Differential expression of oestrogen receptor and oestrogen inducible genes in gastric mucosa and cancer

S Singh, R Poulsom, N A Wright, M C Sheppard, M J S Langman

Abstract

Background—Evidence exists for a role for oestrogen in gastric cancer. The incidence of gastric cancer is much higher in men than in woman, and a similar sex difference is also seen in a rat experimental model of gastric cancer.

Aims—Evidence for a functional oestrogen receptor in normal gastric mucosa and cancer has been sought.

Methods—Oestrogen receptor and the oestrogen inducible genes pS2 and ERD5 were sought by northern blot analysis and in situ hybridisation and immunohistochemistry. Oestrogen receptor protein was studied by enzyme immunoassay.

Results—mRNA for oestrogen receptor was detected in cancer and normal gastric mucosa. Enzyme immunoassay for oestrogen receptor showed a mean of 1.8 fmol/mg protein in cancer and 13.7 fmol/mg in paired normal mucosa. The oestrogen inducible genes pS2 and ERD5 were also detected in both cancer and normal mucosa, with expression localised to epithelium. Expression of pS2 was lower in cancer compared with normal mucosa, whereas ERD5 expression was higher in cancer.

Conclusions—Significant amounts of oestrogen receptor were expressed in gastric mucosa with a lower amount in cancer. Oestrogen inducible genes were also expressed differentially but were not found to correlate closely with oestrogen receptor. Oestrogen receptor functionality remains to be demonstrated in the stomach.

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The aetiology of gastric cancer is largely unknown, but is likely to be multifactorial. A consistent sex difference is seen in areas of high and low incidence of gastric cancer; males have twice the incidence of females.¹ A male preponderance is also seen in a rat experimental model of gastric cancer; MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) induced gastric cancers are more than twice as common in male rats than in female rats.² Furthermore, an early menopause predisposes women to gastric cancer.³ It is possible, therefore, that sex steroid hormones have a role in gastric cancer

with oestrogen having a protective role or androgens increasing the risk.

If oestrogen is to have a role in gastric cancer, then functional oestrogen receptor should be present in gastric mucosa. Previous studies have found evidence of oestrogen receptor in normal mucosa and cancer,^{4,5} but the localisation and functionality of the receptor remains unclear. We have looked for oestrogen receptor expression in normal and cancerous gastric mucosa using northern blot analysis, in situ hybridisation, and immunohistochemistry. To provide evidence of oestrogen receptor function we have looked for the expression of the oestrogen inducible proteins pS2 and ERD5^{6,7} because the expression of these in breast cancer correlates with expression of functional oestrogen receptor.^{8,9}

Methods

Tissue samples were collected into liquid nitrogen within 20 minutes of resection of surgical specimens. Non-necrotic cancer was identified and removed, and a sample of normal mucosa was dissected from the muscularis propria about 5 cm from the cancer. Tissue was also collected from surgical resections for non-gastric disease. Tissue samples were stored at –70°C.

NORTHERN BLOT ANALYSIS

Total RNA was extracted by the single step guanidinium isothiocyanate method.¹⁰ Integrity of the RNA was assessed by the visualisation of distinct ribosomal 28S and 18S bands on a 1% agarose gel stained with ethidium bromide. Northern blot analysis was performed by electrophoresis of about 30 µg of total RNA in a 1% agarose gel containing 15% formaldehyde in 20 mM morpholinopropane-sulphonic acid (MOPS buffer). RNA was transferred for three hours in 0.05 M sodium hydroxide to Hybond N+ nylon membranes (Amersham International, Buckinghamshire, UK).

cDNA PROBES AND MEMBRANE HYBRIDISATION

The partial cDNA clone pOR3 of the human oestrogen receptor¹¹ was obtained from the Medical Research Council UK DNA Probe Bank, Harrow, England. Probes for cDNA were also used for ERD5,¹² and pS2.⁶ Probes were validated by multiple restriction enzyme

Department of
Medicine, Queen
Elizabeth Hospital,
Edgbaston,
Birmingham
B15 2TH

S Singh
M C Sheppard
M J S Langman

Histopathology Unit,
Imperial Cancer
Research Fund,
Lincoln's Inn Fields,
London WC2A 3PN
R Poulsom
N A Wright

Correspondence to:
Dr Sukhdev Singh,
Department of Medicine,
Queen Elizabeth Hospital,
Edgbaston, Birmingham
B15 2TH.

