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Insulin Secretion by Fetal Human Pancreas in Organ Culture

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Summary. Whole fetal human pancreases of 12–22 weeks gestation, showed histological growth and differentiation in vitro over 3 weeks. At glucose concentrations of 1–4 g/l, there was no difference in insulin secretion into culture medium over 1 h. There was no stimulation of insulin release by D-glyceraldehyde, thus defective glucose-stimulated insulin release was probably not due to impairment of an early step in glycolysis. In the presence of 0.5 mmol/l dibutyryl cyclic AMP, insulin secretion was enhanced (0.188 ± 0.030 versus 0.100 ± 0.012 mU · mg tissue^{-1} · h^{-1}, $p < 0.001$) independently of glucose concentrations. It thus appears that impairment of glucose-stimulated insulin release was unlikely to be due to insufficient intracellular cyclic AMP. Insulin release increased in response to tolbutamide and theophylline. Insulin secretion was stimulated in the presence of a fivefold increase in amino acid concentration (0.118 ± 0.018 versus 0.031 ± 0.008 mU · mg tissue^{-1} · h^{-1}, $p < 0.001$). There was a fourfold increase in basal insulin secretion from islets previously grown in high concentration of amino acids compared with standard culture medium, (0.284 ± 0.052 versus 0.067 ± 0.011 mU · mg tissue^{-1} · h^{-1}, $p < 0.001$), emphasizing the important role of amino acids as substrates for *B* cell metabolism and development.

Key words: Fetal pancreas, morphology, insulin secretion, secretagogues.

Pancreatic islet transplantation has potentially wide application in the treatment of insulin-dependent diabetes mellitus. However, the limited availability of suitable donors, the poor recovery of isolated islets from adult human pancreas, and rejection of foreign tissue by the host, are problems still requiring solution [1]. Since fetal tissue has a large potential for proliferation, the fetal pancreas may provide a way of obtaining sufficient islet cells from each donor [2]. In organ culture of pancreas, selective endocrine differentiation and simultaneous degeneration of acinar cells occurs [3] and provides a potentially efficient way of harvesting islets. Evidence is also accumulating for modification of immunogenicity of pancreatic islets in organ culture [4–6].

We have described previously an organ culture technique whereby whole fetal mouse thymus [7] and pancreatic islets [8] will differentiate and proliferate in vitro for prolonged periods. Diabetes was reversed [9, 10] and the renal complications were prevented [11] following transplantation of the tissue. This culture method has now been extended to study the growth and differentiation of fetal human pancreas.

Materials and Methods

Organ Culture Technique

Ten human fetal pancreases of 12–22 weeks gestation were obtained from hysterotomies or prostaglandin-induced abortions; the cold ischaemia time for the specimens being up to 10 h. The pancreas was dissected under sterile conditions in phosphate buffered saline and was cut into fragments of 0.5–1 mm^3. These were placed on to strips of millipore filter (0.45 μm pore size) lying on blocks of surgical gelatin foam (Gelfoam, Upjohn, Kalamazoo, USA) in 9 cm Petri dishes containing 15 ml of Dulbecco's Modified Eagle's Medium (DMEM) (Flow Laboratories, North Ryde, NSW, Australia) supplemented with 15% fetal calf serum. The tissue was maintained at the gas/medium interface. The medium contained penicillin (60 mg/100 ml) and streptomycin (100 mg/100 ml) and glucose (1 g/l), unless otherwise indicated. Media were changed twice weekly. Each dish contained fragments equivalent to approximately one-fifth of one pancreas. The dishes were maintained at 37 °C in a humidified incubator in 10% CO_2 in air. Catalogue numbers of all reagents are available on request.

