Glucagon-like-peptide-1 receptor expression in normal and diseased human thyroid and pancreas

Beatrice Waser, Annika Blank, Eva Karamitopoulou, Aurel Perren and Jean C Reubi

¹Cell Biology and Experimental Cancer Research, Institute of Pathology, University of Berne, Berne, Switzerland

Glucagon-like-peptide-1 (GLP1) analogs may induce thyroid or pancreatic diseases in animals, raising questions about their use in diabetic patients. There is, however, controversy regarding expression of GLP1 receptors (GLP1R) in human normal and diseased thyroid and pancreas. Here, 221 human thyroid and pancreas samples were analyzed for GLP1R immunohistochemistry and compared with quantitative *in vitro* GLP1R autoradiography. Neither normal nor hyperplastic human thyroids containing parafollicular C cells express GLP1R with either method. Papillary thyroid cancer do not, and medullary thyroid carcinomas rarely express GLP1R. Insulin- and somatostatin-producing cells in the normal pancreas express a high density of GLP1R, whereas acinar cells express them in low amounts. Ductal epithelial cells do not express GLP1R. All benign insulinomas express high densities of GLP1R, whereas malignant insulinomas rarely express them. All ductal pancreatic carcinomas are GLP1R negative, whereas 6/20 PanIN 1/2 and 0/12 PanIN 3 express GLP1R. Therefore, normal thyroid, including normal and hyperplastic C cells, or papillary thyroid cancer are not targets for GLP1 analogs in humans. Conversely, all pancreatic insulin- and somatostatin-producing cells are physiological GLP1 targets, as well as most acini. As normal ductal epithelial cells or PanIN 3 or ductal pancreatic carcinomas do not express GLP1R, it seems unlikely that GLP1R is related to neoplastic transformation in pancreas. GLP1R-positive medullary thyroid carcinomas and all benign insulinomas are candidates for *in vivo* GLP1R targeting.

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The receptors for the gut hormone glucagon-like-peptide-1 (GLP1) are physiologically expressed in the endocrine pancreas as well as in very large quantities in specific endocrine tumors such as benign insulinomas.^{1–3} These receptors have increasing clinical importance as, on one hand, they mediate the action of commercially available GLP1 analogs such as exenatide or liraglutide to help treat diabetes patients^{4–6} and, on the other hand, represent the targets for radiolabeled GLP1 or exendin to visualize *in vivo* insulinoma tumors.^{7–10}

In recent years, there have been numerous discussions about potential problems with incretin used for diabetes treatment as it may induce thyroid and/or pancreas pathologies.^{11–13} On one hand, it

was observed in preclinical animal studies that a significant number of animals developed medullary thyroid carcinomas after long-term treatment with GLP1 analogs.^{14–16} On the other hand, there have been reports suggesting that GLP1 analogs may induce pancreatitis or pancreatic cancers.^{12,17,18} These studies are presently taken very seriously and weighted with regard to the benefits of incretin therapy in diabetes.^{11,19,20} It is important to find a consensus in understanding the molecular mechanisms of the postulated pathologies and to know to which extent the animal data can be extrapolated.¹¹

An important prerequisite is to assess exactly the GLP1 receptor (GLP1R) expression in human thyroid and pancreas, as the presence of these receptors in these tissues may be the molecular basis for the deleterious actions of GLP1 analogs. Unfortunately, to date, the various reports on that subject do not provide a clear consensus.^{11,13}

There have been early studies by Körner *et al*² that found species differences for GLP1R expression in thyroids and lungs, with a much higher expression

Correspondence: Professor JC Reubi, MD, Cell Biology and Experimental Cancer Research, Institute of Pathology, University of Berne, PO Box 62, Murtenstrasse 31, CH-3010 Berne, Switzerland.

E-mail: reubi@pathology.unibe.ch

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in the thyroid and lungs of rat and mouse than in the corresponding human organs. According to these *in vitro* receptor autoradiography studies, no GLP1Rs were detected in normal human thyroids, whereas C cells of normal rat and mice thyroids expressed GLP1R. Moreover, rat thyroids with C-cell hyperplasia as well as two rat medullary thyroid carcinoma tumor models showed a high density of GLP1R in all tested cases.²¹ In humans, however, using the same method, approximately 1/3 of the cases of medullary thyroid carcinomas, but none of the normal thyroids, had GLP1R.^{2,21} These species differences were confirmed by Bjerre-Knudsen *et al*¹⁵ and Boess *et al*.¹⁶ A subsequent immunohistochemical study by Gier *et al*²² in human thyroid tissues, however, has suggested that normal human thyroid and various thyroid pathologies can often express GLP1R. The authors concluded that caution was necessary when treating patients with incretins. Unfortunately, the immunohistochemistry protocol and the antibody used in the mentioned study did not appear to have the necessary specificity for GLP1R immunohistochemistry in human tissues;²³ a recent paper challenged the specificity of the used antibody,¹¹ questioning the validity of the data of this study.

