Autoantibodies to Pancreatic hsp60 Precede the Development of Glucose Intolerance in Patients with Cystic Fibrosis

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Persons expressing the genetic disease cystic fibrosis (CF) suffer from a high risk of developing impaired glucose tolerance and diabetes. The development of diabetes in CF has been attributed, in the past, to the destruction of pancreatic islets and their resident β-cells secondary to the destruction of the surrounding tissue by mechanical clogging of the pancreatic exocrine ducts. However, the discovery that autoimmunity to the 60-kDa heat shock protein (hsp60) may cause type I diabetes in NOD mice raises the possibility that hsp60 autoimmunity may be involved in CF diabetes too; could the hyperimmunization to bacterial hsp60 characteristic of CF spread to self-hsp60 and hence to autoimmune diabetes? We now report that rising levels of IgG autoantibodies to hsp60 do indeed precede the appearance of glucose intolerance and diabetes in CF patients. We produced a recombinant human pancreatic hsp60 protein and investigated the IgG antibody response to hsp60 in prediabetic and non-diabetic patients with CF. To detect hsp60 autoantibodies in the presence of high levels of antibodies to bacterial hsp60, we absorbed test sera with the 60-kDa GroEL of Pseudomonas aeruginosa and used an immunostaining technique. Using this technique, 32 prediabetic CF patients were evaluated over a five-year period, three years, on the average, before the onset of glucose intolerance. We found that a significant increase in hsp60 autoantibody preceded impaired glucose tolerance (P=0.042, n=17), diabetes (P=0.011, n=15) and glucose intolerance (P=0.005, n=32). As has been observed in NOD mice and in type I diabetic patients, the hsp60 autoantibodies decline at the outbreak of glucose intolerance in the CF patients. The association of CF diabetes with the rise and fall of hsp60 autoimmunity suggests that the pathogenesis of the diabetes may not be merely mechanical, but arise in the wake of bacterial hyperimmunisation.
The purpose of this study was to investigate longitudinally the level of serum IgG autoantibodies to a human pancreatic hsp60 protein in prediabetic CF patients during their development of IGT and CFDM. The study was based on an examination of sera that had been collected earlier during two longitudinal studies carried out over a five-year period, initiated three years prior to the time of onset of impaired glucose tolerance and diabetes.

Patients, Materials and Methods

Patients

The CF patients were routinely tested for glucose tolerance using an oral glucose tolerance test (OGTT) according to the 1985 WHO recommendations [11]. The blood glucose level (capillary plasma glucose concentrations) was determined before, 1 h and 2 h after a glucose intake of 1.75 g glucose monohydrate per kg body weight (maximum 75 g) using a glucose dehydrogenase test, as previously described [12]. A 2-h post-load blood glucose concentration below 8.8 mmol/l was considered normal (NGT); a value of 8.9–12.1 mmol/l indicated impaired glucose tolerance (IGT); and a value above 12.2 mmol/l was diagnostic of diabetes (CFDM).

Cloning and expression of pancreatic hsp60

Amplification of a human hsp60 gene was performed by PCR using a human pancreatic cDNA library (HL1069b, Clontech Laboratories, CA, USA) as a template. The DNA sequence of a gene encoding the 63-kDa human mitochondrial heat shock protein P1 [13] was used for the design of two PCR primers PR5449: 5’-GAG TTA AGA ATT GGA TCC ACC ACC TCC CAT ACC CAT ACC-3’ and PR5450: 5’-CGC AAT TTA GTG TAT TCA TAT GGT TCG GTT ACC CAC AGT CTT TCG-3’, which covered the coding sequence of the entire human p1 gene. Each primer included one recognition site for the restriction enzymes Bam HI or Nde I (underlined). PCRs with 0.25 μM of each primer and human pancreatic cDNA library (HL1069b) as template were performed under standard PCR conditions. The PCR product (~1.75 kbp) verified by the internal EcoR I site (nt 712) was purified from an 0.7% agarose gel (SeaKem GTG, FMC BioProducts, Rockland, ME, USA) using Spin-X filter units (Costar, Cambridge, MA, USA). The purified PCR product was ligated on to a pET16b vector (Novagen, Madison, WI, USA) and transformed into competent Escherichia coli Novablvue (Novagen). Cells were plated on Luria-Bertani (LB) agar plates containing 0.2 mg/ml carbenicillin and incubated overnight at 37°C. Clones containing the pET16b/hsp60 construction were verified by plasmid digestion using Nde I, Bam HI and Eco RI were transformed into competent E. coli BL21 (DE3) (Novagen).

