FULL PAPER *Surgery*

Xenotransplantation of Porcine Pancreatic Endocrine Cells to Total Pancreatectomized Dogs

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ABSTRACT. Xenotransplantation of porcine pancreatic endocrine (PE) cells in a diffusion chamber, a bioartificial endocrine pancreas (Bio-AEP), was conducted to total pancreatectomized dogs. Six pancreatectomized dogs were divided into two groups of 3 dogs each. In three dogs of the control group, exogenous insulin was administered twice a day for 30 weeks to maintain fasting blood glucose (FBG) levels within the normal range. The remaining three dogs were implanted with Bio-AEPs (implantation group), in addition to daily insulin administration. In the implantation group, Bio-AEPs containing 1.3 to 1.8×10^7 cells per kg of body weight of the recipient were implanted without fixation into the abdominal cavity. In the control group, exogenous insulin requirements did not decrease during the experimental period, whereas it significantly decreased for a certain period (3, 11, 17 weeks) after implantation in all implanted dogs. In the implantation group, laparotomy was performed after FBG and the exogenous insulin requirement increased again and Bio-AEPs were removed. Two Bio-AEPs were completely destroyed, and the remaining one was encapsulated by thin fibrous tissue. In this dog, effusion was present within the capsule, but the Bio-AEP was not destroyed. Histopathologically, the necrosis, presumably caused by hypoxia, of the PE-cells was observed on transmission electron microscopy. In conclusion, Bio-AEP could function for a certain period after implantation in this study. However, more preclinical researches should be needed to apply this technique for the treatment of diabetic dogs.

KEY WORDS: bio-artificial pancreas, canine, islet, swine, xenotransplantation.

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Most diabetic dogs are insulin-dependent (IDDM) and require daily insulin administration to maintain their glucose levels [8]. However, insulin therapy is a symptomatic treatment and does not prevent the progress of microangiopathy. In human medicine, the transplantation of pancreas or islet cells has been developed and is the most dramatic and effective therapy for patients with IDDM. Unfortunately, the limited availability of donor organs is currently the most significant problem [13, 21, 23]. In addition, the recurrence of immune-mediated diabetes with failure of the pancreatic allograft has been reported [33]. Therefore, a potential solution to these problems is the use of non-human donor islets. Pigs are considered to be the species of choice as a potential xenogeneic organ or tissue graft donor to humans, because pigs 1) are easy to breed, 2) grow and mature rapidly, 3) have a physiology remarkably like that human, 4) have established specific pathogen free (SPF) breeding, and 5) porcine insulin differs from human insulin only as regards one amino acid and 6) had routinely used to treat diabetic patients [9, 12, 21, 23].

In canine medicine, there have been only a few reports regarding transplantation of the pancreas or of islet cells for spontaneous diabetic patients [1, 19, 37, 38]. These transplantations would be a potential treatment method for diabetic dogs. In allotransplantation of islets to dogs, it must be difficult to obtain the donor dog from the ethical point of view. In the kidney transplantation in the cat, the donor must be kept by recipient cat owner, however, the islet transplantation requires the whole pancreas removal from the donor. The destruction of transplanted islet is also speculated in the dog with autoimmune IDDM although there has been no report about destruction of transplanted allo-islets by autoimmune response. Fortunately, the amino acid sequence of insulin in the dog is completely the same as that of the pig [11] and porcine insulin has been routinely used to treat dogs with IDDM. From these reasons, we chose the porcine islets as the xeno-islet source for the treatment of the dog with IDDM.

