Endocrine (1995) 3, 661-665

Biphasic actions of estrogen on colon cancer cell growth: possible mediation by high- and low-affinity estrogen binding sites

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The present experiments were carried out to investigate the possible direct effects of estrogens (E) on the growth of colon cancer cells. Estradiol exhibited a concentration-dependent biphasic growth effect on a mouse colon cancer cell line (MC-26). Low concentrations of estradiol (10^{-10} M to 10^{-8} M) had a growth-stimulatory effect, while higher concentrations (10^{-7} M to 10^{-6} M) were growth-inhibitory. Estrogen receptor (ER) mRNA as well as specific, saturable binding of estradiol to ER (K_d = 0.3 nM, B_{max} = 0.72 fmol/μg DNA) was identified in these cells. In addition to the classical high affinity ER, lower affinity, higher capacity estrogen binding sites (K_d = 35 mM, B_{max} = 30 fmol/μg DNA) were also characterized in MC-26 cells. These two types of estrogen binding sites exhibited distinct binding specificities for E and antiestrogens. Treatment of MC-26 cells with an oligodeoxynucleotide antisense to the translation start codon of ER mRNA did not alter the grown-inhibitory effect of 10^{-6} M estradiol, demonstrating that the growth-inhibitory effect of high concentrations of E was not mediated by ER; we have previously shown that under the same conditions, ER antisense oligonucleotides do block the growth-stimulatory effects of 10^{-9} M E_2 in MC-26 cells. The data suggest that physiological concentrations of estradiol acting via the classical ER may have a proliferative effect on the growth of colon cancer cells. However, in situations where there are high luminal concentrations of estrogenic compounds, they may act on low affinity estrogen binding sites that mediate the growth-inhibitory effect.

Keywords: estrogen; cell growth; colon cancer; estrogen receptor; type-II estrogen binding site

Introduction

With the detection of estrogen receptors (ER) in both normal colon epithelium (Singh *et al.*, 1993; Thomas *et al.*, 1993) and colon cancer tumors (McClendon *et al.*, 1977; Alford *et al.*, 1979; Singh *et al.*, 1993), we have become interested in the possible role of estrogen (E) in the etiology and/or growth of colon cancer. Estrogens have been implicated in the etiology of cancers in reproductive organs of humans (Henderson *et al.*, 1991). In breast cancer, the role of E has not only been supported by epidemiological data indicating a correlation between E status and tumor incidence, but also by *in vitro* experiments indicating that estradiol (E_2) is a powerful mitogen in breast cancer cells cultured in short-term E-free conditions (Katzenellenbogen *et al.*, 1987). Substantial similarities in the descriptive epidemiology of colorectal cancer and breast cancer (McMichael & Potter, 1983; Howell, 1976; Willett, 1989; Schoenberg *et al.*, 1969) suggest a role for sex hormones in the development and/or growth of colon cancer. Also, ovariectomy was found to reduce the mitotic rate in dimethylhydrazine-induced tumors of rat colon. This effect was reversible by treatment of the ovariectomized animals with estradiol, but not treatment with progesterone (Tutton & Barkla, 1982). MC-26 cells, a 1,2-dimethylhydrazine-induced mouse colon adenocarcinoma cell line (Waldrop *et al.*, 1989), inoculated into female mice produced tumors with higher tumor weights than similarly inoculated male mice, ovariectomy results in descreased tumor weight compared to sham-operated female control mice (Narayan *et al.*, 1992). These data suggest that E is mitotic to colon cancer cells *in vivo*.

ER has been detected in colorectal carcinomas with varying percentages of positivity (McClendon *et al.*, 1977; Alford *et al.*, 1979; Sica *et al.*, 1984; Francavilla *et al.*, 1987; Waldrop *et al.*, 1989; Stopera *et al.*, 1992). Recently, ER mRNA has also been identified in colorectal cancers, corresponding normal mucosa, and in adenomatous polyps (Singh *et al.*, 1993). The presence of ER in normal as well as cancerous tumor epithelium suggests that E may have direct regulatory effects on colon cancer cells.

