Oestrogen-induced apoptosis in colonocytes expressing oestrogen receptor β

Y Qiu, C E Waters, A E Lewis, M J S Langman and **M C Eggo**

Division of Medical Sciences, University of Birmingham, Birmingham B15 2TT, UK

(Requests for offprints should be addressed to M C Eggo, The Medical School, University of Birmingham, Birmingham, B15 2TT, UK; Email: M.C.Eggo@bham.ac.uk)

(C E Waters is now at Department of Medicine, University of Manchester, UK)

(A E Lewis is now at Department of Pharmacology, University of Pennsylvania, USA)

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 (ERα) isoform was absent in the COLO205 cell line in contrast to CACO-2, LoVo and SW620 cells, but mRNAs for ERβ1, -β2, -β5 and -β6 isoforms were detected. Western immunoblotting results showed full-length ERβ protein but no detectable ERα in COLO205 cells. In normal human colon tissue samples immunoreactive ERβ was found but ERα was barely detectable. Expression of ERβ was lost in some colon cancer specimens and reduced in others. We conclude that E_2, through ERβ, 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 (Smirnoff *et al.* 1999).

The effects of E_2 are mediated by two specific, high affinity oestrogen receptors, ERα and ERβ, 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, ERβ 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 ERβ protein expression.

Multiple variant forms of ERα and ERβ 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 ERβ isoforms, which differ from each other in the C-terminal sequences, can form DNA-binding homodimers and heterodimers with each other and with ERα (Moore *et al.* 1998). A variant of ERβ which lacks exon 5 has been shown to have dominant negative activity on both ERα and ERβ, 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,

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increases in the ratios of ERβ2 and ERβ5 to ERβ1 mRNA expression have been observed (Leygue *et al.* 1999). In colon cancer cells functional ERβ, measured by E_2 induction of progesterone binding and by E_2 effects on cell viability and growth, have been found, but no ERα 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 ERα, but ERβ1, -β2 and -β5 isoforms were present in HT-29, CACO-2, T84 and SW1116 colon cancer cell lines.

The mechanisms by which E_2 might protect against colon cancer are unclear. Contributory roles for ERβ 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 E_2 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 D_3-induced apoptosis in colon cancer cell lines. The mechanism of action of E_2 is further complicated by the findings that non-genomic effects occur in the vasculature, breast, bone, uterus and neuronal tissues. In these tissues, E_2 can induce extremely rapid increases in the levels of intracellular second messengers, including Ca^{2+} 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, E_2 protects against colon cancer, we have assessed the effects of E_2 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 ERβ only and therefore results with this cell line have been compared with cells expressing ERα. 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.

E_2 (Sigma Chemicals Corporation) was dissolved in ethanol at a stock concentration of 10^{-2} mol/l. Cyclodextrin-encapsulated E_2 (water-soluble E_2 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-E_2, Sigma Chemical Corporation) was dissolved in Milli-Q distilled water to give a stock concentration equivalent to 10^{-2} mol/l E_2, and dialysed for 24 h against 0·9% NaCl in Milli-Q distilled water to remove unconjugated E_2. 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 μg/ml streptomycin sulphate (Sigma Chemical Corporation) in a humid atmosphere at 37 °C, with 5% CO_2. Cells were passaged twice a week and plated at a density of 5×10^4 cells per cm^2 and cultured for 3 days in 10% charcoal-stripped FCS (CSFCS), followed by treatment with E_2, or BSA-conjugated E_2, 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

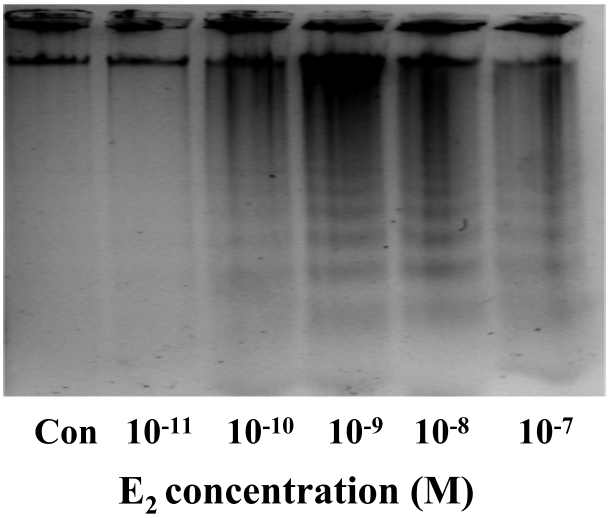

Figure 1 DNA fragmentation in COLO205 cells following treatment with E_2. COLO205 cells were treated for 48 h with E_2 at the indicated concentrations in serum-free RPMI medium subsequent to culture in 10% CSFCS for 3 days. DNA in the apoptotic, floating fraction of COLO205 cells was examined. Cells 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

