Oestrogen-induced apoptosis in colonocytes expressing oestrogen receptor β

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Abstract

Epidemiological studies of postmenopausal hormone replacement therapy show a reduction in the risk of developing colon cancer, and animal studies using 17β -oestradiol (E_2) demonstrate a decreased incidence of chemically-induced colon cancer. Using the colon cancer cell line, COLO205, we found that E_2 induced a dose-dependent increase in DNA fragmentation and nuclear condensation, significant effects being seen at 10^{-12} mol/l. BSA-conjugated E_2 , which cannot enter cells, was ineffective at inducing apoptosis in COLO205 cells, indicating that E_2 was not acting through a cell-membrane receptor. E_2 did not induce the morphological changes characteristic of differentiation.

Using RT-PCR we found that the oestrogen receptor α (ER α) isoform was absent in the COLO205 cell line in contrast to CACO-2, LoVo and SW620 cells, but mRNAs for ER β 1, - β 2, - β 5 and - β 6 isoforms were detected. Western immunoblotting results showed full-length ER β protein but no detectable ER α in COLO205 cells. In normal human colon tissue samples immunoreactive ER β was found but ER α was barely detectable. Expression of ER β was lost in some colon cancer specimens and reduced in others. We conclude that E_2 , through ER β , at concentrations found during replacement therapy, may inhibit the development of colon cancer by inducing apoptosis.

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Introduction

The epidemiological finding that the risk of colorectal cancer is reduced among postmenopausal hormone users was unexpected, but has been consistently confirmed. Thus, in their meta-analysis Grodstein *et al.* (1999) found a one-fifth reduction in the frequency of cancer of the colon and rectum in women who had ever used postmenopausal hormones (predominantly conjugated oestrogen), compared with never users. There are also animal models showing that male rats have a higher risk of developing colon cancer compared with their female counterparts when exposed to dimethylhydrazine, an experimental carcinogen. That effects are likely to be direct is indicated by evidence that 17β -oestradiol (E_2) treatment can reduce significantly the frequency of dimethylhydrazine-induced large intestinal tumours in rats (Smirnov *et al.* 1999).

The effects of E_2 are mediated by two specific, high affinity oestrogen receptors, ER α and ER β , ligand-activated or ligand-independent transcription factors which modulate gene expression by interaction with

promoter response elements or other transcription factors (Kuiper *et al.* 1997, Muramatsu & Inoue 2000). Although both receptors have been identified in breast, bone, cardiovascular tissue, the urogenital tract and the central nervous system, ER β is the form that predominates in the gastrointestinal tract (Gustafsson 1999, Campbell-Thompson *et al.* 2001). Foley *et al.* (2000) showed that malignant transformation in the colon is associated with a marked diminution of ER β protein expression.

Multiple variant forms of ER α and ER β mRNAs have been identified (Murphy *et al.* 1997, Lu *et al.* 1998, Yang *et al.* 2000). These show differential expression in human tissues suggesting different functions and may explain the heterogeneous response of tissues to E_2 . The ER β isoforms, which differ from each other in the C-terminal sequences, can form DNA-binding homodimers and heterodimers with each other and with ER α (Moore *et al.* 1998). A variant of ER β which lacks exon 5 has been shown to have dominant negative activity on both ER α and ER β , illustrating the potential of these multiple isoforms to regulate tissue responsiveness to E_2 (Moore *et al.* 1998, Ogawa *et al.* 1998). In breast tumours,

increases in the ratios of ER β 2 and ER β 5 to ER β 1 mRNA expression have been observed (Leygue *et al.* 1999). In colon cancer cells functional ER β , measured by E₂ induction of progesterone binding and by E₂ effects on cell viability and growth, have been found, but no ER α was detected in HCT116, HCT8, DLD-1, and LoVo colon cancer cell lines (Fiorelli *et al.* 1999). Campbell-Thompson *et al.* (2001) also found little or no ER α , but ER β 1, - β 2 and - β 5 isoforms were present in HT-29, CACO-2, T84 and SW1116 colon cancer cell lines.