We have recently shown that E directly stimulates MC-26 cell growth *in vitro* (Xu & Thomas, 1994). In these experiments, the growth-stimulatory effect of E was eliminated in cells containing deceased levels of ER following treatment with an oligodeoxynucleotide (oligo) directed against the ER mRNA. This demonstrated that the growth-stimulatory effect of E is ER-dependent. In the present study, we report an ER-independent growth-inhibitory effect of concentrations of E that are higher than normal circulating levels, as well as the presence of low-affinity estrogen binding sites (EBS) in MC-26 cells. The growth-inhibitory effect of higher concentrations of E may be particularly relevant to intestinal cells and cancers due to the potential for exposure to high luminal concentrations which may occur following dietary or oral pharmacological E.

Results

The effects of E_2 on MC-26 cell growth were biphasic depending on E_2 concentrations (Figure 1). Low concentrations (10^{-10}–10^{-8} M) of E_2 stimulated cell growth, whereas higher concentrations (10^{-7}–10^{-6} M) inhibited cell growth. The growth-stimulatory effect of E_2 peaked at 10^{-9} M; the inhibitory effect approached maximum at 10^{-7} M. Both the growth-stimulatory and -inhibitory effects of E_2 were most prominent after continuous treatment with E_2 for 6 days under our culture conditions. The cell viability after 6 days in culture did not vary between control and treated cells, both being greater than 90%. Therefore, the growth-inhibitory effect of E_2 at relatively high concentrations was not attributable to enhanced cytotoxic effects. Furthermore, the inhibitory effect appeared to be reversible, since when 10^{-6} M E_2 was removed after 4 days of treatment, the cell number increased relative to cells which continued to be maintained in the presence of E_2 (Figure 2).

Northern blot analysis of MC-26 cell mRNA revealed a single band of ER transcript approximately 6.4 Kb in size (Figure 3), identical to the mouse uterus ER mRNA, which has been previously described (White *et al.*, 1987). Furthermore, MC-26 cells exhibited binding characteristic of classical

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Received 18 April 1995; accepted 20 June 1995

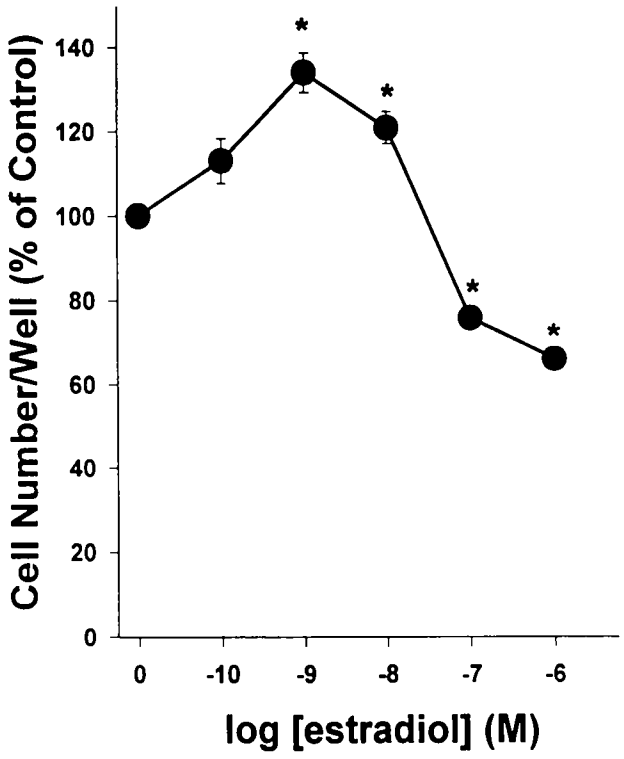

Figure 1 Dose-dependent effect of E_2 on the growth of MC-26 cells. MC-26 cells were treated with different concentrations of E_2 for 6 days. Control cells were treated with the same amount (0.1%) of ethanol vehicle alone. The presented data were obtained from a single experiment representative of three replicate experiments yielding similar results. Each bar represents mean ± SD for four wells expressed as % of control. $^*P<0.05$ compared to control

