in colon cancer tissue, our results strongly support ERβ-reduced expression as a relevant biomarker of tumor promotion and progression. Similar conclusions were drawn in a recent study on azoxymethane-induced intestinal carcinogenesis in male and female Sprague–Dawley rats, lifetime exposed to dietary soy isoflavones (40). It could be inferred that down-modulation of ERβ expression is a key point in tumor-facilitated growth, irrespective of the experimental mouse model. In our experimental conditions, the dietary-managed increase in migration of cells skipping programmed cell death on the adenomatous mucosa could reflect an attempt for clearance of tumorigenic cell phenotype, aimed to a healthier epithelial renewal. The involvement of ERβ in growth, organization and maintenance of the normal colonic crypt-villus architecture has been demonstrated in vivo (41). ERβ-deficient mice show a downregulation of transforming growth factor-β pathway and a consequent reduction of the cyclin-dependent kinase inhibitors p21 and p15 (14). On the other hand, ERβ stimulation in colon cancer cell lines induced p38/mitogen-activated protein kinase phosphorylation, which, in turn, drives cells into the apoptotic cycle (39,42). In humans, ERβ and its isoforms could exert a protective role on colon mucosa regarding cellular turnover during tumor development, derived from p53 control loss on the cellular cycle (43). In addition to the prospective evidence as an effective chemopreventive compound in ApcMin/+ and azoxymethane-induced intestinal neoplasia (24,44), this study now demonstrates that SIL, either as single agent (0.02%) or combined at 0.005% with LIG, is an ERβ and apoptosis inducer on the intestinal cells with Apc gene mutation. SIL has been shown to induce apoptosis involving increased tumor suppressor p53 expression and caspase activation, and caspase-mediated cleavage of cyclin-dependent kinase inhibitors, thus driving growth arrest at the G1 and G2 checkpoints in various tumoral tissues (45), including human colon cancer, notably with no effect on cyclooxygenase-2 expression (46).

So far as LIG and its tumor-inhibiting property is concerned, two groups of mechanisms have been proposed: a direct role into the intestinal lumen as an adsorbent of carcinogens with genotoxic activity toward intestinal epithelia, including bile acids and heterocyclic aromatic amines (47), and an indirect effect through its degradation by colonic bacterial enzymes to enterolignans (31). Enterolignans, namely enterolactone and enterodiol, are phytoestrogens with modest ERβ-binding capacity, previously reported to display chemopreventive properties against CRC (33) and breast cancer (34). Enterolignan-induced cytostatic and apoptotic mechanism has been described in human colon cancer Caco-2 (48) and SW480 cells (49) involving upregulation of the CC-3 (apoptosis-enhancing protein), downregulation of proliferation-related proliferating cell nuclear antigen protein, whereas p53, Bax, Bcl-xL and S and Caspase-8 levels were unchanged.

Our data support appropriate dietary management as a feasible approach to counteract polypl development on the Apc mutated mucosa, targeted to the selective upregulation of ERβ. This observation confirms and extends the SIL dose-dependent increase in ERβ mRNA and protein in syngeneic WT mice fed the tumorigenic diet (supplementary Figure II is available at Carcinogenesis Online). In our experimental conditions, ERβ increased with no significant change in ERα expression, thus supporting ERβ as an inhibitory mediator of aberrant intestinal proliferative phenomena, favoring the maintenance of proper pro-apoptotic activity. ERα involvement in the reduction of Apc-dependent tumorigenesis has been recently advocated, as per an increased tumor formation in ApcMin/+ and ERα−/− mice (50). However, whereas a physical association of functional APC protein with ERα in healthy conditions has been described (51), such an association is abolished when APC mutations occur (51). However, ERβ could interfere on ERα expression and/or function by other mechanisms, including heterodimerization, depending on ERβ abundance (50).

As far as tumor parameters are concerned, our dietary-managed ApcMin/+ mice produced a reduced tumor multiplicity and size, an increased epithelial cell migration, a reduced cell proliferation and an increased apoptosis and a low-grade dysplasia. High-grade dysplasia is known to correlate with CRC risk development in humans (52), enhancing the probability of genomic mutations and tumor progression (53). The full recovery of pro-apoptotic activity and ERβ on the non-adenomatous mucosa in the SIL + LIG dietary-supplemented ApcMin/+ mice is worth of note. The concept of a higher efficacy in combining phytochemicals with different mechanisms of actions, or at concentrations much lower than the ones needed to exert the same biological activity as single agents, has been advocated as a strategy to potentiate bioactivity particularly in long-term administrations (54). This concept was supported by the efficacy of the SIL + insoluble fiber-LIG diet in our study, with SIL at a low concentration otherwise ineffective on ERβ expression (supplementary Figure II is available at Carcinogenesis Online). Some familial forms, and ~80% of the sporadic CRCs are associated with mutations of the APC tumor suppressor gene (2), and CRC develops through the adenoma–cancer pathway. In developed countries, the majority of individuals are expected to develop premalignant colon epithelial lesions over a 70 year lifetime (55). Adenomas frequently recur after polypectomy because conditions favoring abnormal proliferation remain in place, and there is sound evidence to include reduced apoptosis and ERβ-reduced expression among them. Chemopreventive agents potentially able to reduce the rate of recurrence could be of great interest, as an adjunct to screening colonoscopy programs aimed at detection and removal of newly formed or recurrent polyps and to control tumor progression.

Supplementary material

Supplementary Figures I and II can be found at http://carcin.oxfordjournals.org/

Funding

University of Bari, Italy.

Acknowledgements

When safety assessment of different dietary combinations was performed M.C.C. was employed at Madaus Srl.

Conflict of Interest Statement: None declared.

References


Received May 11, 2009; revised October 28, 2009; accepted October 30, 2009