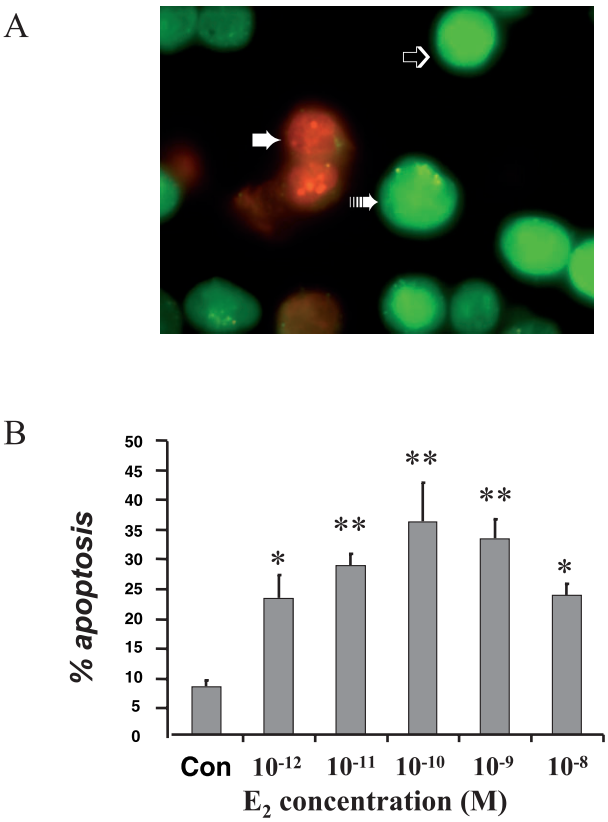

Figure 2 Effect of E_2 on the apoptosis in COLO205 cells assayed by acridine orange/propidium iodide staining. (A) Representative view of acridine orange/propidium iodide staining of COLO205 cells (× 1000). ⇨, indicates viable cells. ⇨, indicates early apoptotic cells and ➡, shows late apoptotic cells. (B) Dose–response effect of E_2 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 ± S.E.M.) was plotted against concentrations of E_2 ($n=3$). *$P<0.05$, **$P<0.01$ 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 × 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 (λ260 nm). ERα was amplified using a pair of appropriate oligonucleotide primers as follows: ERα 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

