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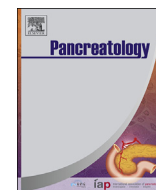

Endocrine pancreas engineered using porcine islets and partial pancreatic scaffolds

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Objectives: Because therapeutic options for severe diabetes are currently limited, there is a continuing need for new therapeutic strategies, especially in the field of regenerative medicine. Collaborative efforts across the fields of tissue engineering technology and islet biology may be able to create functionally engineered islets capable of restoring endocrine function in patients with insulin-dependent diabetes.
Methods: This engineered scaffold was seeded with isolated primary porcine islets via the pancreatic duct using a multi-step infusion technique. Endocrine function of perfusion-cultured islets in the native scaffold was analyzed by immunohistochemical staining of insulin and glucagon as well as by the insulin stimulation test.
Results: The pancreas in this large animal could be uniformly decellularized by perfusion with trypsin and TritonX-100 via the pancreatic duct, as shown by positive staining of extracellular matrix (ECM) components. These scaffolds derived from porcine pancreas were able to maintain the cellular integrity of islets that had repopulated the parenchymal space, which is fundamental for the restoration of endocrine function. Insulin release up to four days after islet infusion was maintained.
Conclusions: This scaffold from a large animal maintained islet survival and function in the short-term, retaining the cells as a solid organ in the parenchymal space after infusion through the pancreatic duct. These results suggest that this scaffold is suitable for further fabrication of fully functional bioengineered endocrine pancreases when implanted *in vivo*. Therefore, it may represent a key improvement in the field of beta-cell replacement therapy. Nonetheless, the facilitation of longer-term islet survival and studies of implantation *in vivo* is required for successful clinical translation.
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1. Introduction

In spite of numerous investigations seeking appropriate treatment options, currently diabetes mellitus is one of the most serious worldwide health problems [1,2]. Although exogenous insulin therapy, islet transplantation or whole pancreas transplantation are effective means for preventing acute metabolic decompensation in patients with severe diabetes [3–7], only limited numbers of patients achieve their therapeutic targets. Hyperglycemia-related organ injury including kidney or heart damage remains a significant cause of morbidity and mortality among these severe diabetics [8,9].

Currently, the most common treatment options for diabetes, such as pharmaceutical interventions and insulin supplementation, have not resulted in a curative effect and can potentially lead to long-term complications [10,11]. Progress in tissue engineering technology has facilitated the development of replacement tissues for the treatment of organ dysfunction [12–14]. Among these, the development of a bioengineered pancreas using an appropriate combination of cells and ECM scaffolds will provide an alternative approach to diabetes therapy. Indeed, cell-ECM interactions have

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been reported to be fundamental for the regulation of islet survival and insulin production. Native tissues are characterized by their unique ECM composition making an appropriate micro-environment [15–17]. Hence, three-dimensional (3D) scaffolds will play a critical role in pancreatic regenerative medicine by providing a similar micro-environmental niche for the seeded islet cells. At present, investigations of pancreatic scaffolds have been focused on artificially constructing those similar to the native ECM environment [18]. Using different synthetic biomaterials, many different types of scaffold have been developed, but none was found to accurately mimic the complexity of the actual pancreatic ECM composition and ultrastructure [19,20]. Very recently, tissue decellularization technology has been explored as an alternative; it was demonstrated that eliminating all the viable cells but preserving the native ECM composition from tissues or organs including vessels [21], valves [22], skin [23], heart [24], liver [25,26], kidney [27] and pancreas [28] is feasible. This unique technology can meet the requirement for functional pancreas tissue engineering by providing a physiologically relevant ECM scaffold that represents the complex *in vivo* micro-environment [29]. Indeed, we have reported the efficacy of this native organ scaffold in the liver context, showing that the decellularized scaffold could support cell function and viability. Importantly, it could be applied on a large scale, such as in porcine-derived organ scaffolds [30]. Investigations by others have also reported improved islet functionality when cultured on decellularized matrices derived from small intestinal submucosa or pancreatic slices [15]. Therefore, we hypothesized that three-dimensional native pancreas ECM from pigs would support the viability, phenotype, function, and tissue assembly of islets freshly isolated from porcine pancreas. Very recently, Mirmaled-Sani et al. demonstrated the efficacy of pancreas scaffolds along these lines [31]. Optimized protocols for production of the scaffold as well as cell infusion remain to be determined, because the pancreas is basically a fragile tissue and the islet itself is too delicate to be efficiently infused into the parenchymal space.

