In-exendin uptake in the pancreas correlates with the beta cell mass and not with the alpha cell mass

Short running title: In-exendin and alpha cell mass

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Abstract

Targeting of the Glucagon-like peptide 1 receptor with $^{111}$In-labeled exendin is an attractive approach to determine the beta cell mass (BCM). Preclinical studies as well as a proof-of-concept study in type 1 diabetic patients and healthy subjects showed a direct correlation between BCM and radiotracer uptake. Despite these promising initial results, the influence of alpha cells on the uptake of the radiotracer remains a matter of debate. In this study we determined the correlation between pancreatic tracer uptake and beta and alpha cell mass in a rat model for beta cell loss. The uptake of $^{111}$In-exendin (%ID/g) showed a strong positive linear correlation with the BCM (Pearson $r = 0.82$). The fraction of glucagon positive cells in the total endocrine mass was increased after alloxan treatment (26% ± 4%, 43% ± 8%, and 69% ±21% for 0, 45 and 60 mg/kg alloxan, respectively). The uptake of $^{111}$In-exendin showed a negative linear correlation with the alpha cell fraction (Pearson $r = -0.76$). These data clearly indicate towards specificity of $^{111}$In-exendin for beta cells and that the influence of the alpha cells on $^{111}$In-exendin uptake is negligible.
Introduction

The role of the beta cell mass in the development and progression of type 1 and type 2 diabetes remains poorly understood. Our knowledge is mainly based on autopsy studies and studies in pancreatic specimen obtained in patients undergoing pancreatectomy [1], representing information obtained at only one point in time during the course of the disease without further follow-up. Therefore, a method enabling longitudinal non-invasive determination of the beta cell mass would represent a major breakthrough for diabetes research as it would allow to better elucidate the pathophysiology underlying the development of both types of diabetes [2]. Such a non-invasive imaging technology for determination of the beta cell mass in vivo has been developed based on a radiolabeled GLP-1 analog (\(^{111}\)In-exendin). We have previously demonstrated that \(^{111}\)In-exendin uptake in the pancreas correlates linearly with the beta cell mass in a rat model for beta cell loss and the first clinical proof-of-principal revealed a clearly reduced uptake of the radiotracer in the pancreas of long-standing T1D patients as compared to healthy volunteers [3]. Despite these promising initial preclinical and clinical results, the specificity of radiolabeled exendin towards beta cells has been a matter of debate. Although relevant GLP-1R expression in the exocrine pancreas was claimed [4-6], recent studies showed only low expression in acinar cells and no expression in ductal cells, while high GLP-1R expression could be demonstrated in the pancreatic islets, as determined by in vitro autoradiography with \(^{125}\)I-GLP-1 [7] and by immunohistochemistry with an extensively validated anti-GLP-1R antibody [8]. We have recently shown by ex vivo autoradiography that \(^{111}\)In-exendin specifically accumulates in the islets of Langerhans of rats and that GLP-1R mRNA expression is much higher in the islets compared to the exocrine pancreas. Moreover, the endocrine:exocrine ratio is even more favorable in humans than in rats in which the validity of the method for non-invasive determination of BCM has been demonstrated [9]. Although these data clearly indicate towards specificity of \(^{111}\)In-exendin
accumulation in islets, they do not rule out the potential influence of other endocrine cells on the accumulation of the tracer. One concern is the presence of the GLP-1R on alpha cells, reported in some studies [10-12], although other studies contradict these findings [13-15]. The expression of the GLP-1R on alpha cells could potentially lead to accumulation of $^{111}$In-exendin in the alpha cells and thus to an overestimation of the beta cell mass as determined by this technique. To date, the influence of the presence of the GLP-1R on alpha cells on the uptake of $^{111}$In-exendin in the endocrine pancreas has not been studied. Therefore, we have examined the contribution of the alpha cell mass on the accumulation of radiolabeled exendin in a rat model of alloxan induced beta cell loss. The alpha and beta cell mass, determined by morphometric analysis, was compared with the uptake of $^{111}$In-exendin in healthy and alloxan-induced diabetic rats.
Materials and Methods

Radiolabeling

Radiolabeling and quality control of [Lys\textsuperscript{40}(DTPA)]exendin-3 (Peptide Specialty Laboratories, Heidelberg, Germany) with \textsuperscript{111}InCl\textsubscript{3} (Mallinckrodt Medical, Petten, The Netherlands) was carried out as previously described [16].