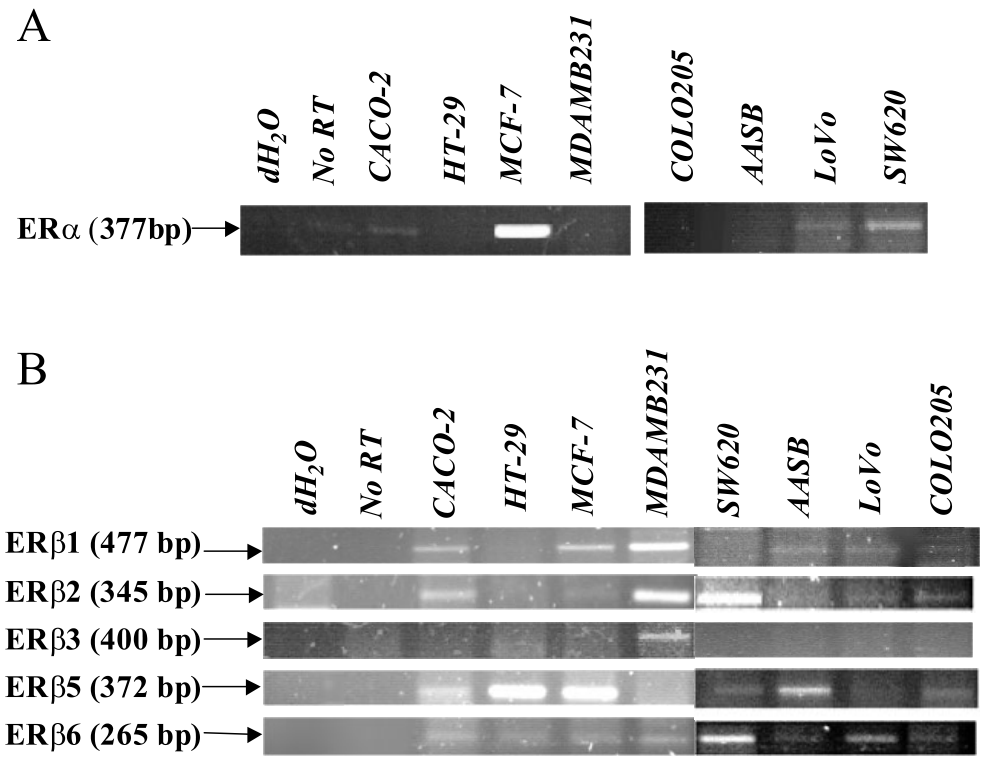

Figure 3 ER expression in colon cancer cell lines. Total RNA was extracted and RT-PCR was performed as described in Materials and Methods. (A) ERα expression in colon cancer cell lines detected by RT-PCR. CACO-2, HT-29, COLO205, LoVo, and SW620 human colon cancer cell lines and AASB, a human colonic adenoma cell line, are indicated on the Figure. MCF-7, used as positive control, and MDAMB231, reported to be ERα negative, are also labelled. The expected size of RT-PCR product is indicated on the left. (B) ERβ isoform expression in colon cancer cell lines detected with RT-PCR. The names and expected sizes of ERβ isoforms are indicated on the left. No ERβ4 is labelled on this Figure because none was detected.

expression of human ERβ isoforms was also determined. The forward primer for the ERβ1–ERβ5 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: ERβ1: 5′-CCA AAT GAG GGA CCA CAC AGC AG-3′ (1560–1638, X99101); ERβ2: 5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3′ (1987–1960, AF051428); ERβ3: 5′-GCA GTC AAG GTG TCG ACA AAG GCT GC-3′ (2094–2069, AF060555); ERβ4: 5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3′ (1987–1960, AF051428); ERβ5: 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 ERβ6. ERβ6 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 μl diethyl pyrocarbonate (DEPC)-treated dH_2O, and 1 μg total RNA template and 2·5 μg 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 μl. 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 dH_2O 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

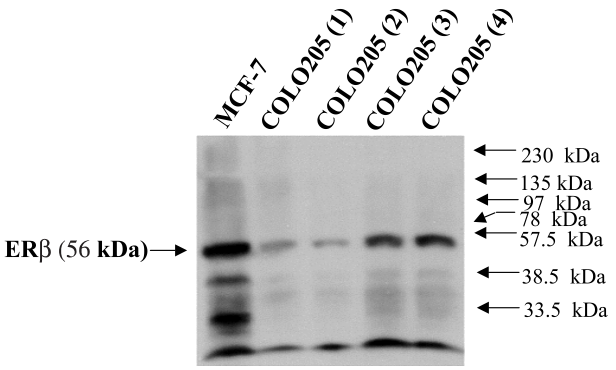

Figure 4 Western immunoblotting analysis of ERβ expression in COLO205 cells. ERβ was expressed in COLO205 and MCF-7 cells as a 56 kDa protein. Duplicate lanes containing 50 ((1) and (2)) and 100 µg ((3) and (4)) COLO205 cell proteins are shown.