One of the major problems in islet xenotransplantation is immunological rejection. Several strategies to avoid the rejection have been investigated. One approach is the use of a device to separate the islets from the host immune system by a semi-permeable membrane barrier that transfers low molecular weight substances such as glucose, oxygen, and insulin, but does not transfer high molecular weight substances such as immunological cells and antibodies. Over the past few decades, various types of biohybrid pancreas using hollow fiber, microcapsulation, or a diffusion chamber, have been described [1–3, 6, 18, 19, 39, 40]. We have been studying islet cell transplantation using a diffusion chamber for a bio-artificial endocrine pancreas (Bio-AEP) [10, 26]. When Bio-AEPs containing MIN6 cells, a mouse insulinoma cell line, were implanted into the abdominal cavities of streptozotocin-induced diabetic rats, normoglycemia was maintained without administration of exogenous insulin and immunosuppressive drugs for more than 50

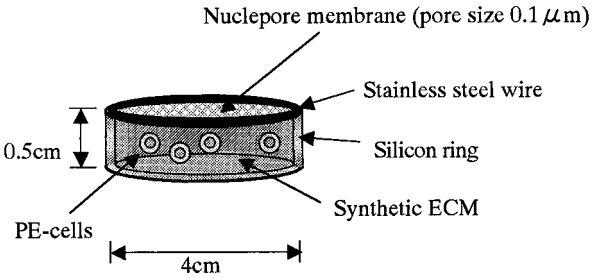

Fig. 1. Schematic drawing of the bioartificial endocrine pancreas (Bio-AEP).

weeks [27], suggesting the efficacy of this chamber for the xenotransplantation of islet cells.

The purpose of this study was to evaluate the *in vivo* efficacy of Bio-AEPs containing adult porcine pancreatic endocrine (PE) cells on total pancreatectomized dogs.

MATERIALS AND METHODS

Bio-AEP: The structure of the Bio-AEP used in this study has previously been reported [5, 10, 26]. Briefly, the Bio-AEP used here was constructed with a silicon ring (4 cm in diameter and 0.5 cm thick) and two selectively semi-permeable membranes (0.1 μm pore size and 10 μm thick; Nuclepore Co., Pleasanton, California, U.S.A.) (Fig. 1). These membranes were adhered to the silicon ring by corona treatment. Within the chamber, PE-cells were cultured three-dimensionally in synthetic extracellular matrix (ECM). The composition of the ECM was 1.0% agarose (Nacalai Tesque, Kyoto, Japan), 0.005% maltose-carrying polystyrene, 10 mM nicotinamide (Nacalai Tesque), and 0.15 mg/m*l* type-I collagen treated to reduce the antigenicity (Cellmatrix I LA; Nitta Gelatin Co., Osaka, Japan) [5, 10, 26, 28, 29].

Preparation of PE-cells: The preparation and purification methods for adult porcine PE-cells have been described elsewhere [30–32, 35]. Briefly, pancreases were obtained from 7- to 10-months-old adult pigs at a local slaughterhouse. The pancreases were trimmed and cut into small pieces with scissors on ice, and the porcine PE-cells were then harvested by the auto-digestion method [31]. The PE-cells were separated from the exocrine tissue using a Histopaque-1077 (Sigma Co., Ltd., St. Louis, Mo., U.S.A.) density gradient method. After the PE-cells were purified, they were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in an incubator for 1 week. At day 7 of the culture, the medium was discarded and replaced with ethylenediaminetetraacetic acid (EDTA: 0.005%) -trypsin (0.125%) (GIBCO, Grand Island, N.Y., U.S.A.), and the PE-cells were then re-cultured. The PE-cells used for the Bio-AEP were collected with EDTA-trypsin and trapped in the ECM immediately before transplantation.

Animals: Six adult male beagle dogs weighing 10.9 ± 1.0 kg were used in this study. All dogs were determined to be normal on the basis of the results of physical examinations, blood counts, blood chemical profiles, and intravenous glucose tolerance tests. This study was performed in accordance with the institutional guidelines of Graduate School of Agricultural and Life Sciences, The University of Tokyo, for the care and use of laboratory animals.