The situation is not much clearer in the pancreas. Although all authors agree for the presence of GLP1R in the pancreatic islets, there are differences at the level of ducts: whereas Körner *et al*,² Pyke *et al*,²⁴ and Drucker,¹¹ using GLP1R autoradiography, immunohistochemistry, or mRNA analysis, respectively, were unable to detect GLP1R in human ducts, Gier *et al*²⁵ found such receptors in rat and human pancreatic ducts by immunohistochemistry. In addition, ductal pancreatic cancers were found positive for GLP1R by the same group using immunohistochemistry,²⁵ whereas Körner *et al*² found no such receptors in pancreatic ductal cancers with receptor autoradiography. Although these discrepancies may be because of the different techniques used to measure the receptor protein, namely autoradiography vs immunohistochemistry, they may also be related to the antibodies used and their level of characterization and validation, as mentioned by Drucker¹¹ and Pyke *et al*.^{23,24}

We have therefore evaluated GLP1R in human thyroid and pancreas tissues using an extensively validated GLP1R monoclonal antibody²⁴ that we used for immunohistochemistry and western blots and that we compared with results of GLP1R autoradiography in the same tissues. In addition to normal thyroid and pancreas, thyroids with C-cell hyperplasia, chronically inflamed pancreas, and also neoplastic thyroid tissues, namely medullary thyroid carcinomas and papillary thyroid carcinomas, as well as neoplastic pancreas tissues, including pancreatic neuroendocrine tumors, ductal pancreatic carcinomas, and pancreatic intraepithelial neoplasia (PanIN), were tested.

Materials and methods

Tissues

A total of 221 formalin-fixed human thyroid and pancreatic tissues (see Tables 1 and 2) were used in this study: normal thyroids ($n=8$), thyroids with C-cell hyperplasia ($n=8$), papillary thyroid carcinomas ($n=8$), sporadic medullary thyroid carcinomas ($n=10$), familial (MEN 2) medullary thyroid carcinomas ($n=10$), chronic pancreatitis tissues ($n=9$), benign insulinomas ($n=31$), malignant insulinomas ($n=7$), pancreatic nonfunctioning NET ($n=10$), ductal pancreatic carcinomas ($n=88$), PanIN 1/2 ($n=20$), and PanIN 3 ($n=12$). From this series, 19 insulinomas, 57 ductal pancreatic carcinomas, and 26 PanIN were tested solely on tissue microarrays. All above-mentioned tissues were used for GLP1R immunohistochemistry and, in all cases with available fresh-frozen samples, also for autoradiography and western blotting (see Tables 1 and 2). The sample collection conformed to the ethical guidelines of the Institute of Pathology, University of Berne, and was reviewed by the Institutional Review of Board (Kantonale Ethik Kommission).

Immunohistochemistry

Immunohistochemistry for calcitonin, synaptophysin, insulin, glucagon, somatostatin, and pancreatic polypeptide (PP) was performed on formalin-fixed tissues. The antibodies were purchased as follow: calcitonin (NCL CALp) from Leica (Newcastle, UK); synaptophysin (MO 776) from Leica (Newcastle, UK), insulin (I-2018) from Sigma (Buchs, Switzerland), glucagon from Sigma (Buchs, Switzerland), somatostatin from Dako (Baar, Switzerland), and PP (RP030) from Diagnostic BioSystem (Pleasanton, CA, USA).

GLP1R Immunohistochemistry

For single-label immunohistochemistry DAB (Diaminobenzidine) staining, paraffin sections were dewaxed and rehydrated to H₂O. Sections were microwave treated for 8 min at 900 W and 30 min at 360 W in Tris-EDTA buffer, pH 9.0, were allowed to cool for 15 min, rinsed in Tris-buffered saline (TBS), pH 7.5, and blocked in 3% H₂O₂ with 0.15 M NaN₃. Slides were incubated overnight with the primary GLP1R monoclonal mouse antibody 7 µg/ml (MAB 3F52; provided by Novo Nordisk, Copenhagen, Denmark) diluted in TBS containing 1% casein, 5% normal goat serum, and 0.1% NaN₃. After a wash in TBS for 10 min, slides were incubated for 45 min in Flex+Linker mouse (Dako) or Post Primary (Leica, Zürich, Switzerland), then washed again in TBS, and incubated for 45 min in Flex+HRP (Dako) or Polymer (Leica). Another wash was performed and the slides were developed with DAB (Sigma) and counterstained with HE, rinsed in water, and mounted with Aquatex.

Table 1 GLP1R in human thyroid tissues

		GLP1R IHC (positive cases vs total n)		Comments
		In glandular tissue	In C cells (identified by calcitonin IHC)	
Normal thyroid	N = 8 (from normal thyroid samples)	0/8	0/8	(Five cases tested by ARG and confirmed as GLP1R negative)
	N = 4 (from hyperplastic thyroid samples)	0/4	0/4	
	N = 7 (from papillary thyroid carcinoma samples)	0/7	0/1	
	N = 4 (from medullary thyroid carcinoma samples)	0/4	0/1	
C-cell hyperplasia	N = 8	0/8	0/8	Five cases tested by ARG and confirmed as GLP1R negative
Papillary thyroid carcinoma	N = 8		In tumor cells 0/8	
Sporadic MTC	N = 10		2/10	
MEN 2 MTC	N = 10		0/10	One case tested by ARG and confirmed as GLP1R positive

Abbreviations: ARG, GLP1R autoradiography; IHC, immunohistochemistry; MTC, medullary thyroid carcinoma.