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

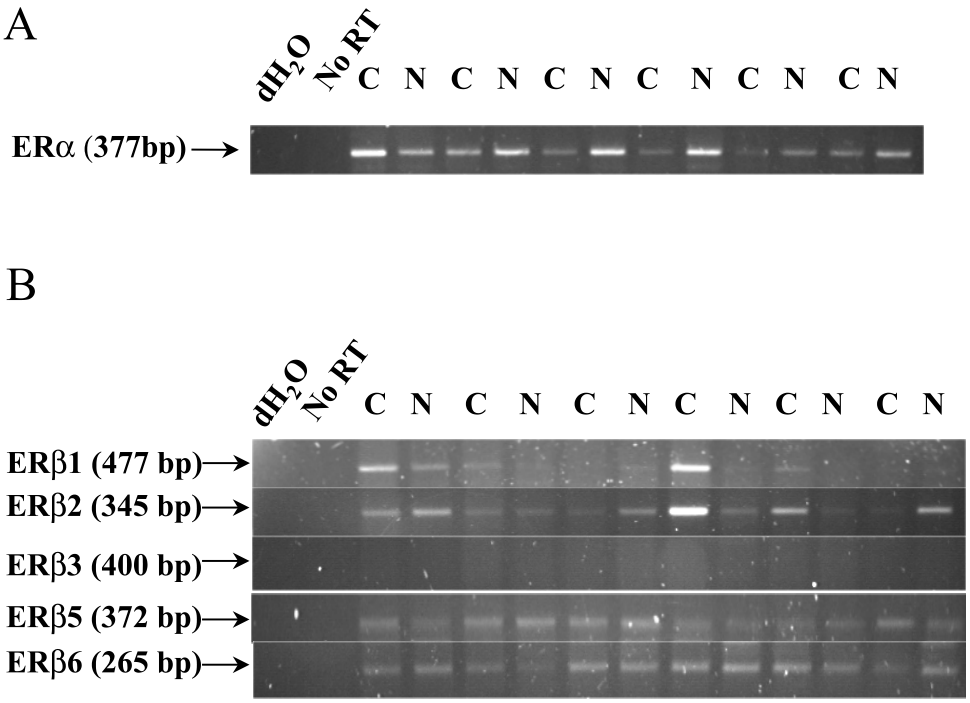

Figure 5 Profiles of ER expression in colon cancer and paired normal tissue samples. Total RNA was extracted from frozen tissue and RT-PCR was performed as described in Materials and Methods. (A) RT-PCR results for ERα in human colon cancer and paired normal tissue samples. (B) RT-PCR results for ERβ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

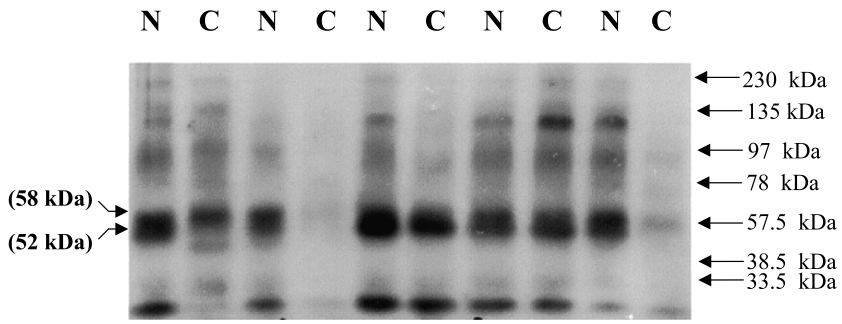

Figure 6 Western immunoblotting analysis of ERβ expression in colon cancer and paired normal tissue samples. ERβ is expressed as a double band of 52–58 kDa detected when using monoclonal anti-ERβ antibody. C, cancerous tissues; and N, corresponding normal tissues.

ERβ1 and most probably other ERβ isoforms. ERα antibody is commercially available from Santa Cruz and shows no cross reactivity with ERβ.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA), and Dunnett's multiple comparison tests. A P value $<0{\cdot}05$ was considered significant.

Results

DNA fragmentation

E_2 was found to induce apoptosis dose-dependently in COLO205 cells at concentrations of 10^{-10}–10^{-7} mol/l (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 E_2 treatment (data not shown).