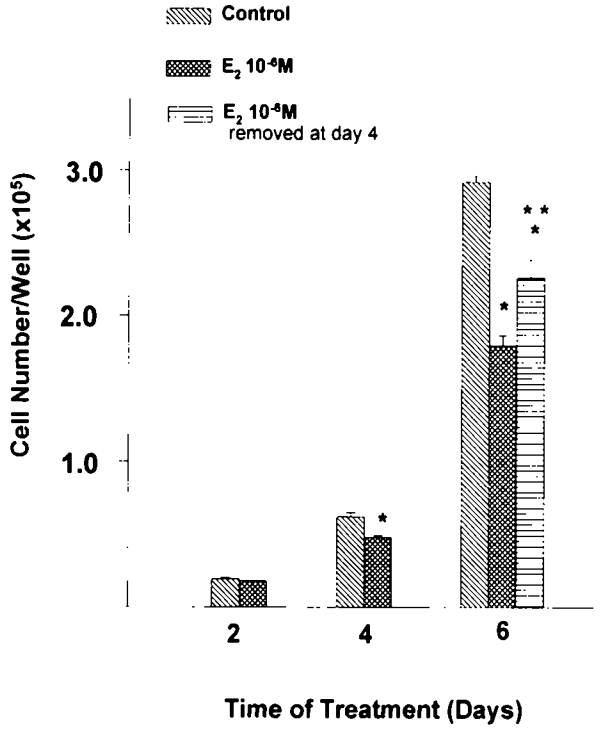

Figure 2 Reversibility of the growth inhibitory effect of E_2 at 10^{-6} M. Cells were plated at 2000/well (− 2 days) and after 48 h (0 day) the medium was replaced with fresh medium containing 10^{-6} M E_2 or vehicle alone (0.1% ethanol). Medium was changed and treatment replaced every 2 days. On day 4, one E_2 group was treated as control without E_2 replacement. Cells in each group were counted on days 2, 4 and 6. Results represent the means ± SD of four wells. $^*P<0.05$ compared to control; $^{**}P<0.05$ compared to the group with continuous E_2 treatment

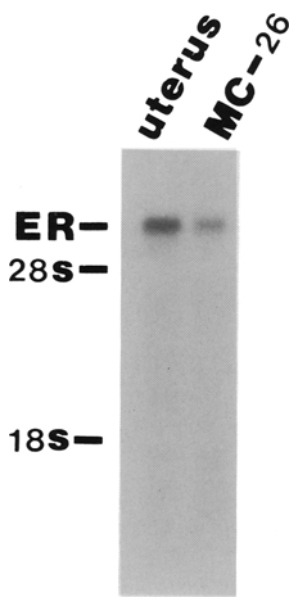

Figure 3 Northern analysis of ER mRNA in MC-26 cells. 3 μg of total RNA from mouse uterus and 15 μg of total RNA from MC-26 cells were run in each lane

ER (Figure 4). Scatchard analysis (Figure 4, inset) of ER binding data suggests a single class of high affinity binding sites, with an estimated K_d of 0.3 nM and B_{max} of 0.72 fmol/μg DNA corresponding to approximately 3400 sites/cell.

When we examined the binding of higher concentrations of $[^3H]E_2$ under conditions which did not favor the measurements of occupied ER (Scambia *et al.*, 1993; Markaverich *et al.*, 1981b), MC-26 cells exhibited a sigmoidal saturation curve (Figure 5), typical of type-II EBS (Ranelletti *et al.*, 1992; Markaverich *et al.*, 1981b). Since no accurate estimate either of K_d or of the number of EBS can be made from a curvilinear Scatchard plot, the number of EBS and the K_d were estimated directly from the saturation curve (Markaverich *et al.*, 1981a) with approximate K_d of 35 nM, and a B_{max} of 30 fmol/μg DNA corresponding to 1.3×10^5 sites/cell.

In MC-26 cells, the ligand specificity of ER (Figure 6A) and the lower affinity EBS binding (Figure 6B) differed. E_2 was an efficacious competitor for both types of binding, while the relatively pure antiestrogen, ICI 182,780, competed successfully for ER binding, but did not compete for the lower affinity binding. The ER antagonist, tamoxifen was similarly competitive for both types of binding. Tamoxifen binding to lower affinity estrogen binding sites (EBS) has been noticed by others (Scambia *et al.*, 1991). Quercetin, a known ligand for type-II EBS (Markaverich *et al.*, 1988b) did not compete for ER binding, but competed efficiently for lower affinity EBS (Figure 6B). Progesterone and testosterone were unable to compete with labeled E_2 for either ER or EBS binding. When tested in a growth experiment, 10^{-6} M concentrations of DES, tamoxifen and quercetin had effects similar to those of estradiol, decreasing cell number by 30–40% after 6 days (data not shown).

