Fetal Pancreas Transplantation in Miniature Swine

II. Survival of Fetal Pig Pancreas Allografts Cultured at Room Temperature

KUMIKO YONEDA, YOKO MULLEN, ELIZABETH STEIN, MICHAEL CLARE-SALZLER, AKIHITO OZAWA, LINDA SHEVLIN, AND JOHN DANILOVS

Islet-allograft survival has been shown to be markedly prolonged in rodents when donor tissue has been precultured at 24°C. In this study, the feasibility of this approach was tested in NIH minipigs transplanted with fetal pancreases. Collagenase-digested fetal pig pancreatic tissues survived in culture at 24°C for 6–7 days and continued to grow in vitro at 37°C after being transferred. These tissues no longer stimulated allogeneic lymphocytes in vitro, although some tissues cultured at 37°C did. This allogeneic stimulation did not correlate to the number of major histocompatibility complex (MHC) class II–positive cells in stimulator pancreatic cultures. When transplanted into an omentum pouch of normal, nonimmunosuppressed minipigs, fresh fetal pancreatic tissues were rejected within 14 days. Tissues cultured at 24°C grew, and β-cells proliferated in minipigs treated daily with cyclosporin A (CsA) and azathioprine. Twelve normal minipigs were transplanted with 24°C-cultured fetal pancreases: 8 pigs received no treatment, 2 received 14 mg · kg^{-1} · day^{-1} CsA for 14 days, and 2 received 6–7 intravenous injections of platelets prepared from pooled farm-pig blood before grafting. Strong lymphocytic infiltration was detected in all grafts removed between 30 and 90 days posttransplantation. However, β-cells were found on day 45 in one of five minipig pancreas grafts incompatible at the MHC loci and on days 60–90 in all three grafts compatible at MHC but incompatible at minor histocompatibility loci. Short-term CsA treatment did not prolong survival of allografts from farm pigs into minipigs. In contrast, some β-cells were detectable on day 30 in farm-pig pancreases grafted into 2 minipigs pretreated with platelets. *Diabetes* 38 (Suppl. 1):213–16, 1989

From the Department of Medicine and Dental Research Institute, University of California, Los Angeles, California.

Address correspondence and reprint requests to Yoko Mullen, MD, PhD, Room 73-017, Center for Health Sciences, University of California, Los Angeles, CA 90024.

We have demonstrated the reversal of experimental diabetes in rats (1) and more recently in minipigs (2; preliminary results) by fetal pancreatic tissue transplants. This approach would be an effective treatment for insulin-dependent diabetes in humans. Using partially inbred NIH miniature swine, we investigated transplantation methods that may reduce requirements of immunosuppressive drugs. For this objective, transplantation of 24°C-cultured donor tissues may have advantages in swine, as has been shown in rats (3). In this study, we examined the effectiveness of this approach for prolongation of fetal pancreas-allograft survival.

MATERIALS AND METHODS

Animals. Miniature swine inbred at the major histocompatibility complex (MHC) were used in this study. The original breeders were provided by D.H. Sachs (National Cancer Institute, Bethesda, MD), and experimental pigs were raised at our pig farm.

Preparation of fetal pancreases. Pancreases were dissected from minipig fetuses ranging in gestational ages from 45 to 55 days (except 1 83-day-old litter) removed from timed-pregnant sows by cesarean section (4). Farm-pig fetuses were collected at the Farmer John slaughterhouse (Clougherty Packing, Los Angeles, CA), and fetal ages were estimated by the crown-rump length based on the previous data (4). Pancreases from each litter were pooled, minced, and then digested in collagenase (1 mg/ml type V, Sigma, St Louis, MO). Digested tissues were centrifuged to bring all cells and cell clumps down and were washed three times in Hanks' balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS). Tissues were cultured in Falcon 1007 plates (Becton Dickinson, Oxnard, CA) with 1.5 ml RPMI-1640 culture medium containing 10% FBS at either 24°C (room temperature) or 37°C in a tissue-culture incubator in 95% air/5% CO_2.

TABLE 1
Abrogation of mixed islet-lymphocyte culture (MILC) responses by 24°C culture of fetal pancreases

Experiment no.	Fetal age (days)	Responder		Stimulation index*				
					24°C culture		37°C culture	
		ID no.	cpm†	Fresh	1 wk	2 wk‡	1 wk	2 wk
1	55–57	56		1.5	1.2 (1.6)	0.1	(3.6)	
2	57–60	56	1693 ± 316	1.7 (2.3)	0.4 (2.5)	0.2 (23.4)	0.9 (14.3)	1.2 (4.2)
3	65–70	56		2.5 (2.3)	0.4 (0.9)		1.1 (6.5)	1.5 (4.8)
4	75–80	39	205 ± 56	17.8 (3.0)	3.2 (3.5)	1.7 (10.2)	4.3 (12.1)	(3.9)
5	75–80	56	1693 ± 316	12.6 (11.7)	3.2 (1.0)	1.1 (4.6)	10.3 (3.9)	0.9 (7.6)

