Expression and functional consequences of oestrogen and progesterone receptors in human insulinomas

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Abstract

The expression of steroid receptors by tumours offers a therapeutic advantage if functionally responsive to exogenous hormones. Insulinomas represent a highly symptomatic group of pancreatic tumours and the steroid receptor status of these tumours is poorly understood. The object of the study was to characterise the sex steroid receptor status of human insulinomas and to investigate whether sex steroids alter insulin expression therein. At our tertiary referral University Hospital, archival and prospective tissues from 25 insulinoma patients collected over 14 years were analysed for oestrogen receptor-α (ERα), oestrogen receptor β (ERβ) and progesterone receptor (PR) expression. Tissue explants of insulinoma and control pancreatic tissue from two new insulinoma patients were cultured and treated with oestrogen and progesterone and insulin expression measured by RT-PCR and ELISA. The main outcome measures were established before data collection and included sex steroid receptor status of tumours and insulin expression in fresh tissue in response to exogenous sex steroids. PR was expressed in 24 out of 25, ERα in 10 out of 25 and ERβ in 21 out of 25 insulinomas. In fresh insulinoma cultures, insulin expression was increased by oestrogen or progesterone, whereas no significant effect was observed in adjacent pancreatic tissue. This study demonstrates widespread expression of sex steroid receptors on human insulinoma tissue and provides *in vitro* evidence of functionality with increased expression of insulin by insulinoma explants in response to exogenous oestrogen or progesterone. Confirmation of these results may provide a therapeutic mechanism for reducing symptomatic insulin secretion by receptor blockade.

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Introduction

Hormonal manipulation using sex steroid hormones has an established role as therapy for tumours of the breast and female genital tract. The hormones exert their effect by interacting with specific receptor proteins that must be expressed by cells for them to be responsive. Progesterone receptor (PR) immunoreactivity has been demonstrated in normal pancreas (Targarona *et al*. 1991) and specifically co-localised to normal insulin-producing β cells in the islets of Langerhans (Doglioni *et al*. 1990). There have been a limited number of studies characterising the steroid hormone receptor profile of pancreatic tumours. PRs have been detected immunohistochemically in normal pancreas and pancreatic tumours, almost exclusively being confined to endocrine pancreas tissue (Doglioni *et al*. 1990, Viale *et al*. 1992). A decade ago, oestrogen receptor (ER) presence was demonstrated in pancreatic tumour tissue using radioligand binding assays and Scatchard analysis (Greenway *et al*. 1981). However, the more recent studies have reported no detection of ER in either normal or pathological pancreatic tissue (Doglioni *et al*. 1990, Targarona *et al*. 1991, Viale *et al*. 1992, Singh *et al*. 1997).

In terms of translational significance, trials of anti-oestrogenic therapy in ductal pancreatic cancer have had disappointing results. Some studies have shown beneficial effect of tamoxifen (Wong & Chan 1993,

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Horimi *et al.* 1996) but the definitive randomised trials of tamoxifen in pancreatic cancer have been unanimously disappointing (Keating *et al.* 1989, Bakkevold *et al.* 1990, Taylor *et al.* 1993). There have been no clinical trials of anti-progestogenic therapy in pancreatic cancer.

Insulinomas are rare neuroendocrine tumours of the pancreas affecting 1–2 per million per year in the UK. Patients usually present with symptoms of hypoglycaemia secondary to insulin hypersecretion. These symptoms may sometimes be episodic and non-specific, and thus misleadingly prompt a barrage of neurological or psychiatric investigations. The tumours show a female preponderance with a female to male ratio of 4:1 (Service *et al.* 1991). Most lesions are small, solitary and benign with only 10% showing size $\geq$2 cm, multiplicity or malignancy. There is a good long-term survival of 88% at 10 years following surgical excision (Service *et al.* 1991).

We present an analysis of the oestrogen and PR status of 25 insulinoma cases. Finally, we demonstrate the effect of exogenous oestrogen and progesterone on insulin mRNA and protein expression in freshly isolated human insulinoma tissue.

Materials and methods

Tissue source

A total of 25 cases of pancreatic insulinoma were analysed in our series, and 23 cases of pancreatic insulinoma were retrieved from the files of the Department of Pathology, Queen Elizabeth Hospital, Birmingham, UK. Clinical details were reviewed from the files of medical records. In addition, we prospectively collected freshly explanted pancreatic tissue from two new cases of insulinoma. These studies were performed with appropriate ethical approval form the South Birmingham Regional Ethics Committee.

