Research Paper

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

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Abstract

Age-related oxidative changes in pancreatic islets are predominantly located in the vascular system. In rodents, the islet 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].

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 glycosylation 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 end-products (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 main-
pancreatic sections were stained with insulin, visualized with 3,3′-Diaminobenzidin and counterstained with hematoxylin as described above. Digital images of the entire pancreas were taken with a MIRA-X-MIDI Scanner (Zeiss, Jena, Germany). Total pancreatic and insulin positive area of each section was quantified with Zeiss ZEN 2.3 imaging software. The software evaluates size of single islets, overall islet size and islet number of a given image.

2.6. Statistical analysis

Statistical analysis was performed by using GraphPad Prism version 7.03 (La Jolla, CA, USA). All data presented in the figures are mean values ± SD. Differences between two groups were assessed by Student’s t-test and one-way ANOVA was used for multiple comparisons of more than two groups. Differences were considered as statistically significant, if p < 0.05 was reached.

3. Results

3.1. Morphological changes of murine pancreatic islets during aging

Aged C57BL/6J mice show increased body weights that remain stable after 10 months of age. In addition blood glucose and plasma insulin levels are unchanged in all age groups, indicating good health conditions also in old C57BL/6J mice. In contrast, the plasma proinsulin level decreases with age. Starting from 10 months of age the levels are significantly lower compared to the youngest mice (2.5 months). Consequently, the proinsulin-to-insulin ratio shifts from 1:5 in young (2.5 months) to 1:14 in old mice (21 months), suggesting a higher conversion rate of proinsulin to insulin in aging to maintain glucose homeostasis (Table 1).

In order to determine age-related morphological changes in whole pancreatic tissue, sections were IHC stained for insulin to visualize the insulin-positive area representing the islets. Islet area was defined as percentage of islet area within the whole pancreatic slice, whereas the islet size represents the area of single islets (in mm²). IHC analysis revealed an age-dependent increase in pancreatic islet size, starting from the age of 10 months. Additionally, islet size doubled when comparing 2.5 and 21 months old mice (Fig. 1A, B). Islet area was only increased in islets of mice aged 2.5 and 5 months (Fig. 2C). In summary, the decrease in Ki-67-positive nuclei is accompanied by reduced levels of the transcription factor PDX-1, and an age-related increase in p16^INK4a^ levels, indicating an association between the induction of cellular senescence and the expansion of pancreatic islet size with age.

3.2. Expansion of islet mass is associated with increased p16^INK4a^ levels

In a next step, replication and proliferation rate of islets was determined. Initially, pancreatic tissue sections were IF stained for the pancreatic duodenal homeobox protein-1 (PDX-1), a specific marker for β-cell replication, differentiation and survival and co-stained for insulin and DAPI. The number of positive-labeled nuclei within the insulin-positive area was quantified and indicated as percentage of the entire nuclei. As shown in Fig. 2A, the relative number of PDX-1-positive β-cells decreases with advancing age. At 10, 15 and 21 months of age, a decline of 25% compared to 2.5 months and 15% compared to 5 months old mice was found. In addition to PDX-1, tissue sections were stained with Ki-67 confirming an age-dependent decrease in proliferation capacity. After 5 months of age the number of Ki-67-positive nuclei was reduced by 50% compared to 2.5 months old mice. Moreover, Ki-67-levels of 15 months old mice were lowered by further 30% (Fig. 2B). At 21 months of age, the average of Ki-67-positive nuclei was approximately 0.3%.

The age-dependent decline in proliferative capacity of pancreatic islets has been previously correlated with the transcriptional activation of the cell cycle inhibitor protein p16^INK4a^, reflecting the induction of cellular senescence [12]. Thus, to investigate the potential connection between proliferation and senescence, pancreatic tissue sections were IF labeled for p16^INK4a^ and co-stained for insulin and DAPI. As expected, a higher number of p16^INK4a^-positive nuclei within the insulin-positive area of islets were observed with advancing age. After 10 months of age, mice showed elevated levels of p16^INK4a^ compared to animals aged 2.5 and 5 months (Fig. 2C). In summary, the decrease in Ki-67-positive nuclei is accompanied by reduced levels of the transcription factor PDX-1, and an age-related increase in p16^INK4a^ levels, indicating an association between the induction of cellular senescence and the expansion of pancreatic islet size with age.

