Upsala J Med Sci 98: 39–52, 1993

In vitro Screening of Putative Compounds Inducing Fetal Porcine Pancreatic β-cell Differentiation: Implications for Cell Transplantation in Insulin-dependent Diabetes Mellitus

Olle Korsgren, Arne Andersson and Stellan Sandler
Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden

ABSTRACT

Successful transplantation of fetal pancreatic β-cells to diabetic recipients requires that differentiation of the immature β-cells is achieved. Animal experiments have shown that this can occur in vivo, but it would be desirable to induce β-cell maturation in vitro prior to transplantation. For that purpose the effect of several putative inducers of β-cell differentiation and/or replication in explant cultures of fetal porcine pancreatic islet-like cell clusters (ICC) were investigated. Initial screening experiments indicated that dexamethasone (DEX; 200 ng/ml) and sodium butyrate (BUT; 2 mM) might promote β-cell differentiation as evidenced by increased insulin/DNA contents in the ICC. In subsequent experiments these two substances, and also nicotinamide (NIC; 10 mM) which previously has been found to promote fetal β-cell differentiation, were added alone or in combinations to the basal control medium consisting of RPMI 1640 + 1 % human serum. All three test agents alone or in combinations increased the insulin content/DNA of the ICC compared with that of the control group. The combination of NIC + DEX increased the insulin mRNA levels of the ICC. No significant stimulation of insulin release was observed in any test group after short-term incubation with high glucose alone. Addition of 5 mM theophylline to high glucose stimulation, however, increased the insulin secretion in most groups of ICC. Finally, ICC in groups of about 600, which had developed in the presence of NIC or NIC + DEX, were transplanted under the kidney capsule of alloxan-diabetic nude mice. However, neither the time for reversal of diabetes (4 weeks) nor the amount of insulin secretion during perfusion from the grafted ICC were further affected by adding DEX to the NIC supplemented medium. The marked increase of the insulin content of the ICC cultured with DEX supplementation, appeared transient and was not manifested after transplantation. In conclusion, the present study demonstrated that some compounds can stimulate porcine fetal β-cells in an in vitro system, but in order to attain terminal differentiation of the β-cells including glucose-sensitive insulin secretion, longer observation periods might be required than used herein. Alternatively, an in vivo environment like that after transplantation is mandatory for this process.

INTRODUCTION

Animal studies have shown that experimentally induced diabetes can be cured by transplantation of syngeneic fetal pancreas preparations (4,5). Undisputable reports showing successful clinical application with human fetal pancreas preparations are essentially lacking (12). The reason for this is both allograft rejection and difficulties in obtaining sufficient amounts of viable human fetal β-cells which will replicate and mature into fully differentiated β-cells in the recipient.

We have described a tissue culture technique for the porcine fetal pancreas, in which the explant forms so called islet-like cell clusters (ICC) in large numbers during a 4 day culture period (17,18). This technique was originally developed for the fetal rat pancreas (10) and then modified for the human fetal pancreas (29). Fetal rat islets obtained by this technique (fetal age day 20-21) are composed of approximately 80 % β-cells (7), whereas human or porcine ICC contain only a minority of insulin-positive cells (5-10 %) (18,29). Therefore, when grafting porcine ICC into diabetic recipents there is a considerable time lag (about 8 weeks) before a normalization of the recipients hyperglycemia is achieved (16). We recently found that nicotinamide supplementation of the culture medium induced an accelerated reversal of diabetes after transplantation of porcine ICC to alloxan-diabetic nude mice, suggesting that nicotinamide might promote fetal β-cell differentiation (15) Thus, in the present study compounds, which could be anticipated to promote fetal β-cell differentiation, were added to explant cultures of fetal porcine pancreas. ICC formed under these conditions were functionally characterized in vitro and some were subsequently examined after transplantation to nude mice.

MATERIALS AND METHODS

Preparation and culture of fetal porcine pancreas

Pregnant sows belonging to a local stock were killed by means of a slaughtering mask. The length of gestation was 65-75 days (full-term ≈ 115 days). The fetuses (8-15 in one litter) were immediately collected from the uterus and placed on ice during transportation to the laboratory. After aseptic removal the pancreatic glands were placed in cold Hanks' solution, minced into 1-2 mm^3 fragments and treated with collagenase from Clostridium histolyticum (≈10 mg/ml, Boehringer Mannheim, Mannheim, Germany) during vigorous shaking as described elsewhere (18). The digested tissue was washed and explanted into culture dishes allowing cellular attachment (Nunclon 50 mm Ø; Nunc, Kamstrup, Denmark). The culture medium was RPMI 1640 (11.1 mM glucose; Flow Laboratories, Irvine, UK) supplemented with either 1% or 10% (v/v) human serum (HS; The Blood Center, Huddinge Hospital, Huddinge, Sweden).