Immunohistochemistry

Light microscopic findings were reviewed and immunohistochemical staining was repeated where necessary. Formalin fixed, paraffin-embedded tissues were cut at 4 μm on to adhesive-coated slides. After dewaxing and rehydration of sections, all slides were treated for 15 min in 3% hydrogen peroxide in water to block endogenous peroxidase activity. Antigens were retrieved by incubation overnight in EDTA buffer on a hot-plate stirrer at 65 °C as previously described (Reynolds *et al.* 2002). Using a 'Sequenzer' for automation, slides were incubated for 1 h with the following primary antibodies diluted in TBS/Tween (pH 7.6):

- anti-gastrin antibody (Novo-Castra, GASp)
- anti-PP (Novo-Castra, NCL-PPp)
- anti-insulin (Novo-Castra, insulin)
- anti-ERα mAb (Dako, 1D5)
- anti-ERβ mAb (Labvision, RB-10658-P)
- anti-PR mAb (Dako, PR636)
- anti-somatostatin (Dako, A0566)
- anti-glucagon (Dako, A0565)
- anti-chromogranin (Dako, A0430)
- anti-synaptophysin (Novo-Castra, Synap-299)
- anti-Ki-67 (Dako, M7240)

(Novo Castra, Newcastle upon Tyne, UK; Dako, Ely, Cambridgeshire, UK; Labvision, Roncorn, Cheshire, UK)

Following a wash in TBS/Tween, primary antibodies were visualised using Dako ChemMate EnVision detection kit (K5007, Dako), counterstained in haematoxylin, dehydrated, cleared and mounted.

Immunoperoxidase-stained slides were reviewed independently. Each slide for gastrin, pancreatic polypeptide, insulin, somatostatin, glucagons, chromogranin, synaptophysin, Ki67, ER and PR was coded for two parameters of staining: intensity (strong, detectable at $\times$40; weak, only detectable at greater than $\times$100) and distribution (diffuse, $>$50% of tumour cells; focal, $<$50% of tumour cells). The labelling index of Ki-67 was evaluated by scoring a minimum of 1000 tumour cells in randomly selected fields. For each case, three different counts were performed and the highest score was chosen as the corresponding index value.

In vitro culture insulinoma tissue and insulin secretion

Pancreatic insulinoma tumour explants surplus to requirements were trimmed from two resection specimens. For each culture condition, equal 5 mm^3 blocks of insulinoma tissue were finely diced and incubated in 2 ml phenol red-free RPMI 1640 medium containing 5% foetal calf serum. In each insulinoma case, macroscopically uninvolved pancreatic tissue was obtained from the resection margins, distant from the insulinoma tumour, under the supervision of the reporting histopathologist (Phillipe Taniere). Equal 5 mm^3 blocks of this tissue were processed in exactly the same way as tumour tissue and served as patient-specific control pancreatic tissue.

Tumour and 'control' tissue were incubated for 24 h in four culture conditions namely: phenol red-free RPMI 1640 medium (Invitrogen) alone, medium plus 10^{-7} M E2 (17β-oestradiol; E2257, Sigma–Aldrich), medium plus 10^{-7} M progesterone (P6149, Sigma–Aldrich) alone, and or medium plus 10^{-7} M of E2 and 10^{-7} M

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progesterone. Culture was carried out in 24-well plates maintained at 37 °C in 5% CO_2. After 24 h in culture, the supernatant was collected for insulin assay and the pancreatic tissue was used for RNA extraction. The supernatant was frozen at −70 °C prior to use in insulin ELISA.

Insulin ELISA

Insulin concentrations in the supernatants were measured using an immunoenzymometric assay which was specific for insulin (BioSource International, Camarillo, CA, USA) and calibrated against IRP 66/304. Fifty microlitres of each standard, control or sample were dispensed into the appropriate number of wells of the insulin antibody-coated 96-well plate. Fifty microlitres of anti-insulin-HRP conjugate were dispensed into all the wells and the plate was incubated for 30 min at room temperature on a horizontal shaker set at 700 r.p.m. The plate was washed thrice with Tween 20 diluted 1:200 in distilled water. Two hundred microlitres of freshly prepared chromogen solution (TMB mixed with H_2O_2 in acetate/citrate buffer) were added into each well within 15 min following the washing step. The plate was then wrapped in tin foil (to exclude direct sunlight) and incubated for 15 min at room temperature on a horizontal shaker set at 700 r.p.m. The reaction was stopped by dispensing 50 μl of 1.8 M H_2SO_4 into each well. The microtitre plate absorbances were read at 450 and 490 nm. The detection range of the ELISA kit was $<$10–3400 pmol/l.