The mechanisms by which E₂ might protect against colon cancer are unclear. Contributory roles for ER β in the regulation of cell differentiation in osteoblast cells (Arts *et al.* 1997) and in the induction of apoptosis in neuronal cells (Nilsen *et al.* 2000) have been documented, where it is suggested that E₂ can function as a survival agent or an inducer of apoptosis depending on the ER subtype present in cells. Apoptotic cell death in epithelial lineages generally occurs after a terminal differentiation event (Counis *et al.* 1989, Wyllie 1992). Thus, terminal differentiation is involved in butyrate- and vitamin D₃-induced apoptosis in colon cancer cell lines. The mechanism of action of E₂ is further complicated by the findings that non-genomic effects occur in the vasculature, breast, bone, uterus and neuronal tissues. In these tissues, E₂ can induce extremely rapid increases in the levels of intracellular second messengers, including Ca²⁺ and cAMP, as well as activation of MAP kinase and phospholipase C (Gu & Moss 1996, Migliaccio *et al.* 1996, Picotto *et al.* 1996, Chieffi *et al.* 2000).

In seeking to determine whether, and if so how, E₂ protects against colon cancer, we have assessed the effects of E₂ on apoptosis and differentiation in a colon cancer cell line (COLO205), at concentrations to be expected during hormone replacement therapy (HRT) (Belchetz 1994). We found that this cell line expresses ER β only and therefore results with this cell line have been compared with cells expressing ER α . We further investigated ER isoform expression in epithelium from normal and cancerous human colon tissues.

Materials and Methods

Materials

Five human colon cancer cell lines, COLO205, LoVo, SW620, CACO-2 and HT-29, were obtained from the European Animal Cell Culture Collection (EACC, Porton Down, Wilts, UK). COLO205 cells were derived from the ascites fluid of a 70-year-old Caucasian male with colorectal cancer. LoVo cells were established from the metastatic nodule resected from a 56-year-old colon adenocarcinoma patient, SW620 cells were established from the lymph node of a 51-year-old Caucasian male, CACO-2 cells were derived from primary colon tumour (adenocarcinoma) of a Caucasian male and HT-29 cells

were established from the primary tumour of a 44-year-old Caucasian. In addition, a human adenoma cell line, AASB, was generously provided by Professor C Paraskeva (University of Bristol, UK) and two human breast cancer cell lines, MCF-7 and MDAMB231, were generously provided by the Department of Surgery, University of Birmingham, UK.

COLO205 cells were examined for apoptotic responses by DNA fragmentation and by acridine orange/propidium iodide staining. These and HT-29, CACO-2, LoVo, SW620 and AASB cell lines were also examined by RT-PCR and Western blotting for ER isoforms, with MCF-7 and MDAMB231 as controls. Results were compared with human colon cancer samples and adjacent normal mucosa.

E₂ (Sigma Chemicals Corporation) was dissolved in ethanol at a stock concentration of 10⁻² mol/l. Cyclodextrin-encapsulated E₂ (water-soluble E₂ from Sigma Chemicals Corporation) was dissolved in Milli-Q distilled water (Millipore Ltd, Watford, UK) at a stock concentration of 10⁻² mol/l, and 2-hydroxypropyl- β -cyclodextrin (Sigma Chemicals Corporation) was used as control for this agent. The β -oestradiol 17-hemisuccinate:BSA conjugate (BSA-E₂, Sigma Chemical Corporation) was dissolved in Milli-Q distilled water to give a stock concentration equivalent to 10⁻² mol/l E₂, and dialysed for 24 h against 0.9% NaCl in Milli-Q distilled water to remove unconjugated E₂. TRIzol (GIBCO BRL) was purchased from Invitrogen Ltd, UK and Ready-To-Go RT-PCR beads were from Amersham Pharmacia Biotech Inc. (NY, USA).

Cell culture and treatment

All cell lines were maintained in phenol red-free RPMI 1640 medium (Sigma Chemical Corporation) augmented with 10% foetal bovine calf serum (FCS, First Link Ltd, West Midlands, UK), 100 U/ml penicillin G and 100 μ g/ml streptomycin sulphate (Sigma Chemical Corporation) in a humid atmosphere at 37 °C, with 5% CO₂. Cells were passaged twice a week and plated at a density of 5 $\times$ 10⁴ cells per cm² and cultured for 3 days in 10% charcoal-stripped FCS (CSFCS), followed by treatment with E₂, or BSA-conjugated E₂, for 24–48 h in serum-free medium in the presence of antibiotics. Charcoal-stripped foetal calf serum was prepared by mixing 50 ml FCS with 5 g activated charcoal (Sigma Chemical Corporation) overnight at 4 °C. Following centrifugation to remove the charcoal, a further 5 g charcoal were added to the supernatant and incubated for 30 min at 37 °C and centrifuged. This was repeated one more time.

