relapsing after stopping treatment for acute lymphoblastic leukaemia depends on the time that has elapsed since they stopped treatment. In children relapsing during the first six months or so there is a high risk of early marrow recurrence and they should be considered for marrow transplantation during second remission if there is a suitable donor. In patients relapsing later prolonged haematological remission is possible. We are presently studying the effect of more intensive induction and consolidation treatment in both groups of patients; whether this approach will increase the proportion of long-term survivors remains to be seen.

Further central nervous system prophylaxis is imperative for all children who have a bone-marrow recurrence after stopping treatment; intrathecal chemotherapy is the most appropriate method because it is less likely to compromise the delivery of systemic chemotherapy and avoids the undesirable effects of further cranial irradiation. The role of further "delayed" irradiation in long-term survivors remains unclear and will perhaps be elucidated by extrapolation from results of trials evaluating the necessity for routine irradiation in all newly diagnosed cases of acute lymphoblastic leukaemia.

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Oestrogen receptor proteins in malignant and fetal pancreas

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Abstract

High levels of high-affinity oestrogen binding were detected in cytosolic and nuclear fractions of tumour tissue from six patients with pancreatic carcinoma. Similar binding was found in fetal but not in normal adult pancreas, making derepression of fetal genes a possibility. Specific androgen binding was not detectable.

These findings suggest that anti-oestrogen treatment may offer a new approach to the treatment of pancreatic carcinoma.

Introduction

High levels of cytosolic oestrogen and androgen receptor binding have been detected in breast carcinoma tissue.^{1,2} High cytosolic oestrogen receptor binding may be used to predict an increased possibility of clinical response to anti-oestrogen treatment or hormone ablation. About 40% of patients with breast carcinoma do not respond to treatment, and this has been attributed to failure of the oestrogen receptor complex to bind to the nucleus. Recent evidence suggests that the detection of nuclear as well as cytosolic binding may give a better prediction of response.³ Cytosolic oestrogen receptors have been reported in several other human tumours⁴⁻⁷ including two cases of

pancreatic carcinoma*; we studied nuclear as well as cytosolic oestrogen binding in six specimens of malignant pancreatic tissue. In view of the possibility that the presence of steroid receptors in malignant pancreas might represent the derepression of fetal genes we also carried out studies with fetal pancreas. Since the specimens might have been contaminated with other competing proteins we estimated both the high-affinity, low-capacity sex-hormone-binding globulin and the low-affinity, high-capacity human serum albumin, major oestrogen binding proteins.

Patients and methods

Six specimens of pancreatic carcinoma tissue were obtained, three at laparotomy (two from men aged 69 and 48 years and one from a woman aged 56) and three at necropsy within six hours after death (two from men aged 73 and 78 years and one from a woman aged 78). All specimens were stored in liquid nitrogen and studied within 10 days except the one obtained from the 73-year-old patient at necropsy, which was stored at -20°C for five months. Histological examination confirmed ductular cell carcinoma in all cases.

Pancreatic tissue was obtained from five fetuses (12-14 weeks) aborted by the use of intrauterine prostaglandin and was used as pooled tissue of three and two specimens. Normal pancreas taken at necropsy within five hours after death from three men and one woman dying of unrelated diseases served as control tissue.

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PREPARATION OF CYTOSOL AND NUCLEOSOL AND ESTIMATION OF RECEPTOR BINDING

Tissue samples were manipulated below 4°C and homogenised in TED buffer (10 mmol TRIS, 1.5 mmol EDTA, and 1 mmol dithiothreitol/l, pH 7.4) using an Ultra-Turrax before centrifugation at

Concentration of oestradiol binding sites in nuclear and cytosolic fractions of pancreatic carcinoma and fetal pancreas, and determination of competing proteins by immunodiffusion and stanolone binding