Operation and postoperative therapy: All dogs were premedicated intravenously with midazolam hydrochloride (0.1 mg/kg) and butorphanol tartrate (0.2 mg/kg). Atropine sulfate (0.05 mg/kg) was administered subcutaneously, if necessary. After epidural administration of morphine (0.1 mg/kg), anesthesia was induced with an intravenous injection of thiopental (12.5 mg/kg) and maintained with 1.5–2.0% isoflurane in 100% oxygen. Total pancreatectomy was performed through a ventral midline incision. Butorphanol tartrate (0.2 mg/kg) was again administered intravenously for postsurgical pain relief immediately after surgery. Antibiotic treatment (25 mg/kg of cefazolin) was given for 3 days postoperatively. Subcutaneous exogenous insulin was administered twice a day to maintain morning fasting blood glucose levels (FBG) at the normal range from the next day of surgery. Pancreatic exocrine enzymes (xcelase®: Meiji Seika, Ltd., Tokyo, Japan and Conczyme®: Hankyu Kyoei Bussan Co., Ltd., Osaka, Japan) were supplemented. Acarbose, an alpha-glucosidase inhibitor, (Glucobay®; Bayer Yakuhin, Ltd., Osaka, Japan) was also administered to attenuate postprandial hyperglycemia. These drugs were mixed with food (w/d and a/d; Hill's Colgate Japan, Ltd., Tokyo, Japan) twice a day from the next day of surgery.

After 3 to 4 weeks of these stabilization procedures, the total pancreatectomized dogs were divided into two groups; three dogs were maintained by the administration of exogenous insulin for 30 weeks (control group), and the remaining three dogs were implanted with Bio-AEP (implantation group). In implantation group (n=3), Bio-AEP containing 1.3 to 1.8 × 10^7 PE-cells per kg was implanted. Through laparotomy, each Bio-AEP was placed within the abdominal cavity without fixation. Anesthesia and postoperative therapies were performed according to the same protocol as that used in the pancreatectomy cases. No immunosuppression was used in this study. After the implantation, the dose of exogenous insulin was adjusted according the FBG levels. When FBG levels of higher than 150 mg/d*l* or lower than 60 mg/d*l* were detected in the two continuous measurements, 0.5 units/head of exogenous insulin was reduced from or added to the basal insulin requirement dose.

Blood chemical analysis: In both groups, FBG, body weight, and insulin requirement were monitored three to seven times per week, and the measurement of fructosamine was performed weekly after pancreatectomy.

Urinalysis: Morning fasting urine samples were collected to detect glycosuria using BM-10 test (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) once a week after pancreatectomy and implantation of Bio-AEP.

Histological evaluation: Bio-AEPs were removed from the dogs of the implantation group when the FBG and insulin requirements increased and returned to the values

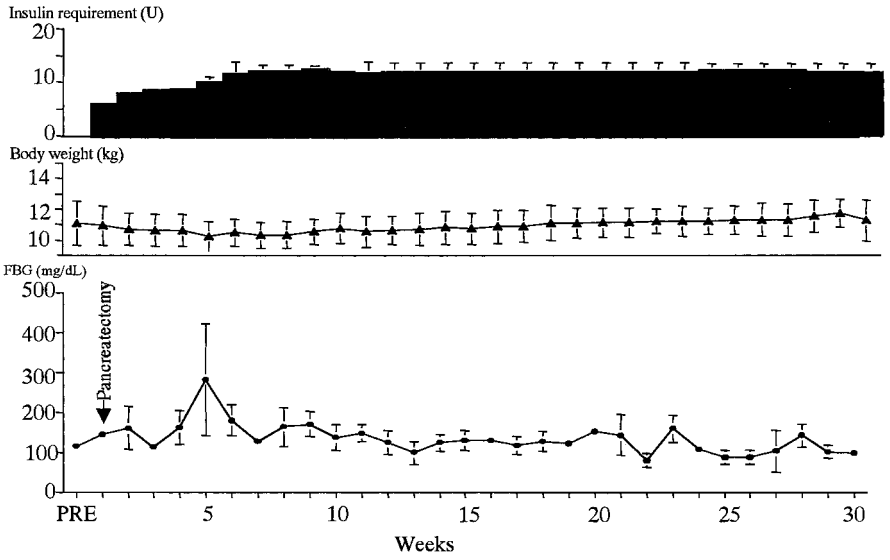

Fig. 2. Changes in fasting blood glucose (FBG) levels, body weights, and doses of exogenous insulin requirements in the control group. Values are expressed as mean +/– S.D.

