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Mechanistic Insights into Ferroptotic Cell Death in Pancreatic Islets

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Introduction

Ferroptosis was identified as a non-apoptotic cell death mechanism, which causes iron-dependent peroxidation of membrane lipids and subsequent membrane rupture [1,2] As a form of necroptotic cell death, ferroptosis has already been shown to play an important role in the pathogenesis of various diseases [3]. Ferroptosis can be experimentally induced by the glutathione peroxidase-4 (GPX4) inhibitor RSL3. The inhibition of GPX4 causes oxidative stress through the release of free iron molecules and induction of lipid peroxidation, followed by a membrane burst with massive necrotic inflammation [1,4]. The effects of ferroptotic cell death can be attenuated by the treatment with ferrostatin-1 (Fer-1) [5,6], a small lipophilic molecule with various functions. Fer-1 mainly acts as an iron scavenger and prevents lipid peroxidation and subsequent membrane rupture [5]

The transplantation of pancreatic islets into the liver represents a therapeutic strategy mainly for patients with type 1 diabetes (T1D) experiencing life-threatening hypoglycemic situations despite optimal diabetes therapy or, in the case of an autologous transplantation setting, to prevent patients undergoing (sub-)total pancreatectomy from iatrogenic diabetes [5,7,8]. The intraportal transplantation of pancreatic islets is less invasive than a complete pancreas transplantation but has comparable effects on the stabilization of glycemic control and diabetes-associated complications [8,9]. However, it is estimated that nearly 70% of the islet graft is lost during the peri- and post-transplant period [10].

To further improve the outcome of this therapy, it is of key importance to minimize islet loss and consequently improve islet functional potency and survival. A better understanding of mechanisms mediating islet death during and after the isolation process and targeted prevention are therefore key issues. Bruni *et al*. first described in 2018 that human pancreatic islets are susceptible to pharmaceutically induced ferroptosis. The aim of our study was to gain closer insight into the mechanism of ferroptosis in pancreatic islets on a cellular and functional level and thereby identify potential interventional strategies.

Materials and Methods

Islet isolation

Pancreatic islets were isolated from female Wistar rats according to guidelines established by the University of Dresden Institutional Animal Care and Use Committee. Animals were euthanized with CO_2. Afterwards, the abdomen was opened, and the pancreatic duct was clamped at the papilla. The digestion solution (1 mg/ml collagenase V (Sigma-Aldrich) and 100 µg/ml DNase (Roche)) was injected *in situ* into the pancreas via the bile duct. The pancreas was carefully dissected and transferred to digestion solution. The digestion was supported by gentle shaking at 37°C for approximately 12 min. After adding cold washing solution (RPMI 5.5 mM glucose, 10% FBS), the digest was filtered through a cell strainer with a pore size of 600 µm (Sigma-Aldrich) and centrifuged for 1 min (277g, Acc6, Dec6, 4°C). The washing procedure was repeated three times. Pancreatic islets were then separated from exocrine tissue by discontinuous density gradient centrifugation (15 min, 1590g, Acc2, Dec2, 4°C) using Ficoll (Sigma-Aldrich) density layers of 1.125 g/cm³, 1.096 g/cm³, 1.08 g/cm³ and 1.06 g/cm³. The interfaces containing the purified islets were collected, washed and cultured in 5.5 mM glucose RPMI 1640 (PAA) supplemented with 10% FBS, 20 mM HEPES, 1x penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere prior to further experimentation.

Islet yield and purity

Dithizone (DTZ), a zinc chelating agent, is known to selectively stain the islets of Langerhans in the pancreas. While exocrine tissue is not stained, the endocrine part appears red. For determination of the islet yield islet particle number (IP) and islet equivalents (IEQ, islet volume normalized to 150 µm diameter) were determined. To this end, representative triplicate samples were stained with dithizone (2% w/v DTZ in 0.25% v/v DMSO in phosphate-buffered saline (PBS)) and examined using an inverted microscope equipped with a 10x objective and eyepiece micrometer. All islets with a diameter >50 µm were grouped into diameter classes of 50 µm segments (i.e., 50–100, 100–150, 150–200, etc.). Each diameter class was converted into the mean volume of 150-µm diameter islets by a relative conversion factor. This method enables the evaluation of the total IEQ number and total IP number of each preparation. The purity and morphology were described descriptively using light microscopy observation at 100X magnification.