Histological Preparation

Some pancreatic fragments were placed in Bouin's fixative immediately after removal from a fetus, and further fragments from the same

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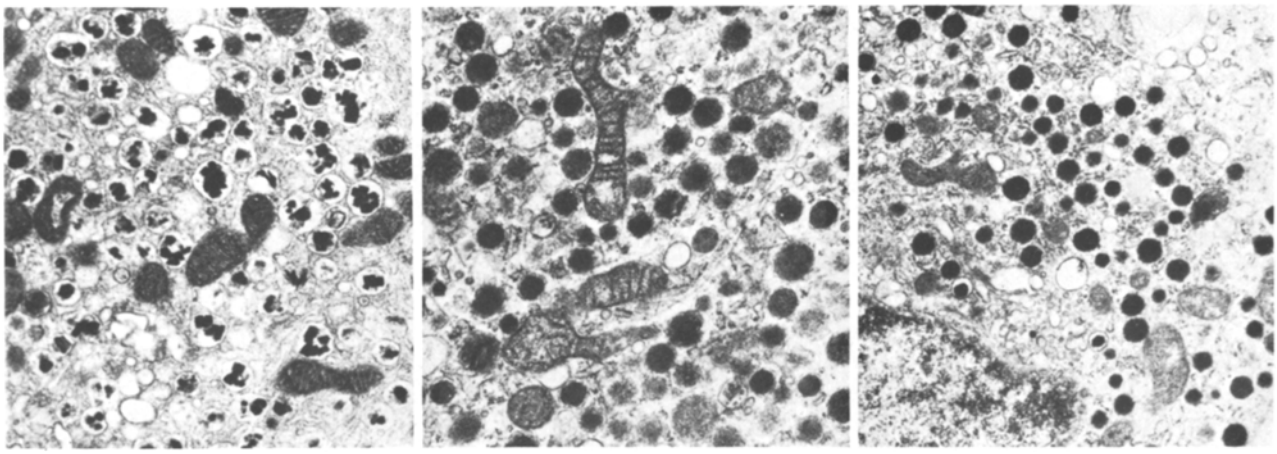

Fig. 1. Electron micrograph showing the characteristic structure of islet cell granules of B, D and A cells (left to right) from a 12 week fetal pancreas after 3 weeks in vitro (× 23,000)

pancreases were fixed after 3 weeks in organ culture. The tissues were processed for light microscopy and 5 μm sections were cut and stained with hematoxylin and eosin, and Gomori aldehyde fuchsin stains. For electron microscopy, tissue was fixed in dilute Karnovsky fixative (1% glutaraldehyde and 1.2% paraformaldehyde in 80 mmol/l cacodylate buffer), washed overnight in cacodylate buffer and post-fixed in 2% osmium tetroxide followed by 2% aqueous uranyl acetate. Following dehydration in acetone, the tissue was embedded in Spurrs' low viscosity resin. Thin sections, stained with saturated uranyl acetate in 70% ethanol followed by lead citrate, were examined in a Philips 300 electron microscope.

Determination of Pancreatic Insulin Content

Insulin was extracted from fragments of seven pancreases of 12, 16 or 22 weeks gestation, by a method similar to that used by Lundgren et al. [12]. Pancreatic fragments were sonicated in 3 ml acid ethanol (15 ml 12 N HC1/l 70% ethanol), extracted for 24 h at 4 °C, and stored at −20°C before radioimmunoassay of insulin [13]. Insulin content was expressed as mU insulin/mg wet weight of tissue.

Determination of Insulin Secretion Under Basal and Stimulating Conditions

Forty-eight pancreatic fragments from a fetus of 16 weeks gestation were grown for 16 days, following which insulin secretion during 1 h in DMEM with 1 g glucose/l was measured by radioimmunoassay [13]. The lower limit of sensitivity of the insulin assay was 2.5 mU/l, the intra-assay coefficient of variation was 3%, and interassay coefficient of variation was 7%. For measurement of insulin release over hourly periods, the fragments on millipore filter strips were transferred from Petri dishes into individual Falcon tubes and submerged in 1 ml of fresh medium [14]. The fragments were divided into four equal groups and insulin release over a second hour was measured in one of the following solutions: (a) DMEM with 1 g glucose/l, (b) DMEM with 4 g glucose/l, (c) D-glyceraldehyde 5 mmol/l and (d) tolbutamide 100 μg/ml (Hoechst, Frankfurt, FRG).