Western Blotting

Approximately 50–100 mg fresh-frozen tumors were cut into small pieces, partially thawed, and washed in ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride, incubated for 30 min at 4 °C in ice-cold lysis buffer (20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 3 mM EGTA, and 4 mg/ml dodecyl β -D-maltoside (pH 7.6)) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml soybean trypsin inhibitor, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 50 μ g/ml bacitracin), and homogenized in a tissue lyser Miller Mix for 2 min at 20 U/s. The lysate was subsequently clarified by centrifugation at 16 000 g and 4 °C for 30 min and subjected to partial purification by wheat germ agglutinin-agarose treatment. The adsorbed glycoproteins were eluted at 37 °C for 30 min with sample buffer (400 mM Tris-HCl, 10% SDS, 0.5 M 2-mercaptoethanol, 50% glycerol, and 0.025% Bromophenolblau) and resolved on a 10% SDS-polyacrylamide gel. Resolved proteins were transferred to polyvinylidene difluoride membrane. The membrane was blocked for 2 h with 5% nonfat dry milk in TBS-Tween (TBS-T: 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.4)), and then incubated overnight at 4 °C with 1.78 μ g/ml GLP1R-specific antibody (MAb 3F52)²⁴ in TBS-T. Immunoreactive proteins were detected with a sheep antimouse IgG conjugated with horseradish peroxidase (1:2000) in TBS-T, and the ECL chemiluminescent antibody detection system (GE Healthcare, Little Chalfont, UK).

In Vitro GLP1R Autoradiography

The *in vitro* GLP1R autoradiography was carried out as described previously.^{2,21} Thick frozen tissue

sections (20 μ m) were incubated for 2 h at room temperature in the incubation solution containing 170 mM Tris-HCl buffer (pH 8.2), 1% bovine serum albumin (BSA), 40 μ g/ml bacitracin, 10 mM MgCl₂, and 15 000 c.p.m./100 μ l ¹²⁵I-GLP1(7–36)amide (2000 Ci/mmol; Anawa, Wangen, Switzerland). Nonspecific binding was determined by incubating tissue sections in the incubation solution containing additionally 100 nM unlabeled GLP1(7–36)amide (Bachem, Bubendorf, Switzerland) that, at this concentration, completely and specifically displaces ¹²⁵I-GLP1(7–36)amide at the receptors. After incubation, the slides were washed five times in ice-cold Tris-HCl buffer (170 mM; pH 8.2) containing 0.25% BSA and twice in ice-cold Tris-HCl buffer without BSA. The slides were dried for 15 min under a stream of cold air and exposed to Kodak Biomax MR films for 7 days at 4 °C. The signals were analyzed in correlation with morphology using corresponding HE-stained tissue slides. The receptor density was quantitatively assessed using tissue standards for iodinated compounds (Amersham, Aylesbury, UK) and a computer-assisted image processing system (Analysis Imaging System, Interfocus, Mering, Germany).

Results

Controls

The detailed sequential description of the figures is given in the legends. Figure 1 depicts two GLP1R-expressing human control tissues as complementary validation of the GLP1R immunoreactivity, as revealed by the MAb 3F52 (Figure 1a and e), together with GLP1R *in situ* ligand binding as shown by autoradiography (Figure 1b–d and f–h). Two established human GLP1 targets, the duodenal

Table 2 GLP1R in human pancreatic tissues

<i>Nonneoplastic tissues</i>	<i>Number of cases</i>	<i>GLP1R IHC (positive cases vs total n)</i>		<i>Comments</i>
Pancreatic islets		<i>In normal islets</i>		
	N = 12 (from insulinoma samples)	12/12		
	N = 9 (from chronic pancreatitis samples)	9/9		
	N = 11 (from ductal pancreatic carcinoma samples)	11/11		
Pancreatic acini		<i>In normal acini</i>		
	N = 12 (from insulinoma samples)	10/12		
	N = 9 (from chronic pancreatitis samples)	3/9		
	N = 11 (from ductal pancreatic carcinoma samples)	2/11		
Pancreatic ducts		<i>In normal ductal epithelial cells</i>	<i>In ductal neuroendocrine cells</i>	
	N = 10 (from insulinoma samples)	0/10	7/10	
	N = 9 (from chronic pancreatitis samples)	0/9	7/9	
	N = 11 (from ductal pancreatic carcinoma samples)	0/11	9/11	
<i>Neoplastic tissues</i>		<i>In tumor cells</i>		
Benign insulinomas	N = 31	31/31		14 Cases tested by ARG and confirmed as GLP1R positive
Malignant insulinomas	N = 7	1/7		Three cases tested by ARG and confirmed as 1 positive, 2 negative
Pancreatic nonfunctioning NET	N = 10	0/10		
Ductal pancreatic carcinomas	N = 88	0/88		Eight carcinomas tested by ARG and confirmed as GLP1R negative
PanIN 1/2	N = 20	6/20		1/7 GLP1R positive by ARG
PanIN 3	N = 12	0/12		0/7 GLP1R positive by ARG

Abbreviations: ARG, GLP1R autoradiography; IHC, immunohistochemistry.