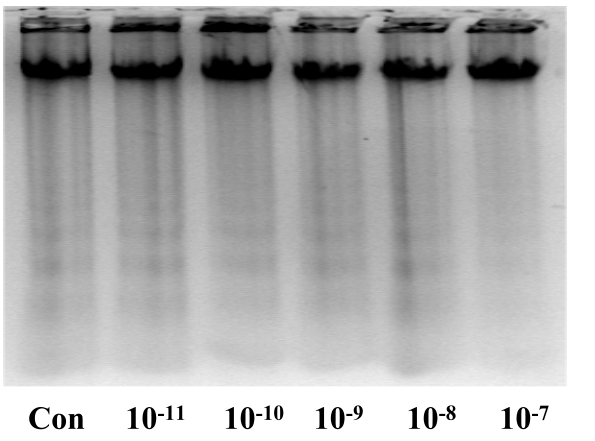

Figure 7 Effect of BSA-conjugated E_2 on apoptosis in COLO205 cells. COLO205 cells were cultured in 10% CSFCS for 3 days prior to treatment with BSA-E_2 in serum-free RPMI medium for 48 h. Floating cells were collected and DNA fragmentation was analysed. Doses shown in the Figure are equivalent to E_2 concentrations. Con, control.

Acridine orange/propidium iodide staining

Cell counting results confirmed the findings of DNA fragmentation with increasing E_2. 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 E_2 induced apoptosis dose-dependently, with a maximum at 10^{-10} mol/l (mean percentage apoptosis=38·5, $P<0{\cdot}01$), 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 ERα mRNA was not detected in COLO205 cells or HT-29 cells, but was detectable in CACO-2, LoVo, and SW620 cells (Fig. 3A). ERα 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 ERα negative (Horwitz *et al.* 1978). Figure 3B shows that transcripts for ERβ1, -β2 -β5 and -β6, with PCR products of 477, 345, 372 and 265 bp fragments respectively, were detected in COLO205 cells, no other ERβ isoforms being found. ERβ3 and ERβ4 were not detected in any of the colon cancer cell lines while the remaining isoforms were variably expressed. HT-29 cells did not express ERβ1 and ERβ2. MCF-7 cells expressed ERβ1, -β5 and -β6, and transcripts for ERβ1, -β2, -β3 and -β6 were detected in the MDAMB231 cell line. All cell lines examined expressed ERβ6.