The effect of dibutyryladenosine 3′, 5′ cyclic monophosphate (dibutyryl cyclic AMP) (Sigma Chemicals, St. Louis, USA) on insulin secretion was studied using 40 pancreatic fragments, previously cultured for 7 days, from a fetus of 14 weeks gestation. Insulin secretion during the first hour in DMEM with 1 g glucose/l was measured for each fragment. During the second hour, insulin release was measured as above in one of the following solutions (10 fragments/group): (a) DMEM with 1 g glucose/l, (b) DMEM with 4 g glucose/l, (c) DMEM with 1 g glucose/l plus 0.5 mmol/l dibutyryl cyclic AMP and (d) DMEM with 4 g glucose/l plus 0.5 mmol/l dibutyryl cyclic AMP. During the third hour, insulin secretion was measured in DMEM with 1 g glucose/l plus 10 mmol/l theophylline.

The effect of a combined amino acid and glucose stimulus on insulin release was studied using 28 pancreatic fragments, previously grown under basal conditions for 21 days, from a fetus of 14 weeks gestation. Augmented concentrations of amino acids above standard DMEM were made by diluting modified essential amino acid solution (50 ×) (Flow Laboratories, North Ryde, NSW, Australia) to a final X5 concentration in DMEM, and by diluting modified non-essential amino acid solution (Flow Laboratories) to a final X5 concentration in DMEM. This resulted in final concentrations of individual amino acids between three- and sixfold higher than in standard medium. The medium with augmented amino acids is hereafter described as '5AA' to distinguish it from the amino acid concentration in standard DMEM ('1AA'). After incubation for 1 h in DMEM with 1 g glucose/l, four groups of fragments (seven/group) were transferred to fresh media of one of the following compositions for a second hour: (a) DMEM with standard glucose and amino acids (1 g, 1AA), (b) DMEM with high amino acids (1 g, 5AA), (c) DMEM with 4 g glucose/l (4 g, 1AA) and (d) DMEM with 4 g glucose/l plus high amino acids (4 g, 5AA). After measurement of insulin release over the 2 h, the 28 fragments were removed from individual Falcon tubes and placed back into one of four Petri dishes, each group of seven fragments being maintained in one dish. The tissue was then grown for one further week in DMEM with one of the following compositions: (a) 1 g, 1AA, (b) 1 g, 5AA, (c) 4 g, 1AA and (d) 4 g, 5AA. At the end of 1 week, the acute 1 h release of insulin in standard DMEM with 1 g glucose/l was measured for each fragment to determine whether there was any difference in the rate of basal insulin secretion induced by the variable culture conditions. In order to measure glucose stimulated insulin release, all fragments were transferred for a second hour to Falcon tubes containing DMEM with 4 g glucose/l. The fragments were then transferred for a third hour into DMEM with 4 g glucose/l plus 10 mmol/l theophylline. Statistical analyses were performed by Student's t-test.

Results

The light microscopic appearance of uncultured fetal pancreas of 12 weeks gestation showed that the tissue was largely composed of poorly differentiated ducts lined by a single layer of cuboidal epithelium and embedded in loose connective tissue. The ducts contained

a few islet cells in their walls. After 3 weeks in culture, no acinar tissue was present but well developed islets containing endocrine cells were apparent. The Gomori aldehyde fuchsin stain revealed well stained *B* cells. Electron microscopic examination confirmed the presence of well differentiated A, B and D cells containing large numbers of typical granules (Fig. 1). Insulin content of seven fetal pancreases increased with gestational age, ranging between 1.992 mU/mg tissue for a 12 weeks pancreas previously cultured for 9 days to 13.776 mU/mg tissue for uncultured 22 week pancreas.