Brunner glands (Figure 1a–d) and the pancreatic islets (Figure 1e–h), known to be GLP1R positive with receptor autoradiography, are also found GLP1R positive by immunohistochemistry (Figure 1a and e). Most importantly, the immunostaining of these tissues is strongly membrane bound (insets, Figure 1a and e). Such a membranous staining is expected as G protein-coupled receptors (GPCRs; GLP1R is a GPCR) are defined as cell surface receptors. In the present study, we considered membranous staining to be an obligatory feature of GLP1R-specific immunoreactivity, whereas diffuse intracellular staining was not considered to be specific for GLP1R.

Thyroid

As shown in Table 1, none of the human thyroid samples with normal or hyperplastic C cells were found positive for GLP1R, neither in the glandular tissue nor in the C cells that were identified by calcitonin immunohistochemistry. These results

were independent of the type of thyroid pathology present in the surroundings of the normal thyroid tissue (Table 1).

Figure 2a–d (normal thyroid) and Figure 2h–k (thyroid and C-cell hyperplasia) illustrate that none of the C cells identified by calcitonin immunohistochemistry were GLP1R positive. Figure 2e–g documents that the normal thyroid is also GLP1R negative by autoradiography.

The nine papillary thyroid carcinomas did not express GLP1R (Figure 2l–p), neither by immunohistochemistry nor by autoradiography in five cases where both formalin-fixed and fresh-frozen tissues were available; the tumor cells in 2/10 sporadic medullary thyroid carcinomas were GLP1R positive by immunohistochemistry (Table 1). The receptor distribution was not homogenous in the tumor sample, as seen in Figure 2q and r. The inset in Figure 2r shows that the GLP1R are membrane bound. Figure 2s–u shows that this particular tumor was also positive in GLP1R autoradiography. Conversely, all 10 familial MEN 2 medullary thyroid

Figure 1 Comparison of GLP1R immunohistochemistry with ^{125}I -GLP1(7-36)-amide receptor autoradiography in two representative examples of GLP1R-expressing human tissues: the Brunner's glands (a-d) and the pancreatic islets (e-h). Brunner's gland: (a) Strong membranous GLP1R immunostaining of the glands. Bar = 0.1 mm. Insert shows strong membranous staining. (b-d) Receptor autoradiography on tissue sections of the same case showing the hematoxylin and eosin-stained tissue (bar = 0.1 mm) in (b), very strong total ^{125}I -GLP1(7-36)-amide binding to the glandular tissue in (c), and complete displacement of ^{125}I -GLP1(7-36)-amide by cold GLP1 in (d), providing evidence of specificity of GLP1R binding. There is an excellent correlation between strong GLP1R immunostaining and high ^{125}I -GLP1(7-36)-amide binding levels. Pancreatic islets: (e) GLP1R immunostaining of the islets (bar = 0.1 mm) is shown. Insert shows the strong membranous staining. This immunostaining corresponds well with the strong specific ^{125}I -GLP1(7-36)-amide binding to the pancreatic islets of the same case in the autoradiography experiment (f-h). (f) Synaptophysin immunostaining. Bar = 0.1 mm. (g) Total ^{125}I -GLP1(7-36)-amide binding. (h) Nonspecific binding.

carcinomas were GLP1R negative by immunohistochemistry (Table 1).

Pancreas

As shown in Table 2, all pancreatic islets were found to be GLP1R positive, irrespective of whether they originate from samples containing insulinoma, chronic pancreatitis, or ductal pancreatic carcinoma. As seen in Figure 3a and b, the GLP1R-expressing cells corresponded to insulin-producing cells. The GLP1Rs were located at the cell membrane (Figure 3a). In areas of the pancreas containing PP-expressing cells (Figure 3h), the GLP1Rs (Figure 3j) were not expressed in the PP cells but only in the few surrounding insulin cells (Figure 3i). The receptors are located at the cell membrane

(Figure 3k). Furthermore, although glucagon cells were not found receptor positive (Figure 3l and m), the somatostatin cells in the islets expressed GLP1R (Figure 3n and o). Moreover, single insulin-producing endocrine cells distributed within the pancreas (Figure 3u) were also GLP1R positive (Figure 3t).