In a first series of experiments, the following agents were tested for their action on the insulin content/DNA of the ICC: epidermal growth factor (EGF; 100 ng/ml), glycyl-L-histidyl-L-lysine acetate (GHL; 100 ng/ml), thyrotropin releasing hormone (TRH; 10 ng/ml), dexamethasone (DEX; 200 ng/ml), thyrocalcitonin (50 μg/ml), retinol acetate (20 ng/ml), fetuin (1 mg/ml), transferrin (30 μg/ml), prolactin (1 μg/ml), cholecystokinin 33 (CCK 33, 10 nM), theophylline (0.5 mM), sodium butyrate (BUT; 2 mM), LiCl (10 mM), transforming growth factor-α (TGF-α; 100 ng/ml), transforming growth factor-β, (TGF-β; 500 pM), putrescine (100 μM) and human placental lactogen (HPL; 100 ng/ml). TGF-β was kindly provided by Genentech, San Francisco, CA, USA. TGF-α was from Bachem, Torrance, CA, USA and theophylline from Apoteksbolaget, Uppsala, Sweden. All other compounds were from Sigma Chemicals, St Louis, MO, USA. In each experiment the test substance was added to duplicate culture dishes also supplemented with 1% HS, and after two days of culture the medium was exchanged for identical medium for another two days of culture. The culture medium was then changed in all groups to RPMI 1640 + 10% HS, without any other addition and the ICC cultured for another four days (Day 8), with medium exchange on day 6. On days 4 and 8 the insulin content/DNA of the ICC was estimated from each culture dish. A mean from the duplicate culture dishes was calculated and considered as one observation.

In a second series of experiments only NIC, DEX and BUT and combinations of these substances at the concentrations given above, i. e. NIC + DEX, NIC + BUT, DEX + BUT and NIC + DEX + BUT, were added to medium RPMI 1640 supplemented with 1% HS.

DNA and insulin content of ICC

In the second series of experiments duplicate groups of 50 ICC were homogenised by ultrasonic disruption in 200 μl redistilled water, whereas in the initial screening experiments a volume of ≈ 1 μl of ICC, corresponding to ≈100 ICC, was analyzed but no attempts were made to count the actual number of ICC. An aliquot of 50 μl was mixed with 125 μl acid ethanol (0.18 M HCl in 95% [v/v] ethanol) and extracted overnight at 4°C. The insulin concentration of the extract was measured by RIA (9). Two 50 μl aliquots of the water homogenate were analyzed by fluorophotometry for their DNA content (13,14).

Insulin mRNA content of ICC

Insulin mRNA was measured by dot blot analysis (31). Duplicate groups of 50 ICC were sonicated, and extracted with phenol-chloroform. RNA precipitates were treated with 1 M glyoxal, diluted with 4 volumes 3 M NaCl + 0.3 M sodium citrate, and bound to Genescreen Hybridization Transfer Membranes (New England Nuclear, Boston, MA, USA). After prehybridization, the samples were hybridized at 42°C with the probe (pRI-7; 6), which was labeled using an oligolabeling kit (Pharmacia Fine Chemicals, Uppsala, Sweden). The filters were then washed and exposed at -70°C to Hyperfilm-MP (Amersham International, Amersham, UK) with an intensifying screen. The

intensities of the spots obtained after autoradiography were quantitated by densitometry and expressed as a percentage of the corresponding controls.

Insulin release of ICC

Duplicate groups of 50 ICC were incubated for three consecutive hours at 37°C (CO_2:O_2; 5:95) in glass vials in a Krebs-Ringer bicarbonate buffer (19) containing 10 mM Hepes (Sigma) and 2 mg/ml of bovine serum albumin (Miles Laboratories, Slough, UK). The medium was supplemented with 1.7 mM glucose during the first hour of incubation and with 16.7 mM glucose during the second hour and with 16.7 mM glucose + 5 mM theophylline during the final hour. The incubation media were kept frozen at -20°C until analysis for insulin by the RIA.

Transplantation of ICC and perfusion of the graft

Athymic, male, inbred C57BL/6J mice (Bomholdtgaard, Ry, Denmark) were transplanted with ICC. The animals had been rendered diabetic by an i.v. alloxan (75 mg/kg body-weight) injection 4 days prior to transplantion. Two grafts consisting of 3 µl ICC (approximately 2 x 300 ICC) were implanted under the left kidney capsule, as described in detail elsewhere (15). The mice became normoglycemic within 4-6 weeks posttransplantation. After 18 weeks the graft-bearing kidneys were perfused according to a previously described procedure (18). After an initial 30 min perfusion with a medium containing 2.8 mM glucose, the grafts were perfused with 16.7 mM glucose for 45 min. This was followed by another perfusion period with 2.8 mM glucose (15 min) and subsequently the grafts were perfused with medium containing 16.7 mM glucose + 10 mM theophylline (20 min). Samples were taken from the effluent medium for determination of insulin by RIA (9). The total insulin release per graft, during perfusion with 16.7 mM glucose or with 16.7 mM glucose + 10 mM theophylline, was calculated from planimetric measurements of the areas under the individual perfusion curves.

