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Research Paper

Age-related oxidative changes in pancreatic islets are predominantly located in the vascular system

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ABSTRACT

Aged tissues usually show a decreased regenerative capacity accompanied by a decline in functionality. During aging pancreatic islets also undergo several morphological and metabolic changes. Besides proliferative and regenerative limitations, endocrine cells lose their secretory capacity, contributing to a decline in functional islet mass and a deregulated glucose homeostasis. This is linked to several features of aging, such as induction of cellular senescence or the formation of modified proteins, such as advanced glycation end products (AGEs) - the latter mainly examined in relation to hyperglycemia and in disease models. However, age-related changes of endocrine islets under normoglycemic and non-pathologic conditions are poorly investigated. Therefore, a characterization of pancreatic tissue sections as wells as plasma samples of wild-type mice (C57BL/6J) at various age groups (2.5, 5, 10, 15, 21 months) was performed. Our findings reveal that mice at older age are able to secret sufficient amounts of insulin to maintain normoglycemia. During aging the pancreatic islet area increased and the islet size doubled in 21 months old mice when compared to 2.5 months old mice, whereas the islet number was unchanged. This was accompanied by an age-dependent decrease in Ki-67 levels and pancreatic duodenal homeobox-1 (PDX-1), indicating a decline in proliferative and regenerative capacity of pancreatic islets with advancing age. In contrast, the number of $p16^{Ink4a}$-positive nuclei within the islets was elevated starting from 10 months of age. Interestingly, AGEs accumulated exclusively in the islet blood vessels of old mice associated with increased amounts of inflammatory markers, such as the inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine (3-NT). In summary, the age-related increase in islet size and area was associated with the induction of senescence, accompanied by an accumulation of non-enzymatically modified proteins in the islet vascular system.

1. Introduction

Pancreatic islets represent a network of endocrine cells, basically divided into two major subgroups (β-cells and non-β-cells). In rodents, β-cells are the most common cell type of the endocrine pancreas (up to 85%) and form the center of the islet. They are surrounded by the non-β-cell fraction (α-, δ-, ε-, and pancreatic polypeptide cells) and penetrated by a large number of blood vessels. In contrast, the human islet architecture exhibits a heterogeneous distribution of endocrine cells, but this remains a matter of discussion. The main function of pancreatic islets is the secretion of hormones (insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide), essential for the maintenance of homeostatic processes [1–4]. During aging, the endocrine pancreas undergoes morphological and metabolic changes, contributing to an inappropriate regulation of glucose levels. These changes mostly affect the insulin-producing β-cells, whereas in other cell types, only a few modifications were observed [5]. The pancreatic β-cell mass, basically representing the islet mass in rodents, declines with age, induced by an imbalance in β-cell turnover (decreased proliferation and replication, elevated apoptosis). This is accompanied by an increase in β-cell dysfunction, together leading to an overall reduction in functional β-cell mass [6–11].

Abbreviations: 3-NT, 3-Nitrotyrosine; AGE(s), advanced glycation end product(s); IF, immunofluorescence; IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; RT, room temperature

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It has been shown that the limitation of the proliferative and replicative capacity of β-cells during aging correlates with the induction of senescence, activated by the transcriptional upregulation of cell cycle inhibitors, such as $p16^{Ink4a}$, preventing the cell cycle entry [12–15]. In addition, Helman and colleagues were recently able to show that an increased expression of $p16^{Ink4a}$ enhances the insulin secretory capacity of β-cells in advanced age, which is in contrast to the previous literature [16–18]. Further well-known age-related changes include accumulation of non-enzymatic modified proteins, such as glycation (formation of advanced glycation endproducts, AGEs), oxidation or nitration of proteins [19–22].

AGEs are formed as products of the Maillard reaction, or precursors are generated as intermediates of glycolysis and lipid peroxidation. Additionally, it is suggested that the development of AGE deposits is accelerated mainly under hyperglycemic conditions and contributes to diabetic complications. However, AGE formation also occurs in normal aging [23–25]. By binding the receptor for advanced glycation endproducts (*RAGE*), AGEs induce the production of reactive oxygen species (ROS) by activating enzymatic processes. This causes a proinflammatory response mediated by the transcription factor Nuclear-factor Kappa B (NFκB) [26–28]. In addition, peroxynitrite as a product of the proinflammatory response is formed, facilitating protein nitration [29,30]. Since age-related changes in pancreatic islets and their major cell type (β-cells) are associated with the amount of circulating glucose, AGE formation and related processes were mainly investigated under hyperglycemic and disease conditions.

Here, we characterized pancreatic islets of wild-type mice (C57BL/6) at various age groups to describe age-related alterations of endocrine islets. C57BL/6J is a widely used inbred strain susceptible to polygenic obesity, type 2 diabetes and atherosclerosis. The observed expansion of islets was associated with the induction of senescence and the maintenance of insulin secretory capacity sufficient for metabolic demand. Additionally, our data show that glycated as well as nitrated proteins are also formed in normal aging, independent of hyperglycemic conditions. We also found that this modified proteins accumulate exclusively in the vascular system of the endocrine pancreas.