Based on these considerations, the objectives of this study were to determine whether porcine pancreas-derived scaffolds could serve as suitable platforms for the reconstruction of an endocrine pancreas, with emphasis on its insulin-producing capacity. Although islet transplantation is already an established therapeutic option, viability of infused islets engrafted mostly in the portal vein decreased to less than 50% soon after their infusion [32,33]. This is due to the methodological limitation inherent in islet infusion via the portal vein, which triggers rapid destruction of the cells not only by mechanical shear stress caused by blood flow but also by an immediate blood-mediated inflammatory reaction (IBMIR) which is defined as an innate inflammatory response activating complement and infiltration of polymorphonuclear cells into “foreign” material [34,35].

To overcome these limitations to clinical translation, we developed an effective method for the decellularization and recellularization of intact porcine pancreases which can maintain freshly isolated porcine islets in the parenchymal space, resulting in the construction of three-dimensional ECM scaffold-based bioengineered tissues with efficient insulin production, at least in the short-term. The methods and techniques established in this study demonstrate an effective approach for pancreas decellularization and recellularization by bioengineering for human-scale transplantation.

2. Material and methods

2.1. Animals

Female LWD pigs weighing 20–23 kg were used for pancreas harvesting to prepare decellularized matrices as a scaffold for islet maintenance. Female pigs were also the source of pancreases harvested for the isolation of islet cells. All protocols were reviewed and approved by the local Ethics Committee of Keio University.

2.2. Whole pancreas harvest and decellularization

The pigs were anesthetized by midazolam 0.2 mg/kg (Astellas, Tokyo, Japan) and medetomidine 0.08 mg/kg (Zenoaq, Fukushima, Japan), followed by isoflurane inhalation to maintain anesthesia during the procedure. They were connected to a standard respiratory system, consisting of endotracheal tube for continuing inhalation. Using a vertical midline incision, the pancreas was excised en-bloc together with the upper duodenal segment. The latter was then removed from the pancreas, which was cannulated via the main pancreatic duct and superior mesenteric vein using 18 Fr Safeed Tubes (Terumo, Tokyo, Japan). The pancreas was flushed with PBS at 20 ml/min for 3–4 h and then frozen at −80 °C for a minimum of 24 h prior to decellularization. The frozen/thawed pancreas was washed with PBS at 20 ml/min for 6 h to remove blood via the main pancreatic duct and then perfused with 0.05% trypsin at 20 ml/min for 6 h, and rinsed with PBS at the same flow rate for a further 3 h. It was then decellularized with 0.1% TritonX-100 and 0.05% EGTA for 24–36 h by perfusion at 20 ml/min via the main pancreatic duct. The resulting pancreas bioscaffold was finally rinsed with PBS for 12 h.

2.3. Vascular corrosion casting

Corrosion casts were prepared using Baton's #17 anatomical corrosion kit following the manufacturer's recommendations (Polysciences, Inc., Warrington, PA, USA). Polymer mixture (20–40 ml depending on pancreas size) was injected via tubes inserted into the main pancreatic duct, superior mesenteric vein, and superior mesenteric artery. Polymerization took at least 2 h at 4 °C and was followed by maceration in 1N KOH solution for 3–4 h at 50 °C.

2.4. Histological analyses

Normal pancreas, decellularized pancreas matrix and recellularized pancreas samples at day 4 were fixed with 10% formalin, embedded in paraffin, and processed for hematoxylin and eosin staining. Additional samples were permeabilized and incubated with rabbit polyclonal anti-collagen IV, rabbit polyclonal anti-fibronectin, and rabbit polyclonal anti-laminin (Abcam, Cambridge, MA, USA). Samples were then incubated overnight at 4 °C with monoclonal anti-insulin (1:50, Sigma) or anti-glucagon (1:3,000, Dako, Glostrup, Denmark) antibodies. The secondary antibody was goat anti-rabbit IgG (Life Technologies, Grand Island, NY, USA). After all procedures, specimens were counterstained with DAPI (DAPI Fluoromount-G, Southern Biotech).

2.5. Porcine islet cell isolation

Immediately after receiving the pancreas, we inserted a cannula into the main pancreatic duct for infusion of modified ET-Kyoto solution consisting of 50,000U of ulinastatin (Mochida Pharmaceutical Co Ltd, Tokyo, Japan) added to 1 L of Kyoto solution (Otsuka Pharmaceutical Co Ltd, Tokyo, Japan) for ductal protection, and placed the pancreas into a two-layer (modified ET-Kyoto solution/PFC) preservation container. Islets were isolated using a simple method optimized for adult pigs, as described elsewhere [36].

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