3.3. AGE formation and nitric oxide production increase with age in the vascular system

Besides cellular senescence and proliferative modifications, aging is also associated with the accumulation of AGEs [31,32]. Therefore, different AGEs were analyzed by IHC staining of pancreatic tissue sections. Comparing 2.5 and 21 months old mice, a 9.5-fold increase for methylglyoxal-derived Arg-Pyrimidine was observed, MG-H1 increased 8-fold (Fig. 3A, B). Equally, the levels of the 3-deoxyglucosone-formed pentosidine were higher in the old mice (Fig. 3C). Interestingly, the age-related formation of all analyzed AGEs was not prominent in endocrine cells but rather located in the blood vessels of the pancreatic islets (Fig. 3D). In addition to glycation, nitration of proteins is another important non-enzymatic modification in aging [19]. In order to investigate this, iNOS and 3-NT-levels were determined by IF labeling. iNOS and 3-NT-levels of old C57BL/6J mice were also increased and located in the blood vessels of the endocrine islets comparable to AGE accumulation (Fig. 4A, B). Altogether, these results suggest that the age-dependent formation of AGEs is associated with higher amounts of nitrated proteins in the vascular system of the pancreatic islets.

4. Discussion

In general, aging affects the endocrine pancreas by a decline in islet turnover [10,33]. This contributes to a decreased islet mass and functionality, associated with deregulated glucose utilization and...
hyperglycemia [34–36]. In contrast, our data show that pancreatic islets of normal aged wild-type C57BL/6J mice were able to secrete adequate amounts of insulin to compensate the metabolic demand, thus preventing hyperglycemic conditions. This was accompanied by an age-dependent increase in islet area and size, whereas the islet number was unchanged, reflecting an expansion of the entire islet mass. Similar results were found by Sone and Kagawa and Tschen et al., both showing an adaptive increase in beta cell and islet mass in aging rodents under standard conditions [37,38]. Several reasons, mainly self-renewal and growth of β-cells as well as replication and differentiation of pancreatic progenitor cells, were used to explain the age-induced expansion of the pancreatic islets [39–41]. Here, the differentiation capacity of murine islets was determined by using the major pancreatic transcription factor, PDX-1, showing a decline with advancing age. This was directly associated with an age-dependent decrease in islet proliferation rate under normoglycemic conditions indicated by Ki-67. Our findings are in agreement with previous investigations, revealing that β-cells of normal aged mice show low PDX-1 expression and a decreased proliferative capacity [7,38]. Additionally, the age-related proliferative limitation of pancreatic islets correlates with the increased expression of the cell cycle regulator p16\(^{ink4a}\), shown by Krishnamurthy and colleagues [12]. By blocking the cell cycle due to the inhibition of the cyclin-dependent kinases 4 and 6, p16\(^{ink4a}\) induces senescence and restrains the proliferative capacity of cells [13,42]. According to that, the decreased proliferation shown in the present study was also accompanied by increased p16\(^{ink4a}\)-levels with advancing age. Consequently, the p16\(^{ink4a}\)-induced reduction of cell cycle progression contributes to limited regenerative potential of pancreatic islets in old mice. However, given that islet expansion continues until advanced age, these results suggest that endocrine cells have a relatively long lifespan and the reduced growth rate seems to be sufficient to maintain an increase in islet size [43,44]. Another factor, possibly contributing to an increase in islet size, is the senescence-induced structural and functional reprogramming of cells. By increasing protein and RNA content including an overproduction of cytoskeleton and membrane proteins, such as vimentin and caveolin-1, senescent cells are known to enlarge [45–47]. Furthermore, a recent investigation [16] revealed a beneficial role of the senescence inducer p16\(^{ink4a}\) towards an increase in β-cell functionality possibly responsible for the maintenance of glucose homeostasis by generating adequate amounts of insulin as seen in this study.