Insulin secretion in response to glucose, D-glyceraldehyde and tolbutamide is shown in Table 1. During hour 2, basal insulin release in control tubes was consistently decreased compared with hour 1. No increase in insulin release above control values was demonstrated in response to either glucose or D-glyceraldehyde. However, tolbutamide significantly stimulated insulin secretion (0.265 ± 0.035 versus 0.110 ± 0.010 mU·mg tissue$^{-1}\cdot h^{-1}$, $p < 0.01$).

The results of the response of fetal pancreas to dibutyryl cyclic AMP and theophylline are shown in Figure 2. Insulin secretion was significantly enhanced by transferring pancreatic fragments from DMEM to standard medium plus 0.5 mmol/l dibutyryl cyclic AMP (0.188 ± 0.030 versus 0.100 ± 0.012 mU·mg tissue$^{-1}\cdot h^{-1}$, $p < 0.001$). Similarly, insulin secretion was enhanced by transferring pancreatic fragments from DMEM to medium containing both 4 g glucose/l plus 0.5 mmol/l dibutyryl cyclic AMP (0.182 ± 0.036 versus 0.096 ± 0.016 mU·mg tissue$^{-1}\cdot h^{-1}$, $p < 0.001$). Insulin secretion during hour 2 in the presence of 0.5 mmol/l dibutyryl cyclic AMP was not different in the presence or absence of the additional hyperglycaemic stimulus. In hour 3, insulin secretion was clearly increased by theophylline in all groups.

Insulin release was significantly increased during hour 2, in '1 g, 5AA' medium compared with that from '1 g, 1AA' medium (0.118 ± 0.018 versus 0.031 ± 0.008 mU·tissue$^{-1}\cdot h^{-1}$, $p < 0.001$) (Table 2). Similarly, insulin secretion was greater in '4 g, 5AA' medium than in '4 g, 1AA' medium (0.090 ± 0.020 versus 0.043 ± 0.005 mU·mg tissue$^{-1}\cdot h^{-1}$, $p < 0.001$). Insulin secretion during hour 2 by pancreatic fragments in medium with high amino acid concentration was not increased by higher glucose.

After the fragments had been maintained for one further week in organ culture under varying culture conditions, insulin secretion under basal conditions was significantly enhanced in the two groups which had been maintained for the preceding week in high amino acids (Fig. 3). Basal insulin release from fragments cultured in '1g, 5AA' medium was 0.284 ± 0.052 mU·tissue$^{-1}\cdot h^{-1}$ compared with 0.067 ± 0.011 mU·mg tissue$^{-1}\cdot h^{-1}$ from fragments which had been grown in '1 g, 1AA', ($p < 0.001$). Similarly, fragments grown in '4 g, 5AA' secreted 0.206 ± 0.028 mU·mg tissue$^{-1}\cdot h^{-1}$ compared with 0.092 ± 0.025 mU·mg tissue$^{-1}\cdot h^{-1}$ from

Table 1. Insulin release from pancreatic fragments from a fetus of 16 weeks gestation

Group of fragments	Basal insulin release in the first hour (mU·mg$^{-1}\cdot h^{-1}$)	Stimulus during the second hour	Insulin release during the second hour (mU·mg$^{-1}\cdot h^{-1}$)
A	0.100 ± 0.035	Glucose (1 g/l)	0.060 ± 0.015
B	0.085 ± 0.010	Glucose (4 g/l)	0.070 ± 0.010
C	0.110 ± 0.030	D-glyceraldehyde (5 mmol/l)	0.080 ± 0.015
D	0.110 ± 0.010	Tolbutamide (100 μg/ml)	0.265 ± 0.035[a]

Results expressed as mean ± SEM of 12 estimations.
[a] Second hour > first hour $p < 0.01$

Table 2. The effect of variable amino acid and glucose concentrations on insulin release from human fetal pancreas of 14 weeks gestation