Insulin PCR

RNA was extracted from the pancreatic tissue (obtained from patient 25 only) using the RNeasy mini kit (Qiagen). A total of 30 mg pancreatic tissue was used as starting material for each sample and tissue was disrupted and homogenised using a conventional rotor–stator homogeniser. The optional on-column DNase digestion was used and the rest of the method was as indicated in the standard manufacturer's protocol for total RNA isolation from animal tissue.

RT was carried out in RNase- and DNase-free conditions. Samples were heated at 75 °C for 5 min. A RT mix comprising RNasin RNase Inhibitor (Promega), dNTPs (Amersham), random hexamers (Amersham), 10× PCR buffer (Qiagen) and 25 mM $MgCl_2$ was added to each sample. After 5-min incubation at 42 °C, Superscript II RNase H–reverse transcriptase (Invitrogen) was added to each sample. Incubation was carried out for 1 h at 42 °C and then for 5 min at 95 °C.

Conventional PCR for insulin was performed using a forward primer (5′-GCA-AGC-AGG-TCA-TTG-TTT-CA-3′) and reverse primer (5′-CAC-TTG-TGG-GTC-CTC-CAC-TT-3′). PCR amplification was carried out according to the following cycling programme: 5-min denaturation at 95 °C, followed by 30 cycles of: 95 °C for 30 s; 60 °C for 30 s and 72 °C for 30 s. Samples were held at 72 °C for 7 min and then at 4 °C. After PCR amplification, samples were electrophoresed in 2% agarose gel and stained with ethidium bromide; the photographic negatives were used for assessment. Under these conditions, the insulin PCR amplification product has a band size of 211 bp on the agarose gel.

Results

Clinical details

A total of 25 patients with insulinomas were studied, 19 were female and 6 were male (female to male ratio was 3.2:1; Table 1). The mean age of all patients with insulinomas was 50 years 8 months ± 17 years 7 months (range = 17 years 4 months–83 years 7 months) and their mean follow-up time was 30.5 ± 32.9 months (range = 1.7–138.1 months). There were five deaths during the follow-up period and four of these were due to recurrent disease. Of the four patients who died of recurrent disease, two had undergone distal pancreatectomy plus concomitant partial liver resection and one underwent distal pancreatectomy plus concomitant cryotherapy for hepatic metastases. The final patient who died of recurrent disease had undergone complete hepatectomy and liver allograft transplantation. One patient (patient 18) is known to have multiple endocrine neoplasia type 1A (MEN-1A) and is currently having pituitary investigations for reported symptoms. Another patient (patient 3 in Table 1) was diagnosed with acromegaly before presenting with his insulinoma, but gene analysis showed that he had no mutations in the menin gene. Predicted malignant behaviour as defined by the presence of metastases, angioinvasion or local infiltration and/or recurrent disease (Rindi *et al*. 1998, Schindl *et al*. 2000) was seen in 6 (24%) of the insulinomas, while the remaining 19 (76%) were benign.

Immunohistochemistry

The neuroendocrine differentiation of our series of insulinomas is confirmed immunohistochemically by the presence of protein gene product 9.5, neuron-specific enolase, chromogranin, synaptophysin and insulin (Lam *et al*. 1999).

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Table 1 Clinic details of the 25 insulinoma patients in the study

