Selective Loss of Estrogen Receptor β in Malignant Human Colon

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Abstract

Epidemiological data suggest a protective effect for estrogen replacement therapy on colon cancer. The estrogen receptor (ER) is required for the action of estrogen. The ER-β isofrom is functionally similar to ER-α but has a distinct pattern of expression and transcriptional response to selective estrogen response modulators. Our goal was to investigate the presence of ER-α and ER-β in normal and malignant colon tissue. Human colon cancer tissue and adjacent normal colon tissue were harvested from five male and six female patients undergoing segmental colon resection for colon cancer. Western blot analysis revealed very low levels of ER-α protein in tumor and normal colon tissue. In both male and female patients, malignant colon tissue showed a selective loss of ER-β protein expression when compared to normal colon tissue in the same patient. Semiquantitative reverse transcription-PCR revealed no difference in ER-β mRNA levels between normal and malignant colon tissue. Malignant transformation of the colon is associated with a marked diminution of ER-β protein expression, possibly through a posttranscriptional mechanism.

Introduction

The protective effect of estrogen replacement therapy on colon cancer is supported by a number of clinical and laboratory observations. In a case-controlled study, Newcomb and Storer (1) found that recent users of HRT had a RR of 0.54 (95% CI, 0.36–0.81) for colon cancer and a RR of 0.91 (95% CI, 0.54–1.55) for rectal cancer compared to postmenopausal women who never used HRT. Calle et al. (2) studied women selected from 676,526 female participants in the Cancer Prevention Study II of the American Cancer Society and found that current or past use of HRT was associated with significantly decreased risk of fatal colon cancer (RR, 0.71; 95% CI, 0.61–0.83). The reduction in risk was strongest among current users (RR, 0.55; 95% CI, 0.40–0.76), and there was a significant (P = 0.0001) trend of decreasing risk with increasing years of use among all users (2). In a cohort study, Persson et al. (3) analyzed cancer incidence and mortality in 22,597 Swedish women who were prescribed HRT and found a reduction in both parameters. Finally, Kampman et al. (4) found an inverse relationship between HRT and colon cancer (odds ratio, 0.71; 95% CI, 0.56–0.89) in a case-control study.

Gender differences in the incidence and behavior of colon cancer suggest that estrogen influences carcinogenesis and possibly tumor spread. Specifically, in women, the proximal colon is the more common site of neoplasia. In contrast, the rectum is more commonly involved in men (5, 6). Male rats exposed to dimethylhydrazine, an experimental carcinogen, have a 2-fold increased risk of developing colon cancer and significantly shorter survival times compared to their female counterparts (7, 8).

These findings have led many investigators to search for the biological mechanisms by which estrogen and estrogen-like compounds may influence the pathogenesis of colorectal cancer. In vitro experiments have shown that the growth of colon cancer cell lines is affected by estrogen and is dependent on ER expression (9–11). The presence of ER-α in normal colonic epithelium, several colon cancer cell lines, and human colon cancer tissue has been confirmed by several investigators (12). However, the function of ER in normal and malignant colon remains unknown. Recently, a second ER, ER-β, has been detected, and its functional domains have been characterized (13, 14). Research addressing tissue distribution of this receptor has localized ER-β to multiple organs including the female genital tract (14).

Kuiper et al. (15) did not find any significant ER-β mRNA expression in rat colon and intestine. To date, there is only one report of ER-β mRNA expression in a single normal human colon specimen (14). In this study, colonic ER-β mRNA was detectable only by in situ hybridization and not by Northern blot (14). More recently, ER-β was the only identified ER in colon cancer cell lines in vitro (16). Distinct effects of ER-β on the transcription of ER-responsive genes have been documented. On AP-1-containing promoters, ER-α and ER-β have opposite effects on transcription in the presence of selective estrogen response modulators (17). Furthermore, the ratio of ER-α:ER-β, which has been demonstrated to vary depending on the tissue type, may be critical in the ultimate physiological response to estrogens and antiestrogens (17).

We investigated the presence and possible differential expression of ER-α and ER-β in normal colon and colon cancer samples from patients. ER-β was the prevalent protein form found in colon, and there was a selective loss of ER-β protein in malignant colon as compared to normal colon from the same individual.