By using an oligonucleotide antisense to the region of the translation start codon of ER mRNA, we have previously demonstrated that inhibition of ER expression could inhibit the growth-stimulatory effect of a physiological concentration of E (10^{-9} M) in MC-26 cells (Xu & Thomas, 1994). Using the same antisense oligo, the growth-inhibitory effect of high concentration E (10^{-6} M) was not altered (Figure 7). There was no difference between control cells and cells treated with the scrambled oligo either in the presence or absence of 10^{-6} M E_2. To verify the effectiveness of oligo treatment, ER binding was determined in parallel culture wells undergoing

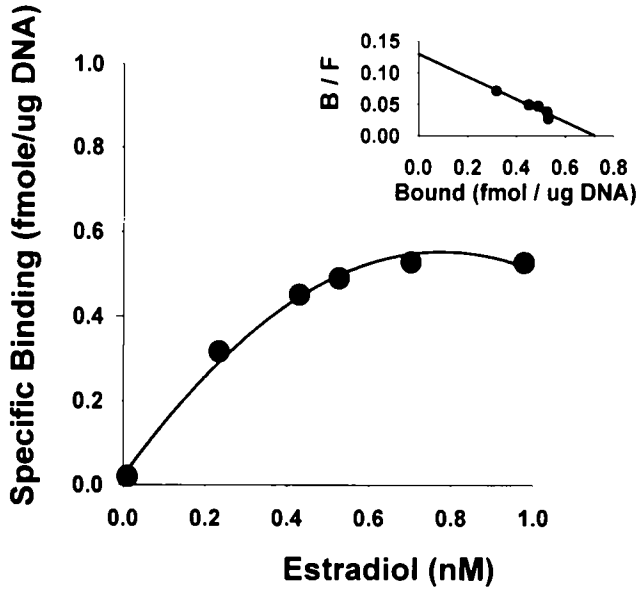

Figure 4 Saturation analysis of E_2 binding to ER in MC-26 cells. Inset, Scatchard analysis of ER binding data. B/F = bound/free ratio. The indicated concentrations of [^{3}H]E_2 (44.5 Ci/mmol) were added to each well in the presence or absence of a 300-fold excess of unlabeled DES for 2 h at room temperature. Each point represents the mean for three wells

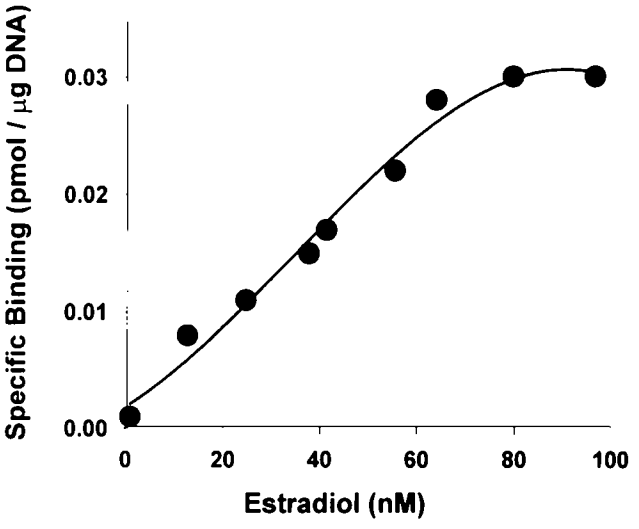

Figure 5 Saturation analysis of E_2 binding to EBS in MC-26 cells. The indicated concentrations of [^{3}H]E_2 (44.5 Ci/mmol) were added to each well in the presence or absence of a 300-fold excess of unlabeled DES for 2 h at 4°C. Each point represents the mean for three wells