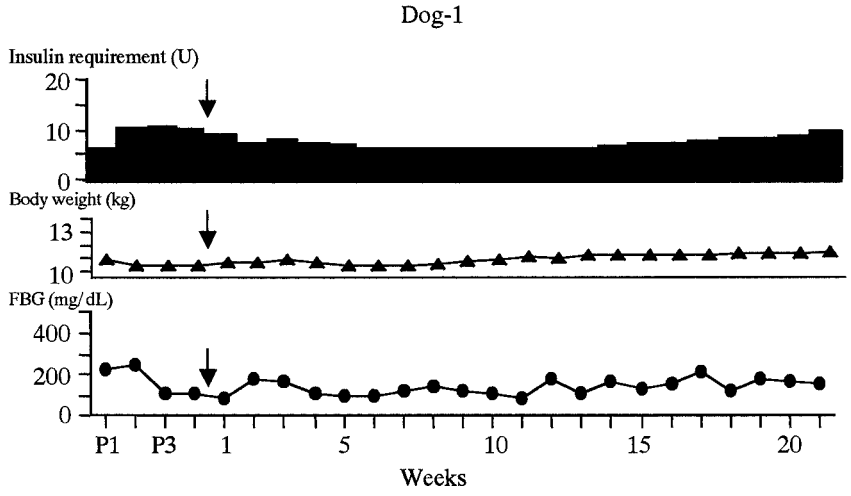

Fig. 3. Changes in FBG levels, body weights, and doses of exogenous insulin requirements before and after implantation of the Bio-AEP in Dog 1. Closed arrows indicate the time of implantation of the Bio-AEP.

observed before implantation. After the Bio-AEPs were removed, the ECM in the chamber was fixed in 10% buffered formalin for light microscopy or 2.0% gluteraldehyde in 0.1 M phosphate buffer for electron microscopy. For light microscopy, tissue sections (4 μm) were stained with hematoxylin-eosin (H-E) and an avidin-biotin-peroxidase complex technique using guinea pig anti-porcine insulin antibody (LSAB 2 kit/HRP, DAKO Japan Co., Ltd., Kyoto, Japan). The ultrastructure of the PE-cells was observed minutely by transmission electron microscopy (TEM).

RESULTS

In the dogs of the control group, exogenous insulin requirements increased until FBG became stable after pancreatectomy (Fig. 2), when the insulin dose was 10 units (1.0 units/kg) to 13 (1.3 units/kg) per a day (12.0 ± 1.7 units/day on average). After stabilization, basal exogenous insulin requirements did not change during the experimental period in this group. No significant weight loss was observed in any of the dogs during the experimental period.

Dog 1 received 1.8×10^8 PE-cells (1.7×10^7 cells/kg). After implantation, the dose of exogenous insulin was gradually decreased and FBG was maintained within the normal

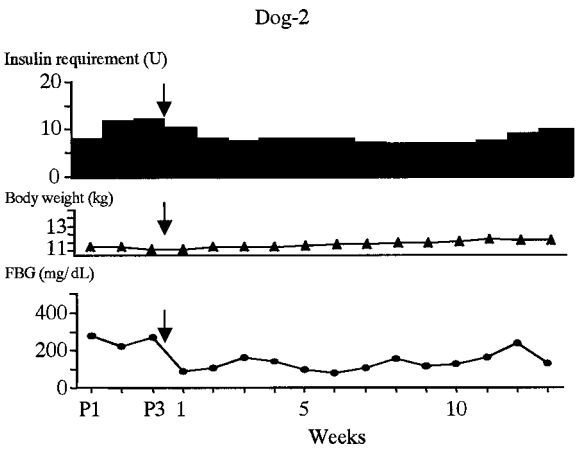

Fig. 4. Changes in FBG levels, body weights, and doses of exogenous insulin requirements before and after implantation of the Bio-AEP in Dog 2. Closed arrows indicate the time of implantation of the Bio-AEP.

