The Research of Acellular pancreatic bioscaffold as a natural 3D platform In Vitro

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Abstract. AIM: To investigate the biochemical and functional properties of a rat acellular pancreatic bioscaffold (APB). METHODS: Fresh pancreata were soaked and perfused. The histological structure, the extracellular matrix (ECM) composition, and the DNA content of the APBs were evaluated. After biocompatibility studies, the proliferation, apoptosis and differentiation of AR42J pancreatic acinar cells cultured on APBs were assessed. RESULTS: The pancreatic tissues became translucent after decellularization. The native macroscopic 3D architecture and the ECM ultrastructure were preserved, with large ductal structures and vascular tissue branching from the greater pancreatic artery, but there were no visible vascular endothelial cells, cellular components or cracked cellular debris. The ECM components, including collagen I, collagen IV, fibronectin, laminin and sGAG, were not decreased after decellularization of the APB (P>0.05); however, the DNA content was decreased significantly (P<0.05). The subcutaneous implantation sites showed low immunological response and low cytotoxicity around the APB. The proliferation rate was higher and the apoptosis rate was lower when AR42J cells were cultured on APB than when they were cultured in media alone, on artificial scaffold or ECM (P<0.05). The gene expression of pancreatic duodenal homeodomain containing transcription factor (PDX-1) and pancreatic exocrine transcription factor (PTF-1) and the protein expression of α-Amy, cytokeratin 7 (CK7) and fetal liver kinase-1 (Flk-1) were higher for the APB group than for the other groups (P<0.001). CONCLUSION: Our findings support the biological utility of whole pancreas APBs as biomaterial scaffolds, which provides an improved approach for regenerative medicine.

1. Introduction

3D scaffolds are of central importance in regenerative medicine and tissue engineering. Although various types of artificial scaffolds have been studied, they cannot simulate the array of functions performed by the native extracellular matrix (ECM) [1–2].

As a biomaterial scaffold with a highly preserved innate extracellular matrix [3], acellular matrix (ACM) provides an ideal platform for regeneration and implantation because it is biocompatible and preserves 3D geometric and spatial architecture [4]. Whole organ ACM has been widely utilized as a scaffold for the repopulation of engineered tissue/organs in RM because of the physiological resemblance of the original tissue, including intact 3D architecture, preserved native ECM components, vascular networks, and biomechanical properties. Furthermore, whole organ perfusion decellularization has recently been proposed as a method to generate ACM scaffolds from complex...
organs. However, pancreas bioengineering has been less well studied, with only a handful of studies reporting the successful generation of pancreatic ACM.

To further evaluate the use of pancreatic ACM, we introduced the soaking-perfusion decellularization technique to produce an acellular whole pancreatic bioscaffold (APB). We compared the biochemical and functional properties of the APB to that of other types of artificial and native scaffold as a platform to support pancreatic tissue engineering in vitro.

2. Materials and methods

2.1 Rat pancreas Harvest and Decellularization

All animal work was approved by Institutional Animal Care of China and performed in accordance with the Animal Welfare Act Institutional Guidelines. After celiotomy, the harvested pancreata were then connected to a peristaltic pump (MasterFlex L/S) to allow retrograde perfusion to flow through the hepatic portal vein at a speed of 480ml/h until the tissues turned completely transparent.

2.2 Histological Evaluation of the APBs

To evaluate the effect of our decellularization process, the hematoxylin-eosin (H&E) staining technique was conducted. The decellularized scaffolds sections were fixed on slides, stained with H&E, and observed by phase contrast microscopy.

2.3 Immunofluorescence Staining of the ECM Component on the APBs

Tissue samples were fixed. After the first primary antibody staining, an additional blocking step was included prior to the addition of a secondary antibody. The slides were washed three times. Images were recorded with a Metamorph 7.5.6.0 (Molecular Device) on an Olympus microscope.

2.4 Scanning Electron Microscopy (SEM) Analysis of the APBs

The samples were placed in a critical point dryer for 10min. Specimens were overgilt to obtain electric conductivity, and samples were visualized using a scanning electron microscope.

2.5 Quantification of the DNA Content of the APBs

Total DNA in the native pancreas and APB was extracted with the QIAamp DNA Mini Kit (Qiagen) and visualized by electrophoresis. The concentration of the DNA in the extracting solution was measured using the Quanti-IT TM PicoGreen® dsDNA assay kit(Invitrogen).

2.6 Quantification of the ECM Component by ELISA Assay

The ECM content, including collagen and glycosaminoglycans (sGAGs) on the APB, was examined using rat collagen and sGAGs ELISA kits (BioSwamp, China) according to the manufacturer’s protocol.