Purification of human pancreatic hsp60

Purification of recombinant protein was performed essentially as recommended by the manufacturer (Novagen), with some modifications. Clones (BL21 (DE3)) containing the pET16b/hsp60 construction, tested for expression of recombinant protein by SDS–PAGE and Coomassie brilliant blue staining, were grown in two steps in LB broth containing 0.2 mg/ml carbenicillin to OD600=1 and induced with 1 mM isopropylthio-β-D-galactoside (IPTG) for 1 h at 37°C and overnight at 4°C. Cells were harvested, sonicated and centrifuged, and the resulting supernatant was sterilised using a 0.2 μm filter. The supernatant was loaded on to a Ni2+-charged his-bind resin column and eluted in fractions. Eluted fractions were subsequently tested by SDS–PAGE and stained with Coomassie brilliant blue. Fractions containing recombinant protein were pooled and dialysed against phosphate buffered saline (PBS, pH 7.2). Protein concentration was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), adjusted to 0.5 mg protein/ml and stored at −20°C until used.

Purification of 60-kDa GroEL of P. aeruginosa

Recombinant 60-kDa GroEL of P. aeruginosa was expressed and purified as previously described [6].

SDS–PAGE and Western bloting

Procedures for SDS–PAGE and Western blotting were performed as described previously [6]. Briefly, prior to protein separation in SDS–PAGE, all samples were treated for 3 min at 100°C in a buffer containing 0.05 M Tris hydrochloride buffer (pH 6.8), 2% (wt/vol) SDS, 10% (wt/vol) sucrose and 0.01% bromphenol blue. The samples were then fractionated on SDS–PAGE containing 4% and 14% acrylamide in stacking and separating gels, respectively. The separated fractions were transferred at 30 V overnight to nitrocellulose papers in transfer buffer (0.02 M Tris, 0.2 M glycine and 20% methanol, pH 8.5).

Gold staining

Gold staining of Western blot transferred proteins was performed as previously described [6]. Briefly, the nitrocellulose paper was incubated 30 min at room temperature in citric acid buffer (55 mM citric acid, pH 3.0), and staining was developed within 2–4 h in 75% (vol/vol) gold solution (25 mM gold chloride, 0.1 mM sodium citrate in water) in citric acid buffer supplemented with 0.1% (vol/vol) Tween 20.

Immunostaining

Immunostaining of Western blot-transferred proteins was performed as previously described [6]. Briefly,
Specific hsp60 antibodies in pre-diabetic CF patients

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Antibody absorption

Patient sera were prior to immunostaining absorbed with recombinant 60-kDa GroEL of *P. aeruginosa* by incubating 1:1 sera with *P. aeruginosa* GroEL (4 mg/ml) in diluting buffer (PBS, 0.1% (vol/vol) Tween 20 and 1.5% (wt/vol) NaCl, pH 7.2) and 0.01 volume 10% (vol/vol) in diluting buffer (PBS, 0.1% (vol/vol) Tween 20 and 1.5% (wt/vol) NaCl, pH 7.2) and 0.01 volume 10% Na3PO4 in dimethylsulphoxide (DMSO), diocetyl sodium sulphosuccinate (4.5 mM DONS) and hydrogen peroxide (0.05% H2O2) in a phosphate citrate buffer (54 mM citric acid, 0.1 M Na2HPO4, pH 5.0). Staining was terminated by incubation with DONS (4.5 mM) in water.