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

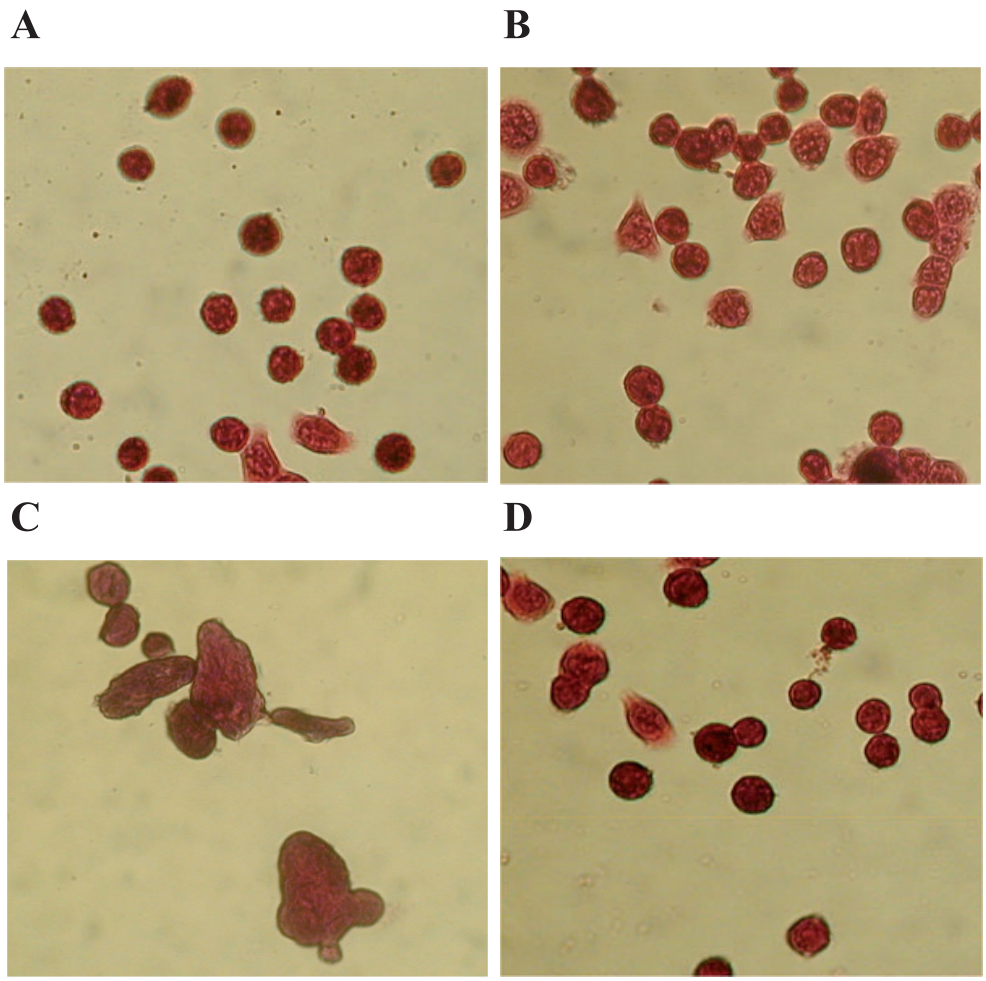

Figure 8 Light microscopy images of COLO205 cells post haematoxylin:eosin (H&E) staining (× 400). COLO205 cells were treated with E_2 and herbimycin A for 48 h prior to fixation and H&E staining. (A) Control (RPMI) and (B) control vehicle-treated cells appeared small, round, and isolated. (C) Herbimycin A (300 ng/ml) treatment for 48 h resulted in the formation of cellular aggregates and organisation into epithelioid colonies. (D) Cells treated with E_2 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. ERβ 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. ERα, ERβ2, -β5 and -β6 were found in both the cancer and the normal tissues but expression did not vary consistently between them. Neither ERβ3 nor ERβ4 was identified. ERβ1 expression was variable, but no obvious difference was found in the frequency of ERβ1 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 ERα was very low in all tissues (data not shown), as previously reported by Foley *et al.* (2000). ERβ 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 E_2 on apoptosis in COLO205 cells

To determine whether E_2-induced apoptosis is mediated by membrane receptors, we treated COLO205 cells for 48 h in serum-free medium with BSA-E_2 with concentrations equivalent to 10^{-11}–10^{-7} mol/l E_2. DNA fragmentation analysis was performed. As shown in Fig. 7, BSA-conjugated E_2 had no effect on apoptosis.

Effect of E_2 on cellular differentiation

The effect of E_2 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 E_2 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). E_2-treated cells did not show these morphological changes (Fig. 8D).

Discussion

We have found that E_2 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 E_2 (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 E_2 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 ERβ 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 ERβ1, -β2, -β5 and -β6 were the only transcripts identified in COLO205 cells and no ERα 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 E_2-induced apoptosis in CACO-2, LoVo, and SW620 cells in which both ERα and ERβ were detectable, further suggesting ERα and ERβ may function in opposite ways in these cells. Paech *et al.* (1997) showed that ERα:E_2 activates whereas ERβ:E_2 inhibits transcription. ERα is postulated to act as a survival factor, while ERβ mediates the induction of apoptosis (Nilsson & Gustafsson 2000). The antiproliferative property of ERβ is also demonstrated in ERβ knockout mice, in which loss of ERβ 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 ERα and ERβ signal in opposite ways from the AP-1 site when complexed with E_2 (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 ERα and selective loss of ERβ in malignant human colon. We found ERα 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 ERα protein (65 kDa) were detected. A double band of 52 and 58 kDa was detected when using monoclonal anti-ERβ 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 E_2-induced apoptosis, probably mediated through nuclear ERβ receptors, particularly ERβ1, ERβ2, ERβ5 and/or ERβ6.

Acknowledgements

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References

Anwar A, McTernan PG, Anderson LA, Askaa J, Moody CG, Barnett AH, Eggo MC & Kumar S 2001 Site-specific regulation of oestrogen receptor-alpha and -beta by oestradiol in human adipose tissue. *Diabetes, Obesity and Metabolism* **3** 338–349.

Arai N, Strom A, Rafter JJ & Gustafsson JÅ 2000 Estrogen receptor beta mRNA in colon cancer cells: growth effects of estrogen and genistein. *Biochemical and Biophysical Research Communications* **270** 425–431.

Arts J, Kuiper GG, Janssen JM, Gustafsson JÅ, Lowik CW, Pols HA & van Leeuwen JP 1997 Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. *Endocrinology* **138** 5067–5070.

Belchetz PE 1994 Hormonal treatment of postmenopausal women. *New England Journal of Medicine* **330** 1062–1071.