Human colon cancer and paired normal tissue

Malignant and paired normal tissues (more than 10 cm away from the tumour) were obtained from patients at the

Con **10⁻¹¹** **10⁻¹⁰** **10⁻⁹** **10⁻⁸** **10⁻⁷**
E₂ concentration (M)

Figure 1 DNA fragmentation in COLO205 cells following treatment with E₂. COLO205 cells were treated for 48 h with E₂ at the indicated concentrations in serum-free RPMI medium subsequent to culture in 10% CSFCS for 3 days. DNA in the apoptotic, floating fraction of COLO205 cells was examined. Cells were collected by centrifugation and DNA fragmentation analysis was performed as described in Materials and Methods. Con indicates control treatment.

time of surgery in accordance with the guidelines of the local ethical committee, and were immediately frozen in liquid nitrogen. Total RNA and protein were extracted from frozen tissues using TRIzol reagent according to the manufacturer's recommendations.

DNA fragmentation

The apoptotic, floating fraction of COLO205 cells was collected by centrifugation and dissolved in DNA sample buffer (50 mmol/l Tris-HCl, 0.5% sodium dodecylsulphate (SDS), 10 mmol/l EDTA, pH 8.0). Incubation with RNase A (100 µg/ml) at 50 °C for 1 h was followed by proteinase K (1 µg/ml) digestion at 50 °C for 1 h and incubation at 70 °C for 10 min. DNA was resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide, and visualised by UV illumination.

Acridine orange/propidium iodide staining

Both floating and attached COLO205 cells were collected and suspended in Hank's Balanced Salt Solution (HBSS) with acridine orange (5 µg/ml) and propidium iodide (5 µg/ml) (Gregory *et al.* 1991). A drop of the stained cell suspension was placed on a microscope slide and immediately viewed by fluorescence microscopy. Early apoptotic cells had bright nuclei with dense green areas, while late apoptotic cells had dense orange nuclei. Necrotic fragments, which stained red but had lost cellular structure

A

B

Figure 2 Effect of E₂ on the apoptosis in COLO205 cells assayed by acridine orange/propidium iodide staining. (A) Representative view of acridine orange/propidium iodide staining of COLO205 cells ($\times 1000$). $\rightarrow$, indicates viable cells. $\rightarrow$, indicates early apoptotic cells and $\rightarrow$, shows late apoptotic cells. (B) Dose-response effect of E₂ on the apoptosis in COLO205 cells. COLO205 cells were incubated as described for DNA fragmentation analysis. Floating and attached cells were pooled and acridine orange/propidium iodide staining was performed as described in Materials and Methods. The percentage of apoptotic cells (means $\pm$ S.E.M.) was plotted against concentrations of E₂ ($n=3$). * $P<0.05$, ** $P<0.01$ compared with control (Con).

due to cell membrane lysis, were ignored. Each treatment was repeated in triplicate and the cell counts were performed on a random field at 100 $\times$ magnification for each sample. The entire field was counted and on no occasion was this less than 100 cells. The data were corrected for percentage of apoptotic cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was quantified by spectrometry ($\lambda 260$ nm). ER α was amplified using a pair of appropriate oligonucleotide primers as follows: ER α forward primer 5'-CCG CTC ATG ATC AAA CGC TCT AAG-3' (sequence position 1243-1266, Genbank accession no. NM000125), reverse primer 5'-GCC CTC TAC ACA TTT TCC CTG GTT-3' (1620-1597, NM000125). The

Figure 3 ER expression in colon cancer cell lines. Total RNA was extracted and RT-PCR was performed as described in Materials and Methods. (A) ER α expression in colon cancer cell lines detected by RT-PCR. CACO-2, HT-29, COLO205, LoVo, and SW620 human colon cancer cell lines and AASB, a human colonic adenoma cell line, are indicated on the Figure. MCF-7, used as positive control, and MDAMB231, reported to be ER α negative, are also labelled. The expected size of RT-PCR product is indicated on the left. (B) ER β isoform expression in colon cancer cell lines detected with RT-PCR. The names and expected sizes of ER β isoforms are indicated on the left. No ER β 4 is labelled on this Figure because none was detected.