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digestions and by sequencing. To determine total RNA loading, northern blots were probed with a cDNA coding for the rat 18S ribosomal RNA.¹³

Probes were radiolabelled with α [³²P] dCTP (deoxycytidine triphosphate, 3000 Ci/mmol) using the random primer extension technique to an activity of 10⁹ cpm/ μ g. Membranes were hybridised overnight in a buffer comprising 0.77 M sodium phosphate, 5 mM EDTA, 7% sodium dodecyl sulphate (SDS), and 100 μ g/ml denatured salmon sperm DNA at 65°C in a rotating oven (Hybaid, Middlesex, UK). Membranes were washed in 1×SSC (0.015 M sodium citrate, 0.15 M sodium chloride), and 1% SDS at 65°C for one hour, followed by 0.1×SSC and 0.1% SDS at 65°C for 15 minutes. 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 removed as demonstrated by autoradiography. Autoradiograms were analysed by laser densitometry (LKB Ultrascan, Pharmacia Biosystems, Milton Keynes, UK).

Total RNA was also analysed from the human breast carcinoma cell line MCF-7 and human uterus to provide a positive control for oestrogen receptor and oestrogen inducible genes.

IN SITU HYBRIDISATION FOR OESTROGEN RECEPTOR AND pS2

In situ hybridisation for oestrogen receptor and PS2 mRNA was performed to localise tissue expression. Briefly, antisense riboprobes were transcribed with SP6 RNA polymerase (Promega Biotechnology, Southampton, UK) from the Eco-RI-Ball fragment of the oestrogen receptor subcloned into pGEM3 (Promega). The antisense probe for pS2 was made using SP6 RNA polymerase and EcoRI linearised pSP73 containing the EcoRI/BgIII fragment of the PCR clone. Probes were labelled with ³⁵SUTP (uridine triphosphate) to a specific activity 0.8–1.7×10⁹ dpm/ μ g RNA. Sections of formalin fixed material (5 μ m) were mounted on 3-aminopropyl-triethoxysilane treated glass slides, dewaxed, and hydrated in phosphate buffered saline (PBS). Sections were permeabilised with proteinase K, postfixed in 4% paraformaldehyde, acetylated, and incubated overnight at 55°C in 10–20 μ l hybridisation mixture (300 mM NaCl, 10 mM Na₂HPO₄, 10 mM Tris-HCl, 5 mM EDTA, 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 50% formamide, 0.5 mg/ml bovine ribosomal RNA, 10% dextran sulphate, 10 mM dithiothreitol). Probe (1–2×10⁶ cpm) was added to each slide. Posthybridisation washes included a ribonuclease A step and reached a final stringency of 0.5×SSC at 65°C. Sections were dehydrated in alcohols containing 300 mM ammonium acetate, dried, dipped in Ilford K5 emulsion, and exposed for three to nine weeks at 4°C before development of silver grains in Kodak D19. Sections were counterstained with Giemsa stain. Hybridisation with oestrogen

receptor sense riboprobe transcribed with T7 polymerase provided a control to estimate non-specific hybridisation. Hybridisation with a β actin antisense riboprobe was performed to show the presence of hybridisable mRNA in the blocks sectioned.

OESTROGEN RECEPTOR ENZYME IMMUNOASSAY
A commercially available 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, 0.5 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 anti-oestrogen receptor monoclonal antibody. The beads were washed to remove unbound material. Bound oestrogen receptor was labelled by incubating with a second monoclonal anti-oestrogen receptor antibody conjugated with horseradish 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 using the Bradford method (Bio-rad Laboratories, Hertfordshire, UK).¹⁴

IMMUNOHISTOCHEMISTRY FOR OESTROGEN RECEPTOR, pS2, AND ERD5

Commercially available monoclonal antibodies were used. Antibody against the human oestrogen receptor was obtained from Dako Ltd (Buckinghamshire, UK). Antibodies against pS2 and ERD5 were obtained from CIS bio International, Gif-sur-Yvette Cedex, France, and Amersham International, Buckinghamshire, UK), respectively.

Tissue was fixed in neutral buffered formalin before being embedded in paraffin wax. Sections were dewaxed and endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol. Sections to be stained for oestrogen receptor were placed in a microwave oven for 10 minutes in 7 mM sodium citrate, as recommended by the manufacturer. Sections were incubated with the primary antibody for 30 minutes, washed, and incubated with biotinylated rabbit antimouse antibody for 35 minutes. Sections were then washed and incubated for 30 minutes in streptavidin-peroxidase. The sections were washed and incubated for two minutes in peroxidase substrate (diaminobenzidine and 0.3% hydrogen peroxide) and counterstained with haematoxylin. Control sections were processed as above omitting the primary antibody.

