Analysis of Islet Regenerating (reg) Gene Polymorphisms in Fibrocalkulous Pancreatic Diabetes

K. Hawrani, *V. Mohan, †A. Bone, and G. A. Hitman

Medical Unit, St. Bartholomew's and The Royal London School of Medicine and Dentistry, Whitechapel, London, England,
*MV Diabetes Specialties Centre, Madras, India, and †Department of Pharmacy, University of Brighton, Brighton, England

Summary: Fibrocalkulous pancreatic diabetes (FCPD) is a form of diabetes associated with tropical chronic calcific pancreatitis, seen mostly in developing countries. FCPD is likely to be a multifactorial disease with both environmental and genetic components. The reg 1A gene encodes a protein associated with regeneration of pancreatic islets and has a sequence identical to that of pancreatic stone protein. Since FCPD is associated with both diabetes and pancreatitis, we tested the hypothesis that FCPD may be the result of mutations in the coding region of the reg 1A gene. Restriction length polymorphisms (RFLPs) and possible sequence variants of the reg 1A gene were studied by RFLP analysis, looking for single-stranded conformational polymorphisms (SSCPs) and direct nucleotide sequencing. In 20 patients with FCPD and 20 control subjects, no RFLPs were detected using 10 restriction enzymes. In 50 patients with FCPD and 50 control subjects, no SSCP variants were detected. Finally, direct nucleotide sequencing of the reg 1A gene from 30 patients with FCPD did not show any differences from the published human reg 1A gene sequence. In conclusion, it seems unlikely that mutations in the coding region of the reg 1A gene are a common cause of FCPD. Keywords: Fibrocalkulous pancreatic diabetes—reg 1A gene—Restriction fragment length polymorphism—Single-stranded conformational polymorphism—Nucleotide sequencing.

The World Health Organization (WHO) classifies diabetes mellitus into three main types: non-insulin-dependent diabetes mellitus (NIDDM), insulin-dependent diabetes mellitus (IDDM), and malnutrition-related diabetes mellitus (MRDM). MRDM can be further subdivided into fibrocalkulous pancreatic diabetes (FCPD) and protein-deficient pancreatic diabetes (PDPD). FCPD is a form of diabetes secondary to tropical chronic calcific pancreatitis, seen mostly in developing countries (1,2). FCPD constitutes ~1% of all diabetic patients in South India and 4% of diabetic patients with an age at diagnoses of <30 years (3). Many studies have suggested an environmental aetiology of the disease (4). However, family studies have shown familial aggregation of the disease, with evidence of vertical transmission of the FCPD from parent to offspring in some families (5). Furthermore, we have previously demonstrated associations between FCPD and polymorphism of HLA-DQB1 and the insulin gene (6). FCPD is therefore likely to be a multifactorial disease with both environmental and genetic components.

The reg 1A gene maps to the short arm of chromosome 2 near the centromere at band 2p12 in humans (7). It spans ~3.0 kb, encodes a 166-amino acid protein, and is associated with regeneration of pancreatic islets (8). The reg protein sequence is also identical to that of pancreatic stone protein (PSP) (9,10). Watanabe and colleagues suggested that the reg protein and PSP are derived from the same gene (11). Pancreatic stone protein accounts for up to 14% of the total protein in normal exocrine pancreatic secretions and is a major component of the protein matrix of the calculi in patients suffering from chronic calcifying pancreatitis (12). Furthermore, PSP is reduced in the pancreatic juice from patients with chronic pancreatitis, which is thought to favour stone formation. Since in FCPD there is both stone formation and evidence of a β cell defect, a mutation of the reg 1A gene might lead to both the principal components of FCPD.

The aim of this study was to investigate the hypothesis
that FCPD may be the result of a mutation(s) in the coding regions of the reg 1A gene.

SUBJECTS AND METHODS

South Indian subjects
The study cohort comprised unrelated South Indian subjects recruited via the MV Diabetes Specialities Centre and MV Hospital for Diabetes in Madras.