expression of human ER β isoforms was also determined. The forward primer for the ER β 1–ER β 5 isoforms was the same because they share the common sequence (5′-AGT ATG TAC CCT CTG GTC ACA GCG-3′, 1666–1643, AF051427), while the reverse primers specific for the isoforms 1 to 5 were as follows: ER β 1: 5′-CCA AAT GAG GGA CCA CAC AGC AG-3′ (1560–1638, X99101); ER β 2: 5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3′ (1987–1960, AF051428); ER β 3: 5′-GCA GTC AAG GTG TCG ACA AAG GCT GC-3′ (2094–2069, AF060555); ER β 4: 5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3′ (1987–1960, AF051428); ER β 5: 5′-CTT TAG GCC ACC GAG TTG ATT AGA G-3′ (372–348, AF061054). In addition, the forward primer 5′-GTT GGC CGA CAA GGA GTT GG-3′ (27–46, AF074598) and reverse primer 5′-ACG GCG TTC AGC AAG TGA GC-3′ (291–272, AF074598) were used to amplify the transcript of ER β 6. ER β 6 was identified and submitted to Genbank by A W Brandenberger in 1998 (Genbank no. AF074598). RT-PCR was performed by standard methods. Briefly,

RT-PCR beads were resuspended in 44 μ l diethyl pyrocarbonate (DEPC)-treated dH₂O, and 1 μ g total RNA template and 2.5 μ g random hexamer primer were added. Incubation was carried out at 42 °C for 20 min, followed by 95 °C for 5 min. Indicated primer pairs (10 pmol) were added to give a final total volume of 50 μ l. PCR was performed for 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 90 s. A single cycle of 72 °C for 7 min completed the reaction. Products were resolved on a 2% agarose gel stained with ethidium bromide, and visualised by UV illumination. For negative controls, total RNA was amplified with heat-inactivated RT-PCR beads to confirm absence of contamination from genomic DNA, and dH₂O was used as a template to determine contamination from the laboratory environment in each RT-PCR reaction.

Western immunoblotting

Floating and adherent COLO205 cells were pooled and washed with HBSS twice, followed by dissolution in lysis

Figure 4 Western immunoblotting analysis of ERβ expression in COLO205 cells. ERβ was expressed in COLO205 and MCF-7 cells as a 56 kDa protein. Duplicate lanes containing 50 ((1) and (2)) and 100 µg ((3) and (4)) COLO205 cell proteins are shown.

buffer (2% SDS, 62.5 mmol/l Tris-HCl, pH 6.8). Proteins were also extracted from frozen tissues using TRIzol reagent according to the manufacturer's recommendations and dissolved in lysis buffer. Protein concentration was assayed using the method described by Bio-Rad (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as standard. One hundred micrograms protein were separated on a 10% SDS-

polyacrylamide gel with a 5% stack under reducing conditions. The separated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech Inc., USA) for 3 h at 450 mA. Non-specific binding was blocked by incubating the membrane in 10% low-fat milk in Tris-buffered saline-Tween 20 (TBS-T, 10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20) for 2 h at room temperature. The blot was incubated with 1:1000 dilution of anti-ERα (Santa Cruz, Autogen Bioclear UK Ltd) or anti-ERβ (DAKO, Cambridgeshire, UK) antibody in TBS-T with 0.25% BSA overnight at 4 °C. After exposure to primary antibody, the blot was washed in TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1: 50 000 dilution in TBS-T with 0.25% BSA, Santa Cruz) or anti-mouse (1:20 000 dilution, Binding Site, Birmingham, UK) for 1 h at room temperature and washed again. LumiGLO Chemiluminescent Substrate (Kirkegaard & Perry Laboratories, MD, USA) and X-ray films (Kodak, UK) were used for detection of immunoreactive species. The monoclonal antibody to ERβ was developed using a full-length recombinant ERβ as an antigen for immunisation of Balb-c mice and has been tested for cross reaction to ERα protein in an ELISA. No cross-reactivity with full length recombinant ERα was observed (Anwar *et al.* 2001). The antibody will recognise

Figure 5 Profiles of ER expression in colon cancer and paired normal tissue samples. Total RNA was extracted from frozen tissue and RT-PCR was performed as described in Materials and Methods. (A) RT-PCR results for ERα in human colon cancer and paired normal tissue samples. (B) RT-PCR results for ERβ isoform expression in colon cancer and paired normal tissue samples. C, cancer tissues; N, normal tissues.

Figure 6 Western immunoblotting analysis of ER β expression in colon cancer and paired normal tissue samples. ER β is expressed as a double band of 52–58 kDa detected when using monoclonal anti-ER β antibody. C, cancerous tissues; and N, corresponding normal tissues.