Results

Thirteen pairs of gastric cancer cells and normal adjacent mucosae were analysed by northern blot analysis. Three were defined as

Figure 1: Expression of mRNA for oestrogen receptor and oestrogen inducible genes pS2 and ERD5 in paired cancer and normal gastric mucosa (C-N). Shown are autoradiographs of northern blots hybridised to cDNA probes for oestrogen receptor (ER), ERD5, pS2, and 18S ribosomal RNA. Cancers were from men and women. 18S ribosomal RNA indicates amount of total RNA loaded. RNA from the breast cancer cell line MCF-7, and from human uterus provide positive controls. Ant=gastric antrum; duod=duodenum; jejun=jejunum; Ca=cancer; N=normal.

“diffuse” according to the Lauren classification, and 10 were “intestinal” in type. In addition, normal gastric mucosa and small bowel were analysed from two patients undergoing radical pancreatic surgery. The mean age of the patients was 72 (range 58 to 82) years. Five patients were women.

OESTROGEN RECEPTOR

Northern blot analysis identified mRNA for oestrogen receptor in all samples of gastric cancer and normal mucosa. Figure 1 shows the results in six representative cancer and normal pairs. The transcript identified is likely to be specific for oestrogen receptor as we had used stringent probe hybridisation and washing conditions, and the transcript was identical in size to that expressed by MCF-7 breast cancer cells (6.2 Kb)¹¹ and uterus. Furthermore, no binding was seen with calf liver ribosomal RNA (negative control; lane 1, Fig 1). After correcting laser densitometry readings for uneven loading by expressing oestrogen receptor mRNA as a ratio of 18S ribosomal RNA, no quantitative difference in oestrogen receptor mRNA expression was seen between cancer cells and normal gastric mucosa. Oestrogen receptor was also found in the two samples of

small bowel mucosa examined (Fig 1). In situ hybridisation for oestrogen receptor was unsuccessful at localising expression, although expression was identified in slides of uterus (positive control)/(data not shown).

Enzyme immunoassay for oestrogen receptor showed much lower expression in cancer compared with adjacent normal mucosa, a mean (range) of 1.8 (0.1–5.0) fmol/mg protein and 13.7 (0.6–32.6) fmol/mg respectively (Table). This difference was significant ($p<0.05$, Mann-Whitney U test). There was no difference in oestrogen receptor expression between men ($n=6$) or women ($n=3$), although the numbers studied were small. Immunohistochemistry for oestrogen receptor was not successful at localising expression, although positive staining was seen on slides of uterus (data not shown).

pS2

Northern blot analysis disclosed a major transcript identical in size to that expressed in the MCF-7 cells (0.6 Kb).⁶ A smaller transcript of approximately 0.4 Kb was also seen in gastric tissues (Fig 1). The expression of the minor transcript varied in parallel with that of the major 0.6 Kb transcript. There was no difference in transcript size between normal mucosa and cancer. However, expression of pS2 was significantly lower in cancer (Table).

In situ hybridisation localised expression to surface epithelium in normal mucosa (Fig 2A). Expression in cancer cells was noted to be heterogeneous with adjacent signets of malignant cells having disparate expression of pS2 (Fig 2B). Immunohistochemistry for pS2 also localised expression to epithelium (data not shown).

Mean ($n=9$) expression of oestrogen receptor protein (SEM) (fmol/mg protein) and mRNA (SEM) for the oestrogen inducible genes, ERD5 and pS2, in gastric cancer and paired normal mucosa ($n=13$) (arbitrary units)

	Oestrogen receptor	ERD5	pS2
Cancer	1.8 (0.6)*	10.0 (4.2)*	10.0 (9.5)***
Normal mucosa	13.7 (4.3)	2.7 (1.0)	16.4 (3.8)

* $p<0.05$; *** $p<0.001$, Mann-Whitney U test v normal controls.

Figure 2: In situ hybridisation for the oestrogen inducible gene pS2, (A) in normal gastric mucosa, and (B) in a moderately differentiated cancer. Expression of pS2 is in surface epithelium in normal mucosa. In cancer pS2 expression is heterogeneous.

ERD5

Northern blot analysis identified a single transcript of approximately 1 Kb in gastric tissue, identical in size to that seen in MCF-7 cells and uterus (Fig 1). Expression of ERD5 was, in contrast to expression of pS2, higher in cancer than in normal mucosa (Table). Immunohistochemistry showed faint staining in normal and malignant epithelium (data not shown).