Animals, alloxan treatment and biodistribution studies

Female Brown Norway rats of 6-8 weeks were purchased from Harlan (Horst, The Netherlands). Animal experiments were performed after approval of the local ethical committee for animal experiments (RUDEC). Alloxan was injected intravenously (45 or 60 mg/kg, n=4 per group) as previously described [3]. A separate group was injected with vehicle only as a control (n=4).

One week after alloxan injection, rats were injected intravenously with 15 MBq \textsuperscript{111}In-exendin (peptide dose: 0.1 µg/rat). One hour after \textsuperscript{111}In-exendin administration the rats were euthanized, the pancreas was dissected, weighed and fixed in formalin. The radioactivity concentration in the pancreas was measured using an automated well-type gamma counter (Wallac 1480-Wizard, Perkin-Elmer, Boston, MA, USA) and the uptake of \textsuperscript{111}In-exendin was calculated and expressed as the percentage of the administered dose per gram of tissue (%ID/g).

Histology and determination of the beta- and alpha cell mass

After 48 h fixation in formalin pancreata were embedded in paraffin and 4-µm sections were cut at 3 levels 100 µm apart. One section of each level was stained for insulin and the beta cell mass was determined by morphometric analysis as previous described [3]. A consecutive
section was stained for glucagon: the sections were rehydrated using xylene for 10 min and washed with xylene, washed twice with 100% ethanol, 75% ethanol, 50% ethanol and water. Antigen retrieval was performed by a microwave treatment in 10 mM sodiumcitrate buffer, pH 6.0, for 10 min. Endogenous peroxidase activity was blocked by 10 min incubation with 3 % H₂O₂ in PBS. The sections were washed twice with demineralized water and three times with PBS and incubated with 5% normal goat serum (Bodinco, Alkmaar, The Netherlands) for 30 min. After removal of the goat serum the sections were washed three times with PBS and incubated with 50 µl anti-glucagon antibody (1:500 diluted in PBS containing 1% BSA w/v) (#2760, Cell Signaling, Leiden, The Netherlands) for 60 min. After washing three times with PBS, 50 µl goat-anti-rabbit IgG-biotin-conjugate (Vector, Burlingame, CA, USA) diluted in PBS containing 1% BSA (1:200) was added and incubated for 30 min. The sections were washed three times with PBS and the sections were incubated with 50 µl ABC-complex ( Vectastain, ABC kit Elite Vector, Burlingame, CA, USA) for 30 min. The bound anti-glucagon antibody was visualized using diaminobenzidine (Bright DAB, Sigma, St. Louis, MO, USA) as a chromogen after washing three times with PBS. All slides were counterstained with hematoxylin and mounted with mounting fluid (Permount, Fisher Scientific, Waltham, MA, USA). All steps were performed at room temperature in the dark. The absolute alpha cell mass was determined analogous with the beta cell mass determination described above. The relative alpha cell mass was calculated by dividing the alpha cell mass by the total endocrine mass (beta cell and alpha cell mass).

**Statistical analysis**

All mean values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using unpaired two-tailed unpaired t-test using GraphPad Prism version 5.00 for
Windows (GraphPad Software, San Diego, California, USA). The level of significance was set at \( p < 0.05 \).

