Syngeneic Transplantation of Cryopreserved Fetal Mouse Proislets

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SUMMARY
Proislets, derived from fetal mouse pancreata by collagenase digestion and subsequent organ culture, can be frozen to −196°C and stored in a viable condition before successful syngeneic transplantation.

Cryopreserved proislets are relatively undifferentiated morphologically, but continue to differentiate into mature islets after transplantation. DIABETES 1984; 33:975-77.

Fetal rodent pancreatic fragments and isolated adult rodent islets have been maintained at −196°C for up to 1 mo, thawed, and successfully transplanted to either the kidney subcapsular site or intraportally. The proislet preparation, generated from fetal pancreas by a combination of collagenase digestion with subsequent organ culture, consists of cellular aggregates made up of undifferentiated ductal-derived epithelial cells and differentiating endocrine cells. We have examined the feasibility of cryopreservation as a means of storage for fetal mouse proislets before syngeneic transplantation.

MATERIALS AND METHODS
Pancreas digestion and culture. Fetal mouse proislets were prepared as previously described. Briefly, 17-day fetal CBA/H mice (vaginal plug = day 0) were sterilely removed and dissected free of surrounding mesenteric connective and lymphoid tissue. Each pancreas was trisected. Twelve pancreata were incubated in 1 ml of isolation medium containing 6 mg/ml collagenase (Boehringer-Mannheim, Indianapolis, Indiana) in a siliconized vial for 5 min at 37°C in a water shaker bath at 200 oscillations/min. After incubation, the digest was agitated by hand shaking for about 1 min, then diluted to 10 ml with isolation medium (no collagenase present) and placed on ice. After a 10-min sedimentation period, the supernatant was removed and discarded and the digest material resuspended in 10 ml of fresh isolation medium and again placed on ice to sediment. This wash procedure was repeated a third time. The particulate material was then suspended in 12 ml of culture medium and 2 ml of this suspension was added to each well of a 6-well hydrophobic culture dish (Linbro 76-247-05). Culture medium was composed of RPMI 1640 with a bicarbonate buffer (1 g/L), antibiotics, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum. The culture dish was agitated gently to disperse the pancreatic digest evenly onto the plastic surface. The dish was then placed in a humidified incubator at 37°C in an atmosphere of 10% CO2 and 90% air for 2 days. After 2 days of culture, 1 ml of medium was removed from each well and replaced with fresh culture medium and incubation continued for an additional 2 days.

Cryopreservation. After the 4-day culture period, numerous proislets were present in each well. The proislets from 2 wells (approximately 300 derived originally from 4 fetal pancreata) were then allowed to sediment in a plastic screw-top vial. All but 200 µl of medium was removed. At room temperature, 100 µl of 2 M dimethylsulphoxide (DMSO) in culture medium was added. The components were mixed by gentle pipetting and incubated for 5 min. The procedure was repeated with a second 100 µl volume of 2 M DMSO. Then 400 µl of 3 M DMSO was added, mixed, and the vial placed on ice for 20 min. Each vial was then placed in a programmable step-rate freezer at a chamber temperature of −8°C. Vials remained in the chamber for 15 min to equilibrate. The outside of each vial was then briefly placed in contact with a steel spring chilled in liquid nitrogen to induce ice nucleation within the fluid. Vials were returned to the chamber and held at −8°C for 20 min.
SYNGENEIC TRANSPLANTATION OF MOUSE PROISLETs

FIGURE 1. Photomicrograph of a section of cryopreserved fetal mouse proislets after thawing and before transplantation. Some cells within the compacted aggregates react positively for islet endocrine hormones (arrows). The majority of cells, however, appear to be hormone negative. Immunoperoxidase-glucagon, x 234.

for an additional 10–15 min. The chamber temperature was then decreased at a rate of 0.25–0.5°C/min until the chamber temperature reached –80°C. At this point, the vials were transferred to liquid nitrogen and stored at –196°C for 10 days.

Thaw. Rapid thawing was accomplished by incubation at room temperature for approximately 10 min. The DMSO in the vial (2 M) was then diluted stepwise by the addition of fresh culture medium at room temperature for 5-min periods in the following sequence: 100 μl, 100 μl, 200 μl, and 200 μl. At this point, the 1.4-ml volume of fluid containing the frozen-thawed proislets was transferred to a 35-mm plastic dish and approximately 5 ml of fresh medium was added to further dilute the DMSO. Proislets were then concentrated at the center of the dish by gyrational motion and transferred in a small volume to a well of a round-bottom multiwell dish (Linbro/Titertek 76-213-05). Excess medium was removed (to about 50 μl) and 200 μl of citrated mouse plasma was added. Proislets were suspended in the plasma and then allowed 10 min to sediment. Excess plasma (to about 10–20 μl) was removed and a small quantity of bovine thrombin powder (1 mg) was added to initiate clot formation. The clot was dislodged by flotation in cold Hepes-buffered Hanks’ and either fixed in formal-saline or Bouin’s solution for histologic study or kept on ice until transplantation.