Discussion

We have found evidence for oestrogen receptor mRNA and protein and oestrogen inducible genes in normal gastric mucosa and cancer cells from the stomach. Although mRNA for oestrogen receptor is equally expressed in normal mucosa and cancer, oestrogen receptor protein itself is expressed in much lower amount by cancer. The oestrogen inducible genes examined, pS2 and ERD5, are also differentially expressed, with pS2 expression being lower in cancer, whereas ERD5 was expressed in higher amount in cancer.

The amount of oestrogen receptor we have detected in normal gastric mucosa is striking, a mean of 14 fmol/mg, an amount greater than the 2–10 fmol/mg seen in normal breast tissue. Wu *et al*⁴ detected a lower amount of oestrogen receptor in normal gastric tissue, and found a similar level of oestrogen receptor in gastric cancer. One possible reason for this discrepancy is that we have used mucosa dissected from the muscularis propria, and not used whole stomach including gastric smooth muscle, which would tend to dilute down the oestrogen receptor content of gastric mucosa.

Our attempts to confirm oestrogen receptor expression in gastric epithelium by in situ hybridisation and immunohistochemistry were

unsuccessful. Kojima *et al*⁵ failed to detect immunostaining for oestrogen receptor in normal mucosa, although they reported positive receptor staining in a minority of cancers after very prolonged incubation with primary antibody. The absence of immunohistochemical staining for oestrogen receptor in normal gastric mucosa may simply reflect a limitation in sensitivity of the technique, and it should be noted that even breast, a classic oestrogen responsive tissue, fails to stain for oestrogen receptor during the luteal phase of the menstrual cycle.¹⁵

Expression of the oestrogen inducible genes pS2 and ERD5 in breast tissue correlates with the presence of functional oestrogen receptor. The correlation between ERD5 and oestrogen receptor is high. Thus antibody to ERD5 can immunoprecipitate ligand bound oestrogen receptor,¹⁶ ERD5 expression in linearly correlated with specific binding for oestrogen in breast tissue,¹⁷ and ERD5 status has similar clinical prognostic relevance to oestrogen receptor expression in breast cancer.⁹ pS2 expression is also closely related to expression of oestrogen receptor in breast tissue. Oestradiol promotes transcription of pS2 mRNA in breast cancer cells,⁶ acting via an oestrogen response element (ERE), which is present upstream of the transcription start site.¹⁸ Expression of pS2 correlates with the presence of oestrogen receptor in breast cancers,⁸ and is predictive of response to anti-oestrogen therapy.¹⁹

We have detected ERD5 and pS2 in normal gastric mucosa and cancer as previously reported.^{20–21} However, pS2 and ERD5 expression was not co-localised nor correlated with oestrogen receptor – for example, we have detected lower amounts of oestrogen receptor protein in gastric cancer compared with normal mucosa; however, ERD5 mRNA concentrations were higher in cancer. Thus other factors in addition to oestrogen may be important in the regulation of these genes in stomach; pS2 is also inducible by the epidermal growth factor and the proto-oncogenes *c-jun* and *cHa-ras*.²²

If oestrogen receptor expression affected the behaviour of gastric cancer then anti-oestrogens might be expected to affect prognosis, and expression of oestrogen inducible genes might also predict outcome. Tamoxifen in combination with chemotherapy has been reported by some to improve survival,^{23–24} but other studies have shown no effect.²⁵ However, in the study of Harrison *et al*²⁵ patients with cancers positive for ERD5 had a significantly lower survival when treated with tamoxifen. The same group also reported that ERD5 status of gastric cancer was an independent prognostic factor; patients with ERD5 positive cancers having a significantly shorter survival.²⁶ The position is therefore unclear.

The antiulcer drug cimetidine has been documented to improve survival in gastric cancer.²⁷ The mechanism is not known, but may be endocrine in action as the drug is recognised to have oestrogenic or antiandrogenic side effects, such as gynaecomastia and impotence.

Other evidence relating sex steroid actions to gastric physiology includes lower serum gastrin concentrations in female animals than male animals,²⁸ with ovariectomy increasing circulating gastrin concentrations to male values. When treated with oestradiol, ovariectomised rats develop lower serum gastrin concentrations.²⁹ It is noteworthy that the oestrogen stilboestrol has been used with benefit for the treatment of duodenal ulcer in humans.³⁰

We have detected significant amounts of oestrogen receptor in normal gastric mucosa with lower amounts in cancer cells. This is consistent with oestrogen having a protective action and may contribute to the sex difference seen in the incidence of gastric cancer. To determine evidence of oestrogen receptor functionality we looked for the oestrogen inducible genes pS2 and ERD5 in gastric tissue. Both genes were detected, but we were unable to show close correlation with oestrogen receptor. Oestrogen receptor functionality remains to be demonstrated in stomach tissue.

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