Materials and Methods

Harvesting of Human Colon and Isolation of Colon mRNA. Malignant tissue and adjacent normal colon tissue were freshly obtained from 11 patients (5 males and 6 females), with the approval of the Human Investigations Committee of the University of Virginia, at the time of surgery and immediately frozen in liquid nitrogen. Total RNA was extracted from half of the human tissue by direct homogenization in TRizol reagent (Life Technologies, Inc., Gaithersburg, MD), according to the manufacturer’s instructions. The remaining half of the tissue was saved for protein extraction. Spectrophotometry was used to quantitate total RNA, and the integrity of RNA was confirmed on an agarose-formaldehyde gel

Semiquantitative RT-PCR. To ensure exponential phase amplification of both ER-β and β2m, the optimal number of PCR cycles was determined and found to be 34. This was accomplished by reverse transcribing 6 µg of cellular patient RNA according to the methods described previously (18) using a GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ). Twenty µl of the resulting total cellular cDNA were then used in each of two PCR reactions for amplifying ER-β and β2m at different cycle numbers ranging from 24–40. To
increase the sensitivity of this assay, digoxigenin-labeled dUTP (at one-fortieth of the concentration of dTTP; Boehringer Mannheim, Indianapolis, IN) was incorporated into each PCR reaction. The primers used for ER-β were HERB5ntrm (5’-CTCGCTGTGATGAAATTACACG-3’) and HERB3ntrm (5’-TTCTCTGTGTCCTGGCACAG-3’). The primers used for β2m were B2 M5’ (5’-ACCCCACTGAAAGGAATGAT-3’) and B2 M3’ (5’-ATCTTCAACACTCCATGATG-3’). Nonradioactive visualization of amplified cDNAs was accomplished in the following manner: PCR products were separated on a 1.5% agarose gel before denaturation and neutralization of the DNA and underwent Southern transfer as described previously (18). The membranes were blocked in a 10% dried milk solution in Tris-buffered saline with Tween-20 (TBS-T) for 1 h before antidigoxigenin primary antibody (2 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) incubation for 60 min at room temperature. Membranes were then rinsed three times in TBS-T and incubated in the appropriate horseradish peroxidase-conjugated secondary antibody (goat antimouse; Jackson ImmunoResearch Laboratories, Inc.). Final visualization was performed using ECL assays according to the manufacturer’s directions (Amersham, Buckinghamshire, England). Medical X-ray film (Fuji Medical Systems USA, Stamford, CT) was used to develop the ECL reactions. Developed radiographs were scanned in and subsequently digitized using a densitometer. Imagequant densitometry software (Molecular Dynamics, Sunnyvale, CA) was used to analyze and compare the ER-β and β2m bands. The area under the curve graph for each cDNA band was plotted and used to form the basis for comparison of ER-β with β2m.

DNA Sequencing. PCR products were subcloned into vector pCR2.1 (Invitrogen, San Diego, CA). All fragments were sequenced by the Sanger method.

Protein Extraction and Western Blot Analysis. The methods for tissue processing, protein extraction, and immunoblotting were identical to those described previously (18). ER-α was detected using the 1D5 antihuman mouse monoclonal antibody (DAKO Corp., Carpinteria, CA), which is specific to amino acids 65–78 of the NH2 terminus of the human ER-α protein. This membrane was incubated in 1D5 for 3 h at room temperature. ER-β was detected using the anti-human rabbit polyclonal antibody directed against amino acids 46–63 of the human ER-β (Upstate Biotechnology, Lake Placid, NY). This membrane was incubated for 1 h at room temperature. Each immunoblot was then incubated in the appropriate horseradish peroxidase-labeled secondary antibody: goat antimouse (Jackson ImmunoResearch Laboratories, Inc.) and donkey antirabbit (Amersham, Arlington Heights, IL) antibody. The immunolabeled proteins were visualized using ECL assays (Amersham). For positive control experiments ER-α protein was obtained from transfection of the HEO plasmid as described previously (18), and ER-β was commercially available (Affinity Bioreagents, Golden, CO).