Islet viability

Islet viability was assessed by double staining with fluorescein diacetate (FDA) and propidium iodide (PI). FDA is a non-fluorescent molecule, which is hydrolysed to green-fluorescent fluorescein in live cells. Dead cells cannot accumulate or hydrolyse FDA. PI only permeates

through the membrane of dead cells resulting in red fluorescence. In detail, islets were washed with PBS and both agents, FDA and PI, were added at a final concentration of 0.5 and 75 µM, respectively. Samples were incubated in the dark for 5 min and evaluated using an inverted fluorescence microscope (100x magnification). For each sample, 100 islets were individually analyzed by calculating the percentage of non-viable cells (red) and viable cells (green). Islets were grouped into 5 categories (0%, 25%, 50%, 75%, and 100% viability) and islet viability was calculated**.**

Glucose-stimulated insulin secretion

The functional secretory capacity of pancreatic islets was analyzed by glucose-stimulated insulin secretion. Therefore, islets were transferred to Modified Krebs Ringer Buffer (MKRB) and equilibrated at 3.3 mM glucose at 37°C for 30 min. Afterwards, islets were divided into two groups containing fresh MKRB with either 3.3 mM or 16.7 mM glucose and incubated for 1 h in a gently shaking water bath at 37°C. For both conditions, five samples containing ten islets each were used. After incubation, islets were collected by gentle centrifugation at 200 g for 1 min. Secreted insulin in the supernatants was measured by ELISA (Mercodia Insulin Elisa Kit). Pellets were resuspended in 200 µl PBS and analyzed for DNA content determined by DNeasy Kit (Qiagen). Stimulation Index (SI) is calculated as the ratio of secreted insulin in high glucose in comparison to low glucose, both normalized to DNA content (SI = [$Insulin_{stim}$/ total DNA_{stim}] / [$Insulin_{res}$ /total DNA_{res}].

Lipid peroxidation

Lipid peroxidation is the degradation of lipids that occurs as a result of oxidative damage, typically by reactive oxygen species, and is a useful marker for oxidative stress. The peroxidation process leads to the production of malondialdehyde (MDA), which can be measured using the Malondialdehyde Microplate Assay Kit (Cohesion Biosiences). The lipid peroxidation was determined by the reaction of MDA with thiobarbituric acid (TBA) to generate the MDA-TBA adduct. The MDA-TBA adduct was quantified colorimetrically (λ = 532 nm and 600 nm). MDA levels were calculated according to the manufacturer's instruction.

Zinc and iron measurement

For the Inductively coupled plasma mass spectrometry analysis (ICP-MS), the islets were thawed and centrifuged. The supernatant was discarded, and the pellet resuspended in 200 µl 50 µM NaOH. For the physical cell lysis, the islets were drawn up six times with a syringe and spread out again. Then 50 µl of each sample was used for protein quantification. The remaining 150 µl was placed in a concentrator at 60°C for 4 h. Afterwards, 200 µl HNO_3 was

added to resuspend the cell extract by thorough vortexing. To complete the digestion, the samples were heated for further 4 h at 80°C.
After digestion, the samples were cooled to room temperature overnight. 2.6 ml of HPLC water was added to the samples and the iron concentration was analyzed by ICP-MS.

Islet treatment with Fer-1 over a period of 7 days

After isolation, all islets were aliquoted equally into two treatment groups: (i) culture media with vehicle (DMSO) as solvent control and (ii) culture media with 10 µM Fer-1 (Ferrostatin-1, Merck Millipore, 341494). Readout assays were performed on days 3, 5, and 7.

RSL3 dilution series

After isolation, all islets were aliquoted equally into five groups with increasing concentrations of RSL3 (5 µM, 10 µM, 15 µM, 20 µM, 40 µM; RSL3, type 2 FIN, Selleck Chemicals, S8155) and a solvent control with DMSO. After 24 h FDA/PI assay was performed, and viability was determined.

Ferroptosis inhibition

Islets were divided equally into four treatment groups: (i) culture media with vehicle (DMSO) as solvent control, (ii) culture media with a single treatment of 10 µM Fer-1 and day 0, (iii) culture media with a single treatment of 20 µM RSL3 on day 1, (iiii) culture media with a combination of 10 µM Fer-1 on day 0 and 20 µM RSL3 on day 1. The day of isolation was defined as day 0. All readouts were performed on day 2 after isolation.