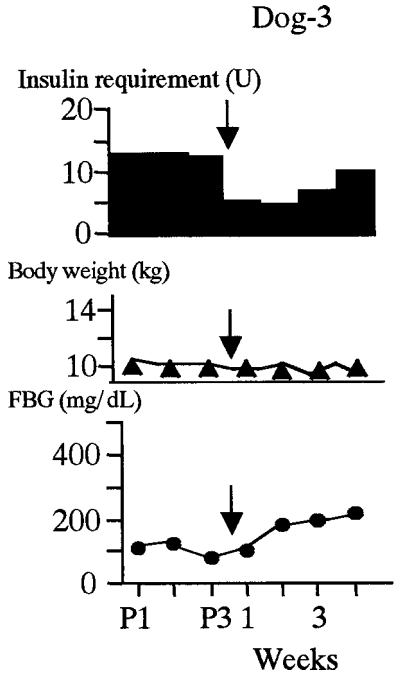

Fig. 5. Changes in FBG levels, body weights, and doses of exogenous insulin requirements before and after implantation of the Bio-AEP in Dog 3. Closed arrows indicate the time of implantation of the Bio-AEP.

range. At 6 weeks after implantation, the exogenous insulin requirements reached 56.1% of the pre-implantation levels (Fig. 3). The minimal exogenous insulin requirement was 6 units (0.6 units/kg) per a day. Fructosamine levels at all stages of the experimental period were within the normal range. Glycosuria was observed at 17 weeks after implantation, when the exogenous insulin requirements started to increase. It returned to the pre-implantation levels at 21 weeks after implantation, and therefore, the Bio-AEPs were removed by laparotomy. The Bio-AEPs were completely destroyed, and the ECM in the Bio-AEP was not identified within the chamber (Fig. 6-A). No adhesion to the intestine nor other abdominal organs, abscess formation, and fibrous tissue encapsulation of the Bio-AEP was observed.

Dog 2 received 1.9×10^8 PE-cells (1.8×10^7 cells/kg). At 2 weeks after implantation, the exogenous insulin requirements decreased to 66.6% of the pre-implantation levels (Fig. 4). The exogenous insulin requirement when FBG was stabilized was 7 to 8 units/day (0.7 to 0.8 units/kg/day), and was lower than that in pre-implantation periods until 11 weeks after implantation. At 12 weeks after implantation, the exogenous insulin requirements increased and returned to the basal value (Fig. 4). Glycosuria was observed between 9 to 13 weeks after implantation. Fructosamine levels were within the normal range during the experimental period. The Bio-AEP was then removed by laparotomy. The chamber was encapsulated with thin fibrous tissue, within which the effusion was retained (Fig. 6-B). No aerobic nor anaerobic bacteria was detected within the fluid, but a small number of macrophages and lymphocytes were observed. The nuclepore membranes of the Bio-AEP were not destroyed. No evidence of infiltration of red blood cells, lymphocytes, or other inflammatory cells into the chamber was observed on light microscopy. A large number of PE-cells were present in the ECM. The cytoplasm of the PE-cells was stained with eosin, but the nuclei of these cells were not stained with hematoxylin (Fig. 7-A). Most of the PE-cells in the ECM were stained by immunological staining against insulin (Fig. 7-B). TEM revealed that the nucleic and cell membranes were disrupted, although insulin granules were observed in the PE-cells (Fig. 7-C). These results were suggestive of cell necrosis.

Dog 3 received 1.3×10^8 PE-cells (1.3×10^7 cells/kg). The exogenous insulin requirement decreased after implantation and was lower than the pre-implantation levels for 3 weeks after implantation. The minimal exogenous insulin requirement was 4.0 units/day (0.4 units/kg/day), 32.0% of the pre-implantation level (Fig. 5). At 4 weeks of implantation, FBG and exogenous insulin requirements suddenly increased and returned to the pre-implantation level. Glycosuria was observed from 4 weeks after implantation. Fructosamine levels were within the normal range during the experimental period. The laparotomy revealed that the Bio-AEP was completely destroyed and that the ECM was lost (Fig. 6-C).