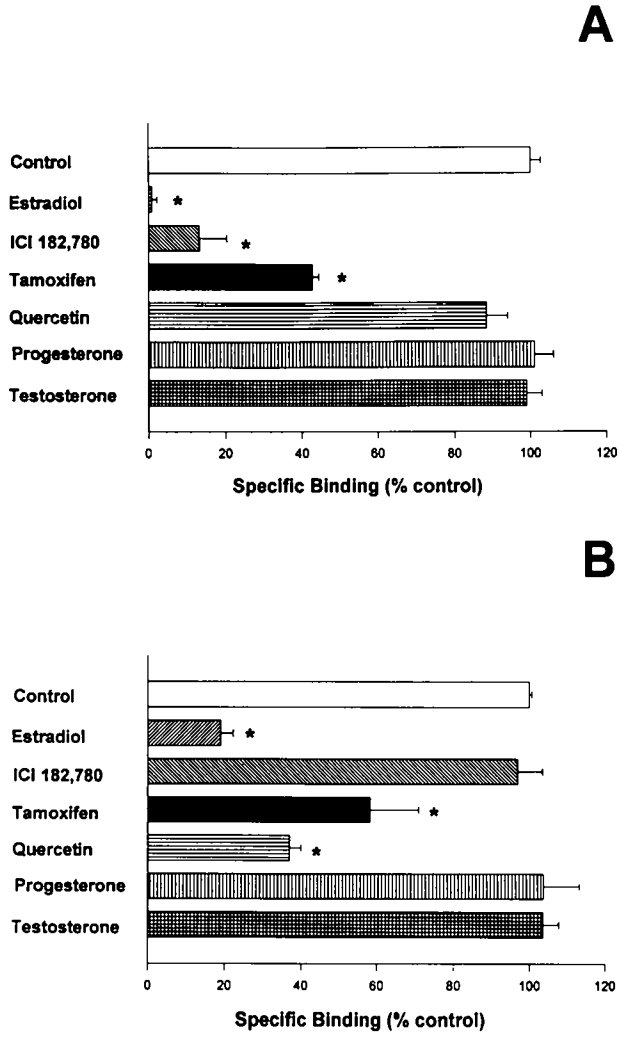

Figure 6 Specificity of E_2 binding to ER (A) and EBS (B) in MC-26 cells. Cells were incubated with 0.3 nM (A) or 35 nM (B) [^{3}H]E_2 and a 300-fold excess of competing agents: Estradiol, ICI 182,780, Tamoxifen, Quercertin, Progesterone (progest.), and Testosterone (testost). The control bar represents specific [^{3}H]E_2 binding using 300-fold excess of DES to define nonspecific binding. Uncompeted specific binding in the presence of each competing agent was expressed as % of control. Each bar represents the mean ± SD for three wells. $^*P < 0.05$ *vs* control

the same experimental treatments as those used to determine cell number. We found that antisense oligo treatment decreased specific, high affinity ER binding to 30% of control, while the scrambled oligo had no effect on binding (data not shown).

Discussion

Our results have demonstrated that low concentrations of E stimulate MC-26 cell growth. This effect occurred in the concentration range of the classical ER K_d. We also found that higher concentrations of E had a net effect to decrease replication of MC-26 cells. This response occurred in a concentration range consistent with being mediated via low affinity EBS, and indeed the cells exhibited binding characteristic of the reported type-II EBS. The results of the present work have led us to the hypothesis that in colon cancer cells, E activation of the ER results in increased cell proliferation, while at higher concentrations E acts via the low affinity, high capacity EBS overriding the growth-stimulatory effect mediated by ER and resulting in a net suppression of cell growth.

Type-II EBS have been found to be present in various normal and neoplastic tissues (Capony & Rochefort, 1977; Panko *et al.*, 1981; Smith *et al.*, 1979; Ranelletti *et al.*, 1992). Given the low affinity of type-II EBS for E and the low levels of circulating hormone it is hard to imagine that these sites can be occupied by E *in vivo*. However, gastrointestinal tissue may be an interesting exception, because gastrointestinal epithelium may be exposed not only to circulating E, but also to higher concentrations of dietary flavonoids and phytoestrogens as well as orally administered pharmaceutical E's and estrogens produced by intestinal microflora (Minelli *et al.*, 1990; Ling, 1995) which are present in the intestinal lumen. There are epidemiological reports that oral contraceptive usage (Potter & McMichael, 1983), and more consistently, postmenopausal E therapy (Chute *et al.*, 1991), have a pro-