ER β 1 and most probably other ER β isoforms. ER α antibody is commercially available from Santa Cruz and shows no cross reactivity with ER β .

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA), and Dunnett's multiple comparison tests. A *P* value <0.05 was considered significant.

Results

DNA fragmentation

E₂ was found to induce apoptosis dose-dependently in COLO205 cells at concentrations of 10^{-10} – 10^{-7} mol/l

BSA-E₂ concentration (M, equivalent to E₂)

Figure 7 Effect of BSA-conjugated E₂ on apoptosis in COLO205 cells. COLO205 cells were cultured in 10% CSFCS for 3 days prior to treatment with BSA-E₂ in serum-free RPMI medium for 48 h. Floating cells were collected and DNA fragmentation was analysed. Doses shown in the Figure are equivalent to E₂ concentrations. Con, control.

(Fig. 1). Results shown are representative of individual experiments repeated in triplicate. Apoptosis measured by this assay was not found in CACO-2, LoVo and SW620 cells with E₂ treatment (data not shown).

Acridine orange/propidium iodide staining

Cell counting results confirmed the findings of DNA fragmentation with increasing E₂. Figure 2A shows examples of viable, early and late apoptotic cells; early apoptotic cells had bright nuclei with dense green areas, while late apoptotic cells had dense orange nuclei. Figure 2B demonstrates that treatment for 48 h with E₂ induced apoptosis dose-dependently, with a maximum at 10^{-10} mol/l (mean percentage apoptosis=38.5, *P*<0.01), but with clearly demonstrable effects at 10^{-12} mol/l, and evidence of diminishing responsiveness at 10^{-8} mol/l.

RT-PCR and Western immunoblotting of ER isoforms

The transcript for ER α mRNA was not detected in COLO205 cells or HT-29 cells, but was detectable in CACO-2, LoVo, and SW620 cells (Fig. 3A). ER α was also present in MCF-7 cells (a human breast cancer cell line serving as a positive control), but absent in MDAMB231, a breast cancer cell line previously found to be ER α negative (Horwitz *et al.* 1978). Figure 3B shows that transcripts for ER β 1, - β 2 - β 5 and - β 6, with PCR products of 477, 345, 372 and 265 bp fragments respectively, were detected in COLO205 cells, no other ER β isoforms being found. ER β 3 and ER β 4 were not detected in any of the colon cancer cell lines while the remaining isoforms were variably expressed. HT-29 cells did not express ER β 1 and ER β 2. MCF-7 cells expressed ER β 1, - β 5 and - β 6, and transcripts for ER β 1, - β 2, - β 3 and - β 6 were detected in the MDAMB231 cell line. All cell lines examined expressed ER β 6.

Western immunoblotting results of expression of ERs further confirmed that ER α was not detected in

Figure 8 Light microscopy images of COLO205 cells post haematoxylin:eosin (H&E) staining ($\times 400$). COLO205 cells were treated with E₂ and herbimycin A for 48 h prior to fixation and H&E staining. (A) Control (RPML) and (B) control vehicle-treated cells appeared small, round, and isolated. (C) Herbimycin A (300 ng/ml) treatment for 48 h resulted in the formation of cellular aggregates and organisation into epithelioid colonies. (D) Cells treated with E₂ for 48 h showed a similar phenotype as controls.

COLO205 or MDAMB231 cells but was present in MCF-7 and CACO-2 cells (data not shown) as a 65 kDa protein. ER β was expressed in both MCF-7 cells and COLO205 cells as a 56 kDa band (Fig. 4).

The expression profiles of ER mRNAs in human colon cancer and paired normal tissues are shown in Fig. 5A and B. ER α , ER β 2, - β 5 and - β 6 were found in both the cancer and the normal tissues but expression did not vary consistently between them. Neither ER β 3 nor ER β 4 was identified. ER β 1 expression was variable, but no obvious difference was found in the frequency of ER β 1 expression between the human colon cancer and paired normal tissues. We examined ER expression further by Western immunoblotting of paired normal and cancer tissue from sites throughout the colon. Specific staining of 65 kDa

ER α was very low in all tissues (data not shown), as previously reported by Foley *et al.* (2000). ER β was detected as a dimer of 52 and 58 kDa as shown in Fig. 6. In two of the cancer samples these bands were absent and in two others they were markedly reduced. One sample showed no change between normal and cancer tissues.