Correlation between the BCM and pancreatic uptake was determined by the Pearson correlation coefficient \( (r) \) using two-tailed analysis of variance with GraphPad Prism. The level of significance was set at \( p < 0.05 \).
Results

Histology
In the pancreas of healthy rats scarce glucagon staining was observed at the periphery (Figure 1A) and abundant insulin staining in the core of the islets (Figure 1B). In alloxan-treated rats the number of insulin-positive cells per islet was markedly reduced (Figure 1D) and the islets mainly consist of glucagon-positive cells (Figure 1C).

Alpha and beta cell mass and $^{111}$In-exendin uptake
The total mass of the endocrine pancreas was reduced in the alloxan treated rats (8.5 ± 1.9 mg, 5.5 ± 1.7 mg, and 2.8 ± 0.8 mg for 0, 45 and 60 mg/kg alloxan, respectively). The loss of endocrine mass is due to beta cell loss in alloxan treated rats (BCM: 6.3 ± 1.7 mg in healthy rats, 3.2 ± 1.5 and 1.0 ± 0.9 in rats treated with 45 and 60 mg/kg, respectively (Figure 2A)), since the absolute alpha cell mass was not significantly different in rats treated with 45 or 60 mg/kg alloxan (2.2 ± 0.4, 2.3 ± 0.3 mg and 1.8 ± 0.3 mg (Figure 2B) for healthy, 45 mg/kg and 60 mg/kg alloxan, respectively (p=0.81 and p=0.21, respectively)). Although the absolute alpha cell mass did not change, the relative alpha cell fraction (represented as the percentage of alpha cells of the total endocrine mass) was increased as a result of beta cell loss (and thereby loss of total endocrine mass) when rats were treated with 45 or 60 mg/kg alloxan (43% ± 8% and 69% ± 21%, respectively versus 26% ± 4% in healthy rats, Figure 2C). The pancreatic uptake of $^{111}$In-exendin showed a linear correlation with the BCM (Pearson r = 0.82, p<0.005, Figure 3A). There was no significant correlation between the absolute alpha cell mass and $^{111}$In-exendin uptake in the pancreas (Pearson r = 0.18, p=0.59, Figure 3B). The relative alpha cell fraction (fraction of alpha cell of the total endocrine mass) showed a
negative linear correlation with pancreatic $^{111}$In-exendin uptake (Pearson $r = -0.76$, $p<0.01$, Figure 3C).
Discussion

In this study we examined the contribution of the alpha cells on the uptake of $^{111}$In-exendin, a radiotracer potentially enabling non-invasive determination of beta cell mass, in a rat model of diabetes. We demonstrated that there is no correlation between the absolute alpha cell mass and pancreatic tracer uptake and a negative linear correlation between the relative alpha cell mass and $^{111}$In-exendin uptake, indicating a negligible influence of the alpha cells on tracer accumulation in the pancreas.

The expression of the GLP-1R on alpha cells remains a matter of debate. Specific in vitro binding of $^{125}$I-labeled GLP-1 to insulin and glucagon immunoreactive cells was found by combined autoradiography and immunohistochemical analysis of pancreatic tissue specimens of rats [11]. A later study confirmed these data by showing that a small portion of the alpha cells express the GLP-1R as determined by RT-PCR and immunohistochemical analysis [10]. However, several other studies showed that the GLP-1R is specifically expressed in beta cells in mice, rats and humans and not in alpha cells [13-15]. Another study showed that GLP-1 has a direct inhibitory effect on glucagon excretion by alpha cells, but that the GLP-1R is only expressed on approximately 1% of the alpha cells [12]. In a more recent study in which a highly specific anti-GLP-1R antibody was validated, the specificity of most (commercially) available GLP-1R antibodies was questioned [8]. Therefore, based on the results presented in the literature it remains a matter of debate whether the GLP-1R is expressed on alpha cells. It should be noted that in the case of peptide-based tracers, such as exendin, the tracer accumulation is not only dependent on the receptor expression density but also the internalization rate [17], meaning that if the expression of the GLP-1R on alpha cells or the internalization rate is low, the receptor expression itself might have a negligible influence on the accumulation of $^{111}$In-exendin in the islets.
The aim of our study was not to demonstrate the presence or absence of the GLP-1R on alpha cells, but to evaluate the potential influence of the presence of alpha cells in the islets of Langerhans on the accumulation of $^{111}$In-exendin in the pancreas. Our findings indicate that, even if GLP-1 receptors are present on alpha cells, they only exert a minor influence on $^{111}$In-exendin uptake. This further supports the idea that measurement of the pancreatic uptake of $^{111}$In-exendin can indeed serve as an imaging biomarker for beta cell mass.