Immunohistochemical analysis

For immunohistochemical analysis, 80-100 islets were fixed in 4% paraformaldehyde (PFA) for at least 1 hr, washed with PBS and embedded in tissue-Tek O.C.T. (Sakura Finetek). Embedded islets were sliced by cryosectioning in 6 µM thin sections onto microscope slides. Sections were rehydrated and washed with PBS. Antigen retrieval was performed with PBS with 3% Triton for 15 min. After blocking non-specific antibody binding sites with background sniper (Biocare Medical) for 11 min at room temperature, the sections were incubated at 4°C with primary antibodies (Insulin/Glucagon/Somatostatin/ACSL4/TUNEL) diluted in PBS with 0,2%Triton, 2% BSA and 2% goat serum overnight. Sections were washed with PBS in 0.5% Tween. Secondary antibodies together with 4′,6-diamidino-2-phenylindole (DAPI) were diluted in PBS with 0,2%Triton,2% BSA, and 2% goat serum. After incubation for 1 h, the sections were finally washed with PBS and 0.5% Tween. Immunofluorescence microscopy was performed using Zeiss Axiovert200M with AxioCamMRc5.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8. For comparison between groups, ordinary one-way-ANOVA ($p<0.05$) test with Tukey´s multiple comparison was used. Results were shown as mean ± SEM from N=X independent experiments.

Results

RSL3 causes massive islet death in a dose-dependent manner and pretreatment with Fer-1 can rescue islet viability and function

We observed that ferroptosis induction with increasing concentrations of RSL3 resulted in a significant reduction in islet viability (Fig. 1 A). At concentrations of 10 µM RSL3, islet viability was reduced to 40.3% compared to 77.3% in the control group. Induction of ferroptosis with 40 µM RSL3 had a fatal effect on islet viability. In addition, TUNEL-staining was performed to detect apoptotic cell death by labeling fragmented DNA and differentiate between apoptotic and ferroptotic cell death (Fig. 5 A). Treatment with 10 µM RSL3 and Fer-1 alone and in combination did not cause an increase in TUNEL positive cells compared to the control group.

The viability of islets challenged with 20 µM RSL3 could effectively be preserved by pretreatment with 10 µM Fer-1 for 24 h (Fig. 1 B). Similar effects were observed regarding islet function (Fig. 2). Pretreatment with Fer-1 increased functional potency compared to RSL3 induction alone. Interestingly, single treatment with Fer-1 led to a reduction of islet stimulation Index (7.25 compared to 20.04 in the control group).

Treatment with Fer-1 alone did not impact on islet survival, viability or islet architecture

To determine whether ferroptotic cell death occurs as a common cell death mechanism in cultured rodent islets, the culture time was prolonged to seven days. We observed that treatment with Fer-1 alone over 7 days had no impact on islet viability (Fig. 3). In order to prove whether the sustained islet viability is the result of a positive selection process during islet culture, or whether Fer-1 has a direct effect on the survival of the islets in culture, we examined cell survival over the period of seven days (quantification of IP; Fig. 3). Both groups showed a steady decrease in IP/ml without significant difference.

With immunohistochemical analysis we could demonstrate that the prolonged treatment with Fer-1 (Fig. 5 B) did not influence islet architecture.

RSL3 and Fer-1 influence the intracellular iron, and zinc concentration of cultured rodent islets

In order to elucidate intracellular changes during ferroptosis, the intracellular iron and zinc concentrations were measured. Culturing of the islets with the ferroptosis activator RSL3 increased the iron concentration to 0.88 ± 0.17 nmol/mg protein compared to 0.59 ± 0.05 nmol/mg protein (Fig. 4 A). Interestingly, pretreatment with the ferroptosis inhibitor Fer-1 could significantly reverse this effect by almost 50% and resulted in an iron concentration of 0.42 ± 0.03 nmol/mg protein. Furthermore, Fer1 treatment showed a slight reduction of the iron concentration in contrast to control. Several studies also describe ferroptosis induction by zinc, so we decided to measure intracellular zinc concentration in addition to intracellular iron concentration [12,13].

Similar to the effect on iron concentrations, a treatment of the islets with RSL3 alone significantly increased the zinc concentration to 2.00 ± 0.30 nmol/mg protein in contrast to control islets with 1.10 ± 0.03 nmol/mg protein. A pretreatment of RSL3 challenged islets with Fer-1 could reduce the zinc concentration of the islets to 1.56 ± 0.05 nmol/mg protein (Fig. 4 A).