Results

We have previously demonstrated ER-α and ER-β expression in the human endometrium, where the former is the predominant ER isoform (19). We first compared ER-α and ER-β protein expression in colon and endometrial tissue (Fig. 1). These results show that in contrast to endometrium, colon tissue is characterized by a predominance of ER-β, with minimal ER-α expression. Colon ER-α and ER-β protein expression was further investigated in normal and malignant tissue from five male and six female patients. Fig. 2 shows the results of Western blot experiments using the ER-α (1D5)- and ER-β (UBR)-specific antibodies. ER-α, which appears as an immunopositive band of approximately Mn 66,000, was found to be detectable at very low levels in colon samples from both men and women (Fig. 2, A and B). There was no difference in the pattern of ER-α expression between normal and malignant colon tissue. ER-β protein, which appears as a double band with a molecular weight of 52,000–58,000, was differentially expressed in normal and malignant colon samples. ER-β protein was dramatically diminished in colon cancer tissue in both men and women (Fig. 2, C and D). No significant difference in ER-α or β-actin protein expression between normal and malignant samples was observed, demonstrating that ER-β protein expression is selectively diminished in colon cancer (β-actin results not shown).

The decrease in ER-β at the protein levels observed in malignant colon tissue was further investigated at the mRNA level using semi-quantitative RT-PCR. Fig. 3 shows a representative radiograph of semi-quantitative RT-PCR using ER-β and β2m primer sets. Densitometry was used to determine relative ER-β mRNA expression by calculating the ER-β/β2m cDNA ratios for each normal and malig-
recently described a proteasome-mediated mechanism for the posttranscriptional regulation of the ER in pituitary cells.

ER-α protein was detectable at very low levels in both normal and malignant colon, with no variation on the basis of gender. This suggests that ER-α does not play a major role in the relationship between HRT and colon carcinogenesis. These observations regarding ER-α expression agree with those reported by other investigators. Singh et al. (23) reported the presence of ER-α in normal and malignant colon samples with no differences in ER-α mRNA expression between cancers, normal mucosa, and polyps. They also found no correlation between ER-α mRNA and protein levels, concluding that the regulation of ER-α protein expression occurs at the posttranscriptional level in large bowel (23). Using enzyme immunoassays, other investigators have reported low-level ER expression in normal and colorectal cancer tissue as well as colon cancer cell lines (24). This may explain why colonic samples have failed to test positive for ER expression in immunohistochemical investigations (25, 26). Furthermore, these immunological methods for colonic ER detection were performed using antibodies directed against ER-α and may have been unable to detect ER-β.

Epidemiological observations from several studies have suggested a protective effect from HRT on the incidence of colorectal cancer. The magnitude of this protective effect has ranged from a RR of 0.54 to a RR of 0.76 (1, 2, 4). Both estrogens and antiestrogens exert their physiological effects through the ER proteins. Functional studies comparing ER-α and ER-β have shown that the two ERs seem to have similar effects on transcription at estrogen response element-containing promoters, whereas they have opposite effects at AP-1 containing promoters. When tested on this promoter, estradiol activates transcription via ER-α but inhibits transcription after binding to ER-β. In addition, antiestrogens, such as tamoxifen, raloxifene, and ICI 164,384, are potent activators of ER-β transactivation at the AP-1 site (17). In light of these opposing effects, the net action of estrogen or selective estrogen response modulators in any given tissue may depend on the balance of ER-α and ER-β in that tissue. One group of investigators recently observed that whereas ER-β was the predominant isoform in normal ovary, its relative expression was markedly diminished in ovarian carcinoma, with 60% of samples exhibiting an ER-α:ER-β ratio of >1 (27). These authors concluded that the change in ER-α:ER-β ratio may be a marker for ovarian carcinogenesis. In the present study, investigation of ER-β expression at both mRNA and protein levels established that a posttranscriptional regulatory mechanism is involved in the expression of this receptor in the colon.

The present study suggests that the protective effects of estrogen replacement therapy against colon cancer may be mediated by ER-β. The role of estrogen and antiestrogens in the pathogenesis of colorectal cancer holds significant public health interest and has yet to be fully elucidated. Further investigation of the ERs at the molecular level needs to be undertaken to gain understanding of this important relationship.

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References


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