ORIGINAL ARTICLE

Diabetes mellitus decreases the expression of calcitonin-gene related peptide, γ-amino butyric acid and glutamic acid decarboxylase in human pancreatic islet cells

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Abstract

OBJECTIVES: The pattern of distribution of calcitonin-gene related peptide (CGRP), a neuropeptide, γ-aminobutyric acid (GABA), a neurotransmitter and GABA-converting enzyme, glutamic acid decarboxylase (GAD) in the pancreas of diabetic patients was investigated to determine whether diabetes mellitus influences the expression of these biological transmitters.
METHODS: Pancreatic tissue samples retrieved, during pancreatectomy, from cancer patients with and without Type 2 diabetes were paraffin embedded. The expression of CGRP, GABA and GAD was examined in pancreatic tissue using immunofluorescence techniques.
RESULTS: CGRP, GABA and GAD were observed in many cells located in the central as well as the peripheral regions of pancreatic islet. The expression of CGRP, GABA and GAD decreased dramatically in pancreatic islet cells of diabetic patients compared to control. CGRP and GABA co-localized with glucagon in some pancreatic islet cells of both normal and diabetic patients. The pattern of distribution of CGRP, GABA and GAD in normal and Type 2 diabetic patients was similar to that of insulin.
CONCUSION: The number of human pancreatic islet cells expressing CGRP, GABA and GAD decreased significantly after the onset of Type 2 diabetes. These neuropeptides and neurotransmitters may play a role in the regulation of pancreatic beta cell function.

INTRODUCTION

Diabetes mellitus (DM) is one of the most common, non-infectious diseases, and is a prime cause of increased cardiovascular morbidity and mortality world wide (Mayor, 2007). It is characterized by hyperglycaemia, resulting from defects in insulin secretion, or action, or both (Mayor, 2007). Insulin, which is synthesized by β-cells of the endocrine pancreas, is controlled by many peptides within and outside of the islet of Langerhans. The tissue and plasma levels of these peptides and neurotransmitters may determine the level of insulin production and the viability of β-cells (Adeghate and Ponery, 2003; Mayor, 2007), hence they can be used as markers for early detection of DM.

Calcitonin gene-related peptide (CGRP) is produced in two isoforms (α and β) by an alternative splicing of calcitonin mRNA (Amara *et al.* 1982). Both α and β human CGRP are present in the central and peripheral nervous systems, and although they differ by three amino acids, they produce similar biological activity (Amara *et al.* 1982). CGRP controls many distinct physiological responses including actions on the cardiovascular system and the central nervous system, reproductive organs, skeletal muscles, calcium metabolism, insulin regulation, and gastric secretion (Di Angelantonio *et al.* 2003; Adeghate & Ponery, 2004). CGRP has been shown to be a potent and long-lasting vasodilator in many species including man. CGRP has also been implicated in the regulation of pain. This role is supported by the fact that substance P with an established role in the transmission of pain has been shown to co-localize with CGRP (Adeghate, 1999).

Gamma aminobutyric acid (GABA), a well-known inhibitory neurotransmitter, is also present in islet β-cells of the pancreas at a very high concentration comparable to that in the brain but less is known regarding its function in islets (Wendt *et al.* 2004). However, GABA has been shown to co-exist with β-cells in pancreatic islets (Adeghate & Ponery, 2002). GABA has been known as a modulator of the endocrine pancreas where they have been shown to stimulate insulin release (Adeghate & Ponery, 2002). Given the identification of both GABA-A and GABA-B receptors in islet endocrine cells (Yang *et al.* 1994), it is largely believed that GABA functions as an autocrine and/or paracrine modulator of islet hormone release. It has been reported that GABA and its agonist inhibit release of somatostatin (Yoshioka, 1986) and glucagon (Wendt *et al.* 2004) whereas it stimulates the release of insulin (Adeghate & Ponery, 2002; Dong *et al.* 2006).