In all dogs of the implantation group, exogenous insulin requirements and FBG did not change after the removal of the Bio-AEP. The exogenous insulin requirements after implantation clearly decreased in all implanted dogs, although implantation of the Bio-AEP did not completely supplant exogenous insulin therapy in this study. Complications such as vomiting and fever and changes in blood count and blood chemistry profiles were not confirmed in all dogs

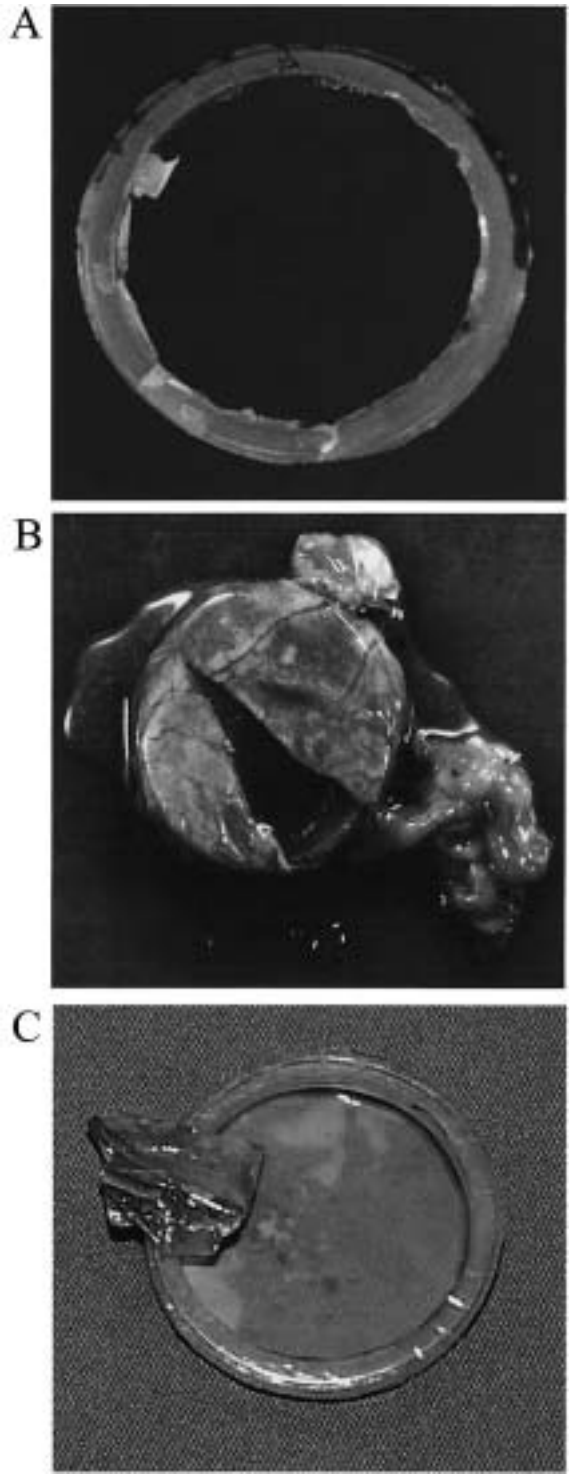

Fig. 6. Gross findings of Bio-AEP after the removal in implantation group. A: Dog 1, B: Dog 2, C: Dog 3. Two Bio-AEPs were completely destroyed (A, C). Fibrous tissue encapsulations with a proliferation of vessels around the Bio-AEP and effusion within the capsule were observed (B).

after implantation.

DISCUSSION

Key factors for a successful biohybrid pancreas may include the protection of islet grafts from immune rejection, the number of islet cells to transplant, and the ability to support long-term islet viability [39]. Since 1990, porcine islet transplantation has been reported in rodents [15, 34]. Although this approach has shown varying degrees of success in diabetic rodents, convincing proof of long-term efficacy in larger animals is generally lacking because rodents are known to have puny immune system [19, 39].