Statistical analysis

Data were computed as means ± SEM and compared, using Student's paired or unpaired t-test.

RESULTS

Effect of different test substances on the insulin content per DNA of ICC

In the stereomicroscope the apparent growth pattern during the first few days of culture was influenced by some of the culture supplements. Thus, DEX, transferrin and thyrocalcitonin all seemed to inhibit fibroblast growth and to promote formation of small ICC. On the contrary, fetuin and also 10% HS stimulated fibroblast growth. After the addition of TGF-β or LiCl and to a lesser extent BUT, there was a tendency for the ICC to disintegrate during the last days of culture.

After four days of culture in the presence of theophylline or LiCl the insulin content per DNA was significantly decreased in the ICC (Table 1; Day 4). Supplementation of the medium with BUT was found to increase the insulin/DNA ratio, whereas the increased value seen after the addition of DEX did not attain statistical significance (p=0.11; n=6).

TABLE 1

Different compounds were tested for their action on the insulin content per DNA of the ICC on days 4 and 8 of culture. For each test substance, duplicate culture dishes supplemented with 1% human serum were used from one litter. The medium was changed to RPMI 1640 + 10% human serum in all groups after four days of culture.

Medium supplementation	Concentration	Day 4 (%)		Day 8 (%)	
Cholecystokinin 33	10 ng	166 ± 61	(5)	123 ± 19	(5)
Dexamethasone; DEX	200 ng/ml	358 ± 134	(6)	1049 ± 108	(6)***
EGF	100 ng/ml	102 ± 19	(6)	88 ± 11	(6)
Fetuin	1 mg/ml	161 ± 39	(6)	53 ± 3	(6)***
GHL	100 ng/ml	168 ± 33	(6)	109 ± 18	(6)
HPL	1 µg/ml	95± 19	(6)	97 ± 16	(5)
Human serum	1%	100	(13)	100	(12)
Human serum	10 %	134 ± 31	(11)	109 ± 15	(10)
LiCl	10 mM	53 ± 9	(7)**	39 ± 5	(6)***
Prolactin	1 µg/ml	206 ± 62	(6)	130 ± 25	(6)
Putrescine	100 µg/ml	109 ± 12	(6)	170 ± 45	(6)
Retinol Acetate	20 ng/ml	132 ± 42	(5)	191 ± 40	(5)
Sodium butyrate; BUT	2 mM	207 ± 25	(7)**	424 ± 154	(7)
TGF-a	100 ng/ml	73 ± 16	(5)	50 ± 10	(4)*
TGF-ß	500 pM	85 ± 9	(5)	62 ± 17	(4)
Theophylline	0.5 mM	59 ± 7	(5)**	251 ± 81	(5)
Thyrocalcitonin	50 µg/ml	203 ± 55	(5)	168 ± 10	(5)**
Transferrin	30 µg/ml	266 ± 89	(6)	121 ± 11	(6)
TRH	10 ng/ml	184 ± 54	(6)	124 ± 17	(6)

The insulin content of the control ICC (1% human serum) after four days was 8.5 ± 1.7 (13) ng/µg DNA and 6.1 ± 1.6 (12) ng/µg DNA after eight days. Data are expressed as a percentage of the ICC cultured in the control medium. Values are means ± SEM. * P<0.05, ** P<0.01 and *** P<0.001, using Student's paired t test compared with the control group. Numbers of pig litters are given within parenthesis.

When ICC were cultured first for four days in the presence of the different test agents and then for another four-day-period in medium supplemented with 10% HS only, it was found that the initial supplementation with LiCl, fetuin and TGF-α caused a persistent decrease in the insulin content/DNA of the ICC (Table 1; Day 8). At this stage the initial DEX supplementation resulted in a marked increase of the insulin content of the ICC, whereas the enhancement induced by BUT was variable and not statistically significant ($p=0.08$; $n=7$). Thyrocalcitonin exerted a minute increase of the insulin content as measured in ICC harvested on day 8 of culture. It should be noted that the decreased insulin content of the ICC, which developed in the presence of theophylline (day 4), was not evident on day 8.

TABLE 2

Effects of culture with different medium supplements + 1% human serum for four days (Day 4) and after another four days in medium RPMI 1640 + 10% human serum (Day 8) on the DNA and insulin content of fetal porcine ICC.