Group of fragments	Basal insulin release in the first hour (mU·mg$^{-1}\cdot h^{-1}$)	Stimulus during the second hour	Insulin release during the second hour (mU·mg$^{-1}\cdot h^{-1}$)
A	0.026 ± 0.004	'1 g, 1AA'	0.031 ± 0.008
B	0.028 ± 0.004	'1 g, 5AA'	0.118 ± 0.018[a]
C	0.051 ± 0.012	'4 g, 1AA'	0.043 ± 0.005
D	0.037 ± 0.006	'4 g, 5AA'	0.090 ± 0.020[a]

Results expressed as mean ± SEM of seven estimations
[a] During the second hour: B > A, D > C, $p < 0.001$

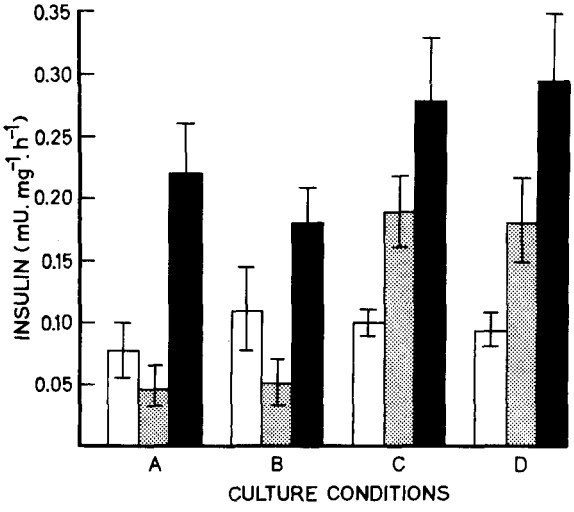

Fig. 2. The effect of dibutyryl cyclic AMP and theophylline on insulin secretion from 14 week fetal pancreas. Hour 1 (□) = 1 g glucose/l; hour 2 (▨): (A) 1 g glucose/l, (B) 4 g glucose/l, (C) 1 g glucose/l plus 0.5 mmol/l dibutyryl cyclic AMP or (D) 4 g glucose/l plus 0.5 mmol/l dibutyryl cyclic AMP; hour 3 (■) = 10 mmol/l theophylline. Results expressed as mean ± SEM of 10 estimations

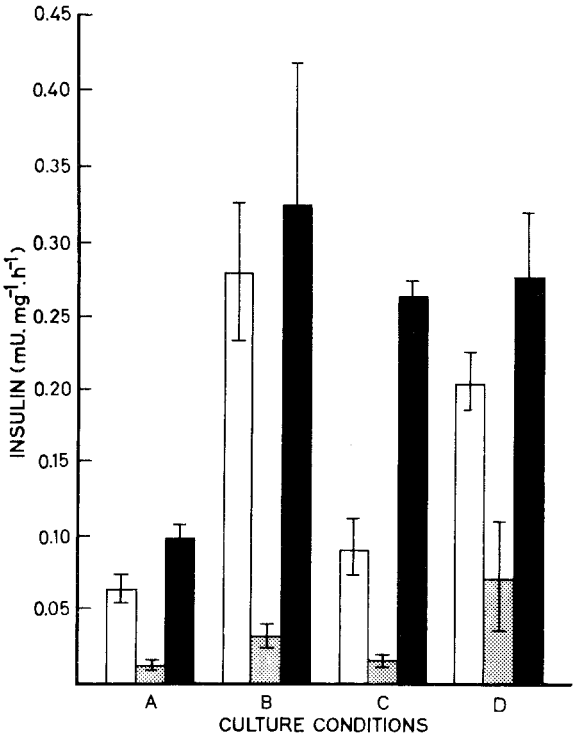

Fig. 3. The effect of previous high amino acid and/or glucose concentration on acute insulin release from 14 week fetal pancreas. Previous culture conditions: (A) '1 g, 1AA', (B) '1 g, 5AA', (C) '4 g, 1AA' and (D) '4 g, 5AA'. Hour 1 (□) = 1 g glucose/l; hour 2 (▨) = 4 g glucose/l and hour 3 (■) = 4 g glucose/l plus 10 mmol/l theophylline. Results expressed as mean ± SEM of seven estimations

fragments previously cultured in '4 g, 1AA' ($p < 0.001$). Despite increased basal insulin secretion from fragments grown in medium with an augmented amino acid concentration, there was no response to a hyperglycaemic stimulus during the second hour of incubation. In all groups insulin release was stimulated by theophylline.