Patient	Sex	Age at surgery (years)	Length of follow-up (months)	Type of surgery	Malignant
1	F	35.7	34.4	Hepatectomy and orthotopic liver transplantation	Yes
2	F	72.2	16.4	Enucleation of insulinoma, cholecystectomy	No
3	M	57.6	66.8	Excision of lesion of pancreas	No
4	F	32.6	34.7	Excision of lesion of pancreas	No
5	F	38.7	46.7	Cholecystectomy, excision of lesion of pancreas, laparotomy	No
6	M	24.5	42.8	Enucleation of insulinoma	No
7	F	63.2	51.5	Excision of insulinoma from tail of pancreas	No
8	F	83.6	6.6	Distal pancreatectomy, splenectomy	No
9	F	34.4	15.9	Local enucleation of insulinoma in head of pancreas	Yes
10	F	38.7	1.7	Excision of insulinoma from tail of pancreas, cholecystectomy	No
11	F	64.4	2.6	Distal pancreatectomy, cholecystectomy	No
12	F	54.1	34.3	Excision of lesion of pancreas, cholecystectomy	No
13	F	41.4	13.4	Distal pancreatectomy, excision of accessory spleen, cholecystectomy	No
14	M	31.0	4.2	Excision of insulinoma in body of pancreas, excision of splenunculus in tail of pancreas	Yes
15	F	51.8	6.9	Enucleation of insulinoma in head of pancreas, cholecystectomy	No
16	M	51.8	18.2	Right extended hemihepatectomy, distal pancreatectomy, splenectomy	Yes
17	M	72.2	13.6	Distal pancreatectomy, L lateral segmentectomy, excision of liver lesion, splenectomy	Yes
18	F	17.4	6.6	Enucleation of insulinoma from pancreatic head, cholecystectomy	No
19	M	67.9	138.1	Distal pancreatectomy, splenectomy, cholecystectomy	Yes
20	F	33.6	94.5	Excision of lesion of pancreas	No
21	F	40.0	46.0	Excision of insulinoma, cholecystectomy	No
22	F	75.6	2.6	Distal pancreatectomy	No
23	F	59.0	2.6	Enucleation of insulinoma, cholecystectomy	No
24	F	73.6	n/a	central pancreatic resection	No
25	F	53.5	n/a	Pylorus-preserving pancreaticoduodenectomy	No

This table shows the relevant descriptive demographics of the 25 patients in our series. Data were collected form patient records and by the review of pathological and radiological reports. The table gives the patients' sex, age at surgery, length of follow-up, type of surgery and tumour malignancy status.

There was expression of PR in 24 out of 25 tumours. ERα was expressed in 10 out of 25, while ERβ was expressed in 21 out of 25 tumours (Table 2, Fig. 1).

Insulin ELISA

As anticipated insulinoma culture samples secreted several fold more insulin than the control pancreatic tissue. We were able to obtain fresh tissue from two patients in our cohort (patients 23 and 25). In both patients, the highest level of insulin secretion achieved (patient 23: 1621 ± 10 pmol/l, patient 25: 2530 ± 82 pmol/l) was seen in the experiments culturing insulinoma tissue in the presence of E2 alone (Figs 2 and 3). Conversely, in both patients the lowest level of insulin secretion achieved was seen in the experiments culturing control pancreatic tissue in the absence or presence of steroid hormones (patient 23: peak level of 89 pmol/l, patient 25: peak level of 90 pmol/l). The culture of insulinoma tissue with progesterone alone, with oestrogen and progesterone in combination or without steroid hormone(s) led to intermediate levels of insulin secretion. However, these levels were still several folds higher than those from control pancreatic tissue cultures. In patient 23, culture of insulinoma tissue with progesterone alone led to insulin secretion of 889 ± 14 pmol/l, with a combination of oestrogen and progesterone led to insulin secretion of 298 ± 16 pmol/l and without steroid hormone led to insulin secretion of 368 ± 24 pmol/l. In patient 25, culture of insulinoma tissue with progesterone alone led to insulin secretion of 1660 ± 35 pmol/l, with a combination of oestrogen and progesterone led to insulin secretion of 1766 ± 34 pmol/l and without steroid hormone led to insulin secretion of 1420 ± 20 pmol/l.

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Table 2 Immunohistochemical analysis of our series of 25 insulinomas

