

Sex differences in the incidence of colorectal cancer: an exploration of oestrogen and progesterone receptors

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Abstract

Sex differences exist in the site specific incidences of colorectal cancer. The increased incidence of colonic cancer in women with breast cancer and the protective effect of increasing parity suggest a role for sex hormones. To explore the molecular basis, the expression of messenger RNA for oestrogen and progesterone receptors in the large bowel has been studied. With northern and dot blot analyses mRNA coding for oestrogen receptor and progesterone receptor in large bowel cancers and corresponding normal mucosa and in adenomatous polyps has been identified. There were no significant differences in receptor mRNA concentrations between males and females or between cancers, normal mucosae, and polyps, except for rectal cancers, which had higher progesterone receptor concentrations than corresponding normal tissue. Oestrogen and progesterone receptor mRNA concentrations were strongly correlated in both cancers and normal tissues. Enzyme immunoassay for oestrogen receptor gave values of 1.2-7.4 fmol/mg total protein, an amount similar to that seen in normal breast tissue. Oestrogen receptor protein and mRNA for oestrogen receptor and progesterone receptor are present in the large bowel.

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Cancer of the colon is more common in women up to 55 years of age but thereafter is more common in men; rectal cancer occurs with almost equal frequency in both sexes up to 45 years of age, but becomes almost twice as common in men after the age of 65 years.^{1,2} There is also a strong correlation within individual countries between breast cancer and colonic cancer mortality.³ In individual women there is a higher than expected concurrence of colonic cancer and breast cancer.^{4,5} Also, multiparous women have a lower risk of colonic cancer than women with only one child, who have a lower risk than women who are nulliparous; nuns also have a higher than expected incidence of colonic cancer.⁷ In men right sided colonic cancer and cancer of the prostate (a condition in which oestrogens and androgens influence disease behaviour) tend to occur together more often than expected.⁸ In a study of men with prostatic carcinoma who subsequently developed colorectal cancer, those treated with stilboestrol had a higher incidence of right sided colonic cancers and a lower incidence of rectal cancers, than those not receiving oestrogen treatment.⁹

These data suggest that sex steroid hormones

influence the development of colorectal cancer. For these hormones to have a direct action, appropriate receptors must exist in the mucosa of the large bowel. Previous studies with the technique of radioligand binding assay have provided limited evidence for the presence of oestrogen and progesterone receptors in a variable proportion of colorectal cancers and normal mucosal samples.^{10,11} Other radioligand studies, however, have shown no characteristic binding or mainly low affinity, high capacity binding sites that may represent non-specific binding only.¹²⁻¹⁴ Also, immunohistological techniques have been unsuccessful in showing oestrogen receptors in the large bowel mucosa or cancers.¹⁵

Before testing any hypothesis regarding sex steroids in the pathogenesis of colorectal cancer, it is essential to show the presence of sex steroid receptors. We have therefore used sensitive molecular biological techniques to seek the presence of messenger RNA (mRNA) coding for oestrogen and progesterone receptors in the mucosa of the normal large bowel, polyps and cancers, and a colonic cancer cell line. We have also sought to show the presence of oestrogen receptor protein by enzyme immunoassay.

Methods

Tissue samples were collected into liquid nitrogen within 20 minutes of resection of surgical specimens. Non-necrotic tissue was identified and removed and a sample of normal mucosa was dissected off the muscularis propria 5-6 cm from the cancer. Colonic polyps were collected after colonoscopic polypectomy, the polyp being bisected axially with one half taken for histology. Tissue samples were stored at -70°C.

Total RNA was extracted by the single step guanidinium isothiocyanate method¹⁶ from about 0.5 g of tissue. Integrity of RNA was assessed by the visualisation of distinct ribosomal 28S and 18S bands after electrophoresis on a 1% agarose gel stained with ethidium bromide. Northern blot analysis was performed by electrophoresis of approximately 30 µg of total RNA in a formaldehyde 15%/agarose 1% gel in 20 mM morpholinopropanesulfonic acid (MOPS) buffer. The RNA was transferred overnight in 20×SSC (0.3 M sodium citrate, 3 M sodium chloride) to Hybond N⁺ membranes (Amersham International, Buckinghamshire, UK). RNA was dot blotted with a hybridot apparatus (Bethesda Research Laboratories, Maryland, USA) to provide quantitative data. RNA was alkali fixed to the membrane according to the manufacturer's instructions.