A low density of GLP1R was found in the pancreatic acini (Table 2). Whereas the great majority of the samples had GLP1R-expressing acini (Figure 3c), their distribution was often heterogeneous. Rare samples even remained negative (Table 2; Figure 3a, at the periphery of the immunoreactive islet). As also seen in Figure 3c and d, the GLP1R in acini are membrane bound. The weak GLP1R immunohistochemical staining is compatible with the low GLP1R density seen in autoradiography (Figure 3e-g), where the acinar structure cannot be seen because of the low resolution of the technique. Interestingly, although the great majority of samples originating from insulinoma cases had GLP1R-expressing acini, the percentage of cases with positively immunostained acini was lower in samples originating from chronic pancreatitis or ductal pancreatic carcinoma (Table 2).

The normal pancreatic duct epithelial cells were always GLP1R negative, irrespective of whether the samples originated from insulinomas, chronic pancreatitis, or ductal pancreatic carcinoma samples. Conversely, single neuroendocrine cells in the ducts, identified by synaptophysin, chromogranin, or insulin staining, were frequently expressing GLP1R (Table 2). Figure 3p-s shows GLP1R-negative ducts. In an otherwise receptor-negative duct, there is a single GLP1R-expressing (Figure 3r) neuroendocrine insulin-secreting cell (Figure 3s). Finally, pancreatic stellate cells (Figure 3v) were always found to be GLP1R negative (Figure 3w).

Pancreatic Neoplasia

As expected from previous *in situ* ligand-binding studies,^{3,26} all 32 benign insulinomas were positive for GLP1R by immunostaining (Table 2). Figure 4a and b shows a representative case of benign insulinoma positively immunostained for GLP1R (Figure 4a). Virtually all cells are stained and the receptors are membrane bound. This illustrated case is also strongly GLP1R positive by autoradiography (Figure 4c-e). Conversely, only one of six malignant insulinomas was positive with GLP1R immunostaining. A typical malignant insulinoma is shown in Figure 4f-j that is negative for both GLP1R immunohistochemistry (Figure 4f) and autoradiography (Figure 4h-j).

Table 3 shows a summary of the semiquantitative GLP1R measurement by immunohistochemistry in all tested insulinomas. The results are expressed in four categories of staining intensity, as percentage of labeled cells, as reported previously.²⁷ These data

Figure 2 GLP1R immunohistochemistry in human nonneoplastic and neoplastic thyroid tissues as compared with GLP1R autoradiography. (a–d) Absence of GLP1R in the normal human thyroid. (a, c) Calcitonin immunohistochemistry shows normal C cells (arrow). Bars = 0.1 mm. (b, d) GLP1R immunostaining is negative in the adjacent serial tissue sections. A high magnification confirms that the two C cells (arrow) (c) do not express GLP1R (d). (e–g) GLP1R autoradiography showing absence of GLP1R in the normal human thyroid. (e) Hematoxylin and eosin staining. Bar = 1 mm. (f) Total binding. (g) Nonspecific binding. (h–k) Absence of GLP1R in C-cell hyperplasia of the thyroid. (h) Calcitonin immunohistochemistry shows a C-cell hyperplastic area (h) that is not immunostained for GLP1R (i). Bars = 0.1 mm. (j, k) At higher magnification, the calcitonin-stained hyperplastic C cells (j) do not immunostain for GLP1R (k). (l–p) Absence of GLP1R in papillary thyroid cancer (PTC). The calcitonin-negative (l) PTC is not immunoreactive for the GLP1R (m). Bars = 0.1 mm. GLP1R autoradiography (n–p) confirms absence of GLP1R in this same tumor case. (n) Hematoxylin and eosin staining. Bar = 1 mm. (o) Total binding. (p) Nonspecific binding. (q–u) GLP1Rs are expressed in a medullary thyroid carcinoma. The calcitonin immunopositive tumor (q) has a focal distribution of GLP1R detected by GLP1R immunohistochemistry (r) (bar = 0.1 mm); membranous staining is shown in the inset. GLP1R autoradiography (s–u) confirms the presence of GLP1R in this tumor. (s) Hematoxylin and eosin staining. Bar = 1 mm. (t) Total binding. (u) Nonspecific binding.

are compared with the quantitative detection of GLP1R by autoradiography, tested in those cases available with both formalin-fixed and fresh-frozen

material. The great majority of the benign insulinomas have a high density of GLP1R, as measured with both methods, whereas malignant insulinomas are