Medium supplement	DNA content (μg DNA/10 ICC)		Insulin content/DNA (ng insulin/μg DNA)	
	Day 4	Day 8	Day 4	Day 8
1 % HS	0.56 ± 0.10	0.62 ± 0.10	7.5 ± 1.6	5.1 ± 0.7
10 % HS	0.48 ± 0.11	0.46 ± 0.04	8.1 ± 1.6	6.1 ± 1.4
NIC	0.43 ± 0.09	0.66 ± 0.07	9.4 ± 1.7*	11 ± 2.5
DEX	0.47 ± 0.10	0.41 ± 0.03	11 ± 1.2*	15 ± 1.9***
BUT	0.42 ± 0.09	0.43 ± 0.05	14 ± 2.5**	11 ± 2.6*
NIC + DEX	0.45 ± 0.09	0.39 ± 0.04	18 ± 2.3***	43 ± 9.3**
NIC + BUT	0.52 ± 0.10	0.45 ± 0.04	21 ± 2.5**	29 ± 8.6*
DEX + BUT	0.36 ± 0.07	0.28 ± 0.02*	17 ± 3.3*	22 ± 3.5***
NIC + DEX + BUT	0.34 ± 0.08*	0.31 ± 0.03*	16 ± 3.6*	40 ± 6.1***

Explant cultures of collagenase digested fetal porcine pancreas were prepared, whereupon islet-like cell clusters (ICC) developed. The cultures were maintained in medium RPMI 1640 plus the supplements given in the first column. The cultures containing NIC, DEX and BUT were also supplemented with 1% HS. ICC were harvested after four days of culture (Day 4) and homogenised in water for measurement of their DNA content by fluorophotometry and for extraction of insulin in acid ethanol. The medium was subsequently changed to 10 % HS during the remaining culture period and the DNA and insulin contents of the ICC remeasured after an additional four days of culture (Day 8). Values are means ± SEM for 5 experiments. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$, respectively, compared with ICC that had developed in 1 % HS only, using Student's paired t test.

Effects of BUT, DEX and NIC on contents of DNA, insulin and insulin mRNA contents of ICC

In the further investigation we chose to study in more detail the effects of supplementation with BUT, DEX and NIC, the latter being based on recent studies with fetal porcine ICC (15). The DNA content of the ICC formed during the 4-day culture period in the presence of the combination of NIC + DEX + BUT was decreased compared with that of the control ICC which developed in 1% HS only (Table 2). This decrease persisted also on day 8, and at that latter time point also the group DEX + BUT showed a lowered DNA content. All groups of ICC formed in the presence of the test substances contained more insulin than the control ICC (Table 2). With the exception of the NIC group, this was also the case on day 8 of culture.

When examined on day 4, the only medium supplementation which significantly stimulated the insulin mRNA content was the combination of NIC + DEX (Fig. 1). Also, ICC developed in the presence of NIC alone tended to contain more insulin mRNA than the control group, but this difference did not attain statistical significance. It should be noted, however, that no corrections have been made in these experiments

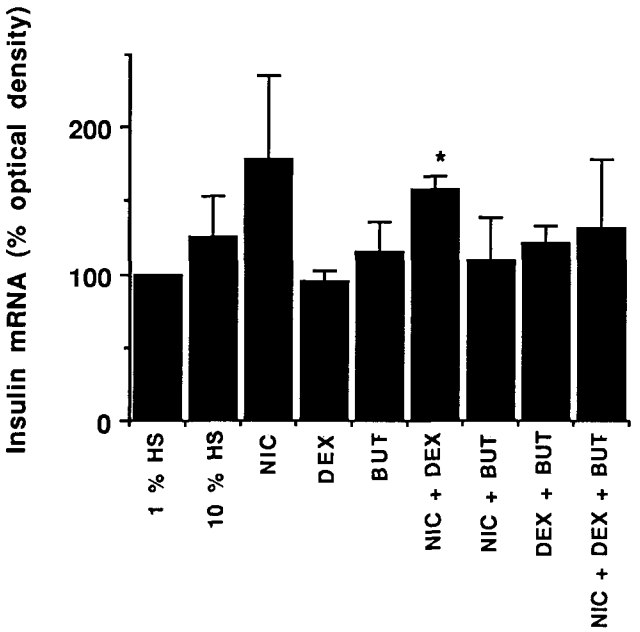

Fig. 1. Insulin mRNA content of porcine ICC on day 4 of culture. The measurements were carried out in groups of 50 ICC by dot blot analysis and quantification by optical densitometry. Bars are means + SEM for 4 culture experiments. * denotes $P<0.05$ compared with ICC which had developed in 1% HS only, using Student's paired t test.

for differences in DNA content of the various groups of ICC. Moreover, since the ICC are fairly immature it could be that the probe used, also hybridized to notable amounts of DNAs in relation to mRNAs for insulin.

Effects of BUT, DEX and NIC on glucose- and theophylline-stimulated insulin release of ICC

In short-term batch type incubations with ICC on day 4, the insulin release at 1.7 mM glucose was decreased after culture with 10% HS (Table 3). In general, within the same medium supplementation group exposure to 16.7 mM glucose during the second hour of incubation did not stimulate the insulin secretory rates either on day 4 (Table 1) or day 8 (Table 4). However, on day 8 the ICC initially formed in the presence of NIC, BUT, NIC + BUT and NIC + DEX + BUT released more insulin at 16.7 mM glucose than the controls (Table 4). Insulin secretion in the presence of 16.7 mM glucose + 5 mM theophylline during the third consecutive hour was higher in most groups of ICC compared with that observed during incubation with high glucose alone. A comparison on day 4 of the secretory rates of different explants after stimulation with 16.7 mM glucose + 5 mM theophylline showed that NIC supplemented ICC released more insulin, and those cultured with 10% HS, DEX, BUT and DEX + BUT released

TABLE 3

Effects of culture of fetal porcine ICC for four days with different medium supplements on glucose and theophylline stimulated insulin release (Day 4).