Transplantation. Details of the transplantation technique have been previously described. Briefly, the left kidney of a syngeneic, CBA, young male mouse was exposed and a plasma clot (containing the cryopreserved proislets derived from 4 fetal mouse pancreata) was placed beneath the kidney capsule. The incision was closed and the animal returned to its cage. A total of 12 mice were transplanted. Frozen-thawed fetal mouse proislets from five separate cryopreservation runs were used. Five weeks posttransplant, 10 animals were killed by chloroform and the graft-bearing kidney removed and fixed in either formal-saline or Bouin’s solution. Grafts were recovered from two other animals at 5 mo posttransplantation.

Histology. Regions of the graft-bearing kidney or clots containing frozen-thawed proislets were embedded in paraffin and sequential 4-μm sections were cut and mounted on glass slides. Routinely, slides of each transplant site were stained with aldehyde-fuchsin and counterstained with hematoxylin. For some blocks, sections prepared from the graft area were processed for immunocytochemical localization of insulin and glucagon by the unlabeled enzyme antibody method. Primary antisera (Accurate-DAKO PAP kit K512) were guinea pig anti-human insulin and rabbit anti-human...
glucagon. Bridge antibody was a porcine anti-rabbit IgG and PAP complex used rabbit antibody. The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin. Specificity controls were run.

RESULTS

Before transplantation, cryopreserved and thawed proislet preparations consisted of single cells as well as varying sized cellular aggregates. The majority of cells within the aggregates appeared epithelioid. Evidence of damage could be found in some cells, characterized by pyknotic nuclei and vacuolated cytoplasm. Immunocytochemical localization of insulin and glucagon confirmed the presence of endocrine cells within the proislets (Figure 1). Not all cells within the aggregates reacted positively for the islet hormones tested and the positive cells that were present did not exhibit any particular organizational pattern.

Posttransplantation, viable endocrine grafts could be found in 10/12 (83%) of syngeneic recipients. Aldehyde-fuchsin-positive cells were frequently found in small multiple clusters or oval masses similar in appearance to in situ islets (Figure 2). Immunocytochemical localization of glucagon in such masses revealed peripherally located alpha cells (Figure 3), while the immunoperoxidase reaction for insulin confirmed the identity of the more centrally located beta cells (Figure 4). Similar islet structures were found at the graft site in both recipients examined at 5 mo posttransplantation.

DISCUSSION

Fetal mouse proislets derived from pancreatic fragments after collagenase digestion and organ culture can be frozen to liquid nitrogen temperature as a means of storage before successful transplantation. As with adult islets and fetal pancreas fragments, DMSO proved to be a suitable cryoprotectant. Slow rates of freezing (0.5 and 0.25°C/min) were compatible with continued endocrine cell survival as previously described. 3,4

The continued differentiation of proislets into adult-like islet structures after transplantation has been demonstrated. 4 Immunoperoxidase studies on cryopreserved proislets after transplantation indicate that both alpha and beta cells are present and that these cells are arranged in a morphologic pattern characteristic of adult murine islets. The presence of such islet structures in cryopreserved proislet grafts suggests that endocrine precursor cells also survive the freezing protocol and continue the process of differentiation and cellular rearrangement to form histologically normal islets after transplantation.

The efficiency of the freezing protocol was not directly examined in these studies. Some cellular damage was observed in the freeze-thawed proislets as has been reported for adult rat islets using a similar protocol. 13 However, the number of adult rat islets required to reverse experimental diabetes was comparable between cryopreserved and fresh islets, suggesting that damage was minimal. 5 Preliminary studies on our part indicate that cryopreserved proislets are functional and can reverse streptozotocin diabetes in syngeneic recipients (unpublished). Detailed functional studies are in progress; however, it can take 6 mo or more for proislet tissue from one fetal donor to reverse streptozotocin-induced diabetes. Consequently, such studies should be carried out over a 9–12-mo study period.

While whole fetal pancreas, even after a period of organ culture, is highly immunogenic, 10,11 fetal proislets appear less so. 3,4 The proislet model offers the opportunity to further examine the efficacy of organ culture and subsequent cryopreservation of islet endocrine tissue/endocrine tissue precursors as a means of altering tissue immunogenicity and tissue banking before transplantation. The demonstration that proislets can be cryopreserved is an important step in the development of this tissue as a source of material for clinical transplantation. Cryopreservation would allow banking of tissue before transplantation. Such a tissue bank would give added flexibility to a transplantation program. It would allow time for tissue typing and, more importantly, allow time for the preparation of antigen that could be used to stabilize the graft 14 or induce specific tolerance before grafting. 3

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REFERENCES