Figure 3 GLP1R immunohistochemistry in the normal human pancreas. GLP1R immunohistochemistry in a pancreatic islet (**a**) localized in the insulin-producing cells (**b**, insulin immunohistochemistry). This area of the sample has no measurable levels of GLP1R in acini. Bars = 0.01 mm. (**c**, **d**) GLP1R immunohistochemistry in the majority of pancreatic acinar cells. Strong membranous staining, also seen in the magnified illustration (**d**). Bars = 0.01 mm. (**e–g**) GLP1R autoradiography of the same pancreas as in (**c**, **d**). (**e**) Hematoxylin and eosin staining. Bar = 1 mm. (**f**) Total binding of ^{125}I -GLP1(7–36)-amide showing a diffuse weak binding in the acinar area and a strong binding in one islet (arrow). (**g**) Nonspecific binding. (**h–k**) GLP1R immunohistochemistry in a pancreatic polypeptide (PP)-rich area (delimited by black line in **h**, **i**, **j**) of the pancreas. (**h**) Immunoreactive PP. (**i**) Insulin immunohistochemistry on adjacent serial section. (**j**) GLP1R immunohistochemistry on adjacent section. Bars = 0.1 mm. GLP1Rs are localized in the insulin cells (arrows), and not in the PP cells. (**k**) The membranous GLP1R staining, magnified from (**j**). Bar = 0.01 mm. (**l**, **m**) Lack of GLP1R immunostaining (**m**; arrows) in glucagon-expressing islet cells (**l**; arrows). Bars = 0.1 mm. (**n**, **o**) GLP1R immunohistochemistry in somatostatin-expressing islet cells (**n**). All somatostatin cells have GLP1R (**o**). Bars = 0.1 mm. (**p**, **q**) Lack of GLP1R immunostaining in normal pancreatic ducts (**p**). Adjacent serial section with hematoxylin staining (**q**). Bars = 0.1 mm. (**r**, **s**) Endocrine cell positively immunostained for GLP1R in an otherwise receptor-negative duct (**r**). This cell is insulin producing (**s**). Bars = 0.1 mm. (**t**, **u**) Single cells in the pancreatic acinar area positively immunostained for GLP1R (**t**). These two cells are insulin producing (**u**). Bars = 0.01 mm. (**v**, **w**) Stellate cells of the human pancreas (area within the black line) do not express GLP1R. (**v**) Hematoxylin and eosin staining. (**w**) GLP1R immunohistochemistry. Bars = 0.01 mm.

usually negative (Table 3). To confirm that the reported immunohistochemistry of benign insulinomas with antibody MAb 3F52 identifies true GLP1R, a western blotting was performed in one of the immunohistochemistry-positive tumors for which fresh-frozen tissue was available. Figure 5a shows a single band at ~53 kDa corresponding to GLP1R in this benign insulinoma. The western blot in

Figure 5d shows a GLP1R-negative malignant insulinoma as negative control.

Pancreatic nonfunctioning neuroendocrine tumors were found to be all GLP1R negative (Table 2). Furthermore, all 88 ductal pancreatic carcinomas were found to be GLP1R negative by immunohistochemistry in the carcinoma cells. An example of an immunohistochemistry-negative ductal pancreatic

carcinoma is shown in Figure 4k and l, with confirmation of the lack of GLP1R by autoradiography (Figure 4m–o). A total of eight ductal carcinomas were tested for GLP1R autoradiography and were confirmed as GLP1R negative (Table 2). Interestingly, however, 6/32 cases (18%) of PanIN were GLP1R positive by immunohistochemistry (Table 1). The six positive PanIN were all diagnosed as PanIN 1 or 2; they belonged to patients with GLP1R-negative ductal pancreatic carcinomas. Two of the PanIN 1/2 receptor-positive samples had adjacent PanIN 3 areas that were GLP1R negative. The other 12 tested cases of PanIN 3 were all GLP1R negative (Table 2). Figure 4p and q shows a GLP1R-positive PanIN 1. From the 14 cases of PanIN tested by GLP1R autoradiography on cryostat sections, 1 of 7 cases of PanIN 1/2 stage was receptor positive, whereas 0 of 7 cases of PanIN 3 stage was positive (Table 2).

Discussion

The present report is based on an extensive immunohistochemical study of the GLP1R status in human thyroid and pancreatic tissues with a specific and well-validated monoclonal GLP1R antibody,²⁴ and also compares and further validates the obtained data with *in situ* ligand binding. Because of previous studies in this field using poorly characterized antibodies,^{22,25} we took particular care to use a thoroughly validated monoclonal antibody that specifically labels GLP1R-expressing cells while being negative for GLP1R-lacking cells,^{23,24} and that has a superior immunohistochemical specificity compared with several other (commercial) GLP1R antibodies.²³ As further proof of specificity and validation, we reported in the present study the results of specific GLP1R immunostaining in established human GLP1R targets such as endocrine pancreas or Brunner's gland and compared these results with the data of an alternative method of GLP1R measurement, namely GLP1R autoradiography in the same tissues. Furthermore, we confirmed that immunohistochemistry of human insulinoma identifies true GLP1R by demonstrating the presence of such receptors in western blots of the same tissue. Finally, we considered only membrane-bound GLP1R immunohistochemistry as truly specific, a basic request for the immunohisto-

chemical characterization of all GPCRs,²⁸ to which family the GLP1Rs also belong.