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cDNA PROBES AND FILTER HYBRIDISATION

Full length cDNA probes coding for the human oestrogen receptor,¹⁷ 2.1 Kb in length, and the human progesterone receptor,¹⁸ 2.6 Kb in length, were used. Membranes were also probed with a 0.7 Kb EcoRI-BalI fragment of the oestrogen receptor and a 1.3 Kb BamHI-HincII fragment of the progesterone receptor. These two fragments correspond to the 5' hypervariable region of the cDNAs, avoiding the DNA binding domain, a region highly conserved in the superfamily of nuclear binding receptors and therefore liable to cause cross hybridisation.¹⁹ Northern blots were also probed with a rat 18S ribosomal RNA cDNA probe²⁰ to determine total RNA loading.

Membranes were prehybridised and hybridised in a buffer comprising 0.77 M sodium phosphate, 5 mM EDTA, 7% sodium dodecyl sulphate (SDS), 100 µg/ml denatured salmon sperm DNA at 65°C in a rotating oven (Hybaid, Middlesex, UK). Hybridisation was performed for 16 hours and then membranes were washed in 1×SSC (0.015 M sodium citrate, 0.15 mM sodium chloride) 1% SDS at 65°C for one hour followed by 0.1×SSC (1.5 mM sodium citrate, 15 mM sodium chloride) 0.1% SDS at room temperature. Probes were radiolabelled with α(³²P) dCTP (deoxycytidine triphosphate (3000 Ci/mmol)) by the random primer extension technique²¹ to an activity of 10⁹ cpm/µg. The membranes were autoradiographed at -70°C with intensifying screens. Membranes were then stripped at 70°C in 0.5% SDS and reprobed after all activity had been shown to be removed by autoradiography. Dot blots were also stripped and reprobed with a 17 mers oligodeoxythymidine probe radiolabelled with α³²P deoxythymidine triphosphate (3000 Ci/mmol) using terminal deoxynucleotidyl transferase (Pharmacia Biosystems, Milton Keynes, UK), to estimate total mRNA.²² Membranes were hybridised at 37°C and the final wash was 2×SSC 0.1% SDS at 50°C. Dot blot autoradiograms were analysed by laser densitometry.²³

To provide a positive control for oestrogen and progesterone receptors, RNA was extracted and analysed from the human breast carcinoma cell line MCF-7, which was cultured in Dulbecco's modified Eagle's medium, (Gibco Laboratories, Renfrewshire, UK) containing 10% fetal calf serum and phenol red indicator. RNA was also analysed from normal breast and endometrial tissues, obtained at operation. Calf liver ribosomal RNA (30–40 µg; Pharmacia Biosystems) was used to detect non-specific binding on both dot and northern blots.

OESTROGEN RECEPTOR ENZYME IMMUNOASSAY

A commercially available oestrogen receptor immunoassay was used according to the manufacturer's instructions (Abbott Laboratories, Berkshire, UK). Briefly, the tissue was homogenised in a buffer comprising 10% glycerol, 10 mM Hepes, 1.5 mM EDTA, 0.4 M potassium chloride, and 5 mM sodium molybdate. The homogenate was centrifuged at 10 000 g. The tissue cytosol and oestrogen receptor standards provided were incubated with beads coated with a rat antioestrogen receptor monoclonal antibody. The beads were washed to remove unbound material. Bound oestrogen receptor was labelled by incubating with a second monoclonal antioestrogen receptor antibody conjugated with horse radish peroxidase. After washing, the beads were incubated with enzyme substrate (hydrogen peroxide and o-phenylenediamine-HCl), and the colour developed was read on a spectrophotometer set at 492 nm. A standard curve was established and specimen values determined. Total cytosolic protein was estimated by the Bradford method²⁴ (Biorad Laboratories, Hertfordshire, UK).

Results

PATIENT DETAILS

Thirty seven normal and cancer tissue pairs and 10 polyps were analysed. Table I shows patient and cancer details. Normal mucosa from a 36 year old woman undergoing colectomy for severe intractable constipation was also analysed. Cancers of the caecum, ascending colon, and transverse and descending colon are analysed together in the 'colonic' group (see Table II). Cancers of the sigmoid colon and at the rectosigmoid junction were analysed together in the 'sigmoid' group. All cancers were adenocarcinoma. There were six polyps less than 1 cm (two women), and four in the range 1–2 cm, all were tubulovillous on histological examination and were from men. All polyps originated from either the sigmoid colon or the rectum.