Medium supplement	Insulin release (ng insulin/50 ICC x 60 min)		
	Glucose (mM)		
	1.7	16.7	16.7 + 5 mM theophylline
1 % HS (Controls)	0.67 ± 0.12	0.61 ± 0.23	4.1 ± 0.85
10 % HS	0.36 ± 0.05 *	0.68 ± 0.44	1.1 ± 0.24 *
NIC	0.70 ± 0.16	0.69 ± 0.12	8.5 ± 1.0 **
DEX	0.46 ± 0.07	0.53 ± 0.16	1.2 ± 0.31 *
BUT	0.56 ± 0.15	0.24 ± 0.03	1.3 ± 0.52 *
NIC + DEX	1.6 ± 0.59	1.2 ± 0.76	3.2 ± 1.16
NIC + BUT	1.8 ± 0.56	1.6 ± 0.71	4.9 ± 0.65
DEX + BUT	0.97 ± 0.55	0.79 ± 0.49	1.2 ± 0.65 *
NIC + DEX + BUT	1.5 ± 0.84	1.2 ± 0.66	2.5 ± 1.30

Insulin release was measured after four days of culture with the various supplements as defined in Table 2. The ICC were incubated for three consecutive hours in KRBH buffer supplemented as given in columns 2, 3 and 4. Values are means ± SEM for 5 litters. * and ** denote $P<0.05$ and $P<0.01$, respectively, using Student's paired t test as compared with ICC which had developed in a medium supplemented with 1% HS only.

TABLE 4

Effects of culture with different medium supplements for four days and for another four days (day 8) in medium RPMI 1640 + 10 % human serum on glucose and theophylline stimulated insulin release of fetal porcine ICC.

Culture supplement	Insulin release (ng insulin/50 ICC x 60 min)		
	Glucose (mM)		
	1.7	16.7	16.7 + 5 mM theophylline
1 % HS (Controls)	0.24 ± 0.07	0.17 ± 0.04	0.90 ± 0.26
10 % HS	0.17 ± 0.02	0.15 ± 0.02	0.77 ± 0.14
NIC	0.29 ± 0.03	0.34 ± 0.06*	4.8 ± 0.39***
DEX	0.19 ± 0.02	0.14 ± 0.02	0.78 ± 0.17
BUT	0.30 ± 0.04	0.30 ± 0.04*	2.8 ± 0.48**
NIC + DEX	0.52 ± 0.11	0.34 ± 0.07	3.3 ± 0.85*
NIC + BUT	0.64 ± 0.17	0.59 ± 0.16*	5.8 ± 0.82***
DEX + BUT	0.25 ± 0.05	0.31 ± 0.06	1.6 ± 0.70
NIC + DEX + BUT	0.38 ± 0.11	0.26 ± 0.02*	1.9 ± 0.55

The insulin release of the ICC was studied after a total of eight days of culture. During the first four days the culture medium was supplemented as given in the first column and defined in Table 1, and subsequently all groups of ICC were cultured in medium RPMI 1640 supplemented with 10% HS. The ICC were incubated in KRBH buffer supplemented as given in columns 2,3 and 4, for three consecutive hours. Values are means ± SEM for 5 experiments. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$, respectively, compared with ICC that had developed in medium supplemented with 1% HS only, using Student's paired t test.

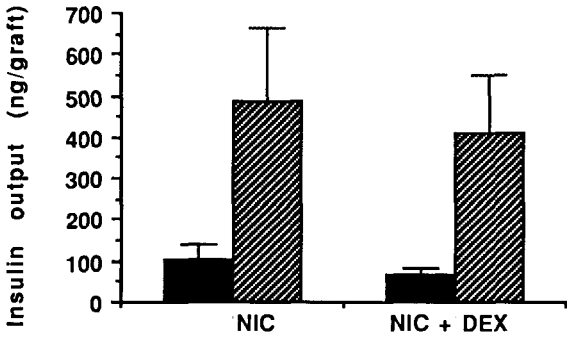

Fig. 2. Insulin secretion in the effluent medium, collected during perfusion of graft-bearing kidneys 18 weeks after transplantation of approximately 600 ICC into alloxan-diabetic nude mice. The ICC had been cultured for four days in medium supplemented with NIC (n=5) or NIC + DEX (n=4). The kidneys were perfused with 16.7 mM glucose for 45 min (filled bars) or 16.7 mM glucose + 10 mM theophylline for 20 min (hatched bars). Bars are means ± SEM.

less insulin, than the control group (Table 3). On day 8 of culture no group exhibited a lowered insulin secretion, whereas ICC initially cultured in the presence of NIC, BUT, NIC + DEX or NIC + BUT showed higher rates of insulin release than the controls (Table 4).