Campbell-Thompson M, Lynch IJ & Bhardwaj B 2001 Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. *Cancer Research* **61** 632–640.

Chieffi P, Colucci-D'Amato GL, Staibano S, Franco R & Tramontano D 2000 Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *Journal of Endocrinology* **167** 77–84.

Counis MF, Chaudun E, Allinquant B, Muel AS, Sanval M, Skidmore C & Courtois Y 1989 The lens: a model for chromatin degradation studies in terminally differentiating cells. *International Journal of Biochemistry* **21** 235–242.

Fiorelli G, Picariello L, Martineti V, Tonelli F & Brandi ML 1999 Functional estrogen receptor beta in colon cancer cells. *Biochemical and Biophysical Research Communications* **261** 521–527.

Foley EF, Jazaeri AA, Shupnik MA, Jazaeri O & Rice LW 2000 Selective loss of estrogen receptor beta in malignant human colon. *Cancer Research* **60** 245–248.

Gregory CD, Dive C, Henderson S, Smith CA, Williams GT, Gordon J & Rickinson AB 1991 Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* **349** 612–614.

Grodstein F, Newcomb PA & Stampfer MJ 1999 Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. *American Journal of Medicine* **106** 574–582.

Gu Q & Moss RL 1996 17β-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. *Journal of Neuroscience* **16** 3620–3629.

Gustafsson JÅ 1999 Estrogen receptor beta - a new dimension in estrogen mechanism of action. *Journal of Endocrinology* **163** 379–383.

Horwitz KB, Zava DT, Thilagar AK, Jensen EM & McGuire WL 1978 Steroid receptor analyses of nine human breast cancer cell lines. *Cancer Research* **38** 2434–2437.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JÅ 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138** 863–870.

Leygue E, Dotzlaw H, Watson PH & Murphy LC 1999 Expression of estrogen receptor beta1, beta2, and beta5 messenger RNAs in human breast tissue. *Cancer Research* **59** 1175–1179.

Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC & Watson PH 1998 Estrogen receptor-beta mRNA variants in human and murine tissues. *Molecular and Cellular Biology* **138** 199–203.

Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Paty PB & Hockenbery DM 1997 Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. *Journal of Cell Biology* **138** 449–469.

Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E & Auricchio F 1996 Tyrosine kinase/p21 ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO Journal* **15** 1292–1300.

Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM & Willson TM 1998 Cloning and characterization of human estrogen receptor beta isoforms. *Biochemical and Biophysical Research Communications* **247** 75–78.

Muramatsu M & Inoue S 2000 Estrogen receptors: how do they control reproductive and nonreproductive functions? *Biochemical and Biophysical Research Communications* **270** 1–10.

Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A & Watson PH 1997 Estrogen receptor variants and mutations. *Journal of Steroid Biochemistry and Molecular Biology* **62** 363–372.

Nilsen J, Mor G & Naftolin F 2000 Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the Fas/Fas ligand system. *Journal of Neurobiology* **43** 64–78.

Nilsson S & Gustafsson JÅ 2000 Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. *Breast Cancer Research* **2** 360–366.

Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998 Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor of estrogen action in human. *Nucleic Acid Research* **26** 3505–3512.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ & Scanlan TS 1997 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277** 1508–1510.

Picotto G, Massheimer V & Boland R 1996 Acute stimulation of intestinal cell calcium influx induced by 17 beta-estradiol via the cAMP messenger system. *Molecular and Cellular Endocrinology* **119** 129–134.

Singh S, Sheppard MC & Langman MJ 1993 Sex differences in the incidence of colorectal cancer: an exploration of oestrogen and progesterone receptors. *Gut* **34** 611–615.

Smirnoff P, Liel Y, Gnainsky J, Shany S & Schwartz B 1999 The protective effect of estrogen against chemically induced murine colon carcinogenesis is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. *Oncology Research* **11** 255–264.

Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson JÅ 2000 Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. *PNAS* **97** 5936–5941.

Wyllie AH 1992 Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer and Metastasis Reviews* **11** 95–103.

Yang J, Liu A, Chiou SK, Guzman R & Nandi S 2000 Estrogen receptor variants are present in many normal human tissues. *International Journal of Molecular Medicine* **5** 223–227.

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