There have been only a few reports on xenografts in dogs *in vivo*. In 1991, Monaco *et al.* transplanted a vascular prosthesis seeded with 2.0 to 3.0×10^4 porcine islets per kg of recipient into total pancreatectomized diabetic dogs [25]. In the study, the device did not completely supplant exogenous insulin therapy in all of the dogs. In 1991, Brunetti *et al.* transplanted microcapsulated porcine islets into spontaneous diabetic dogs with IDDM in which only one of five dogs showed complete reversal from hyperglycemia [1]. In 1996, Maki *et al.* transplanted porcine islets into total pancreatectomized dogs [21, 22]. Only two of the seventeen dogs maintained good glycemic control for 8 months, but all dogs required a small amount of daily insulin administration to maintain normoglycemia.

In this study, the exogenous insulin requirement after implantation of Bio-AEP decreased for a certain period in all pancreatectomized dogs. These results provided the evidence for the potential efficacy of Bio-AEP with porcine PE-cells in diabetic dogs. However, Bio-AEP did not completely replace exogenous insulin therapy.

One potential cause of graft failure may be structural defects in the Bio-AEP chamber. In two dogs, the Bio-AEPs were completely destroyed and the nuclepore membranes had ruptured and/or detached from the silicon ring. We employed corona treatment to adhere the polycarbonate membrane to the silicon ring. High-voltage corona treatment is a technique used to improve adhesion by modification of the surface of the polymers. Corona treatment could lead to the physical weakness of the nuclepore membrane. Our preliminary data showed that the Bio-AEP possessed sufficient strength when it was shaken in a medium *in vitro* at room temperature for several weeks. Therefore, the cause of rupture and/or detachment of the membrane might be the *in vivo* kinetic influence of movements in the abdominal cavity, such as peristalsis.

The fact that the Bio-AEP did not completely supplant exogenous insulin therapy might be explained by an insufficient number of PE-cells in the Bio-AEP. It is difficult to compare the results of this study with those of past reports regarding the number of implanted cells, because single pancreatic endocrine cells, but not islets, were employed in the present experiment. One porcine islet contained approximately 2 to 3×10^3 PE-cells from our histological study using continuous sections of porcine islets (data not shown).