2.7 Biocompatibility Assessment of the APBs by Subcutaneous Implantation

For the study of biocompatibility, a 1cm2 APB was sutured subcutaneously in dorsal pockets of male C57BL/6 mice. At 14 d and 28 d, the mice were euthanized and the APB and surrounding tissue were harvested and fixed in formalin for H&E staining. The remold score was utilized for histologic evaluation.

2.8 Recellularization of APBs and AR42J Acinar Cells Cultured on Different 3D media

The AR42J acinar cell line (ATCC) was used at passage 18–25. The AR42J cell suspension was cultured on 4 different kinds of 3D media: the blank control media; the ECM media; the PLGA media, and the APB media. The samples were placed in RPMI 1640 medium supplemented with 20% FBS (Life Technologies) and 100 U/ml penicillin/ streptomycin in a standard CO2 (5%) cell incubator at a constant temperature (37°C). The medium was changed every day, and the proliferation and the differentiation of AR42J cells were evaluated at the 3rd, 5th, 7th, and 10th days after seeding.

2.9 Statistical Analysis

Data were expressed as means ± SD. Significant differences among groups were determined by the T-test for 2-group comparisons and analysis of variance of repeated measure followed by post-hoc
analysis for multiple group comparisons. Probability values at \( P < 0.05 \) indicated statistical significance.

3. Results

3.1 Histological Evaluation and Ultrastructure of the APB

After perfusion, a gradual change of color was observed during the decellularization process. Macroscopically, the color of the rat pancreata changed from pink-brown to translucent (Figure 1A, subpanels a–d) with the gross anatomical structure of the native pancreas retained. Perfusion was continued until whole pancreata turned totally transparent (about 6h) as depicted in Figure 1A, subpanel d. Histological examination by H&E staining showed native pancreata with acinar and islet structures prior to decellularization (Figure 1B, subpanels a–b), but no cellular material or remnants with preservation of the pancreatic collagen fibers and matrix after the completion of decellularization (Figure 1B, subpanel c).

By SEM analysis, the ECM were well preserved within the APB, while cellular material and debris were removed. Hollow and void spaces observed within the APB were attributed to the “footprints” of removed pancreatic cells during the decellularization process (Figure 1C). The vessel structures and large ducts (yellow arrow) were retained without any vascular endothelial cells and no ductal cracked cellular debris lining the ducts. The recognizable scalloping pattern of arteries also indicated the preservation of vascular structure, likely branched from the greater pancreatic artery (white arrows).
These results indicate that the APB is effectively decellularized, but maintains pancreatic structural features.

3.2 The ECM Composition of the APB

Figure 2. ECM characterization of the APB.

To evaluate the ECM component of the APBs, we performed immunofluorescent staining. Collagen I, IV, fibronectin and laminin were preserved after the decellularization process, but no cellular materials or cytoskeletal elements were detected. Furthermore, the ECM structure orientation of the APBs resembled that of the native pancreas (Figure 2).

Compared to the weight of the native pancreas (84.13 ± 11.94 ng/mg dry weight), the total collagen weight of the APB was slightly less (75.72 ± 9.42 ng/mg dry weight), but the difference was not significant (P = 0.217). Similar results were observed for sGAG (normal pancreas: 33.6 ± 9.38 ng/mg dry weight; APB: 27.2 ± 7.31 ng/mg dry weight; P = 0.209). These results suggest that the ECM components are maintained within the APB.

3.3 Quantification of DNA on the APB

As an additional measure of the efficacy of decellularization, we assessed the DNA content of APBs. The DNA content decreased from 8364.16 ± 1641.15 ng/mg dry weight in normal pancreas to 28.28 ± 11.09 ng/mg dry weight in APB (P < 0.001). Further study revealed that the residual DNA fragments of APBs were less than 200 bp, whereas the DNA fragments of native pancreata were obviously larger. These results provide verification of effective decellularization of the APB.

3.4 In Vivo Response to the Decellularized Pancreas

In order to evaluate biocompatibility, APBs were implanted subcutaneously in C57BL/6 mice in vivo. At 14 days post-surgery, the subcutaneous implantation site showed the appearance of mononuclear cells surrounding the partially degraded APB with active angiogenesis (Figure 3A, arrows). At 28 days after implantation, the number of mononuclear cells decreased to a large extent.
and no multinucleate giant cells or other pathological signs of the foreign body response could be detected. Furthermore, ordered collagen bundles were visible around degraded APB with active angiogenesis at the APB implantation site (Figure 3B, arrows).

The histological remold score was 10.4 ± 1.78 at 14 days post-surgery and 13.8 ± 1.32 at 28 days, which verifies the enhanced remodeling after implantation (Figure 3C). These results suggest that the APB is capable of integrating within host tissue.