Not only the beta cell mass, but also the number of other endocrine cells might change during the progression of diabetes. Non-invasive methods to longitudinally determine the endocrine cell mass and confirmation might provide important information of the role of these cells in the pathogenesis of diabetes. Recently, Eriksson et al. showed that accumulation of the serotonin receptor tracer [$^{11}$C]5-HTP is reduced in the pancreas of T1D patients as compared to healthy volunteers implying that this tracer could also be a useful non-invasive marker to determine the total mass of endocrine cells in the pancreas [18]. Since the serotonin receptor is expressed in all endocrine cells, a combination of [$^{11}$C]5-HTP imaging of serotonin activity and $^{111}$In-exendin imaging could provide useful complementary information about the beta cell and total endocrine mass as well as changes in endocrine cell conformation during the development of diabetes.

In conclusion, we have demonstrated that the uptake of $^{111}$In-exendin correlated with beta cell mass, and not with alpha cell mass. The present data clearly indicate the specificity of $^{111}$In-exendin for beta cells and a negligible influence of the alpha cells on pancreatic $^{111}$In-exendin uptake. This observation further supports the idea that $^{111}$In-exendin uptake may indeed be a measure for beta cell mass in vivo.
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Author contribution: M.B. researched data and wrote the manuscript. L.J. and C.F. researched data and reviewed/edited manuscript. O.B. and M.G. contributed to the discussion and reviewed edited manuscript. M.G. is responsible for the overall integrity of the study.

Conflict of interest: MG is consultant for Boehringer Ingelheim and is patent holder in the field. The other authors have no conflicts of interest to declare.

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References

**Figure legends**

**Figure 1:** Immunohistochemical staining of pancreatic sections for glucagon (A and C) and insulin (B and D) of healthy (A and B) rats and rats treated with 60 mg/kg alloxan (C and D). In healthy rats scarce glucagon staining in the periphery of the islet (A) and abundant insulin staining in the core of the islet (B) is observed. In severely diabetic rats the islets consist predominately of glucagon producing cells (C) and only a few insulin positive cells could be observed (D).

**Figure 2:** Absolute beta (A) and alpha (B) cell mass in control and alloxan treated Brown Norway rats. The relative alpha cell fraction (C) was determined by dividing the absolute alpha cell mass by the sum of the absolute alpha and beta cell mass.

**Figure 3:** Correlation between the pancreatic uptake of $^{111}$In-exendin and the absolute beta cell mass (A. uptake on y-axis in percentage of the injected dose per gram of tissue (%ID/g), BCM in mg on x-axis as determined by morphometric analysis after immunohistochemical staining with anti-insulin antibody). The correlation as determined by Pearson test is $r = 0.82$. No correlation between tracer uptake and absolute alpha cell mass was observed (B) Pearson $r = 0.18$. A negative correlation (Pearson $r = -0.76$) was shown between the pancreatic $^{111}$In-exendin uptake and the relative alpha cell fraction calculated by dividing the alpha cell mass by the total endocrine mass (C). Circles = control rats, squares = rats treated with 45 mg/kg alloxan and triangles = rats treated with 45 mg/kg alloxan.
Figure 1
563x400mm (72 x 72 DPI)
Figure 2
88x354mm (300 x 300 DPI)
Figure 3
88x243mm (300 x 300 DPI)