Patients with FCPD
FCPD patients (n = 50) were defined by the following criteria of Mohan and colleagues (2):
(1) diabetes mellitus as defined by the WHO criteria,
(2) history of recurrent abdominal pain from childhood,
(3) radiological evidence of calculi and/or pancreatic fibrosis and ductal dilatation on ultrasonography, and
(4) absence of a known cause of pancreatitis, e.g., alcoholism, gallstone, etc.

The mean age at onset of FCPD was 27.9 ± 9 years, the mean body mass index (BMI) was 18.8 ± 2.5, and 59% were male. Seven percent of FCPD patients were treated with diet alone, 18% with oral hypoglycaemic agents, 49% with insulin, and 26% with both insulin and oral hypoglycaemic agents.

Control subjects
Fifty control subjects without a personal or first-degree family history of diabetes were recruited from among blood donors (n = 27) or from staff or spouses of patients at the MV Hospital for Diabetes (n = 23). All the latter subjects had random blood glucose estimations <6.7 mmol.

Laboratory methods
Restriction fragment length polymorphism (RFLP) analysis. DNA was extracted from blood samples and studied by Southern blot hybridisation methods. The patients’ DNA was digested with TaqI, PvuII, EcoRI, MapI, HindIII, PstI, BglII, RsaI, SacI, and BamHI restriction enzymes. A [α-32P]dCTP-labelled reg 1A gene probe was used for hybridisation. The probe was a 4.3-kb cDNA reg gene fragment (kindly supplied by Professor H. Okamoto, Tohoku University School of Medicine, Tohoku, Japan). The probe was excised from plasmid vector pBS using PstI and XhoI restriction endonucleases.

Polymerase chain reaction (PCR)—single-stranded conformational polymorphism (SSCP) analysis. All six exons of the reg gene were amplified using the PCR and primers specific for each exon (Table I). PCR reactions were performed in 10-μl volumes containing 100 ng of genomic DNA, 15 pmol each of forward and reverse primers, a 200 μM concentration of each dNTP, 1 μl of 10× PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KCl, 15 mM MgCl2, 1% Triton X-100, and 1 μCi α-32P]. The mixture was overlaid with mineral oil. After initial denaturation of the template DNA at 94°C for 8 min, 0.1 μl (0.5 U) of Taq DNA polymerase (Cetus, Norwalk, CT, U.S.A.) was added and 20 cycles of amplification were carried out, comprising 1 min at 94°C (denaturation), 1 min at 55–60°C (annealing), and 1 min at 72°C (extension). A final extension of 7 min at 72°C was carried out. After the PCRs, 2 μl of the PCR product was mixed with 8 μl of loading dye buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated to 95°C for 3 min, and samples were placed immediately in an ice bath. Two microliters of each sample was loaded onto a 6–8% polyacrylamide nondenaturing gel (29:1). Each sample was applied to four gels, two containing 10% glycerol and two without; one of each type of the gel was subjected to electrophoresis at 4°C and one at room temperature. Electrophoresis was performed in 89 mM Tris-borate/2 mM EDTA, pH 8.3 (TBE), buffer at 30 W for 5 h. Gels were dried and exposed to x-ray film (XAR-5; Kodak, Rochester, NY, U.S.A.) from 2 to 24 h, using an intensifying screen at −70°C.

Direct sequencing analysis. Templates for sequence analysis were prepared by nonradioactive PCR amplification, using primers and conditions described above except for the following conditions: 100-μl PCRs were prepared by adding 0.5 μg of genomic DNA, 45 pmol each of forward and reverse primers (Table I), a 200 μM concentration of each dNTP, and 10 μl of 10× PCR buffer. After initial denaturation, 1.5 U of Taq DNA polymerase (Cetus) was added and 32 cycles of amplification were carried out. The PCR products were purified using micron-100 (Amicon Ltd., U.S.A.) according