Patient	Gastrin	PP	Ins	Somat	Glucag	Chromo, synapto	Ki-67 (%)	PGP 9.5, NSE	ERα	ERβ	PR
1	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	Neg	WFP	WFP
2	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	SFP	SDP	SDP
3	Neg	Neg	SDP	SDP	Neg	SDP	<3	SDP	Neg	SDP	SDP
4	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	SFP	SDP	SDP
5	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	Neg	SDP	Neg
6	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	SDP	SDP	SFP
7	Neg	Neg	SFP	Neg	SDP	SDP	<2	SDP	Neg	SDP	SDP
8	WFP	Neg	SDP	SFP	SDP	SDP	<2	SDP	SDP	SDP	SDP
9	Neg	Neg	SDP	SFP	SDP	SDP	<5	SDP	SFP	SDP	SFP
10	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	SFP	SDP	SDP
11	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	WFP	SDP	SDP
12	Neg	Neg	SDP	Neg	Neg	SDP	<2	SDP	SDP	SDP	SDP
13	Neg	Neg	SDP	Neg	Neg	SDP	<1	SDP	Neg	Neg	SDP
14	Neg	SDP	SDP	Neg	Neg	SDP	2–5	SDP	Neg	SDP	SDP
15	Neg	Neg	SDP	Neg	SDP	SDP	<2	SDP	Neg	SDP	SDP
16	Neg	Neg	SDP	Neg	SFP	SDP	<2	SDP	Neg	Neg	SDP
17	SDP	SDP	SDP	Neg	SDP	SDP	<2	SDP	WFP	Neg	SDP
18	±	±	SDP	±	SDP	SDP	1–2	SDP	Neg	SDP	SDP
19	Neg	Neg	SDP	Neg	Neg	SDP	<2	SDP	Neg	SDP	SDP
20	Neg	±	SDP	Neg	Neg	SDP	<3	SDP	Neg	Neg	SDP
21	Neg	Neg	SDP	Neg	SDP	SDP	<2	SDP	SDP	SDP	SDP
22	Neg	Neg	SDP	SDP	SDP	SDP	<3	SDP	Neg	SDP	SDP
23	Neg	Neg	SDP	Neg	Neg	SDP	<3	SDP	Neg	SDP	SDP
24	Neg	Neg	SDP	Neg	Neg	SDP	>10	SDP	Neg	SDP	SDP
25	Neg	Neg	SDP	Neg	Neg	SDP	<2	SDP	Neg	SDP	SDP

This table shows the immunohistochemical characterisation of the 25 insulinomas in our series. Data on staining were collected by examination of archived slides, re-staining of archived slides and by staining of prospective cases. We have graded staining as negative, weak focal positive, strong focal positive and strong diffuse positive. Staining was scored on two parameters: intensity (strong, detectable at ×40; weak, only detectable at >×100) and distribution (diffuse, >50% of tumour cells; focal, <50% of tumour cells). The labelling index of Ki-67 was evaluated by scoring a minimum of 1000 tumour cells in randomly selected fields. For each case, three different counts were performed and the highest score was chosen as the corresponding index value. Neg, negative; WFP, weak focal positive; SDP, strong diffuse positive; SFP, strong focal positive; ±, equivocal; ERα, oestrogen receptor alpha; ERβ, oestrogen receptor beta; PR, progesterone receptor; Ins, insulin; PP, pancreatic polypeptide; PGP 9.5, protein gene product 9.5; NSE, neuron-specific enolase; chromo, chromogranin; synapto, synaptophysin and somato, somatostatin.

Insulin PCR

RT-PCR confirmed expression of insulin mRNA in all the samples of control pancreatic tissue and insulinoma tissue. There was relatively higher expression of insulin mRNA in the sample of insulinoma tissue that was cultured in the presence of exogenous E2 (Fig. 4).

Discussion

Insulinoma remains a rare but important medical condition that can be resistant to treatment. The average age of the patients in this series was 50 years, which is similar to that in published series from the Mayo Clinic where the average age was 47 years (Service *et al*. 1991), the Cleveland Clinic where the average age was 55 years (Dizon *et al*. 1999) and from the University of Milan where the average age was 49 years (Viale *et al*. 1992).

Our data confirm that there is ER and PR expression in insulinoma tissue. There was strong expression of ERβ and PR in the two patients from whom we obtained fresh tissue for *in vitro* culture. Neither ER nor PR was present in normal pancreatic tissue except for some weak expression of ERβ in some of the 'normal' margins of pancreatic tissue distant from insulinoma tumour (data not shown). It is evident that fresh insulinoma tissue cultured in the presence or absence of oestrogen and/or progesterone secretes significantly more insulin than control pancreatic tissue in any of these conditions. The highest level of insulinoma-secreted insulin was seen with the addition of oestrogen alone and was evidently consistent in both prospective insulinoma cases. The other culture conditions of insulinoma led to the secretion of lower levels of insulin but the hierarchical order of secretion was not consistent in both patients. In addition, we have demonstrated that there is expression of insulin mRNA in both control pancreatic tissue and insulinoma tissue.