The present results show definitely that the normal human thyroid, in particular the C cells, do not express GLP1R. C-cell hyperplastic human thyroids do not express GLP1R either. This is now well documented with two different methods: immunohistochemistry (in the present study) or autoradiography.^{2,21} It confirms previous findings suggesting a strong species difference in the GLP1R expression in the thyroid, with high levels in normal and hyperplastic rat thyroids but with complete absence of such receptors in the human organ.^{2,21} The lack of GLP1R in the human thyroid in the present study is in contrast with the immunohistochemistry study by Gier *et al*²² that showed the presence of immunostained GLP1R in the human thyroid. The explanation for the discrepancy is likely to be that the antibody used by Gier *et al*²² was not sufficiently specific, therefore eliciting questionable results.^{11,23}

We could not find GLP1R in any of the human papillary thyroid carcinomas, neither by immunohistochemistry nor by autoradiography, again as opposed to Gier *et al*,²² who found immunoreactivity attributed to GLP1R in 18% of the cases, but based on an insufficiently validated immunohistochemical protocol.^{11,23}

Conversely, a minority of sporadic medullary thyroid carcinomas, namely 2/10 (20%), did express a measurable amount of GLP1R by immunohistochemistry in our study. Interestingly, the 10 familial medullary thyroid carcinomas of the MEN 2 type, however, were all found to be negative for these receptors. The low percentage of GLP1R-positive medullary thyroid carcinomas corresponds well to previous data obtained with autoradiography, with 28% positivity.^{2,21} These low numbers, however, differ markedly from the high incidence (11/12; 92%) found by Gier *et al*.²²

The present study indicates that not only insulin-producing cells in the islets express GLP1R, but that the latter can also be expressed by virtually all insulin-producing cells in the pancreas. This is true for the insulin cells in the PP-rich areas (the PP cells, however, are GLP1R negative); it is true for the single insulin cells distributed throughout the pancreas and it is also true for the insulin-producing endocrine cells localized in the pancreatic ducts.

Figure 4 GLP1R immunohistochemistry in neoplastic human pancreas tissues compared with GLP1R autoradiography in the same cases. (a–e) GLP1R-expressing benign insulinoma. (a) GLP1R immunohistochemistry showing strong membranous staining in all tumor cells. (b) Positive insulin immunohistochemistry in an adjacent serial section. Bars = 0.01 mm. (c–e) GLP1R autoradiography showing strong GLP1R expression in the whole tumor of the same case. (c) Hematoxylin and eosin staining. Bar = 1 mm. (d) Total binding. (e) Nonspecific binding. (f–j) GLP1R-negative malignant insulinoma. (f) GLP1R immunohistochemistry shows absence of GLP1R. (g) Insulin immunohistochemistry on adjacent serial section. Bars = 0.01 mm. (h–j) GLP1R autoradiography of the same case showing absence of GLP1R. (h) Hematoxylin and eosin staining. Bar = 1 mm. (i) Total binding. (j) Nonspecific binding. (k–o) GLP1R-negative pancreatic ductal carcinoma. (k) GLP1R immunohistochemistry shows absence of GLP1R. (l) Hematoxylin and eosin-stained adjacent section. Bars = 0.1 mm. (m–o) GLP1R autoradiography showing absence of GLP1R. (m) Hematoxylin and eosin staining. Bar = 1 mm. (n) Total binding. (o) Nonspecific binding. (p, q) PanIN 1 case positively immunostained for GLP1R (p). Membranous staining. (q) Hematoxylin and eosin stained adjacent serial section. Bars = 0.01 mm.

It is therefore probable that GLP1 will have a similar physiological role on insulin in the islet cells, as currently believed, as in all the other nonislet

insulin-producing pancreatic cells, through specific GLP1R. This statement can also be extended to the role of the GLP1-like drugs used for diabetes

Table 3 GLP1R in insulinomas: semiquantitative GLP1R immunohistochemistry and quantitative ARG in corresponding cases

Staining intensity ^a	GLP1R IHC			GLP1R ARG
	No. of cases	Membranous staining No. of cases	% Of positive cells (mean)	R density (d.p.m./mg tissues) (mean $\pm$ s.e.m.)
<i>Benign insulinomas</i>				
High receptor density/staining intensity (3 +)	15	15	97%	7132 $\pm$ 817 (<i>n</i> = 8)
Moderate receptor density/staining intensity (2 +)	11	11	83%	4590 $\pm$ 1443 (<i>n</i> = 5)
Low receptor density/staining intensity (1 +)	5	5	37%	3104 (<i>n</i> = 1)
No receptor/no staining	0	—	—	0
<i>Malignant insulinomas</i>				
High receptor density/staining intensity (3 +)	1	1	100%	6480
No receptor/no staining	2	—	—	0

Abbreviation: ARG, autoradiography.

^aStaining intensity according to Körner *et al.*²⁷.

treatment, targeted to these different types of insulin-producing cells. In addition to insulin-producing islet cells, the somatostatin-producing islet cells also express GLP1R, whereas glucagon-producing cells appear to lack such receptors.