Half of the plates containing fetal pancreatic tissues were cultured at 24°C, and the remaining half were cultured at 37°C in 95% air/5% CO_2. MILC assays were performed in a protocol similar to mixed lymphocyte culture with 5×10^6 irradiated single pancreas cells as stimulators and the same number of allogeneic lymphocytes as responders. Percentage of class II–positive cells in islet cells is given in parentheses.
*(cpm in wells of islet cells + responder lymphocytes)/cpm in wells of lymphocytes alone.
†Counts per minute of responder alone.
‡After a 1-wk culture at 24°C, plates were moved into a 37°C tissue-culture incubator.

Insulin contents in cultured tissues and medium. Insulin was extracted from pancreatic tissues by acid-alcohol (pH 1.5), and culture medium was collected daily from each plate. Insulin was measured by a double-antibody radioimmunoassay. Detailed procedures have been described elsewhere (4).

Mixed islet-lymphocyte culture (MILC). Pooled pancreases from two to three litters of farm-pig fetuses of similar age were prepared for culture. Half of the plates were placed at 24°C, the remaining half at 37°C. MILC assays were performed on fresh (after overnight culture at 37°C) and cultured tissues by preparing single pancreas cells with dispase (cat no. P-6141, Sigma) digestion. Half a million irradiated (3000 rad) single cells were cultured together with the same number of adult pig peripheral blood lymphocytes (PBLs) in a tissue-culture incubator. [^{3}H]thymidine was added on day 5, and the cells were harvested after 6 h. The same minipig was used as a responder in each series of experiments. Stimulation indices (SIs) were calculated as the counts per minute (cpm) of wells containing both lymphocytes and irradiated islet cells divided by the counts per minute of wells with lymphocytes alone.

TABLE 2
Survival of fetal pig pancreas allografts cultured at 24°C

Incompatibilities	Donor	Recipient	ID no.	Treatment*		Histological observation of graft†			
				Donor‡	Recipient§	Removal	β	Donor type‖	Infiltrate
MHC I + II + Minor	cd	cc	515	+	–	45 days	+	+	+ + +
MHC II + Minor	ag	ac	218	+	–	36	0	+	+ + +
	ag	ac	521	+	–	90	0	0	+ +
MHC I + Minor	dg	dd	217	+	–	35	0	+	+ + +
	dg	dd	215	+	–	90	0	+	+ + +
Minor	cd	cd	327	+¶	–	58	+	+	+ +
	cd	cd	512	+	–	60	+	+	+ +
	cc	cc	723	+	–	93	+	+	+ +
MHC I + II + Minor	Farm	cc	212	+	CsA, 14 days#	30	0	0	+ + +
	Farm	cc	322	+	CsA, 14 days	30	0	0	+ + +
	Farm	cd	513	+	Platelets	30	+	+	+ + +
						60	0	0	+ +
	Farm	cd	516	+	Platelets**	35	+	+	+ + +
						79	0	0	+ +
Minor	cd	cd	322	–††	–	10	0	Ducts	+ + +
	cd	cg	753	–	–	10	0	Ducts	+ + +

Fetal pancreatic tissues were transplanted into an omentum pouch of normal minipigs after culture at 24°C for 6–7 days. MHC, major histocompatibility complex; minor, minor histocompatibilities.
* +, Present; –, absent.
†Presence of different cell types within the graft: 0, none observed; +, small amount; + +, moderate amount; + + +, many.
‡Fetal pancreases 45–55 days old were collagenase digested and cultured at 24°C for 7 days.
§Normal NIH minipigs were used as recipients.
‖Donor cell types were not identified.
¶Donor tissues were taken from 83-day-old fetuses.
#Fourteen mg/kg cyclosporin A was given orally daily.
**One to 2×10^{10} prepared from pooled farm-pig blood were injected intravenously 6–7 times weekly.
††Fresh pancreatic tissues.

Detection of MHC class II antigens. Class II–positive cells were detected in single–pancreas cell preparations by staining with 40D monoclonal antibody (gift from D.H. Sachs; 5) with biotinylated anti-mouse antibody and avidin–fluorescein isothiocyanate. Either pig or horse serum was used to block nonspecific staining, and normal mouse serum was used instead of 40D monoclonal antibody in negative controls. At least 500 cells were counted under the microscope to obtain percentages of positive cells. Special care was taken to distinguish positive staining and autofluorescence.