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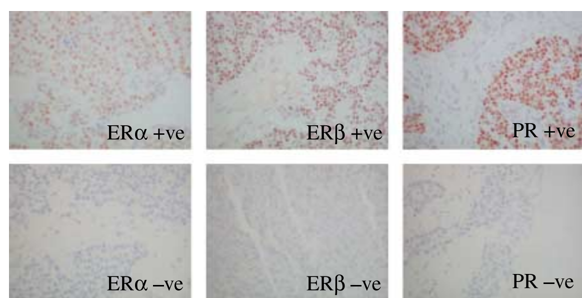

Figure 1 Sample insulinoma tissue slides from our series depicting negative and positive immunostaining for oestrogen receptor alpha (ERα), oestrogen receptor beta (ERβ) and progesterone receptor (PR). The slides are obtained from paraffin-embedded tissue blocks, processed by antigen retrieval and stained, fixed in acetone, incubated with the relevant antibody and finally colour is developed using DAB substrate.

The human ER exists in two forms, ERα and ERβ. Most of the previous studies characterising the ER status of pancreatic tissue have focused on the 'traditional' ER, which is now called ERα. The identification and characterisation of ERβ in humans was first reported 10 years ago (Mosselman *et al*. 1996). Both of the isoforms have been shown to be functional and have different roles in gene regulation as suggested by their contrasting effects on the nuclear transcription factor AP-1 (Paech *et al*. 1997). Ligand-binding experiments have shown that E2 has the same specific binding affinity for ERβ as it does for ERα (Kuiper *et al*. 1997). There are a limited number of published studies looking at the expression of ERβ in pancreatic tissue, but there it appears that ERβ has relatively higher expression in pancreatic tissue than ERα. The use of 'traditional' ER antibodies with no anti-β activity could explain why ER has previously been scarcely found in pancreatic tumours. ERβ is reported to be relatively more expressed than ERα in papillary cystic neoplasms of the pancreas (Morales *et al*. 2003), solid pseudopapillary tumours (Geers *et al*. 2006) and human pancreatic cancer cell lines (Konduri & Schwarz 2007). Our study shows for the first time that there is a considerably high expression of ER in human insulinomas and it is predominantly of the ERβ isoform.

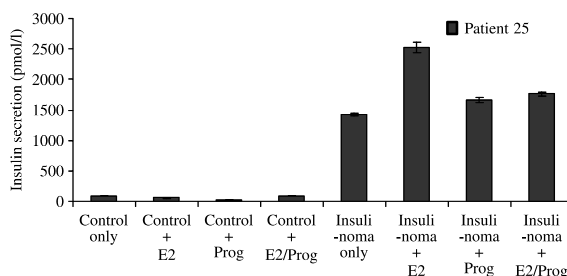

Figure 3 Levels of insulin secreted from cultures of control (macrosopically uninvolved distant tissue margins) pancreas or insulinoma tissue obtained from one of our two prospective cases (patient 25). E2, oestrogen; Prog, progesterone. Insulinoma tissue and control pancreatic tissue were cultured for 24 h under four different conditions: cultured with media only, with media plus E2, with media plus synthetic progesterone and with media plus E2 and progesterone. The supernatants from the culture wells were assayed for insulin using an ELISA kit (each experiment done in triplicate). The detection range of the ELISA kit was <10–3400 pmol/l. Insulin concentrations from all cultures were in the detection range of the ELISA kit.

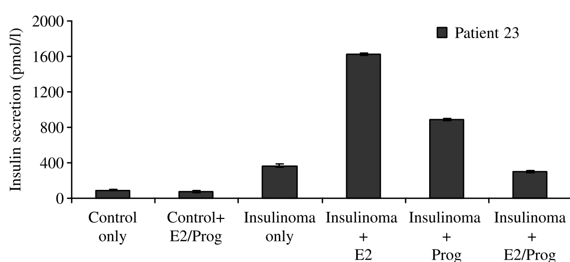

Figure 2 Levels of insulin secreted from cultures of control (macrosopically uninvolved distant tissue margins) pancreas or insulinoma tissue obtained from one of our two prospective cases (patient 23) E2, oestrogen; Prog, progesterone. Insulinoma tissue and control pancreatic tissue were cultured for 24 h under four different conditions: cultured with media only, with media plus E2, with media plus synthetic progesterone and with media plus E2 and progesterone. The supernatants from the culture wells were assayed for insulin using an ELISA kit (each experiment done in triplicate). The detection range of the ELISA kit was <10–3400 pmol/l. Owing to the small size of the specimen from this patient, we were only able to obtain sufficient tissue to perform cultures of 'control only' and 'control+E2/Prog'. Insulin concentrations from all cultures were in the detection range of the ELISA kit.

