Effects of Culture Conditions on Formation and Hormone Content of Fetal Porcine Isletlike Cell Clusters

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To establish methods for stimulation of the growth and differentiation of fetal endocrine pancreatic cells, a technique for the in vitro production of fetal porcine isletlike cell clusters (ICCs) was used. By varying the composition of the culture medium with different glucose concentrations and the addition to the culture medium of insulin, growth hormone (GH), amino acids, or nicotinamide, we estimated the formation of ICCs and their hormone content. High glucose content (28.0 mM) stimulated the formation of abundant ICCs that contained decreased amounts of insulin. In contrast, culture at a low (5.6-mM) glucose concentration increased the ICC insulin content but decreased the number of ICCs formed. Addition of seven times the normal amount of amino acids hampered both the formation of ICCs and their insulin content. Neither insulin nor GH supplementation of the medium influenced the ICC insulin content, but GH stimulated an abundant outgrowth of ICCs containing relatively high insulin concentrations. However, ICCs formed under these circumstances contained <10% of the insulin content of adult islets, and further work has to be carried out to identify factors responsible for further differentiation of the fetal porcine pancreas.
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Experimental diabetes in rodents has been cured after transplantation of human fetal pancreases (1,2). One major problem when applying these results clinically to islet transplantations is to obtain sufficient amounts of islet tissue. This problem may be solved by designing models in which the growth and differentiation of the fetal pancreas could be stimulated in vitro before transplantation. We have described a culture technique for the human fetal pancreas that leads to the formation of isletlike cell clusters (ICCs; 3). ICCs contain few differentiated insulin-positive β-cells with a low degree of glucose-stimulated insulin release. However, after transplantation of ICCs to nude mice, a marked increase in the number of endocrine cells was observed after 4 wk. In a recent study a similar culture technique for the fetal porcine pancreas was described (4). The porcine ICCs exhibited functional characteristics almost identical to those of humans. One major advantage of the use of porcine material is the almost unlimited supply of highly viable preparations of endocrine pancreases; such availability enables detailed studies of ICC growth and differentiation.

This study was designed to evaluate the influence of different culture media and supplements on the formation and hormone content of porcine ICCs. We used a culture period of 8 days and estimated hormone and DNA contents in homogenates of harvested ICCs at the end of the culture period.

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MATERIALS AND METHODS

Chemicals. RPMI-1640 and 100x basal modified Eagle's medium amino acids were purchased from Flow (Irvine, UK). Tissue culture medium 199 (TCM 199) and Hanks' balanced salt solution (HBSS) were from Statens Bakteriologiska (Stockholm). Benzylpenicillin was from Astra Läkemedel (Södertälje, Sweden), and streptomycin was from Gibco (Grand Island, NY). Nicotinamide was from Sigma (St. Louis, MO), and porcine monocomponent insulin was from Novo (Copenhagen). Growth hormone (GH; Somatonorm; sterile lyophilized methionyl human somatotropin) was kindly provided by Drs. A. Skottner and R. Gunnarsson (Kabi Vitrum, Stockholm). Heat-inactivated pooled human serum (HS) was provided by The Blood Center of Huddinge Hospital (Huddinge, Sweden). Collagenase was from *Clostridium histolyticum* by Boehringer Mannheim (Mannheim, FRG).

Preparation and culture of fetal porcine pancreas. Eight pregnant sows belonging to a local stock were killed by means of a slaughtering mask. The length of pregnancy varied between 35 and 81 days (term ~115 days). The

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crown-rump length of the fetuses ranged from 12 to 24 cm and the body weight ranged from 104 to 525 g. Dissection and collagenase digestion of the porcine fetal pancreas were performed as previously described (4). After repeated washings, the digest was resuspended in 6 ml RPMI-1640 medium. Aliquots of 0.2 ml were distributed in duplicate culture dishes containing 4.5 ml medium and supplement (Tables 1 and 2). Finally, 0.5 ml HS was added. The culture dishes were maintained at 37°C in a gas phase of 5% CO_2/95% humidified air. Culture media were changed every 2nd day. During the last 2 days of culture, all groups of ICCs were cultured in RPMI-1640 with 11.1 mM glucose and 10% (vol/vol) HS.

Number of ICCs formed in culture. At the end of the culture period, the approximate number of ICCs formed in the different culture media was estimated by observing the culture dishes containing the explants with a stereomicroscope immediately before harvest. The number of ICCs formed were classified into three groups: + for low, ++ for moderate, and +++ for abundant.

