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Original article

Composition and organization of the pancreatic extracellular matrix by combined methods of immunohistochemistry, proteomics and scanning electron microscopy

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ABSTRACT

The epidemic expansion of diabetes is a major concern of public health. A promising treatment is the transplantation of islets of Langerhans isolated from the whole pancreas but the yields of islets isolation and the rates of successful engraftments still have to be improved to make this therapy effective. The extracellular matrix (ECM) of the pancreatic tissue is partially lost during the isolation process and a comprehensive knowledge of the pancreatic ECM composition and organization could identify targets to improve islets isolation and transplantation or highlight new therapeutics for pancreatic diseases. The organization, composition and three-dimensional architecture of the pancreatic ECM were analysed in mouse and pig by three different techniques. Laminin α-4 and β-2 chains are localized by immunohistochemistry in the exocrine tissue and inside islets of mouse pancreas but not around islets that are surrounded by an ECM made of collagen type IV and type V. Collagen type I, III, and VI were identified by proteomics as specific constituents of the pig pancreatic ECM along with the low-abundance isoforms α3(IV) α4(IV) α5(IV) and α1(V) α2(V) α3(V) of collagen type IV and type V respectively. The three-dimensional ECM architecture is analysed on decellularized mouse pancreas by scanning electron microscopy and is organized in honeycomb structures made of thin ECM fibers assembled in thicker bundles. The combination of immunohistochemistry, proteomics and scanning electron microscopy gives complementary perspective on the pancreatic ECM composition and organization. It represents a valuable toolbox for deeper investigations of ECMs and proposes clues in tissue engineering of the pancreas.

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1. Introduction

Diabetes is a metabolic disease where blood glucose concentration is dysregulated and can lead to multiple complications like cardiovascular diseases, kidney failure or blindness. In the pancreas, the islets of Langerhans contribute to glucose homeostasis by the release of insulin and glucagon hormones into the bloodstream. To date, diabetic patients are treated by insulin injections and can only be cured by pancreas transplantation. An alternative method surgically less invasive is to transplant islets of Langerhans isolated from the whole pancreas [1,2] but the yields of islets isolation and the rates of successful engraftments still have to be improved to make this therapy effective [3,4]. The extracellular matrix (ECM) is a complex multimolecular network that forms the framework of tissue and organs. During the isolation process of islets the pancreatic ECM is partially lost [5,6]. This loss is thought to be involved, among others, in the failure of islets engraftment and function after transplantation because both are enhanced when isolated islets are exposed *in vitro* to purified ECM proteins or when the damaged cell-ECM interactions are restored [7–9]. The pancreatic ECM is organized as a basement membrane (BM) made of a collagen type IV network intertwined by nidogen proteins with a laminin-based network [10,11]. However, some questions remain on its exact composition, in particular on the localisation of some ECM molecules within the endocrine and the exocrine tissue [12–15] and on the ECM organization between animal species like rodents, pigs and humans [16–18].

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To supplement the actual scheme of pancreatic ECM composition and organization in order to identify parameters liable to improve islets isolation and transplantation, we have combined three complementary techniques to unravel some specificity on the organization, composition, and three-dimensional structure of the pancreatic ECM in mice and pigs. Mouse is a wide spread animal model on diabetes and pancreatic functions, and pig is more and more considered for pancreatic xenograft, a foreseen solution for organ donors shortage [19,20]. By the mean of commercially available antibodies against EHS-laminin, laminin-α4, laminin-β2, collagen type IV and collagen type V, we have analysed by immunohistochemistry their localization within the exocrine and endocrine pancreas of both mouse and pig. To bypass some of the limitations of immunohistochemistry we have developed a non-*a priori* identification of ECM proteins of pig pancreas by sequential solubilisation of the ECM and identification of solubilized proteins by mass spectrometry [21,22]. Finally, we have used a decellularization method to expose the pancreatic ECM network and to investigate its three-dimensional organization by scanning electron microscopy.