Discussion

Results of histological studies confirmed that the organ culture system used provided conditions suitable for growth and differentiation of fetal human pancreatic islets. The insulin content of fetal pancreas of 12 weeks gestation was similar to the insulin content of adult cadaver pancreas [15]. Measurement of insulin content from 22 week fetal pancreas was comparable per mg tissue to the reported insulin content of infant human pancreas [15].

The fetal human pancreas did not secrete insulin in response to glucose, a finding consistent with previous studies in vivo [16] and in vitro [17]. Recent reports have suggested that the fetal human pancreas can respond to glucose in vitro [18–20]. However the responses were minimal despite a big glucose load. It is possible that the glucose-insulin dose response curve is different in fetal and adult pancreas. We have not demonstrated altered insulin secretion with exposure of fetal human pancreas to 0.25–1.0 g glucose/l and nor have we seen any difference in the response of individual pancreases between 12 and 22 weeks gestation to each of the secretagogues studied (unpublished data).

Deficiencies in glycolysis, such as impaired phosphorylation of glucose, have been implicated as a cause of 'glucose blindness' by studies in the neonatal rat [21]. Supporting a defective step in glycolysis is the report of diminshed responsiveness to 16.7 mmol/l glucose of newborn rat islets but enhanced insulin secretion in the presence of 10 mmol/l glyceraldehyde [22]. Others have suggested a more generalized defect in recognition of secretagogues, with absent insulin responses by the neonatal rat pancreas to glucose, leucine and D-glyceraldehyde [23]. In the present study, the inability of D-glyceraldehyde to stimulate insulin secretion by fetal human pancreas that responded to other secretagogues provides evidence against a major block in the glycolytic pathway above the triose phosphate step.

The effect of dibutyryl cyclic AMP was studied because it has previously been suggested that lack of glucose-stimulated insulin release by fetal islets may relate to inadequate accumulation of cyclic AMP [24]. The results of the present study confirmed that, in the presence of dibutyryl cyclic AMP, insulin secretion from the fetal human pancreas is markedly stimulated. However, in contrast to the findings in adult tissue [25], the effect of dibutyryl cyclic AMP was not dependent upon the extracellular glucose concentration. It is, therefore, unlikely that impaired glucose-stimulated insulin release results from insufficient intracellular cyclic AMP.

The present studies of the responses of fetal *B* cells to amino acids confirm two previous reports of the response of the fetal human pancreas to stimulation by arginine or leucine [19, 26]. The highly significant increase in basal insulin secretion from fetal human pancreas which was grown in medium with high amino acids compared with standard medium is evidence of an important role of amino acids as substrates for *B* cell metabolism and development [27].

There was no stimulation of insulin secretion by glucose after incubation for 7 days with high amino acid concentrations. Similarly, previous exposure to 4 g glucose/l did not affect acute insulin release by glucose. However it is possible (Fig. 3) that there was an effect of previous glucose incubation on the insulin release induced by theophylline.

The mechanisms which govern the development of full secretory maturation of the fetal *B* cell are obscure, although the present data suggest that a single defect in the early steps of glycolysis, or deficiency of cyclic AMP, is unlikely. It remains to be determined whether cultured fetal human pancreas can develop the capacity to respond to a wider variety of stimuli in vivo. Despite failure to respond to some secretagogues, the use of fetal pancreas remains an important potential source of

islets for transplantation to overcome the problems of limited availability of, and poor islet yield from, adult tissue [15].

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