Hormone and DNA content. On days 4 and 8 of culture, 40 ICCs were collected from each culture dish, washed in HBSS, and then sonicated in 200 μl redistilled water. A sample of 50 μl was transferred to a tube containing 125 μl acid-ethanol [0.18 M HCl in 95% (vol/vol) ethanol] and extracted overnight at 4°C. The insulin, glucagon, and somatostatin contents were analyzed by radioimmunoassay (5–7). Duplicate samples of 40 μl each of the aqueous homogenate were analyzed for DNA content by fluorophotometry (8,9).

Statistical analysis. The hormone content of the ICCs in each medium was calculated as the mean of the values for the duplicate culture dishes. These values were then expressed as a percentage of the corresponding values for ICCs that had developed in RPMI-1640 medium supplemented with 11.1 mM glucose in each experiment. The results were calculated as means ± SE and compared by Student's *t* test for paired samples.

RESULTS

Culture characteristics. During the first days of culture, there was an outgrowth of fibroblastlike cells, and on top of this bottom layer, we observed the formation of isletlike cell clusters. In general, the growth activity reached a plateau on day 4 or 5, when no further formation of ICCs took place. There was an obvious difference in the growth patterns of the explanted pancreatic digests in the different media used. Thus, high-glucose media as well as medium containing GH seemed to stimulate the formation of ICCs. Addition of amino acids to the medium stimulated the growth of fibroblasts to the extent that the number of ICCs formed was markedly decreased (Table 1). Neither the addition of nicotinamide nor of insulin affected the number of ICCs formed.

DNA and hormone content of harvested ICCs. ICCs harvested on day 4 or 8 contained comparable amounts of hormones and DNA when produced in RPMI-1640 with 11.1 mM glucose. The insulin content was 11.2 ± 2.3 ng/μg DNA after 4 days and 8.5 ± 1.8 ng/μg DNA after 8 days, the glucagon content was 5.5 ± 1.0 and 8.8 ± 2.9 ng/μg DNA, the somatostatin content was 183 ± 65 and 582 ± 238 pg/μg DNA, and the DNA content 0.58 ± 0.08 and 0.61 ± 0.13 μg/10 ICCs, respectively. In general, there was an inverse correlation between the glucose concentration of the culture medium and the insulin content of the ICCs when RPMI-1640 or TCM 199 was used as the basal medium (Table 1). This effect, observed after 4 days of culture, was still present during the last 2-day culture period, when all ICCs were cultured in a glucose concentration of 11.1 mM. Furthermore, ICCs produced in TCM 199 tended to contain more insulin than those cultured in RPMI-1640. An addition of insulin or GH did not affect the insulin content of the ICCs, but culture with high levels of amino acid significantly decreased the insulin content during the last days of culture. Finally, addition of 10 mM nicotinamide increased the insulin content of the harvested ICCs on day 8 of culture. ICCs cultured in TCM 199 contained significantly less glucagon on day 4 of

TABLE 1
Effects of culture conditions on insulin content and formation of fetal porcine isletlike cell clusters (ICCs)

Culture medium	Glucose concentration (mM)	Supplementation	Insulin content (% of RPMI-1640 + 11.1 mM glucose) Day 4	Day 8	Number of ICCs formed
RPMI-1640	5.6		137.8 ± 13.5 (8)*	135.9 ± 11.3 (7)*	+
	11.1		100.0 (8)	100.0 (7)	++
	28.0		75.0 ± 7.6 (8)*	78.8 ± 8.7 (7)	+++
	11.1	1 μg/ml insulin	112.4 ± 9.0 (8)	100.1 ± 12.2 (7)	++
	11.1	7 times normal amino acid concentration	101.6 ± 24.5 (7)	81.6 ± 4.3 (6)†	+
	11.1	1 μg/ml growth hormone	100.4 ± 5.7 (8)	91.1 ± 14.3 (7)	+++
	11.1	10 μg/ml growth hormone	107.0 ± 9.4 (8)	122.0 ± 21.5 (7)	+++
	11.1	10 mM nicotinamide	130.6 ± 21.5 (8)	251.9 ± 57.6 (7)*	++
	11.1	10 mM nicotinamide + 1 μg/ml growth hormone	151.7 ± 33.4 (8)	260.5 ± 48.4 (7)*	+++
Medium 199	5.6		142.2 ± 13.0 (8)*	180.1 ± 22.4 (7)*	++
	11.1		130.6 ± 13.7 (8)	156.8 ± 23.8 (7)	+++
	28.0		87.1 ± 13.4 (8)	82.2 ± 6.0 (7)*	+++

Medium insulin content of cultured pancreatic digests from 8 consecutive pig litters and number of ICCs formed according to arbitrary scale (+, low; ++, moderate; +++, abundant). All explant cultures were supplemented with 10% human serum. Data are means ± SE and are expressed as a percentage of corresponding values for ICCs developed in RPMI-1640 (11.1 mM glucose). The number of litters is given within parentheses.