2. Material and methods

2.1. Pancreatic tissue

Mice were obtained from the animal facilities of university of Sherbrooke. All experimental procedures on animals were conducted under the protocol 249-10 approved by the animal care and ethics committee of the university of Sherbrooke. Mouse pancreases were harvested from 3 months old C57Bl/6 males (n = 5). Mice were anesthetised with isoflurane (Abott laboratories, Abbott Park, IL) and then euthanized by cervical dislocation. Pancreases were quickly resected, washed in PBS and then fixed in 4% paraformaldehyde (PFA) in PBS for 24 h at 4 °C. Pig pancreases (6–12 months) were obtained and resected from a local slaughterhouse ($n = 3$). They were kept in ice-cold PBS, washed in PBS and cut in small pieces. Samples processed for histology were fixed in 4% PFA in PBS for 24 h at 4 °C. Samples analysed by mass spectrometry were stored at −80 °C before ECM purification and proteins solubilisation.

2.2. Immunohistology

Pancreases from both mouse and pig were fixed in 4% PFA in PBS for 24 h at 4 °C and washed in a large excess of PBS. They were then dehydrated in serial aqueous ethanol baths (70–90–100%), softened 48 h in butanol and embedded in paraffin. Paraffin sections (4 μm) were dewaxed in xylene, rehydrated in distilled water and processed for antigen retrieval. Heat-induced antigen retrieval was performed for 20 min at 95 °C (plate heater) in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6) or in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween-20, pH 9). Enzymatic antigen retrieval was performed at 37 °C with 0.5% pepsin in 5 mM HCl (P7000, Sigma-Aldrich; Oakville, Canada) or with 20 μg/mL proteinase K in 50 mM Tris pH 8, 1 mM EDTA, 0.5% Triton X-100 (P6556, Sigma-Aldrich; Oakville, Canada). Slides were then immersed in PBS and cooled down for 15 min at room temperature. Sections were blocked for 1 h with 10% goat serum in blocking buffer (1% bovine serum albumin, 0.1% cold fish gelatin, 0.1% Triton X-100, 0.05% Tween-20, 0.05% sodium azide, in PBS pH 7.2) and finally incubated with primary antibodies, at their respective dilution (antibody diluent K8006, Dako; Mississauga, Canada), overnight at 4 °C as depicted in Table 1. Secondary antibodies were diluted in PBS and incubated 1 h at room temperature. Nuclei were stained with DAPI (Invitrogen; Burlington, Canada).

Anti-EHS-laminin antibody was purchased from Sigma-Aldrich (L9393, rabbit against mouse). Anti-laminin α4 (MBS855508) and anti-collagen type IV (α2) (MBS853194) antibodies were purchased from MyBioSource (rabbit against human with cross-reaction with mouse; San Diego, CA). Anti-laminin β2 (LS-C88433, rat against mouse) antibody was purchased from Lifespan Bioscience (Seattle, WA) and anti-collagen type V antibody (ab7046, rabbit against human with cross-reaction with mouse) was purchased from Abcam (Cambridge, MA). Primary antibody to insulin was purchased from Abcam (ab7842). Secondary antibodies goat anti-mouse (IgG Alexa-Fluor 555; A110011) and donkey anti-rat (Alexa-Fluor 488; A21208) were from Invitrogen (Burlington, Canada). Observations were performed with a Nikon Eclipse TE2000-S fluorescent microscope. Images were post treated using ImageJ freeware: merging channels, brightness and contrast adjustment, background reduction (rolling radius: 10-25 pixels).

2.3. Scanning electron microscopy (SEM)

Mouse pancreases were fixed 48 h in 4% PFA in PBS at room temperature, washed in large excess of PBS. Fixed tissues were frozen in liquid nitrogen and crushed in smaller pieces in a mortar with a pestle. Samples were post-fixed in 1% osmium tetroxide (OsO_4), washed in PBS, dehydrated in 100% ethanol solution and then critical-point dried against CO_2.

Decellularization of mouse pancreas samples was adapted from [23,24]. Pancreas were fixed 48 h in 4% PFA in PBS at room temperature, washed in large excess of PBS and then incubated in 2 M NaOH at room temperature for 7 days. NaOH solutions were changed every 2 days. Samples were washed in distilled water for 3 days and incubated 2 h in 1% tannic acid. After washing, samples were post-fixed in OsO_4, dehydrated in 100% ethanol solution and critical-point dried against CO_2. All samples were coat-sputtered with Au/Pd and observed under a Hitachi S3000 N VP microscope operating at 5 to 20 kV.