The glutamic acid decarboxylase (GAD) enzyme catalyzes the formation of GABA from glutamate in neurons and islet endocrine cells (Sorenson *et al.* 1991). There are two main forms of GAD encoded by unique genes in mammals, GAD65 and GAD67; only trace amounts of either enzyme are found in adult human tissues other than the brain and endocrine pancreas (Mally *et al.* 1996). Expression of GAD in islets of Langerhans exhibits species differences for the two isoforms. The human pancreatic α, β, and δ cells express only GAD65, rat islets produce both GAD65 and GAD67, whereas mouse islets synthesize GAD67 almost exclusively (Mally *et al.* 1996). Auto-antibodies to GAD65 are detected in up to 80% of Type 1 diabetes mellitus (T1DM) patients and can be considered predictive of the disease (Lernmark, 1996). The triggering events leading to GAD65 auto-antibody formation in T1DM are still not entirely clear. One plausible hypothesis proposes an initial insult to beta-cells causing the release of GAD65 into the extracellular space, thus activating immune T cells; however, molecular mimicry of viral epitopes has also been suggested as a mechanism of autoimmunity (Kaufman *et al.* 1992). Pancreatic β-cell injury and death are observed in both T1DM and Type 2 diabetes mellitus (T2DD). Although there is a debate over the similarities and differences of mediators of β -cell death in the two subtypes of diabetes (Cnop *et al.* 2005), it is generally accepted that programmed cell death (apoptosis) underlies the loss of β-cell mass in T1DM and the Type 1-like pathology observed in some late stage T2DM patients. GAD 65 was found to be released into the circulation from β-cell after their injury or death and can be used as a marker for early detection of DM (Waldrop *et al.* 2007). In the present study we investigate the pattern of expression of CGRP, GABA and GAD, in normal and diabetic human pancreas in order to determine whether DM influences the expression of these transmitter substances in the pancreas.

MATERIALS AND METHODS

Pancreatic tissue obtained after pancreatectomy (following cancer surgery) were immediately fixed in 10% formalin, embedded in paraffin wax and processed for immunofluorescence according to a previously described method (Adeghate & Ponery, 2004). The pancreatic tissue samples were taken from normal, non-cancerous part of the pancreas. Briefly, sections of normal and diabetic rat pancreas were deparaffinized with xylene (2 × 5 min) and rehydrated with descending concentrations of ethanol. The sections were treated with a blocking agent for 30 min at room temperature after washing in phosphate-buffered saline (PBS). The sections were later incubated with sheep CGRP polyclonal antibody (1:1000) overnight at room temperature. The sections were subsequently incubated in Ig conjugated-TRITC (1:100, Jackson, ImmunoResearch Laboratories, Inc, West Grove, PA, USA) for 1 h at room temperature. After washing several times, the same sections were later incubated overnight at room temperature with glucagon antibody (Prediluted from Dako, Copenhagen). After washing in PBS, the sections were incubated for 1 h at room temperature in Alexa Fluor® 488 dye (1:100), with nearly identical spectral properties and quantum yield as fluorescein isothiocyanate (Invitrogen Corporation, Carlsbad, CA 92008, USA). Co-localization of either GABA or GAD with glucagon was performed in a similar way. In addition, the pancreata of normal and diabetic patients were also processed for insulin immunofluorescence alone using in Ig conjugated-TRITC as the tracer. GABA and GAD were used at a concentration of 1:200. The sections were mounted in Immunomount® (Shandon, Pittsburgh, PA, USA) and viewed with Nikon Axiophot Fluorescence microscope. The experiment was performed according to the guidelines of the Ethics Committee.