NORTHERN ANALYSES

For both the oestrogen and progesterone receptors the full length cDNA probes and the fragments to the hypervariable region produced the same result confirming that hybridisation was specific. Northern blot analysis with the oestrogen receptor cDNA revealed a band of approximately 6 Kb in cancers and normal tissue. This band was identical in size to the band seen in the breast carcinoma line MCF-7 (Fig 1), which has previously been described as 6.2 Kb.¹⁷ Figure 1 also shows the same blot probed for progesterone receptor. A band of approximately 5.5 Kb was seen in normal tissues and cancers, corresponding to the band seen in the MCF-7 line. The progesterone receptor mRNA in the normal uterus displays bands of a similar size of 5.9 and 5.1 Kb, but also lesser bands of 4.3, 3.7, and 2.9 Kb size.¹⁸ When grown in stripped serum and stimulated with oestradiol MCF-7 cells display bands of 11.4, 5.8, 5.3, 3.5, and 2.8 Kb size on northern blots.²⁵ The absence of the larger size

TABLE I Details of patients and cancers

Site of cancer	Men No mean age	Women No mean age	Histological state of differentiation			Dukes stage			
			Well	Moderate	Poor	A	B	C	D
Caecum and ascending colon	4 (64)	5 (78)	0	8	1	0	5	4	0
Transverse and descending colon	1 (72)	1 (57)	0	2	0	0	0	2	0
Sigmoid colon	7 (69)	5 (70)	4	7	1	2	3	5	2
Rectosigmoid colon	2 (62)	3 (77)	0	4	1	1	3	1	0
Rectum	5 (66)	4 (77)	0	8	1	1	3	5	0

TABLE II Mean (SEM) values of oestrogen and progesterone receptor expression in large bowel cancers and corresponding normal mucosa

	Progesterone receptor mRNA (arbitrary units)		Oestrogen receptor mRNA (arbitrary units)		Oestrogen receptor protein (fmol/mg)	
	Normal	Cancer	Normal	Cancer	Normal	Cancer
Men (n=19)	4.6 (0.7)	4.2 (0.7)	2.2 (0.3)	2.2 (0.3)	3.3 (0.4) (n=10)	2.9 (0.3)
Women (n=18)	4.0 (0.3)	4.5 (0.9)	1.6 (0.1)	1.7 (0.4)	3.4 (0.9) (n=6)	2.6 (0.4)
Dukes stage:						
A+B (n=18)	4.4 (1.5)	5.9 (1.0)	2.2 (0.2)	2.4 (0.3)	3.0 (0.4) (n=7)	2.5 (0.4)
C+D (n=19)	4.1 (0.6)	3.0 (0.3)	1.7 (0.2)	1.6 (0.2)	3.5 (0.6) (n=9)	3.0 (0.3)
Differentiation:						
Good (n=4)	3.1 (0.7)	3.7 (1.6)	1.4 (0.2)	1.7 (0.6)	2.9 (0.2) (n=4)	3.0 (0.4)
Moderate (n=29)	4.1 (0.3)	4.3 (0.7)	1.9 (0.4)	2.0 (0.3)	3.3 (0.6) (n=11)	2.8 (0.3)
Poor (n=4)	7.2 (1.5)	5.5 (1.5)	2.7 (0.8)	2.0 (0.8)	4.9 (n=1)	2.2
Site:						
Colonic (n=11)	4.8 (1.7)	4.1 (1.0)	2.0 (0.4)	2.1 (0.7)	3.4 (0.6) (n=7)	2.3 (0.4)
Sigmoid (n=17)	4.5 (0.5)	3.7 (0.5)	2.0 (0.2)	1.7 (0.2)	3.0 (0.3) (n=6)	3.2 (0.3)
Rectal (n=9)	3.9 (0.6)*	5.9 (1.1)*	1.6 (0.2)	2.3 (0.5)	3.8 (1.8) (n=3)	3.0 (0.4)
Polyps (n=10)		5.0 (1.4)		1.4 (0.5)		

*p=0.01 (passed t test).

PR=progesterone receptor; OR=oestrogen receptor; N=normal; C=cancer.

transcript in the MCF-7 RNA in Fig 1 may be because the cells were not grown in stripped serum but in medium containing 10% fetal calf serum, and also no additional oestradiol was added.