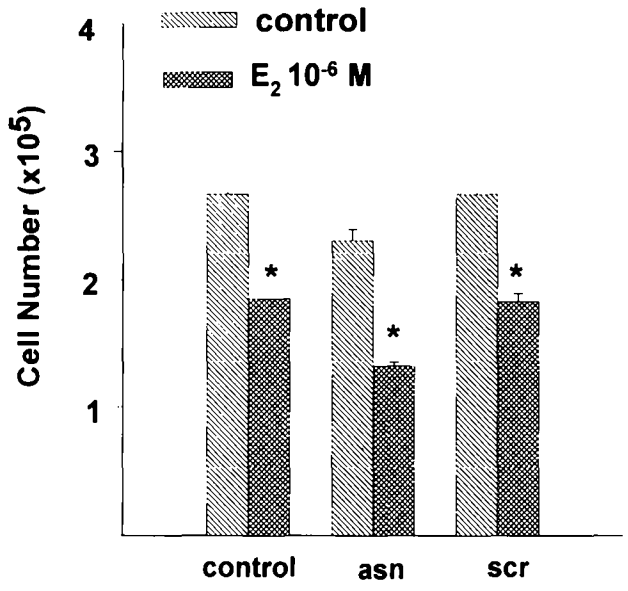

Figure 7 The effect of ER antisense oligo treatment on E-induced growth-inhibition in MC-26 cells. MC-26 cells were treated with 10^{-6} M of either antisense (ASN) or scrambled (SC) oligo in the presence or absence of 10^{-6} M E_2 for 6 days. Control cells were treated with the same amount of ethanol (0.1%) and elution buffer (10 µl) or 10^{-6} M E_2 and elution buffer. Each bar represents mean ± SD for 4 wells. $^*P<0.05$ compared to control without E_2. $^{**}P<0.05$ compared to control with 10^{-6} M E_2

tective role with regard to the risk of large bowel cancer. These findings appear to be in conflict with other epidemiological data showing that premenopausal women have a higher rate of colon cancer than their male cohort (McMichael & Potter, 1983; Doll, 1980). It is possible that the sex differences in colon cancer may be due to the effects of circulating E acting on the ER, while the protective effects of oral E are due to the EBS-mediated response to high luminal concentrations of E.

The biphasic growth effect observed in this study is not unique for E or MC-26 cells. It has been found that retinoic acid at 10^{-8} M stimulated proliferation of the prostatic carcinomas cell line LNCaP, but inhibited their growth at 10^{-7} M (Fong *et al.*, 1993). A similar observation was also made in breast cancer cells (Butler & Fontana, 1992). The growth inhibitory effect of relative high concentrations ($>10^{-6}$ M) of retinoic acid seems to be related to the low affinity cellular retinoic acid-binding protein and retinol binding protein levels (Kaleagasioglu *et al.*, 1993). Breast cancer cells also appear to exhibit a balance of E-regulated effects on growth depending upon the relative amounts of ER and type-II EBS present in a specific cell type as well as the concentrations of E. Physiological concentrations of E can stimulate breast cancer cell proliferation (Lippman *et al.*, 1976). However, high concentrations of E cause regression of some human breast cancers (Adair *et al.*, 1949; Binnie, 1944). It was found that in an ER-negative MCF-7 human breast cancer cell line, both DES and quercetin compete with similar potency for [^{3}H]E_2 binding to EBS and both exert a dose-dependent inhibition of cell proliferation in the range of concentrations between 10^{-9} M and 10^{-6} M (Scambia *et al.*, 1991). Similarly, E at 10^{-8} to 10^{-6} M concentrations inhibits the growth of an ER-negative human colon cancer cell line LoVo (Lointier *et al.*, 1992).

In MC-26 colon cancer cells, the presence of both high affinity ER and lower affinity, higher capacity EBS allows these cells to display a biphasic growth response to E, depending upon the concentration. It is possible that the presence of lower affinity, higher capacity binding sites of hormones is a natural protective mechanism to counteract the effects of estrogenic compounds present at higher than physiological concentrations. This is supported by the finding that quercetin, one of the flavonoids, is a powerful growth inhibitor in human breast (Markaverich *et al.*, 1988b), leukemic (Larocca *et al.*, 1990), ovarian (Scambia *et al.*, 1990) and colorectal (Ranelletti *et al.*, 1992) tumor cells via its interaction with the EBS, and that EBS are occupied *in vivo* by a flavonoid-like endogenous ligand which inhibits normal and malignant cell growth (Markaverich *et al.*, 1988a).