2.4. Purification and solubilisation of ECM proteins from pig pancreas

Protocol for purification of pancreatic ECM and solubilisation of ECM proteins was adapted from [25]. Pig pancreases were diced with a scalpel in 1 to 2 cm^3 pieces and poured into 10 mM Tris buffer (pH 7.5) with 0.5 M NaCl and supplemented with a protease inhibitor cocktail (cOmplete Protease Inhibitor Tablets, Roche; Oakville, Canada) at 4 °C with constant stirring for 2 days. Solution was replaced 2 times per day. The remaining tissue was then incubated at room temperature in PBS with 1% sodium dodecyl sulfate (SDS) for 7 days under constant stirring. The SDS-PBS solution was changed daily. To remove excessive amounts of SDS, the tissue was thoroughly washed with PBS and the remaining tissue (considered as purified ECM) was solubilized in 4 M guanidium chloride (GnHCl) in 50 mM acetate buffer (pH 5.5) for 3 days at room temperature with constant stirring. After centrifugation, the supernatant including solubilized ECM proteins was precipitated with 5 volumes of cold acetone and then finally solubilized in 150 mM NaCl, 50 mM acetate buffer pH 6.5 (fraction I). The remaining clot was subjected to deglycosylation with 10 U of chondroitinase ABC (C3667, Sigma-Aldrich) and 10 U of Heparinase II (H6512, Sigma-Aldrich) overnight at 37 °C in 150 mM NaCl, 50 mM acetate buffer (fraction II). The final ECM insoluble clot was subjected to protein extraction in 4 M GnHCl, 50 mM acetate buffer for 24 h under stirring followed by precipitation in cold acetone (5 volumes) and was then solubilized in 150 mM NaCl, 50 mM acetate buffer pH 6.5 (fraction III). Protein concentrations were quantified by the Bradford assay. Fractions I (2 μg), II (3 μg) and III (5 μg) were separated on a 4-15% polyacrylamide gradient gel (Bio-Rad; Mississauga, Canada) and proteins were stained with Coomassie blue (R250, Bio-Rad).

2.5. Mass spectrometry and database search

In-gel trypsin digestion was performed as described elsewhere [26]. Briefly, bands were excised from the gel with a scalpel and equilibrated in 100 mM ammonium bicarbonate (NH_4HCO_3) for 15 min at room temperature followed by dehydration in acetonitrile. The proteins were reduced by adding 50 μL of 100 mM NH_4HCO_3 containing 5 mg/mL dithiothreitol (Promega; Madison, WI) to the dehydrated gel spots. After 45 min at 37 °C, the buffer was replaced with 200 mM ammonium bicarbonate buffer containing 25 mg/mL iodoacetamide, and the carbamethylation was allowed to proceed for 30 min in the dark at room temperature. Excess reagent was replaced with 400 μL of 100 mM NH_4HCO_3/50% acetonitrile and the gel slices were incubated for 30 min. The solvent was removed and the gel slices were lyophilized for 2 min. The gel slices were rehydrated for 15 min at room temperature in 25 μL of trypsin (V5113, Promega) dissolved at

Table 1
List of primary antibodies and conditions of use for immunohistochemistry.

Antigen (species)	Host species – Supplier (ref)	Antigen retrieval	Dilution
EHS-Laminin (mouse)	Rabbit – Sigma-Aldrich (L9393)	Citrate buffer pH 6, 95 °C, 20 min	1:50
Laminin α4 (human)	Rabbit – MyBioSource (MBS855508)	Proteinase K 20 μg/mL pH 8 37 °C, 20 min	1:100
Laminin-beta 2 (mouse)	Rat – LifeSpan Biosciences (clone A5; LS-C88433)	Tris buffer pH 9 95 °C, 15 min followed by 0.5% Pepsin 5 mM HCl, 37 °C, 20 min	1:100
Collagen type IV alpha-2 (human)	Rabbit – MyBioSource (MBS853194)	Pepsin 0.5% 5 mM HCl, 37 °C, 20 min	1:100
Collagen type V (human)	Rabbit – AbCam (ab7046)	Citrate buffer pH 6, 95 °C, 20 min followed Pepsin 0.5%, 37 °C, 20 min	1:100

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