<table>
<thead>
<tr>
<th>Exon</th>
<th>5' primer sequence 1</th>
<th>Annealing (°C)</th>
<th>Product (bp)</th>
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<tr>
<td>1 and 2</td>
<td>AAGCTTCTAGAAGCTTGTAC</td>
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<td>500</td>
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<tr>
<td>3</td>
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<tr>
<td>5</td>
<td>CACAGGGCCGCAGTGGTC</td>
<td>60</td>
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</tr>
<tr>
<td>6</td>
<td>GTCTCTAAAGCCGAGAAGGTC</td>
<td>55</td>
<td>441</td>
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to the manufacturer's instructions. The amplified products were sequenced on both strands using the same primers used for PCR amplifications by the DyeDeoxy chain terminator methods, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, U.S.A.). The samples were analysed on a 6% polyacrylamide gel at 75 W for 12 h. DNA sequences were determined using an ABI 373A automated DNA sequencer (Applied Biosystems).

RESULTS

Autoradiography revealed no polymorphic sites for all the used enzymes (TaqI, PvuII, EcoRI,MspI, HindIII, PstI, BglII, Rsal, SacI, KpnI) in 20 patients with FCPD and 20 control subjects. The entire coding region of the reg IA gene was examined for single-base changes in 50 patients with FCPD and 50 control subjects, directly from genomic DNA by PCR-SSCP analysis. No variants were observed. Direct nucleotide sequencing of the PCR product from 30 patients with FCPD did not show any differences from the published human reg IA gene sequence.

DISCUSSION

In this study, RFLP analysis using 10 restriction enzymes failed to show any polymorphism of the reg IA gene. The entire coding sequence of the human reg IA gene (six exons, including exon-intron boundaries) was examined by PCR-SSCP and no abnormal conformers were found. Finally, by direct sequencing, we were unable to detect any nucleotide substitutions.

Although deficiency of PSP, also known as lithostatin, has been implicated in the pathogenesis of temperate zone pancreatitis (12), there are no data on its role in tropical pancreatitis leading to FCPD. Furthermore, in one family with hereditary pancreatitis and one case of idiopathic chronic calcifying pancreatitis with diabetes mellitus in which the reg IA gene was sequenced, no mutations were detected (13,14). There is still considerable debate as to the role of the reg gene in modulating changes in islet cell turnover and/or function. Several studies have indicated a direct correlation between increased reg gene expression and islet cell growth (15-17), whilst other investigators have failed to show a direct link between changes in beta-cell proliferation and expression of the reg gene (18,19). Furthermore, recent findings have provided evidence to support a hypothesis that reg protein may be involved in the processes of islet cell repair and regeneration (20). Thus, reg gene-encoded products have been demonstrated in the pancreatic islets of BB rats during temporary remission of diabetes (21) and administration of reg protein to 90% pancreatectomised rats has been shown to increase beta-cell mass and prevent subsequent development of surgical diabetes (16).

These apparently conflicting data may be due at least in part to the existence of two nonallelic reg genes in several animal species including human (22), rat (23), and mouse (24). The two independent reg genes (reg1A and reg1B) are located on different chromosomes and have been reported to be differentially regulated in the pancreas during normal aging in the mouse (25). This problem of gene specific regulation of reg1A and reg1B will obviously serve as a limiting factor in interpreting findings from studies employing reg probes that are unable to hybridize selectively to each of the two nonallelic genes. In this study we have studied only the reg 1A gene. The human reg 1B gene encodes a 166-amino acid protein that differs from reg 1A by 22 amino acid substitutions. A possible link between the reg 1B gene and FCPD would be worth studying by nucleotide sequencing or exclusion-mapping in families. Recently, autosomal dominant hereditary pancreatitis has been mapped to chromosome 7q in a French pedigree (26). Once the gene has been identified, this would also be a good candidate gene for FCPD.

In conclusion, results from this study indicate that mutations of the coding region of the reg 1A gene are unlikely to contribute to the pathogenesis of FCPD, although regulatory variants of this locus cannot be excluded.

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REFERENCES

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