In contrast, it has been reported that functional impairments of pancreatic β-cells with age are associated with the formation of AGEs [48]. Zhao et al. and Coughlan et al. showed that circulating AGEs are associated with a decline in insulin secretory capacity of β-cells, mainly mediated by impaired mitochondrial functionality [49,50]. In addition, it has been shown by Puddu et al. that AGEs downregulate the protein expression of PDX-1 [51]. Thus, the observed reduction in PDX-1 levels together with the accumulation of AGEs in advanced age indicates that AGEs may contribute to the decline in regenerative potential of pancreatic islets. AGEs are formed under hyperglycemic conditions, but occur also as part of the normal aging process and contribute to complications in age-related diseases [50,52]. Here, we observed an accumulation of AGEs, such as pentosidine, Arg-Pyrimidine and MG-H1, in old mice confirming their increased formation at an advanced age. Interestingly, AGEs were found only in the blood vessels of the pancreatic islet, contrary to a recent finding [53]. Our observations demonstrate a local limitation of AGE deposits within the vascular system of the endocrine pancreas. In accordance, other investigations demonstrate an AGE accumulation in the vessel wall [54–57]. Extracellular matrix proteins, especially their most common form collagen, constitute the scaffold of the vascular system. Due to the slow turnover of collagen, this structural protein increases constantly with age [58–60]. Moreover, the formation of cross-linking products such as pentosidine diminishes the protein turnover contributing to AGE accumulation as well as
vascular stiffening [61–63]. In addition to protein modifications, AGEs directly mediate their detrimental effects by binding to their major receptor, RAGE that is expressed, among others on the surface of endothelial cells [64,65]. It has been shown that this interaction activates the NADPH oxidase and causes intracellular ROS production [27,66]. As a feedback mechanism, the formation of AGEs is increased followed by an activation of NF-κB and its downstream pathways [67]. It was shown in different cell types that both processes contribute to the induction of iNOS expression [68–70]. This promotes the formation of nitric oxide able to react with superoxide anions to form peroxynitrite, leading to the production of nitrated proteins such as 3-NT [30,71,72]. This is in accordance with our findings showing that age-related generation of AGEs is accompanied by higher levels of iNOS in the blood vessels. Furthermore, we observed an accumulation of nitrated proteins, quantified via the typical product 3-NT. This modification was again only found in the blood vessels of pancreatic islets, indicating an association between the formation of AGEs and the generation of nitrosative stress in the vascular system of the endocrine pancreas during aging.

In summary our data show an age-related expansion of endocrine islets associated with increased p16<sup>INK4a</sup>-levels and the induction of cellular senescence. This is accompanied by an accumulation of AGEs and nitrated proteins occurring exclusively in the islet vascular system of normal aged wild-type C57BL/6J mice. Further investigations with isolated islets are necessary, to unravel the mechanism behind these age-related changes.

**Declaration of interest**

The authors declare that there is no conflict of interest associated with this manuscript.
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Fig. 3. Formation of AGEs in pancreatic islets of 2.5 and 21 months old C57BL/6 mice. Quantitative analysis of IHC stained (A) Arg-Pyrimidine, (B) MG-H1 and (C) pentosidine. (D) Representative images of each staining in a magnification of 20× and 60× (inset). For better visibility, white lines mark the islet area. Data are presented as mean values ± SD. Statistical significance was assessed by student’s t-test (unpaired), *p < 0.05.

Fig. 4. Induction of nitrosative stress and nitration products in pancreatic islets of 2.5 and 21 months old C57BL/6J mice. Quantitative analysis and representative images of IF labeled (A) iNOS and (B) 3-NT sections. For better visibility of iNOS and 3-NT, the islets are outlined in green (according to insulin staining, not shown). White arrows show iNOS and 3-NT positive stainings. Green: islet-outline, red: iNOS/3-NT, blue: DAPI. Data are presented as mean values ± SD. Statistical significance was assessed by student’s t-test (unpaired), *p < 0.05.
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