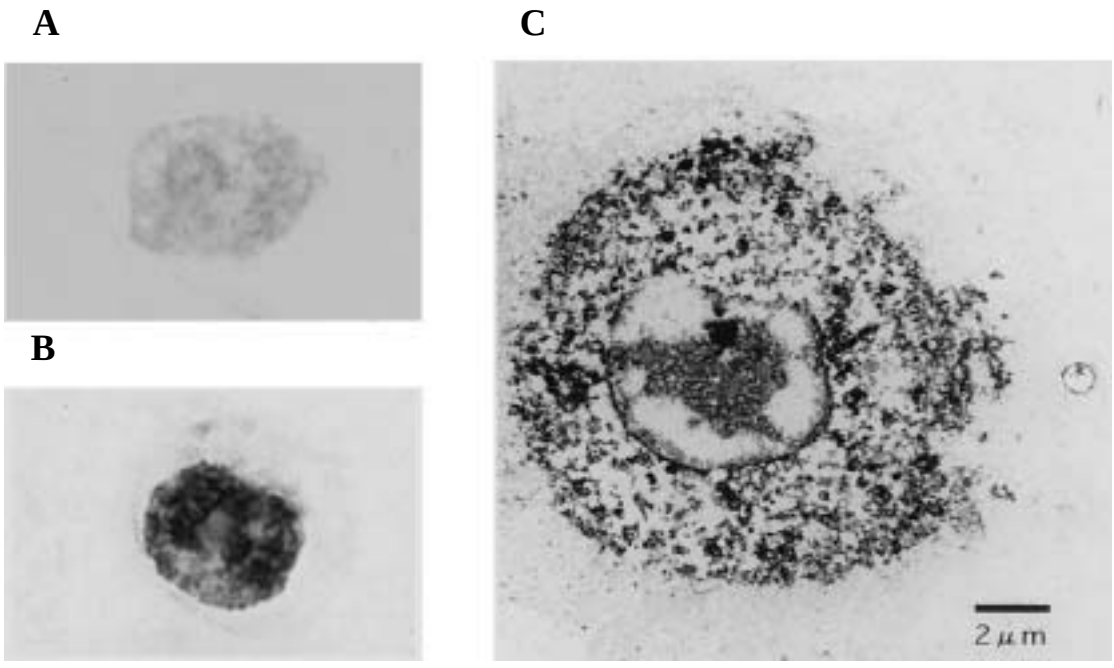

Fig. 7. Light or electron micrographs of PE-cells present in the ECM of the Bio-AEP in Dog 2. A: H-E stain, × 1000. B: Indirect immunoperoxidase staining against insulin, × 1000, C: × 3000 on TEM. Scale bar = 2 μm

Three thousands PE-cells were estimated as being contained in one porcine islet in this study. In other recent studies, 0.9 to 8.8×10^5 allogenic islets were transplanted into chemically-induced or pancreatectomized diabetic dogs [16, 17, 20, 25, 36–39]. Canine islet transplantation completely supplanted exogenous insulin therapy in almost all of the diabetic dogs that received more than 2.4×10^4 islets per kg of body weight [20, 25]. On the contrary, when 2.0 to 3.0×10^4 porcine islets per kg of body weight of the recipient were transplanted into total pancreatectomized diabetic dogs, no dogs maintained normoglycemia, although the exogenous insulin requirements did significantly decrease after implantation [25].

In the present study, the number of the PE-cells implanted was 1.3 to 1.8×10^7 PE-cells per kg of body weight of the recipient (approximately 0.4 to 0.6×10^4 porcine islets per kg of body weight), which was a smaller number than that of previous reports. The difficulty of isolating and preserving a large number of adult porcine islets for a long period was responsible for the smaller number of cells used. An increase in the number of more viable PE-cells through further improvement of isolation and preservation would help this xenograft replace exogenous insulin therapy in diabetic dogs.

The Bio-AEP was encapsulated by thin fibrous tissue in Dog 2 upon removal. The fibrous tissue was filled with effusion, and a small number of macrophages and lymphocytes, but no neutrophils, were observed in the effusion on light microscopy. These findings might be associated with some type of immune response to the Bio-AEP. In our previous investigation on the biocompatibility of the Bio-AEP without PE-cells in normal dogs, immunological reactions to the silicon ring, nuclepore membrane, and the ECM with type-I collagen treated to reduce the antigenicity were not detected for at least 3 months after implantation [4, 5]. The present findings suggested that PE-cells might induce some type of immune response in the recipient, leading to fibrous tissue encapsulation. However, the mechanism for this fibrous tissue encapsulation remains unclear, and further research will be needed.

In Dog 2, the exogenous insulin requirement increased and returned to the pre-implantation levels, although the Bio-AEP was not destroyed. Immunological cells were not observed within the chamber on light microscopy, but TEM indicated necrosis of the PE-cells. The membrane used in this study allowed the passage of humoral immunological factors such as antibodies and complements, but did not allow the passage of immunological cells. However, the agarose employed for the ECM prevented the passage of antibodies and complements. Hyperacute rejection usually occurs immediately after transplantation. Gal-α 1–3 Gal epitope appeared to be the main target in case of hyperacute humoral rejection of pig organs to humans [7]. However, it has been demonstrated in recent studies that porcine islets do not express Gal-α 1–3 Gal epitope [14, 24]. Unlike human, dogs possess the Gal-α 1–3 Gal epitope. It has been reported that the destruction of discordant islet xenografts was mediated by immunological cells [41]. The necrosis observed here may have been due to hypoxia occurring after the encapsulation of the fibrous tissue around the Bio-AEP, rather than to an immunological rejection. However, the influence of humoral immunity could not be completely

denied in this experiment.

In conclusion, these results clearly indicated the potential usefulness of the Bio-AEP as a therapy for diabetes. However, more preclinical researches should be needed to apply this technique for the treatment of diabetic dogs.

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