Cell death induction by RSL3 causes an increase in biomarkers of lipid peroxidation in ferroptosis

Since there is no specific detection method of ferroptosis, we measured the malondialdehyde (MDA) concentration as a surrogate parameter. MDA occurs as an endproduct of ferroptotic lipid peroxidation (Fig. 4 B). Furthermore, the expression of ACSL4 (Fig.6) was determined as a membrane enzyme that incorporates arachidonate lipid acids into the lipid membrane and extremely sensitive to ferroptotic lipid peroxidation [2,3,14]. RSL3 challenged islets showed an increased MDA concentration to 3.49 ± 0.79 nmol/mg compared to 1.63 ± 0.10 nmol/mg

protein in the control group. Pretreatment with Fer-1 (10 µM) was able to attenuate RSL3 induced MDA concentrations to 2.27 ± 0.06 nmol/mg protein.

Immunostainings were performed to visualize the expression of ACSL4 in the lipid membrane of the islets (Fig. 6). All imaged islets showing a positive staining for ACSL4, independently of the treatment group. However, 10 µM RSL3 enhanced ACSL4 protein level as indicated by a strong and pronounced signal compared to control group. Whereas islets treated with 10 µM Fer-1 alone or Fer-1 and RSL3 in combination led to reduced ACSL4 protein level compared to the control group.

Discussion

The aim of the study was to better understand ferroptosis mechanisms in pancreatic islets and thereby identify potential targets for protecting the islets from cell death in the context of transplantation. Minimizing and preventing cell death processes in islet transplantation to improve islet graft function has long been in the focus of islet research. The process of preparation of the pancreas, followed by enzymatic and physical isolation of the islets, leads to cellular stress with increasing radicals.[15,16] These could induce ferroptosis at any stage of preparation and transplantation. We know that apoptotic cell death peaks 5 days after culture and mainly affects beta cells. [17] For ferroptosis, it is still unclear at which stage it occurs, but Desferroxamine (DFO) has been shown to improve islet and graft function.

Since ferroptosis requires available cellular iron, we and others have suggested that ferroptosis plays at least some role for the functional limitations of islets after isolation and transplantation. [15]

Along this line, Bruni *et al*. first discovered that pancreatic islets were sensitive to RSL3-induced ferroptosis [6].

The current study addressed further, the significance of the ferroptosis pathway and the potential of its inhibition on isolated pancreatic islets in a primary cell culture system. We focused on the cellular changes caused by RSL3 and the subsequent induction of ferroptotic

cell death as well as the effects of Fer-1 on pancreatic islets. While the literature is replete on the mechanism of action of RSL3 and Fer-1 on ferroptosis for several cell types, that for pancreatic islets is just evolving [5,18]. The current study showed that pharmaceutical induction of cell death by RSL3 causes an increase in typical ferroptosis-associated biomarkers such as iron, MDA and the membrane protein ACSL4. The findings on the features and the mechanism of ferroptosis in pancreatic islets are consistent with the observation in other cell types [1,12,13]. Moreover, we showed that Fer-1 attenuated the toxic consequences of RLS3 and thus protect the islets from ferroptosis. Moreover, TUNEL-staining with RSL3-challenged islets did not show an increase in apoptosis thereby clearly differentiated between apoptosis and ferroptosis cell death processes. Our results allow for better understanding the effects of ferroptosis in islets and to differentiate more precisely between ferroptosis and other cell death mechanisms such as apoptosis. In our *in vitro* setting, islets did not show significant benefit due to the treatment with Fer-1 alone. Neither islet viability nor survival did improve due to the treatment with Fer-1 alone. Hence, islet isolation and culture per se seem not to substantially induce ferroptosis. However, islet function was indeed impaired due to treatment with Fer-1. As Fer-1 accumulates in the membrane of the endoplasmic reticulum (ER) one could speculate that Fer-1 is anchored in the ER membrane and thereby deteriorates islet function [19] Anchoring Fer-1 in the ER membrane may reduce the function of the ER lipid membrane, which causes a reduction of insulin maturation and misfolded proinsulin. This could be one explanation of the reduced insulin stimulation capacity of Fer1 treated islets in contrast to control islets.

However, in the setting of intraportal islet transplantation with known drawbacks associated with hypoxic and inflammatory environmental conditions [20], it seems likely, that protective mechanisms of ferroptosis mediated through Fer-1 may have a relevant positive effect. Moreover, hepatocytes are the major iron storage in the human body, and this could further affect the impact of ferroptosis (and its inhibition) on pancreatic islet grafts [21].

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