Insulin release by ICC transplanted into diabetic nude mice

Based on the findings of a markedly increased insulin (Table 2) and insulin mRNA contents of the ICC (Fig. 1), we compared the in vivo function of ICC transplanted into diabetic nude mice cultured for 4 days in the presence of NIC alone with the combination NIC + DEX. There was no difference in the time required to reach normoglycemia (about 4 weeks) between the two groups (data not shown). When the graft-bearing kidneys were perfused the insulin secretory response to 16.7 mM glucose of the grafts was also similar (Fig. 2). The rates of insulin secretion were markedly increased when the kidneys were perfused with 16.7 mM glucose + 10 mM theophylline, but again there was no difference between the two types of grafted ICC.

DISCUSSION

Early expansion of the β-cell mass in the fetus is most likely due to cell differentiation, rather than cell replication. This process is completed in the fetal rat pancreatic islets prepared on day 20-21 of gestation (7) and further expansion of this β-cell population depends mainly on cell replication. For evaluation of the process of early β-cell differentiation, human or porcine ICC prepared at mid-gestation are more suited than fetal rat pancreatic islets. Due to the scarce availability of human fetal pancreatic glands obtained at legal abortions, a comparative study of different compounds capacity to promote human fetal β-cell differentiation is precluded in most countries. Using fetal porcine pancreas, however, a study may be designed which allows simultaneous screening of a number of such substances.

Previously, we have demonstrated that porcine ICC transplanted under the kidney capsule of nude mice matured, both from a functional and morphological point of view, within one to two months after implantation (15,16). This suggests that fetal pancreatic endocrine cells have the potential to differentiate even when removed from their normal localization in the pancreas. In order to test the impact of various compounds on β-cell differentiation an in vitro system should conceivably be applied. We used an initial culture period of four days, during which the test substances were added, and a putative stimulation of cell replication and/or differentiation eventually initiated. During the following four-day-period all groups of ICC were cultured under the same conditions with only 10% HS as medium supplementation, which upon reexamination on day 8 might disclose a persistent effect on β-cell function of the different culture supplements used. Extended culture periods would probably allow assessment of β-cell differentiation more accurately. This is, however, partly

precluded because with time the ICC tend to attach and become overgrown by fibroblasts. Unfortunately, the maintenance of fetal ICC free-floating in culture, in contrast to isolated rodent islets (2), for more than a few days has been found to be unsuitable (unpublished observation).

Among the compounds tested herein there was no substance that could induce a maturation of the fetal β-cells to the function of adult β-cells over the 8 days culture period. Only minor effects were seen with factors, which previously have been shown to stimulate cell replication in adult islets (GH, HPL, prolactin, LiCl, EGF, TGF-α; 11,20,22,23,30), whereas factors known to induce differentiation (DEX, BUT, NIC; 1,24,28,35) were more potent in increasing the insulin content of the ICC. Thyrocalciotonin also seemed to induce a late (Day 8), but consistent, increase in the insulin/DNA ratio of the ICC. The mechanism of this action, however, remains unknown. Concerning the substances that reduced the insulin/DNA content of the ICC, the decrease on day 4 in ICC supplemented with theophylline probably reflects a stimulation of insulin secretion to the culture medium. Thus, when these ICC were then cultured for 4 days in the absence of theophylline the insulin content was restored. Prolonged culture with LiCl appeared directly toxic and the number of ICC formed in culture was clearly reduced. The same held true for TGF-α supplementation of the medium. As described above fetuin stimulated fibroblast growth and it is likely that the fibroblast content of such ICC was increased and as a consequence the ratio insulin/DNA decreased.

NIC has previously been attributed β-cell trophic effects, i.e. to increase islet cell replication in vitro and in vivo (26,27), ameliorate diabetes in partially depancreatized rats (36), increase the insulin content and yield of ICC from the cultured human fetal pancreas (28) and retain C-peptide levels in newly diagnosed IDDM patients (21,33,34). There are several possibilities by which NIC may promote fetal β-cell function, e. g. to increase the mass of β-cells or induce differentiation of the existing immature β-cells or stem cells. An increased β-cell mass during culture could be achieved either by stimulation of the replicatory rate of the cultured β-cells or by a protective effect against unidentified compounds noxious to β-cells in vitro. The latter would thus allow a larger population of already existing β-cells in the explanted pancreas to survive the culture period.