2. Material and methods

2.1. Experimental model

Male C57BL/6J mice (2.5, 5, 10, 15 and 21 months) from The Jackson Laboratory were housed in a controlled environment at a temperature of 20 ± 2 °C, with a 12/12 h light/dark cycle and obtained a standard diet (Ssniff, Soest, Germany) as well as water ad libitum. Blood samples were collected before sacrificing the mice, cooled on ice and centrifuged. Subsequently, pancreatic tissues were isolated and fixed in 4% paraformaldehyde solution for 24 h, followed by paraffin embedding according to standard procedures. Mice were kept in agreement with the National Institutes of Health guidelines for care and use of laboratory animals. All procedures are verified and approved by the ethics committee for animal welfare of the State Office Environment, Health, and Consumer Protection (State of Brandenburg, Germany).

2.2. Determination of blood glucose

Blood glucose levels were determined by using an automated analyzer (Cobas Mira S, Hoffmann-La Roche, Basel, Switzerland) and a commercially available reagent kit (Glucose HK CP, Horiba ABX Pentra, Montpellier, France). The method is based on a 2-step enzymatic reaction with Hexokinase followed by Glucose-6-phosphate-dehydrogenase leading to the quantifiable end product D-gluconate-6-phosphate.

2.3. Plasma insulin and proinsulin ELISA

The concentration of insulin and proinsulin in murine plasma was determined by using the Mouse High Range Insulin ELISA (ALPCO, Salem, USA) and carried out according to the manufacturer's instructions.

2.4. Immunohistochemistry and immunofluorescence

Longitudinal serial sections (2 µm) were processed for immunohistochemistry (IHC) and immunofluorescent (IF) analysis. The sections were de-paraffinized and re-hydrated in Roti-Histol (Carl Roth, Karlsruhe, Germany) and decreasing serial solutions of ethanol. Heat-mediated antigen retrieval was performed by placing the slides in citrate-buffer (10 mM citrate acid, 0.05% Tween 20 in distilled water) for 20 min at 95–99 °C in a water bath, followed by a cooling step of 15 min at room temperature (RT). Pancreatic tissue samples were incubated with blocking solution (Antibody Diluent, Agilent, Waldbronn, Germany) containing 10% goat serum for 1 h. For IHC, sections were blocked with 0.03% hydrogen peroxide (Peroxidase block; Agilent, Waldbronn, Germany) for 10 min at RT. Sections were incubated with primary antibodies, diluted in blocking solution, for 1 h in a lightproof humidified chamber at RT. Rabbit anti-insulin antibody (ab181547, Abcam, Cambridge, United Kingdom), rabbit anti-Ki67 antibody (ab16667, Abcam Cambridge, United Kingdom), mouse Methylglyoxal-AGE (Arg-Pyrimidine) (AGE06B, BiLogo, Kiel, Germany), mouse anti-methylglyoxal antibody (MG-H1) (STA-011-CB, BioCat, Heidelberg, Germany), and anti-pentosidine antibody (PEN012, BiLogo, Kiel, Germany) were used for IHC staining of Insulin, Ki-67, Methylglyoxal-derived AGEs and pentosidine, followed by a 30-min incubation with HRP-labeled polymer. Before mounting with Entellan (Merck Millipore, Darmstadt, Germany), tissue sections were incubated with substrate-chromogen solution, 3,3′-Diaminobenzidin (EnVision+ system-HRP, Agilent Waldbronn, Germany) and counterstained with hematoxylin (Sigma-Aldrich, Taufkirchen, Germany). Rabbit anti-PDX-1 antibody (07-696, Merck Millipore, Darmstadt, Germany), mouse anti-CDKN2A/$p16^{INK4a}$ antibody (ab54210, Abcam, Cambridge, United Kingdom), rabbit anti-iNOS antibody (ab178945, Abcam, Cambridge, United Kingdom) and mouse anti-3-Nitrotyrosine antibody (ab110282, Abcam, Cambridge, United Kingdom) were used as primary antibodies for IF staining of PDX-1, $p16^{Ink4a}$, iNOS and 3-NT. All pancreatic slices were co-stained with mouse or rabbit anti-insulin antibodies (L6B10, Cell Signaling, Cambridge, United Kingdom; ab181547, Abcam, Cambridge, United Kingdom) to visualize the β-cell area. Visualization was performed by incubation with secondary antibodies conjugated to AlexaFluor 488 and 594 (Invitrogen, Darmstadt, Germany) and FluorCare including DAPI (Carl Roth, Karlsruhe, Germany) was used as mounting media.

2.5. Quantitative analysis of pancreatic islets

Microscopic analysis was performed by digital imaging of pancreatic sections using an Olympus IX53 microscope (Olympus, Hamburg, Germany) for IHC or Zeiss LSM 780 confocal microscope (Zeiss, Jena, Germany) for IF. To count the number of positive stained nuclei (Ki-67, PDX-1, $p16^{Ink4a}$) and measure the positive stained area (iNOS, 3-NT, pentosidine, Arg-Pyrimidine, MG-H1) within the pancreatic islets for the morphometric analysis, Zeiss ZEN 2.3 imaging software (Zeiss, Jena, Germany) was used. Pancreata of 6–8 mice were used for quantification of each staining. At the time of tissue sectioning, weight of the pancreas was not measured routinely. Thus, the islet mass (pancreas weight x islet area) could not be determined. As equivalent marker the islet area was used (%-islet area/slide). Islet size is an absolute parameter in mm^2 (in the meta-data of the original microscopic image files, both the number of pixels is included and the area of a single pixel in μm^2). To measure islet area, number and size of islets,