3.5 Cell Proliferation and Apoptosis Among 4 Groups

MTT assays indicated that AR42J cells proliferated in all 4 groups, with time-dependent growth from day 3 to 10. Furthermore, the cell proliferation rate was higher in the APB group than in the other 3 groups with significant difference at all timepoints tested (P=0.011).

Further study by flow cytometry indicated the apoptosis rate was lower in the APB group than in the other 3 groups at all timepoints tested with significant difference (P=0.017). The dramatically reduced level of apoptosis of AR42J cells when grown in the presence of APB may account for the decreased proliferation rate. These results suggest that the APB efficiently facilitates pancreatic cell growth.

3.6 Evaluation of the Expression of Pancreatic Acini Genes and cytokeratins Among 4 Groups

The mRNAs for PDX-1 and PTF-1 increased significantly from day 3 to 10 for all samples. Furthermore, expression was higher for cells cultured on the APB than for the other 3 groups with significant difference at all timepoints (P_{PDX-1}=0.009, P_{PTF-1}=0.009).

The protein expression of α-Amy, CK7, and Flk-1 was increased significantly from day 3 to 10 for all groups. Additionally, expression was higher for cells cultured on the APB than for the other 3 groups with significant difference at all timepoints (P_{α-Amy}=0.015, P_{CK7}=0.012, P_{Flk1}=0.013). These results suggest that pancreatic cell differentiation is enhanced in the presence of APB.

4. Discussion

The intact ACM produced by perfusion decellularization offers a promising alternate approach for tissue engineering and functional organ replacement in RM. We have demonstrated the compliance of the APBs in this study to each of these criteria. Furthermore, we have demonstrated that the APBs
produced by this method are biocompatible and are amenable to cell repopulation, which allows proliferation and differentiation of pancreatic acinar cells.

An ideal ACM should have tissue-specific biological and mechanical characterization with biocompatible properties that can promote cell adhesion, viability, proliferation and differentiation [5]. An intact vascular system with endothelial cells on ACMs can prevent thrombosis and hemorrhaging [6]. We demonstrated that the APB scaffold generated in this study retained a perfusable vascular tree, a ductal network and intact 3D architecture, though no cellular remnants were detected by H&E staining or SEM. Also, we examined ECM components which indicated that the ECM microstructure and collagen fibers were preserved with no nuclear material detected by DAPI staining. Further analysis by quantitative ELISA showed non-significant decreases in either total collagen or sGAG. Therefore, our APB maintained the physiological organization of ECM proteins with removal of cellular material after decellularization.

Total DNA levels in the APBs compared with innate pancreata were greatly decreased, with the low levels of residual DNA consisting primarily of fragments less than 200bp. Therefore, our DNA results further support the successful decellularization of the APB. These findings are noteworthy because cellular remnants in ACMs can result in cytocompatibility issues and adverse immunological response after implantation [7], which may influence the biological utility of the APB [8].

Further results suggested that each culture group could promote cell survival as supported by a time-dependent increase in cellular proliferation. However, over 10 days cultivation the APB group better improved AR42J cell proliferation than did the other 3 groups. Consistently, the APB group showed a marked decrease in cell apoptosis as compared to the other 3 culture groups. Therefore, our APB provided a necessary environment for regenerative support and enhanced pancreatic cell proliferation that is more effective than artificial scaffolds.

We also assessed AR42J differentiation on the APB by examining the expression of genes that are known to be important to pancreatic function, including PDX-1 and PTF-1 mRNAs. RT-PCR results demonstrated that both of these pancreatic acini genes sustained expression in a time-dependent manner with significant difference in all 4 groups. However, over the 10 days of cultivation the expression of both PDX-1 and PTF-1 was more highly elevated in the APB group than in the other 3 groups. This indicates that the APB can promote pancreatic organogenesis and exocrine function.

To identify duct cells, we also assessed the expression of the cytoketatinα-Amy, CK7 and Flk-1 by western blotting. Our results demonstrate that the expression of all 3 proteins increased in a time-dependent manner with significant difference in all the 4 groups. However, the expression in the APB group was more markedly increased than in the other 3 groups over the 10 days of cultivation. This indicates that the APB can promote AR42J cell transdifferentiation. Therefore, the APB can support and enhance pancreatic cell proliferation and differentiation more efficiently than other scaffolds.

5. Conclusion

The APB in our study met the stringent requirement for successful decellularization. The technique allowed the generation of an APB with preserved 3D architecture, vasculature and ductal channels, as well as integral ECM composition. We also demonstrated that the APB represents a biocompatible scaffold capable of integrating within host tissue. The APB supported and enhanced pancreatic AR42J cell proliferation and differentiation. Our APB acts as a suitable template for pancreatic tissue engineering and whole organ regeneration in future studies.

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