Case No	Sex	E ₂ binding (fmol E ₂ /mg protein)		Equilibrium constant (l/mol)		R1881 binding		Sex-hormone-binding globulin binding (nmol stanolone bound/l)		Equilibrium constant in cytosol	Human serum albumin binding	
		Cytosol	Nucleosol	Cytosol	Nucleosol	Cytosol	Nucleosol	Cytosol	Nucleosol		Cytosol	Nucleosol
Pancreatic carcinoma												
1	M	1900	2753	3.6 × 10 ¹⁰	3.4 × 10 ¹⁰	ND	ND	ND	ND	ND	ND	ND
2	M	500	1016	6.2 × 10 ¹⁰	8.0 × 10 ¹⁰	0	0	0.20	0	2.47 × 10 ⁹	BP	BP
3	F	724	2066	5.4 × 10 ¹⁰	5.0 × 10 ¹⁰	ND	ND	ND	ND	ND	ND	ND
4	M	546	1224	5.4 × 10 ¹⁰	6.1 × 10 ¹⁰	0	0	0.26	0	1.0 × 10 ⁸	BP	BP
5	F	640	1478	6.6 × 10 ¹⁰	6.1 × 10 ¹⁰	0	0	0.19	0	2.35 × 10 ⁸	BP	BP
6	M	430	835	1.8 × 10 ⁹	1.4 × 10 ⁹	0	0	0.27	0	1.2 × 10 ⁸	BP	BP
Fetal pancreas												
Pool 1		175	157	1.22 × 10 ⁹	9.81 × 10 ¹⁰	ND	ND	+	0	ND	BP	BP
Pool 2		157	ND	1.3 × 10 ⁹	ND	0	0	+	0	ND	BP	BP

ND = Not determined. 0 = Not detected. BP = Binding present in blue gel. + = Present by immunodiffusion.

200 000 *g* for one hour. The supernatant was retained as cytosol. The pellet remaining when the cytosol was decanted was washed in TES buffer (10 mmol TRIS, 1 mmol EDTA, and 250 mmol sucrose/l, pH 7.4), centrifuged at 800 *g* for 10 minutes, and the supernatant discarded. The remaining pellet was homogenised in TSMK buffer (10 mmol TRIS, 250 mmol sucrose, 5 mmol magnesium chloride, and 25 mmol potassium chloride/l, pH 7.5) before filtration through a cellulose acetate plug, centrifuged for 15 minutes at 800 *g*, and the supernatant discarded. The pellet was washed twice in TSMK buffer and then resuspended in TKED buffer containing 0.5 mol potassium chloride/l. The suspension was kept at 4°C for one hour and then centrifuged for 40 minutes at 15 000 *g*, the supernatant being retained for the study of high-affinity nuclear receptor binding.

Aliquots of cytosol and nucleosol (500 µl) were incubated with increasing concentrations of either radioinert oestradiol or radioinert R1881 (a high-affinity synthetic androgen⁹) in the presence of a constant amount of tritiated oestradiol or tritiated R1881. The degree of non-specific binding was estimated in parallel experiments by incubating the 500 µl aliquots with 700 nmol of either radioinert oestradiol or R1881/l. The cytosol samples were incubated for four hours and the nucleosol for 18 hours, both at 4°C. Bound and free steroid were separated by a method based on the technique of Ginsburg *et al*¹⁰: 200 µl aliquots were applied in duplicate on to Sephadex LH20 columns, the dimensions of which allowed for the minimum dissociation of the high-affinity steroid receptor as described by Iqbal and Johnson.¹¹ A discontinuous flow technique was used, the eluate being collected in phials containing scintillation mixture before being counted in a Packard-Tricarb liquid scintillation counter (efficiency 20%). The sensitivity of the assay is approximately 5 fmol steroid bound/mg total soluble protein.