By comparison with the 18S ribosomal band in Fig 1, it can be seen that there was equal expression of oestrogen and progesterone receptor mRNA in paired normal and malignant tissues. No regional differences in receptor expression were seen in the normal mucosal samples analysed from the patient with constipation. Also seen in Fig 1 is the lack of significant cross hybridisation to ribosomal RNA.

QUANTITATIVE ANALYSIS

Oestrogen and progesterone receptor dot blot densitometer readings were expressed as a ratio of the oligodeoxythymidine reading to correct for the total amount of RNA loaded, in arbitrary

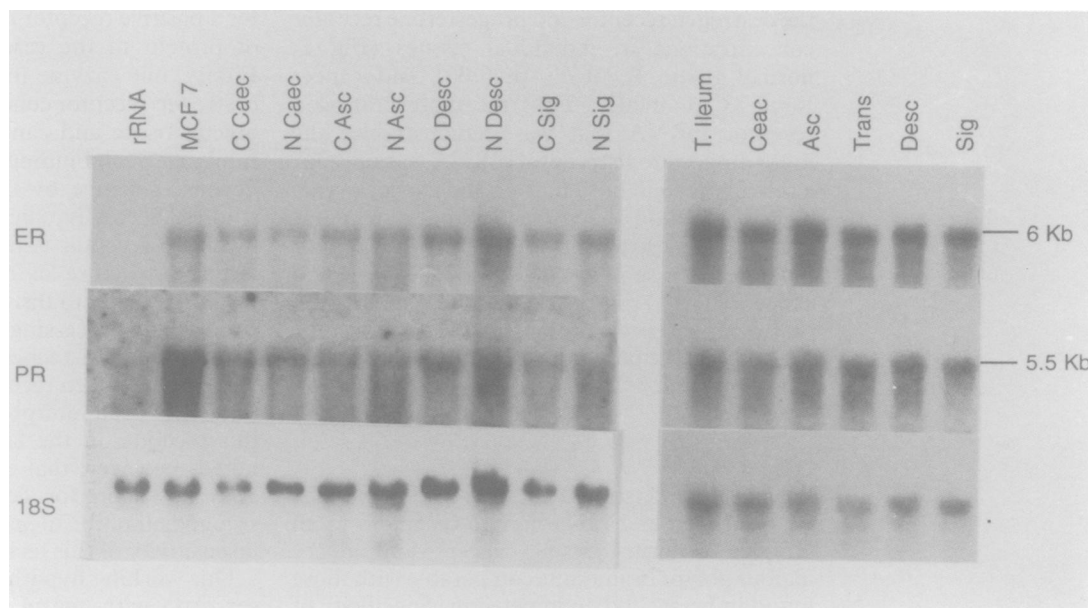
units. Positive controls of normal breast and normal endometrium had oestrogen and progesterone receptor values in the range 0.5–3.0 units and 1.0–5.0 units respectively. No non-specific hybridisation was seen to ribosomal RNA. mRNA for both oestrogen and progesterone receptor was detected in all large bowel specimens in amounts comparable with that seen in breast and endometrial tissues. Oestrogen receptor enzyme immunoassay results were available for 16 pairs of normal and cancer tissues and ranged between 1.2–7.4 fmol/mg of total protein. This amount is comparable with that found in normal breast tissue of between 2–10 fmol/mg depending on the phase of the menstrual cycle,²⁶ but is much less than the 300 fmol/mg seen in the endometrium.²⁷

Table II shows that there were no statistically significant differences in receptor expression between large bowel specimens from men and women (Mann-Whitney test). The differences in oestrogen and progesterone receptor mRNA concentrations between Dukes's stage C and D cancers and Dukes's A and B cancers were not statistically significant, and were not supported by a similar difference in oestrogen receptor protein by enzyme immunoassay. Table II also shows a trend of increasing oestrogen and progesterone receptor mRNA expression in cancers and corresponding normal tissue as the cancers become more poorly differentiated. This trend is supported by a similar trend in the oestrogen receptor immunoassay value in the case of the normal tissue only. Because of the small numbers in the well and poorly differentiated groups, statistical significance was not attained.