Transplantation and recipient's treatment. Pancreases from tissue-typed minipig or farm-pig fetuses were cultured at 24°C for 6–7 days for transplantation. After culture, cells and cell clusters were suspended in 100 μl HBSS and pipetted into a small pouch made with the omentum. A total of 12 normal minipigs of both sexes, ranging in age from 3 to 15 mo, were tissue typed and used as recipients: 8 received no treatment other than transplants; 2 received cyclosporin A (CsA) at a dose of 14 mg/kg for 14 days, starting on the day of grafting; and the remaining 2 received pooled platelets before transplantation. These platelets were prepared from blood collected from several farm pigs, stored at 4°C for >1 mo to eliminate contaminating cells, and injected intravenously in a dose of 1 to 2 × 10^{10} platelets weekly. At various intervals after transplantation, grafts were removed and examined histologically by hematoxylin and eosin (HE) staining and immunoperoxidase staining for insulin.

RESULTS

Viability of fetal β-cells during 24°C culture. Five experiments were performed on 55- to 60-day fetal pig pancreases to determine viability of fetal β-cells during culture at 24°C. Insulin contents in cultured tissues and those released into the medium had decreased considerably during culture for 6 days at either 24 or 37°C. Tissue insulin contents per pancreas decreased from 12.7 ± 3.3 to 6.7 ± 2.3 mU in 24°C culture and to 3.9 ± 2.2 mU in 37°C culture. Insulin released into the culture medium also decreased from 4.6 ± 2.2 mU · pancreas^{-1} · day^{-1} on day 1 to 2.9 ± 2.4 mU · pancreas^{-1} · day^{-1} at 24°C and from 7.3 ± 3.8 to 0.6 ± 0.2 mU · pancreas^{-1} · day^{-1} at 37°C.

In vitro stimulation of allogeneic lymphocytes by cultured fetal pancreas cells. The results of MILC assays are summarized in Table 1. Counts in wells of irradiated pancreas cells were always <250 cpm. Pig 39 was a low responder, but 56 was a high responder. Cells obtained from fresh pancreases of older pig fetuses (>70 days old) strongly stimulated allogeneic lymphocytes. After a 1-wk culture at 24°C, SIs had dropped to 3.2, and after an additional 1-wk culture at 37°C, they dropped to 1.7. By contrast, cells from younger fetal pancreases did not stimulate in MILC only 1 day after harvesting (1.5–2.5). SIs dropped further to <1.5 after culture at 24 or 37°C. The residual cell debris and tissue fragments produced in the process of single-cell preparation were also tested in the same assay system but gave no positive responses. Percentages of class II–positive cells in pancreas cells are also shown in Table 1.

Survival of 24°C-cultured fetal pig pancreas allografts. Results are summarized in Table 2. β-Cells in collagenase-digested cultured fetal pancreases continued to grow and proliferate in recipients immunosuppressed with CsA and azathioprine (results not shown). Ten days after transplantation, pancreatic tissue fragments cultured overnight showed strong lymphocytic infiltration with a few ducts, but no cells stained for insulin. Survival of pancreatic tissues cultured at 24°C for 6–7 days was significantly different depending on immunogenetic barriers between donor and recipient. Grafts across the MHC barrier were rejected within 1 mo. However, one of the five grafts contained β-cells on day 45. Contrary to these grafts, β-cells were present in all three grafts removed between days 58 and 93 that differed only at non-MHC loci. However, lymphocytic infiltration was strong in all grafts. Fully allogeneic grafts from farm pigs to minipigs did not survive for 1 mo even though the recipients were treated with CsA for 2 wk. In contrast, considerable numbers of β-cells were detected on day 30 in farm-pig allografts that were transplanted into two minipigs treated with pooled platelets from farm pigs before transplantation. However, these β-cells were totally destroyed by day 60.

DISCUSSION

Although the appearance of 24°C-cultured fetal pancreatic tissues was different from that of 37°C-cultured tissues, β-cell viability was essentially the same for both tissues. These cells continued to grow in vitro after being transferred to 37°C culture or in vivo after transplantation. However, culture at 24°C for >8 days was deleterious to these cells (data not shown). Younger fetal pancreases (<70 days old) did not cause lymphocytic proliferation after 6–7 days in 24°C culture, although older tissues may need to be cultured for a longer period. Except in fresh pancreatic tissues, the number of class II–positive cells in stimulator tissues did not correspond with their ability to stimulate allogeneic lymphocytes (Table 1). We noticed that cells that stained for class II antigens in the cultured tissues were larger than those in fresh tissues. Characterization of these cells is still in progress. During transplantation, strong lymphocytic infiltration destroyed 24°C-cultured fetal pancreatic tissues. Despite the severe rejection reaction, β-cells were detectable in some grafts after >2 mo. Whereas a 2-wk CsA administration was ineffective, pretreatment of recipients with pooled platelets clearly prolonged β-cell survival. These results may indicate the possibility of suppressing host alloimmunity by immunotherapy.

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