$^*P < .05$ and $^\dagger P < .01$, by Student's *t* test for paired samples.

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TABLE 2
Effects of culture conditions on glucagon content of fetal porcine isletlike cell clusters

Culture medium	Glucose concentration (mM)	Supplementation	Glucagon content (% of RPMI-1640 + 11.1 mM glucose) Day 4	Day 8
RPMI-1640	5.6		109.3 ± 10.7 (8)	98.5 ± 8.1 (7)
	11.1		100.0 (8)	100.0 (7)
	28.0		86.4 ± 10.4 (8)	108.5 ± 13.3 (7)
	11.1	1 μg/ml insulin	106.3 ± 11.6 (8)	110.3 ± 15.8 (7)
	11.1	7 times normal amino acid concentration	93.3 ± 28.3 (7)	122.5 ± 13.8 (6)
	11.1	1 μg/ml growth hormone	71.6 ± 10.9 (8)*	107.7 ± 16.2 (7)
	11.1	10 μg/ml growth hormone	99.9 ± 27.5 (8)	146.5 ± 17.0 (7)*
	11.1	10 mM nicotinamide	86.3 ± 16.5 (8)	161.9 ± 26.8 (7)
	11.1	10 mM nicotinamide + 1 μg/ml growth hormone	82.0 ± 17.7 (8)	168.8 ± 30.0 (7)
Medium 199	5.6		62.3 ± 5.3 (8)†	86.6 ± 16.7 (7)
	11.1		50.8 ± 5.8 (8)†	76.2 ± 12.8 (7)
	28.0		52.2 ± 6.3 (8)†	78.5 ± 10.9 (7)

Medium glucagon content of cultured pancreatic digests from 8 consecutive pig litters. All explant cultures were supplemented with 10% human serum. Data are means ± SE and are expressed as a percentage of corresponding values for isletlike cell clusters developed in RPMI-1640 (11.1 mM glucose). The number of litters is given within parentheses.
$^*P < .05$ and $^\dagger P < .001$, by Student's *t* test for paired samples.

culture than those cultured in RPMI-1640 with 11.1 mM glucose. However, this discrepancy disappeared after the final culture in the control medium (Table 2). There were no significant differences in the somatostatin or DNA contents of the ICCs produced under the various culture conditions (data not shown).

DISCUSSION

The experimental design of this study, including a 6-day culture period in different media and a final 2-day culture for all groups in RPMI-1640 (plus 11.1 mM glucose), was chosen to allow a valid comparison between the different groups of ICCs based on insulin content. However, additional morphometric analyses are needed to resolve whether the apparent observed differences reflect an altered fraction of β-cells per ICC or merely differences in the insulin content per cell. Culture medium RPMI-1640 supplemented with 10% HS was used as control medium, and the lack of effect of GH or insulin supplementation on the insulin content probably reflects a sufficient supply of these factors by the addition of HS. However, the glucose concentration of the culture medium influenced the insulin content of ICCs in a way similar to that described for adult rodent islets in tissue culture (10). The decrease in ICC insulin content observed in explants maintained in media containing 28.0 mM glucose for 4 days persisted even after the final culture period in the control medium (RPMI-1640 with 11.1 mM glucose). This result suggests that the observed decrease was due not only to degranulation of the β-cells but also to a lower frequency of β-cells within the ICC. The finding that ICCs kept in low-glucose–containing media led to production of ICCs that contained more insulin should, however, not be taken to indicate that such a medium is preferential for production of fetal β-cells. Thus, culture in media containing high glucose produced higher numbers of ICCs to the extent that the total yield of β-cells exceeded that seen after low-glucose culture.

Except for the effects exerted by the different glucose concentrations on the insulin content of ICCs, the most pronounced effects were observed after culture in the presence of nicotinamide. However, the increase in insulin content seemed to develop at a slower rate and did not reach statistical significance until the end of the 8-day culture period. A similar beneficial effect of nicotinamide addition has been observed with regard to the production of human ICCs (Sandler et al., p. 168). Despite the high insulin content of the nicotinamide-exposed ICCs, the number of ICCs formed did not decrease. When both nicotinamide and GH were added to the basal medium, the insulin content remained similar to that obtained with nicotinamide only. Because the GH addition effected an increase of the number of ICCs formed, this combination led to an abundant formation of ICCs with a high insulin concentration. However, the insulin content of these ICCs was still <10% that of adult islets (10). On the other hand, the ICC glucagon content was similar to that of adult mouse islets cultured in RPMI-1640 (11). It thus seems that β-cell differentiation has so far been difficult to influence in vitro and that the differentiation mainly occurs when the ICCs are transferred into a more "mature" environment such as that provided by transplantation into nude mice (4).

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