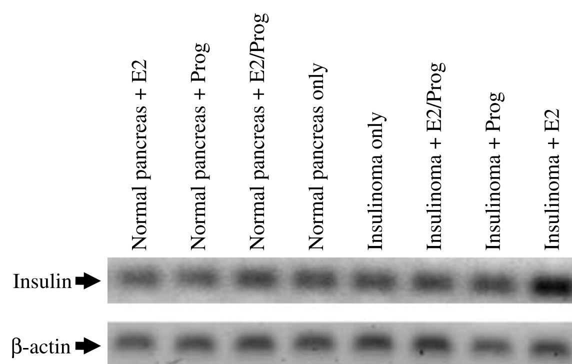

Figure 4 Following 24 h culture with or without exogenous oestrogen and/or progesterone, control pancreas and insulinoma tissue were used for RNA extraction to perform RT and conventional PCR for confirmation of the presence of insulin mRNA in the tissue samples. 'E2' is oestrogen and 'Prog' is progesterone. The insulin PCR amplification product was 211 bp. The corresponding amplification of the housekeeping gene β actin is also shown for each of the samples to demonstrate that the 'E2' and 'Prog' effects are specific for insulin.

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There are two distinct subtypes of the human PR: PR-A and PR-B (Clarke *et al*. 1987). The anti-PR antibody (clone 636) we used has been demonstrated to react with the PR-A and PR-B forms. We have thus reported the overall level of PR that may comprise both subtypes. There are no data available on the relative expression of the different subtypes of PR in pancreatic tissue, and there are no data available to suggest that any subtype is more predominant. Unlike the case for ER, the single PR antibody employed was immunoreactive for both PR subtypes such that we did not have to interrogate separately with two isoform-specific antibodies. PR subtype tissue expression has been reported in breast (Mote *et al*. 2002) and endometrial cancers (Saito *et al*. 2006) where a higher the ratio of PR-A to PR-B affects prognosis. This suggests that both PR isoforms are functional and their expression ratio may have an impact on biological responses with potential important clinical consequences. In our current study, we have shown that there is high expression of PR in human insulinomas but we have not specifically delineated the relative preponderance of the different PR isoforms.

In a series of 156 pancreatic endocrine tumours, Viale *et al*. characterised 42 insulinomas for expression of oestrogen and PRs. Some differences have emerged between the study by Viale *et al*. and our study. In their series, they found that the expression of PR was 66% but this was higher in our series with PR being detected in 96% (24/25) of insulinomas. Secondly, in contrast to their study where ER was undetectable in the series of insulinomas, we detected the expression of ER in a significant number of the insulinomas in our study. We found expression of the ERα and ERβ isoforms in 40 and 84% of tumours respectively. Finally, they were able to show that PR expression correlated with the absence of malignant features but our data do not suggest the same. In our series, the expression of PR seen in malignant and benign insulinomas was not significantly different with values of 100% (6/6) and 95% (18/19) respectively. The analysis of our insulinoma dataset did not reveal any distinct patterns of steroid hormone receptor status and tumour behaviour.

There are no reported cases of insulinomas where glycaemic control has been shown to be affected by exogenous oestrogen and progesterone, although in our own series the index case that led to this study showed just such features (data not shown). Our data show that compared with progesterone alone or a combination of oestrogen and progesterone, stimulation with exogenous oestrogen alone causes a more profound increase in insulin secretion from insulinoma tissue *in vitro*.

The rarity of insulinoma, even in a tertiary referral centre, places unavoidable limitations on studies into this condition. Notwithstanding this limitation, the patterns of receptor expression were consistent and in many cases the high levels of expression allowed easy distinction between insulinoma tissue and adjacent 'normal' pancreatic tissue quite apart form the different histological appearance. The data relating to functionality of sex steroid receptor expression and insulin secretion are limited to two patients and in view of this must be treated with some caution although the insulin response to oestrogen in both cases was substantial. Further studies using concomitant receptor blockade will be required to substantiate these findings and are in progress.

Insulinomas express functional ER and PR which increase tumour insulin secretion when stimulated by oestrogen and progesterone *in vitro*. Hormonal manipulation of insulinomas could thus provide an avenue for the management of the neurogenic and neuroglycopenic symptoms experienced by patients prior to tumour excision or in the event of tumour recurrence.

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