There were no statistically significant differences in receptor expression between normal tissue, cancers, or polyps except for rectal cancers. As seen in Table II, rectal cancers had higher concentrations of progesterone receptor mRNA compared with corresponding normal tissue (p=0.01, paired t test). The difference in oestrogen receptor mRNA concentration was not significant (p=0.07) and was not supported by a corresponding difference in oestrogen receptor protein concentration by immunoassay.

Figure 1: Expression of oestrogen receptor (ER) and progesterone receptor (PR) mRNA in human large bowel. Shown is northern analyses of approximately 30 µg total RNA, with cDNA probes.

Hybridisation with a ribosomal 18S probe estimates total RNA loading. The first lane contains calf liver ribosomal RNA (rRNA) to detect non-specific hybridisation. Next is the human breast carcinoma cell line MCF7, acting as a positive control for oestrogen receptor and progesterone receptor. Next are shown paired cancer (C) and normal mucosa (N) samples from men and women. On the right are normal mucosal samples from the same patients showing the regional distributions of receptors. Rect=rectum; Sig=sigmoid colon; Desc=descending colon; Trans=transverse colon; Asc=ascending colon; Caec=caecum; T Ileum=terminal ileum.



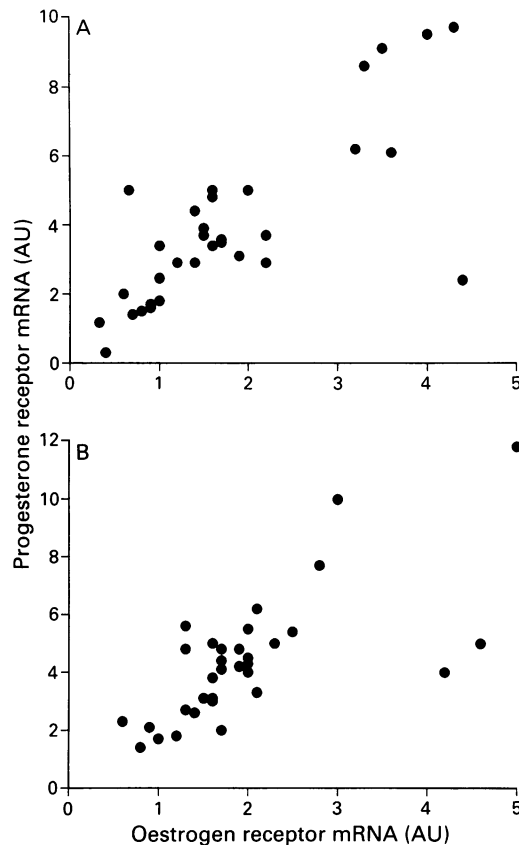


Figure 2: Correlation between progesterone receptor mRNA and oestrogen receptor mRNA in (A) large bowel cancers, and (B) corresponding normal mucosae. Spearman rank correlation coefficients are 0.73, $p < 0.001$ for cancers and 0.74, $p < 0.001$ for normal tissues. AU = arbitrary units.

CORRELATION BETWEEN RECEPTOR CONCENTRATIONS

Oestrogen receptor mRNA concentration in normal tissue correlated with oestrogen receptor mRNA concentration in corresponding cancer tissue (Spearman rank coefficient (R_s) 0.45, $p = 0.005$). This relation was also seen with progesterone receptor mRNA (R_s 0.40, $p = 0.02$). The oestrogen receptor immunoassay also showed this correlation between corresponding normal and cancer tissue (R_s 0.52, $p = 0.04$). There was an even stronger correlation between the oestrogen receptor and progesterone receptor concentrations in individual tissues (Fig 2; normal tissue $R_s = 0.74$, $p < 0.001$ and cancer $R_s = 0.73$, $p < 0.001$). Thus the more oestrogen receptor mRNA that was demonstrable, the more progesterone receptor mRNA was present, a relation seen in both normal and cancer tissue. The correlation between oestrogen receptor mRNA and oestrogen receptor protein was poor both in the case of normal tissue (R_s 0.1), and cancers (R_s 0.1) suggesting that the regulation of oestrogen receptor protein expression may be occurring at the post-transcriptional level in large bowel mucosa.