ESTIMATION OF SEX-HORMONE-BINDING GLOBULIN AND HUMAN SERUM ALBUMIN

Sex-hormone-binding globulin was detected by the double immunodiffusion technique with an antibody to the globulin raised in rabbits using the purified protein.¹² Specificity was confirmed by the absence of precipitin lines after treatment with concanavalin A, which binds glycoproteins such as sex-hormone-binding globulin. In preparations in which sex-hormone-binding globulin was detected immunologically it was measured by means of the two-tier technique of Iqbal and Johnson.¹¹ Stanolone (dihydrotestosterone), rather than oestradiol, is used as the steroid ligand since it has a higher affinity for the globulin and does not bind to corticosteroid-binding globulin. The presence of human serum albumin was detected indirectly by determining the amount of stanolone bound to this protein; this entails counting the Cibacron Blue 3GA Sepharose in the upper tier of the columns as described previously.¹¹ Although this gel is highly specific for its binding of albumin, it may also bind the oestrogen receptor; this possibility was excluded by selective denaturation of the oestrogen receptor by heating. Cytosolic preparations were heated to 40°C for 40 minutes in the presence of 100 mmol calcium chloride/l, a procedure that denatures the oestrogen receptor but not sex-hormone-binding globulin. Specimens were then reanalysed by the two-tier technique for sex-hormone-binding globulin and albumin and also for receptor binding.

Scatchard plots were analysed by the method of Chamness and McGuire¹³ when there was evidence of more than one set of equivalent and homogeneous binding sites. Protein concentration was measured

by the method of Lowry *et al*¹⁴ with human serum albumin as standard.

Results

High levels of high-affinity (equilibrium constant 10⁹–10¹⁰ l/mol) oestrogen receptor binding were found in all six cytoplasmic and nuclear fractions of pancreatic carcinoma and fetal pancreas but not in normal pancreas (table). After the specimens had been heated to 40°C for 40 minutes high-affinity receptor binding was no longer detectable.

Sex-hormone-binding globulin, assessed by the immunodiffusion technique, was present in all cytosolic preparations examined; after the same preparations had been treated with concanavalin A, however, precipitin lines were absent. The amount of sex-hormone-binding globulin present, in terms of its ability to bind stanolone, was between 0.19 and 0.27 nmol stanolone bound/l, and did not change after the oestrogen receptor had been denatured by heating. Sex-hormone-binding globulin could not be detected in any of the nuclear fractions.

There was appreciable steroid binding in the Cibacron Blue gel in all nuclear and cytosolic fractions. This remained after the receptor had been inactivated by heating and can therefore be attributed to the presence of human serum albumin, which is characteristically unsaturable (figure).

No androgen receptor binding was detected in any of the malignant, normal, or fetal pancreatic tissue tested.

Discussion

The highly specific, high-affinity oestradiol receptor binding in neoplastic and fetal pancreas, determined by means of Scatchard analysis, indicates the presence of a single set of equivalent and homogeneous binding sites, similar to the oestrogen receptors found in tissues such as breast or uterus. The loss of this high-affinity binding with heating to 40°C for 40 minutes is further evidence of a true oestrogen receptor.

The concentrations of oestrogen receptor sites in the cytosol of the order of 10²–10⁴ fmol E₂/mg total soluble protein are similar to those found by Stedman *et al*⁸ in two specimens of pancreatic carcinoma and comparable with levels found in many samples of breast carcinoma tissue. The appreciable oestrogen binding found in tissue obtained at necropsy, even after storage in one case at –20°C for five months, was unexpected, since the receptor is considered to be highly labile. Namkung *et al*¹⁵ found that 80% of the receptor activity in breast cancer was destroyed when the tissue was stored at –70°C for one month, suggesting that if fresh tissue had been obtainable in all cases in the present study the concentration of binding sites might have been higher.

The failure to find specific binding in normal pancreas is at variance with the findings of Sandberg and Rosenthal.¹⁶ Their equilibrium constant of 10⁸ l/mol suggests competition by sex-hormone-binding globulin, and the detection of this protein by immunodiffusion in all samples of normal pancreatic cytosol examined in our series lends support to this view.