Effect of BSA-conjugated E₂ on apoptosis in COLO205 cells

To determine whether E₂-induced apoptosis is mediated by membrane receptors, we treated COLO205 cells for 48 h in serum-free medium with BSA-E₂ with concentrations equivalent to 10^{-11} – 10^{-7} mol/l E₂. DNA fragmentation analysis was performed. As shown in Fig. 7, BSA-conjugated E₂ had no effect on apoptosis.

Effect of E₂ on cellular differentiation

The effect of E₂ on differentiation was initially assessed by alkaline phosphatase (ALP) and dipeptidyl-aminopeptidase IV (DPP IV) assays. We compared effects with those of butyrate which is known to be a differentiation inducer in some colon cancer cell lines, e.g. CACO-2 and HT-29, but there are no data of its effects in COLO205 cells. We also used herbimycin A, a tyrosine kinase inhibitor, which has been demonstrated to induce differentiation in COLO205 cells (Mancini *et al.* 1997) assayed by a morphological change. Neither herbimycin A nor butyrate induced changes in the activity of ALP and DPP IV in COLO205 cells. Morphological changes characteristic of differentiation were therefore used to identify the effects of E₂ on differentiation. In Fig. 8A and B, control cells appeared as isolated, small round cells with a high nucleus-to-cytoplasm ratio. Following herbimycin A treatment (300 ng per ml) for 48 h, cells became larger, forming cellular aggregates, and appeared organised in epithelioid colonies (Fig. 8C). E₂-treated cells did not show these morphological changes (Fig. 8D).

Discussion

We have found that E₂ consistently induced apoptosis in COLO205 cells at concentrations equivalent to those attained in women receiving HRT for post-menopausal symptoms. Effects were demonstrable by DNA fragmentation and propidium iodide staining although apoptosis was detectable at lower concentrations of E₂ (10^{-12} mol/l) by the propidium iodide technique than by DNA fragmentation (10^{-10} mol/l). This is probably due to the ability of propidium iodide to detect early and late apoptotic cells, while the DNA fragmentation technique is an indicator of late apoptosis. The lack of effect of BSA-conjugated E₂ indicates that a membrane receptor is unlikely to be involved.

Nuclear ERs are well described in the large bowel (Singh *et al.* 1993). We found that ER β was the prevalent subtype of ER in colon cancer cell lines, which is consistent with the results shown by other groups (Arai *et al.* 2000, Foley *et al.* 2000). Since ER β 1, - β 2, - β 5 and - β 6 were the only transcripts identified in COLO205 cells and no ER α was seen, it follows that if nuclear ERs are responsible for apoptosis induction, then at least one of the four may be critically necessary. We did not find E₂-induced apoptosis in CACO-2, LoVo, and SW620 cells in which both ER α and ER β were detectable, further suggesting ER α and ER β may function in opposite ways in these cells. Paech *et al.* (1997) showed that ER α :E₂ activates whereas ER β :E₂ inhibits transcription. ER α is postulated to act as a survival factor, while ER β mediates the induction of apoptosis (Nilsson & Gustafsson 2000). The antiproliferative property of ER β is also demonstrated in ER β knockout mice, in which loss of ER β function in

the immature uterus causes increased expression of Ki-67, a marker of cell proliferation, and induction of insulin-like growth factor-I and vascular endothelial growth factor (Weihua *et al.* 2000). Furthermore, *in vitro* studies have shown that ER α and ER β signal in opposite ways from the AP-1 site when complexed with E₂ (Paech *et al.* 1997).

Examination of the expression of ERs in human colon cancer and paired normal tissues showed similar results to those of Foley *et al.* (2000), who found low levels of expression of ER α and selective loss of ER β in malignant human colon. We found ER α was expressed in both cancerous and normal human colon tissues using RT-PCR but whether this is derived from colonocytes or other contaminating cell types is impossible to resolve with RT-PCR. With Western immunoblotting, very weak signals of ER α protein (65 kDa) were detected. A double band of 52 and 58 kDa was detected when using monoclonal anti-ER β antibody. These bands were differentially expressed in colon cancer and normal tissues with complete loss in two, a marked reduction in two and no change in one of the colon cancer specimens.

We conclude that a likely mechanism underlying protection by HRT against colorectal cancer is by E₂-induced apoptosis, probably mediated through nuclear ER β receptors, particularly ER β 1, ER β 2, ER β 5 and/or ER β 6.

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