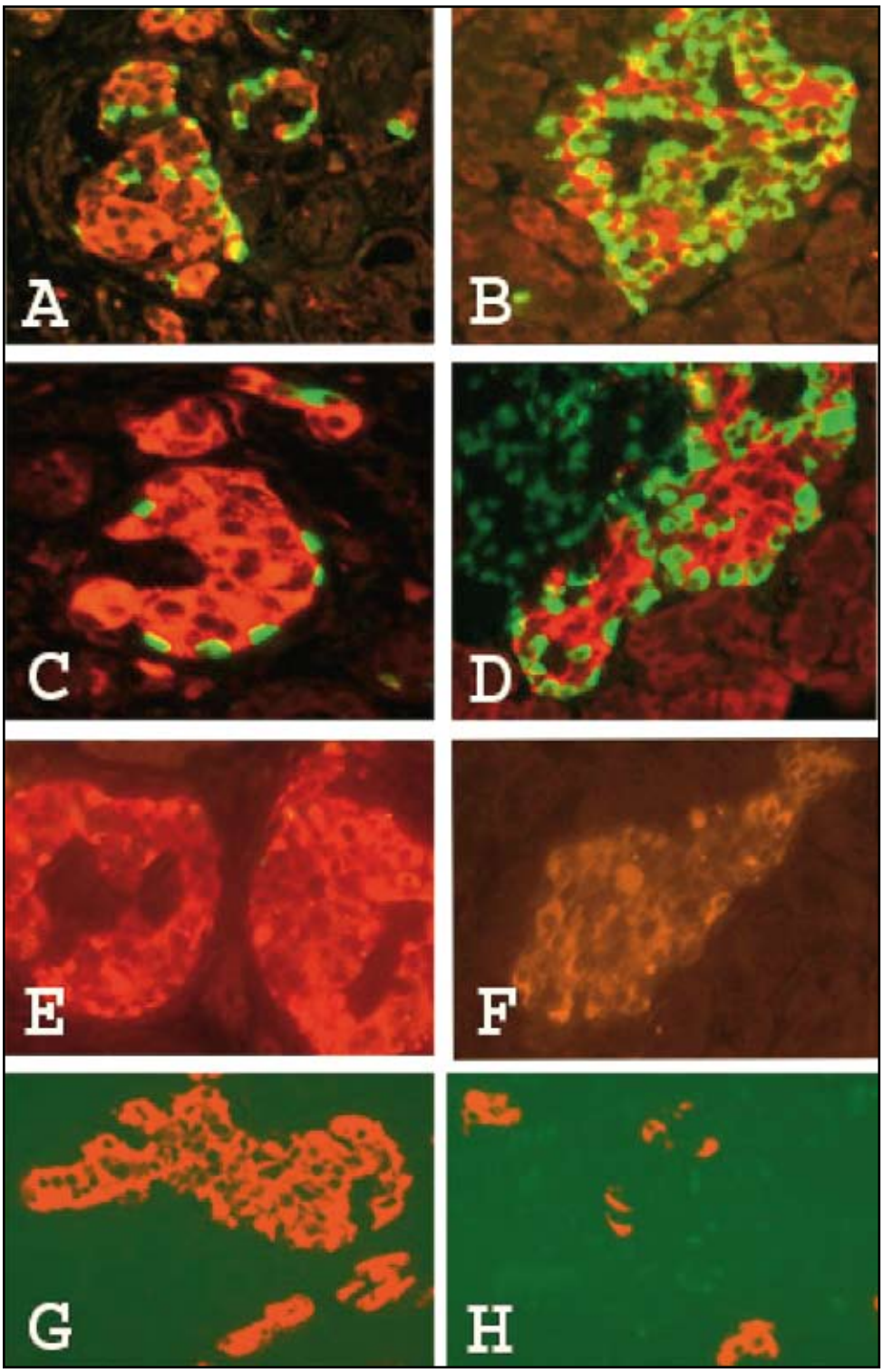

Figure 1: Immunofluorescence images of A, B: calcitonin-gene related peptide (CGRP); C, D: Gamma aminobutyric acid (GABA); E, F: glutamic acid decarboxylase (GAD) and G, H: insulin in human pancreatic islet cells. Note that CGRP- [red coloured cells, (B)]; GABA- [red coloured cells, (D)]; GAD- [red coloured cells, (F)]; and insulin-positive cells (H) is significantly reduced in the islets of patients with Type 2 diabetes. The number of glucagon-immunoreactive cells (green) is significantly higher in the islets of patients with Type 2 diabetes (B, D).
(A: CGRP in normal human pancreas; B: CGRP in diabetic human pancreas; C: GABA in normal human pancreas; D: GABA in diabetic human pancreas; E: GAD in normal human pancreas; F: GAD in diabetic human pancreas; G: insulin in normal human pancreas; H: insulin in diabetic human pancreas]. Magnification: × 400. (Publisher's note: 130% from original size)

RESULTS

Distribution of calcitonin gene-related peptide (CGRP) in islets of normal and diabetic patients

CGRP-immunoreactive cells were observed in both the central as well as peripheral regions of the islets of Langerhans of the pancreas of non diabetic patients. This was associated with a peripheral localization of glucagon-positive cells in the periphery of the islets of normal patients (Fig 1A). There was a significant reduction in the number of CGRP-immunopositive cells in the islets of patients suffering from Type 2 diabetes. In contrast to the peripheral location of glucagon-immunopositive cells seen in the islet of non-diabetic patients, glucagon-immunopositive cells are numerous and were now located in both the central as well as the peripheral regions of the islet of Langerhans in patients with Type 2 diabetes (Fig. 1B). The pattern of distribution of insulin in the islet of normal and diabetic patients was similar to that of CGRP. The islet of diabetic pancreas exhibited a significant reduction in the number of insulin-positive cells compared to control. Some islet cells in the pancreas of non-diabetic and Type 2 diabetic patients contain both CGRP and glucagon.

Distribution of gamma-aminobutyric acid (GABA) in islets of normal and diabetic patients

GABA-positive cells were observed in the central part of the islet of Langerhans of non-diabetic patients. The number and pattern of distribution of GABA-immunoreactive cells changed dramatically after the onset of Type 2 diabetes. Diabetes mellitus was associated with a large reduction in the number of GABA-positive cells in pancreatic islets. The number of GABA-positive cells was significantly higher in normal (Fig. 1C) compared to diabetic (Fig. 1D) patients. A few islet cells contain both GABA and glucagon.