Discussion

We have demonstrated the presence of mRNA for oestrogen and progesterone receptors in all samples of normal tissue, cancers, and adenomatous polyps in amounts comparable with those found in breast and endometrium. Specificity of

hybridisation has been assured by using both full length cDNA probes and cDNA probes specific to the 5' hypervariable region of the respective receptor mRNA. Also, we have been able to show the presence of oestrogen receptor protein by enzyme immunoassay in an amount lower than but comparable with that found in normal breast tissue, suggesting that the receptor mRNA is not as efficiently translated into protein in large bowel mucosa as it is in more classic target tissues. Indeed, we found that the amount of oestrogen receptor mRNA present did not correlate with the amount of receptor protein present. We are not aware of other studies that have compared relative amounts of sex steroid receptor mRNA with amounts of receptor protein present, or amounts of radioligand binding in other than breast tissue. In breast cancers there is a positive correlation between the amount of oestrogen receptor mRNA present and the level of radioligand binding.²⁸ By contrast, oestrogen responsive and unresponsive breast cancer cell lines have similar amounts of oestrogen receptor mRNA, but the amount of oestrogen receptor protein is much less in the oestrogen unresponsive line.²⁹

Previous studies with radioligand binding assays have reported receptors in a variable proportion of colonic mucosal samples. d'Istria *et al*¹² detected no specific oestrogen binding in 28 large bowel cancers and Dawson *et al*¹⁵ found oestrogen receptor in only one cancer out of 29. Alford *et al*,¹⁰ however, found 30% of cancers positive for oestrogen receptor and Meggouh *et al* found 60% positive¹¹; both studies reported 75% of normal mucosal samples positive for oestrogen receptor. Agrez *et al*³⁰ found oestrogen receptor in only two out of 30 polyps. Progesterone binding has been described in between 10%¹¹ and 50%¹⁰ of large bowel cancers. Meggouh *et al*¹¹ found only 10% of normal mucosal samples to be positive. These studies are inconsistent among themselves and discrepant with our own findings. Two factors may explain the differences. Sex hormone binding globulin and other proteins can bind oestradiol with high affinity ($K_d 10^{-9}$ M)³¹; the presence of this non-specific binding has led investigators to choose a high threshold for a positive receptor result, usually 10 fmol/mg of protein in the case of oestrogen receptor. Firstly, our enzyme immunoassay results show oestrogen receptor concentrations in normal colorectal tissue and cancer in the range 1.2–7.4 fmol/mg. Such binding concentrations would be deemed negative by the above ligand binding studies. Secondly, successful binding of radioligand to receptor is likely to need a preserved tertiary structure of receptor proteins, which would be liable to disruption during collection, storage, and processing of specimens. The inability of immunohistochemical techniques to demonstrate oestrogen receptor in cancers or normal tissue has been interpreted to signify absence of this receptor in the large bowel. It should be noted, however, that even normal breast tissue does not stain for oestrogen receptor in the second half of the menstrual cycle, reflecting the insensitivity of this technique.³²

Our working hypothesis that variations in the sex ratio with age of patients with colonic or

rectal cancer correlates with sex steroid effects has received support in that we have consistently been able to detect oestrogen and progesterone mRNA receptor expression in large bowel mucosa. We have failed to show any clear differences in expression between normal and neoplastic tissue, although concentrations of progesterone receptor mRNA in normal rectal mucosa tended to be rather low, and those in neoplastic tissue rather high, a difference not supported by the oestrogen receptor protein assay. The situation therefore differs from that found in breast cancer, where some cancers have far higher oestrogen receptor expression than normal breast tissue.

One limitation of our study on homogenised tissue is that the normal mucosal samples contain a composite of tissues including epithelium, stroma, and muscularis mucosae. We are currently undertaking *in situ* hybridisation to analyse the tissue localisation of oestrogen receptor mRNA.

In breast cancer cells the progesterone receptor is induced via the oestrogen receptor by oestradiol,²⁵ and *in vivo*, binding of oestrogen and progesterone are positively correlated in breast cancers.³³ The detection of a correlation in our study between the two argues that the receptors that we have found may be inducible and therefore functional. Little is known about the function of oestrogens in the large bowel. *In vivo* experiments in mice have produced contradictory effects on growth; both an increase³⁴ and a retardation³⁵ have been seen. *In vitro*, colonic cancer cell lines have shown little response in growth after oestradiol stimulation.³⁶ In breast cancer cells, oestrogen has a prominent growth stimulatory action, and induces proto-oncogene expression and expression of other growth factors.^{37,38} We are currently exploring the action of oestradiol on these same genes in colonic cell lines.

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