In this study NIC was compared with DEX and BUT, since the latter two compounds had been found to be of potential interest in the initial screening study (cf. Table 1). NIC inhibits poly(ADP-ribose) synthetase and this enzyme affects nuclear cellular events such as DNA repair and differentiation (32). It can be envisaged that by altering chromatin structure through inhibition of ADP-ribosylation, genes involved in β-cell differentiation become accessible for substances that regulate gene transcription. Moreover, experiments with adult rat islets in vitro (35) and in vivo treatment of adult rats (8) have shown that DEX induced an increase in the level of mRNA content. In line with this, there was an elevation of the transcript on day 4 after

posure to the combination NIC + DEX. On the other hand, BUT or combinations thereof did not affect the insulin mRNA, although BUT might also influence chromatin structure (3). We also examined if the combination NIC + DEX could be of benefit compared to supplementation with NIC alone, when ICC were implanted to diabetic nude mice. However, neither the time for reversal of diabetes nor the amount of insulin secretion from the grafted ICC were further affected by adding DEX to the NIC supplemented medium. Thus, the marked increase of the insulin content of the ICC cultured with DEX addition (cf. Table 1) appeared transient and was not manifested after transplantation.

In conclusion, the present study has shown that some compounds can stimulate porcine fetal β–cells in an in vitro system, but in order to attain terminal differentiation of the β-cells with a glucose-sensitive insulin secretion, longer observation periods might be required than used herein. Alternatively, an in vivo environment, like that obtained after transplantation, is mandatory for this process.

ACKNOWLEDGEMENTS

The skilled technical assistance by M. Engkvist, E. Forsbeck and A. Nordin and secretarial assistance by A. Snellman is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (19P-8982; 12X-109; 12X-8273; 12X-9237), the Swedish Diabetes Association, the Swedish Childhood Diabetes Fund, the Nordic Insulin Fund, the Aage-Louis Hansens Mindefond, the Procordia Foundation, the Hoechst Diabetes Foundation, the Juvenile Diabetes Foundation International and the Family Ernfors Fund.

REFERENCES

1. Althaus, F.R., Lawrence S.D., He, Y.-Z., Sattler, G.L., Tsukada, Y. & Pitot, H.C.: Effects of altered [ADP-ribose]$_n$ metabolism on expression of fetal functions by adult hepatocytes. Nature 300:366-368, 1982.
2. Andersson, A. : Isolated mouse pancreatic in culture: effects of serum and different culture media on the insulin production of the islets. Diabetologia 14:397-404, 1978.
3. Bohr, V. A. & Hanawalt, P.C.: DNA repair genes. Phamac Ther 38:305-319, 1998.
4. Brown, J., Danilovs, J.A., Clark, W. & Mullen, Y.: Fetal pancreas as a donor organ. World J Surg 8:152-157, 1984.
5. Brown, J., Molnar, I.G., Clark, W. & Mullen, Y.: Control of experimental diabetes mellitus in rats by transplantation of fetal pancreases. Science 184:1377-1379, 1974.
6. Chan, S.J., Noyes, B.E., Agarwal, K.L. & Steiner, D.F.: Construction and selection of recombinant plasmids containing full-length complementary DNAs corresponding to rat insulin I and II. Proc Natl Acad Sci USA 76:5036-5041, 1979.
7. Dudek, R.W., Freinkel, N., Lewis, N.J., Hellerström, C. & Johnson, C.: Morphologic study of cultured pancreatic fetal islets during maturation of the insulin stimulus-secretion mechanism. Diabetes 29:15-21, 1980.
8. Giddings, S.J., Orland, M.J., Weir, G.C., Bonner-Weir, S. & Permut, A.P.: Impaired insulin biosynthetic capacity in a rat model for non-insulin-dependent diabetes. Studies with dexamethasone. Diabetes 34:235-240, 1985.
9. Heding, L.G.: Determination of total serum insulin (IRI) in insulin-treated patients. Diabetologia 8:260-266, 1972.
10. Hellerström, C., Lewis, N.J., Borg, H., Johnson, R & Freinkel, N.: Method for large-scale isolation of pancreatic islets by tissue culture of fetal rat pancreas. Diabetes 28:796-777, 1979.
11. Hellerström, C. & Swenne, I.: Growth pattern of pancreatic islets in animals. In: The Diabetic Pancreas (ed. B.W Volk & E.R. Arquilla),vol 2, pp. 53-79. Plenum, New York, 1985.
12. Hering, B.J., Bretzel, R.G. & Federlin, K.: Current status of clinical islet transplantation. Horm Metab Res 20:537-545, 1988.
13. Hinegardner, R.T.: An improved fluorometric assay for DNA. Anal Biochem 39:197-201, 1971.
14. Kissane, J.M. & Robins, E.: The fluorometric measurments of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J Biol Chem 233:184-188, 1958.
15. Korsgren, O., Andersson, A. & Sandler, S.: Pretreatment of fetal porcine pancreas in culture with nicotinamide accelerates reversal of diabetes after transplantation to nude mice. Surgery 113:205-214, 1993.
16. Korsgren, O., Jansson, L., Eizirik, D. & Andersson, A.: Functional and morphological differentiation of fetal porcine islet-like cell clusters after transplantation into nude mice. Diabetologia 34:379-386, 1991.
17. Korsgren, O., Sandler, S., Jansson, L., Groth, C.G., Hellerström, C. & Andersson, A.: Effects of culture conditions on formation and hormone content of fetal porcine isletlike cell clusters. Diabetes 38 (suppl 1): 209-212, 1989.
18. Korsgren, O., Sandler, S., Schnell Landström, A., Jansson, L. & Andersson, A.: Large-scale production of fetal porcine pancreatic islet-like cell clusters: an experimental tool for studies of islet cell differentiation and xenotransplantation. Transplantation 45:509-514, 1988.
19. Krebs, H.A. & Henseleit, K.: Untersuchungen über die Harnstoffbildung im Tierkörper. Hoppe-Seylers Z Physiol Chem 210:33-66, 1932.
20. Lyons, R.M. & Moses, H.L.: Transforming growth factors and the regulation of cell proliferation. Eur J Biochem 187:467-473, 1990.