Distribution of glutamic acid decarboxylase (GAD) in islets of normal and diabetic patients

GAD, the enzyme that catalyzes the formation of GABA from glutamate in neurons and islet endocrine cells was well presented in pancreatic islet cells of normal patients (Fig. 1E), where it is found in cells located in the central as well as the peripheral parts of the islets of Langerhans. The number of GAD-positive cells and the intensity of GAD staining were significantly lower in the islets of patients with Type 2 diabetes compared to control (Fig. 1F)

Distribution of insulin in islets of normal and diabetic patients

As expected, the pancreatic islet of normal patients contained large number of insulin-positive cells (Fig. 1G) compared to that of diabetic (Fig. 1H) patients. The pattern of distribution of insulin in the islet of normal and diabetic patients is similar to that of CGRP (Figs A, B), GABA (Figs. C, D) and GAD (Figs. E, F). In contrast, the number of glucagon-positive cells increases in the islets of diabetic patients.

DISCUSSION

Distribution of calcitonin gene-related peptide (CGRP) in islets of normal and diabetic patients

The findings of this study showed that CGRP is present in the endocrine cells of human pancreas. The number of CGRP-positive cells was particularly numerous in the islet of Langerhans of normal human pancreas. This number decreased significantly in Type 2 diabetic patients. In addition to the diabetes-associated decrease in the number of CGRP after the onset of Type 2 diabetes, CGRP was also co-localized with glucagon in few cells of the islet of Langerhans. It is well known that the number of insulin-positive cells decreases after the onset of diabetes (Adeghate & Ponery, 2001). The results of this study on the pattern of distribution of CGRP in the islets of normal and diabetic patients corroborate those obtained in normal and diabetic rats (Adeghate & Ponery, 2004).

Some islet cells of normal and diabetic patients contain both CGRP and glucagon. This is perhaps not surprising because previous studies in our laboratory have shown that CGRP was co-localized with both insulin and somatostatin (Adeghate & Ponery, 2004). It appears therefore that in addition to the colocalization of CGRP with insulin, some islet cells in normal and diabetic subjects contain both CGRP and pancreatic hormones such as glucagon, somatostatin and possible pancreatic polypeptide but to a much lesser degree compared to CGRP and insulin. The co-localization of CGRP with insulin in normal pancreas has been reported by other investigators (Edwin & Leigh, 1999).

These findings in both animal model of Type 1 diabetes and human Type 2 diabetes suggest that insulin metabolism may be regulated by CGRP because of the co-localization of CGRP with insulin. It may also suggest an autocrine role of CGRP.

It is worth noting that CGRP is not confined to the endocrine pancreas alone. We have shown previously that CGRP is localized to the nerves innervating the pancreas of normal and diabetic rats (Adeghate 1999; Adeghate & Ponery, 2004) and several other tissues (Clague *et al.* 1985). The presence of CGRP in the endocrine pancreas and nerves shows that CGRP may play an important role in neural regulation of the pancreas in addition to its possible humoral effects in the endocrine pancreas.

Distribution of gamma-aminobutyric acid (GABA) in islets of normal and diabetic patients

Our study showed that GABA is located in a large number of cells in pancreatic islets of non-diabetic human. We also observed that the number of pancreatic islet cells expressing GABA decreased significantly in patients with Type 2 diabetes. The pattern of distri-

bution of GABA in the endocrine pancreas of normal, non-diabetic patients and in patients with Type 2 diabetes is similar to that of insulin. This result supports the findings of Garry *et al.* (1986) that GABA co-localizes with insulin in normal pancreatic beta cell. The present study corroborates those reported from our laboratory on the pattern of distribution of GABA in the endocrine pancreas of normal and Type 1 diabetic rats (Adeghate & Ponery, 2002). This indicates that the pattern of distribution of GABA in human and rat are similar. It also shows that the pattern of expression of GABA is similar in Type 1 as well as Type 2 diabetes. The presence of GABA in the endocrine pancreas is not surprising because, it has been shown that GABA can regulate insulin secretion (Adeghate & Ponery, 2002).

Distribution of glutamic acid decarboxylase (GAD) in islets of normal and diabetic patients

We showed that GAD is present in pancreatic islet cells of normal, non-diabetic patients. In contrast, the expression of GAD was significantly reduced in pancreatic islets of patients suffering from Type 2 diabetes. GAD, a GABA metabolizing enzyme, has been implicated in the pathogenesis of Type I diabetes. GAD is a major target for auto-antibodies linked to the development of Type 1 diabetes (Baekkeskov *et al.* 1990). A high correlation of anti-GAD titer and incidence of Type 1 diabetes has also been reported (Aanstoot *et al.* 1994). The decrease in the number of GAD-positive cells in the pancreas of Type 2 diabetic patients showed that the expression of GAD is not only deranged in Type 1 but also in Type 2 diabetes, indicating some similarity in the pathogenesis of these 2 types of diabetes. It also shows that GAD may be used as marker to test the progression of Type 2 diabetes.

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