As reported previously, we confirm autoradiography and immunohistochemistry data^{2,24} showing that human pancreatic acini do express GLP1R, although in much lower density than the insulin-producing cells. As opposed to the small population of GLP1-expressing endocrine cells, the fact that a large part of the pancreatic acinar population expresses GLP1R, even in low density, indicates that there is a considerable amount of nonendocrine GLP1R in the pancreas, for which a physiological role is not yet known. The presence of GLP1R in acini may jeopardize the effort to successfully perform imaging on β -cell mass using radiolabeled GLP1 analogs in the human pancreas.¹⁰

A marginal observation of this study is that acinar GLP1Rs seem to be reduced in chronic pancreatitis and in ductal pancreatic carcinoma samples as opposed to insulinoma samples. A similar observation was made previously with quantitative GLP1R autoradiography.² Interestingly, in an animal model of pancreatitis, it was recently found that, contrary to the present data, GLP1Rs were increased in acini and ducts.²⁹ This discrepancy may suggest once more that animal models may differ from settings in humans, making extrapolations from animals to humans difficult.^{11,21} Interestingly, Gier *et al.*²⁵ did not detect GLP1R in human acini with their immunohistochemistry protocol.

The pancreatic duct epithelial cells in any of the tested samples did not express GLP1R, with the exception of occasional endocrine insulin-producing cells. The absence of GLP1R is in agreement with the results of another immunohistochemistry study,²⁴ an autoradiography study,² and of an mRNA study,¹¹ but is in conflict with the study by Gier *et al.*²⁵ showing GLP1R in ducts by immunohistochemistry. As the validation of the antibody used in

the study by Gier *et al.*²⁵ was incomplete, we may assume that the convergent results of the four other studies showing absence of GLP1R in human duct epithelial cells correspond more closely to the reality. This implies that the normal ducts would not be a potential target for GLP1 drugs during diabetes therapy.

The tissues that by far express the highest levels of GLP1R are specific neuroendocrine tumors of the pancreas, the benign insulinomas. This was discovered a decade ago by receptor autoradiography.³ It led subsequently to the successful development of *in vivo* targeting of insulinomas by using radio-labeled exendin analogs.^{7–9} The present study shows for the first time that it is possible to perform immunohistochemistry to easily diagnose the presence of GLP1R in these tumors in formalin-fixed tissues. For specificity control and antibody validation, we directly compared GLP1R immunohistochemistry with quantitative GLP1R autoradiography in a large number of these insulinomas and further provided a western blot of one of the immunohistochemistry-positive insulinoma, revealing a single band corresponding to GLP1R. GLP1R immunohistochemistry with antibody MAb 3F52 may therefore become a useful tool to measure GLP1R in tissue samples of neuroendocrine tumors submitted for histopathologic examination, as it is already the case for somatostatin receptor immunohistochemistry in neuroendocrine tumors.²⁷ As also reported in the present study, there are considerable differences in GLP1R expression within pancreatic endocrine tumor types, with large amounts being present in benign insulinomas, a low incidence in malignant insulinomas, and a lack of GLP1R in nonfunctioning pancreatic neuroendocrine tumors.

Confirming previous GLP1R autoradiography data,² we did not find any ductal pancreatic carcinoma expressing GLP1R in the tumor cells. However, because Gier *et al.*²⁵ claimed that PanIN may express GLP1R in a large percentage of cases, we examined a large number of PanIN cases.

Figure 5 Western blot analysis providing evidence of the specificity of staining for GLP1R in a benign insulinoma (a), found to be strongly positive in autoradiography (b, total binding; c, nonspecific binding). There is a single broad band migrating at ~53 kb, the expected size of GLP1R. In the right column, as negative control, absence of a band (d) in a malignant insulinoma, also negative by autoradiography (e, f).

Whereas the present results show that 6/32 cases were GLP1R positive, representing 18% of all our PanIN cases, it should be stressed that the 6 positive cases were all of the PanIN 1/2 type, and that the 12 cases with PanIN 3 were all negative. As neither normal ducts nor PanIN 3 lesions nor pancreatic carcinoma cells express GLP1R in a measurable way, the GLP1R in PanIN 1/2 may be understood as a rare and probably transient phenomenon that is not necessarily involved in neoplastic transformation.

What do the findings of the present study mean with regard to the strategy of treating diabetes with GLP1 analogs? The absence of GLP1R in normal and

hyperplastic C cells of the thyroid clearly indicates that these tissues are unlikely targets for GLP1 analogs in humans. However, diabetic patients with concomitant sporadic medullary thyroid carcinomas that express GLP1R in 20–30% should probably be excluded from any incretin treatment for their diabetes. Such patients may be identified by *in vivo* imaging using radiolabeled exendin, a procedure also performed for the detection of insulinomas.^{7,8} Normal human pancreas as well as the chronically inflamed pancreas express GLP1R, as expected, primarily in the therapeutic targets of GLP1 analogs, namely the insulin-producing cells. The normal ducts, however, lack GLP1R and are therefore not a likely target for GLP1 analogs. The only other exocrine compartment with a consistent GLP1R expression is constituted by the acini, in which no clear function for GLP1 has been found up to now. Finally, the fact that normal ducts, ductal pancreatic carcinomas, or PanIN 3 do not express GLP1R indicate that GLP1Rs are unlikely to be related to neoplastic transformation in the pancreas.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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