21. Mendola, G., Casamitjana, R. & Gomis, R.: Effect of nicotinamide therapy upon B-cell function in newly diagnosed Type I (insulin-dependent) diabetic patients. Diabetologia 32:160-162, 1989.
22. Nielsen, J.H.: Effects of growth hormone, prolactin and placental lactogen on insulin content and release and deoxyribonucleic acid synthesis in cultured pancreatic islets. Endocrinology 110:600-606, 1982.
23. Nielsen, J.H.: Mechanisms of pancreatic β-cell growth and regeneration. Exp Clin Endocrinol 93:277-285, 1989.
24. Powers, A.C., Philippe, J., Herman, H. & Habener, J.F.: Sodium butyrate increases glucagon and insulin gene expression by recruiting immunocytochemically negative cells to produce hormone. Diabetes 37:1405-1410, 1988.
25. Rorsman, P., Arkhammar, P., Bokvist, K., Hellerström, C., Nilsson, T., Welsh, M., Welsh, N. & Berggren, P.O.: Failure of glucose to elicit a normal insulin secretory response in fetal pancreatic beta cells results from glucose insensitivity of the ATP-regulated K^+ channels. Proc Natl Acad Sci USA 86:4505-4509, 1989.
26. Sandler, S. & Andersson, A.: Long-term effects of exposure of pancreatic islets to nicotinamide in vitro on DNA synthesis, metabolism and B-cell function. Diabetologia 29:199-202, 1986.
27. Sandler, S. & Andersson, A.: Nicotinamide treatment stimulates cell replication in transplanted pancreatic islets. Transplantation 46:30-31, 1988.
28. Sandler, S. & Andersson, A., Korsgren, O,. Tollemar, J., Petersson, B., Groth, C.G. & Hellerström, C.: Tissue culture of human fetal pancreas. Effects of nicotinamide on insulin production and formation of isletlike cell clusters. Diabetes 38 (suppl 1):168-171, 1989.
29. Sandler, S., Andersson, A., Schnell, A., Mellgren, A., Tollemar, J., Borg, H., Petersson, B., Groth, C.G. & Hellerström, C.: Tissue culture of human fetal pancreas. Development and function of B-cells in vitro and transplantation of explants to nude mice. Diabetes 34:1113-1119, 1985.
30. Sjöholm, Å., Welsh, N. & Hellerström, C.: Lithium stimulates DNA replication, polyamine content and insulin secretion by rat pancreatic β-cells. Am J Physiol 262:C391-C395, 1992.
31. Thomas, P.S.: Hybridization of denaturated RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201-5205, 1980.
32. Ueda, K. & Hayashi, O.: ADP-ribosylation. Annu Rev Biochem 54: 3-100, 1985.
33. Vague, Ph., Picq, R., Bernal, M., Lassman-Vague, V. & Vialettes, B.: Effect of nicotinamide treatment on the residual insulin secretion in type 1 (insulin-dependent) diabetic patients. Diabetologia 32:316-321, 1989.
34. Vague, Ph., Vialettes, B., Lassman-Vague, V. & Vallo, J.: Nicotinamide may extend remission phase in insulin-dependent diabetes. Lancet i:619-620, 1987.
35. Welsh, M., Weber, T., Wrange, Ö., Nielsen, D.A., Matthieu, M. & Steiner, D.F.: Regulation of insulin gene expression by dexamethasone, Ca^{2+} and a phorbol ester. Biomed Biochim Acta 47: 299-303, 1988.
36. Yonemura, Y., Takashima, T., Miwa, K., Miyazaki, I., Yamamoto, H. & Okamoto, H.: Amelioration of diabetes mellitus in partially pancreatectomized rats by poly(ADP-ribose) synthetase inhibitors. Evidence of islet B-cell regeneration. Diabetes 33:401-404, 1984.

Address for reprints:
Dr. Olle Korsgren,
Department of Medical Cell Biology,
Biomedicum, P.O. Box 571,
S-751 23 Uppsala, Sweden.
Telephone + 46 (0)18 174396
Telefax + 46 (0)18 156648