We have previously shown that inhibition of ER expression by an ER antisense oligo abolished the growth-stimulatory effect of 10^{-9} M E, demonstrating that ER mediate direct stimulation of MC-26 cell growth (Xu & Thomas, 1994). Using the same antisense oligo, the growth-inhibitory effect of 10^{-6} M E was not altered by the oligo treatment, demonstrating that the growth-inhibitory effect of higher concentrations of E is most likely ER-independent.

In summary, the data presented here are consistent with the hypothesis that E is able to act via at least two mechanisms to regulate growth of colon cancer cells. Relatively physiological concentrations of E, acting through the classical intracellular ER, function to stimulate cell replication, while higher E concentrations, perhaps acting via the type-II EBS, inhibit cell growth. At this stage, we can not rule out the possibilities of direct estrogen actions via other receptor- or nonreceptor-mediated intracellular mechanisms. Further testing of our hypothesis that the lower-affinity estrogen binding site in MC-26 cells is a similar protein to that described as type-II EBS in uterus and other tissues will be facilitated by the cloning and further characterization of the type-II EBS.

Materials and methods

Cell Culture: MC-26 cells, obtained from the Department of Surgery at the University of Texas Medical Branch, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). In experiments studying the effects of E on MC-26 cell growth, cells were plated into 24-well plates (2000 cells/well) in phenol red-free DMEM supplemented with 5% charcoal-treated FBS, and were allowed to attach for 48 h without treatment. On treatment day 0, fresh medium containing hormones (in absolute ethanol, final ethanol concentration<0.1%) was added and replaced every two days thereafter. MC-26 cells in the control group were treated with ethanol vehicle only. After 6 days of hormone treatment, cells were harvested by treatment with 0.025% trypsin/0.02% EDTA, and cell numbers were counted using a Coulter counter (Model ZF, Coulter Electronics, Hialeah, FL). Cell viability was determined by trypan blue exclusion using a hemocytometer. In experiments using oligos, they were introduced as the medium was changed every 2 days.

Radioligand Binding Analysis of ER and EBS: ER and EBS in MC-26 cells were measured by whole cell assays as described previously (Thomas *et al.*, 1993; Scambia *et al.*, 1993). Briefly, ER binding was determined after 2 h incubation with [^{3}H]E_2 (Amersham, specific activity = 44.5 Ci/mmol) at room temperature (Thomas *et al.*, 1993). The EBS assay was performed at 4°C for 2 h with other procedures the same as used in the ER binding assay. The nonspecific binding was defined in the presence of a 300-fold molar excess of diethylstilbestrol (DES).

Northern Blot Analysis of ER mRNA: Total RNA from cultured MC-26 cells or mouse uterus (positive control) was isolated as described by Chomczynski (Chomczynski & Sacchi, 1987). The Northern blot analysis was carried out using standard procedures (Thomas, 1980) as described previously (Thomas *et al.*, 1993). The ER probe was a random primer-labeled RT-PCR cDNA fragment generated from rat uterine ER mRNA (Thomas *et al.*, 1993), which had 95.4% sequence homology to the mouse ER cDNA sequence.

Oligonucleotides: A 15-mer antisense oligo (5′ GGGTCATGGTCATGG 3′) and a scrambled oligo (5′GTGGTGGATCGTGAC 3′) modified as phosphorothioates were synthesized by Genosys (Woodlands, TX). The antisense oligo spanned the common translation start codon for mouse and human ER mRNA. The scrambled oligo contained the same 15 base components as the antisense oligo, but had little or no homology to any known gene sequences in the GenBank. The oligos were further purified by NAP-5 column (Pharmacia, Uppsala, Sweden) prior to use in the cell culture experiments.

Statistics: Statistical significance was determined using one-way analysis of variance with *post hoc* Bonferroni *t*-test. A P value < 0.05 was considered significant.

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