Fetal and malignant tissue share behavioural properties, including the production of the proteins α -fetoprotein and carcinoembryonic antigen. This has been attributed to de-repression of fetal genes during the neoplastic process.¹⁷ The presence of oestrogen receptors in both fetal and neoplastic pancreatic tissue, as shown in the present study, may be another example of this phenomenon. Our data are also consistent, however, with the hypothesis of Jamieson *et al*¹⁸ that pancreatic carcinoma arises from undifferentiated embryonic stem cells. They have shown in the rat that neoplastic acinar cells have cell surface characteristics in common with those of the 19-day-old embryonic pancreas. Furthermore, hormone receptors (such as those for insulin and prolactin) are in the fetal rat breast analage as early as 19 days.¹⁹

Scatchard analysis of (a) nuclear and cytoplasmic fractions of pancreatic tumour (case 2) and the competing proteins sex-hormone-binding globulin (SHBG) and human serum albumin (HSA) and (b) nuclear and cytoplasmic fractions of fetal pancreas (pool 1). The equilibrium constants for fetal and malignant pancreas were derived by the method of Chamness and McGuire.¹³

At first, the finding of oestrogen receptors in fetal pancreas might seem surprising since oestrogens have only minor effects on organs outside the reproductive tract. It is well known, however, that testosterone, which may be more important to the growth of extragenital tissues, may act via the oestradiol receptor after being metabolised to oestrogen by the enzyme aromatase.²⁰ The presence of specific cytoplasmic and nuclear oestrogen receptors in pancreatic carcinoma tissue suggests, as with breast carcinoma, that the growth of pancreatic carcinoma may be oestrogen dependent, which in turn implies that hormone manipulation might be of value in treatment.

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ADDENDUM—Since this paper was completed we have examined three further specimens of malignant pancreatic tissue, including one nodal metastasis, of which two showed both cytosolic and nuclear oestrogen receptors. The third specimen, which was not the metastasis, was examined at five different sites and found not to contain any oestrogen receptors.

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CLARY, OR MORE PROPERLY CLEAR-EYE. Our ordinary garden Clary has four square stalks, with broad, rough, wrinkled, whitish, or hoary green leaves somewhat evenly cut in on the edges, and of a strong sweet scent, growing some near the ground, and some by couples upon stalks. The flowers grow at certain distances, with two small leaves at the joints under them, somewhat like unto the flowers of Sage, but smaller, and of a whitish blue colour. The seed is brownish, and somewhat flat, or not so round as the wild. The roots are blackish and spread not far, and perish after the seed time. It is usually sown, for it seldom rises of its own sowing.

This grows in gardens. It flowers in June and July, some a little later than others, and their seed is ripe in August, or thereabouts.

It is under the dominion of the Moon. The seed put into the eyes clears them from mores, and such like things gotten within the lids to offend them, as also clears them from white and red spots on them. The mucilage of the seed made with water, and applied to tumours, or swellings, disperses and takes them away; as also draws forth

splinters, thorns, or other things gotten into the flesh. The leaves used with vinegar, either by itself, or with a little honey, doth help boils, felons, and the hot inflammation that are gathered by their pains, if applied before it be grown too great. The powder of the dried root put into the nose, provokes sneezing, and thereby purges the head and brain of much rheum and corruption. The seed or leaves taken in wine, provokes to venery. It is of much use both for men and women that have weak backs, and helps to strengthen the reins: used either by itself, or with other herbs conducing to the same effect, and in tansies often. The fresh leaves dipped in a batter of flour, eggs, and a little milk, and fried in butter, and served to the table, is not unpleasant to any, but exceedingly profitable for those that are troubled with weak backs, and the effects thereof. The juice of the herb put into ale or beer, and drank, brings down women's courses, and expels the after-birth. (Nicholas Culpeper (1616-54) *The Complete Herbal*, 1850.)