Scanning densitometry

The precipitating bands in immunostaining assays were measured by (i) laser scanning densitometry and (ii) imagine densitometer scanning. (i) Photographic diags of immunoblots were scanned with a helium–neon laser scanner (UltrascanXL, LKB 222, Bromma, Sweden) and the geometric peak area of intensity (measured by OD at 633 nm) was integrated automatically. The calculation was \( \frac{\text{abs}}{\text{abs}_{\text{baseline}}} \) in units, base-line determined by the 16 lowest points in the scan and mm is the peak width at baseline. The result was calculated as peak area and given in units. (ii) Immunoblot were scanned with an Imagine Densitometer Scanner (GA 700, Bio-Rad) and Multi-Analyist®/PC Version 1.1 software (Bio-Rad). The result was calculated as area of band (cm×OD) and given as a percentage of bands in the scan.

Calculation of mean antibodies=100%
(normalisation)

The results were ‘normalised’ for each patient by calculating the mean of all antibody results in units obtained during the observation period (mean=100%). Each observation was then expressed as a percentage of the mean value for each patient.

Statistics

The Mann–Whitney U-Test was used with a 5% level of significance, and the software programs StatView and CricketGraf were used for statistics and graphic presentation, respectively.

Results

The DNA sequence of a gene encoding the human mitochondrial hsp60 protein P1 [13] enabled us to express and purify an hsp60 protein from the human pancreas using a human pancreatic cDNA library and two PCR primers covering the coding sequence of the human *hsp60* gene. The pET16b/hsp60 construction was verified by the internal EcoRI site and DNA sequenced. The cloned pancreatic hsp60 gene showed high homology to the mitochondrial *p1* [13]; we found only seven minor nucleotide substitutions (position 199 G→A; 273 G→A; 337 A→G; 1183 G→C; 1492 T→G; 1700 G→T; and 1703 T→A) of which one was silent (273) and one was a deletion (1672-83 GGT). This indicated the following amino acid substitutions at positions: 67 G→P, 256 S→A, 395 A→P, 506 S→A, 567 M→K and 566 G→V, and the deletion of 557–560 GGGG. The expressed recombinant product is N-terminally linked with a 21 amino acid leader sequence of which 12 residues are the histidines we attached for purification.

We used gold staining and immunostaining to test the purified product. The human hsp60, similar to the 60-kDa *P. aeruginosa* GroEL we previously produced [6], was found to be free of contaminating proteins, giving only one band in goldstaining (Figure 1). The preparation contained no *E. coli* proteins detectable using a polyclonal *E. coli*-positive rabbit serum, and the protein was recognised by the human hsp60–specific monoclonal antibody, LK-1 (H-4149, Sigma) in immunostaining (not shown).

Serum from a prediabetic CF patient was used in a preliminary study of peripheral antibody reactivity against the purified recombinant human pancreatic hsp60 in the immunostaining assay. In immunostaining, the immune response against the pancreatic hsp60 in CF patient sera was very weak. However, by absorption of the patient’s serum with the 60-kDa GroEL of *P. aeruginosa* prior to immunostaining, the absorbed serum lost reactivity to *P. aeruginosa* GroEL, and gained specificity to the hsp60 protein (Figure 2). Preabsorption with GroEL appeared to remove bacterial-specific antibodies and shared epitope-reactive antibodies leaving a population of hsp60-specific antibodies. The weak binding of serum to hsp60 was completely blocked by preabsorption with hsp60 at all concentrations (from 4 μg to 56 μg protein/10 μl serum) of the hsp60 protein (not shown).

Using this absorption technique, sera from three CF patients were examined longitudinally for hsp60-specific IgG antibodies and glucose tolerance. The first patient, a 17-year-old girl who developed CFDM at an age of 13 years (Figure 3A), showed an elevated level
of hsp60-specific IgG antibodies 9 months before the onset of CFDM and a 36.9% increase in the 3-month period before the onset of CFDM. The second patient, a 16-year-old girl who developed CFDM at the age of 11 years (Figure 3B), manifested an elevated level of hsp60-specific IgG antibodies 1.5 years before the onset of CFDM and an increase of 27.8% in a 4-month period prior to the onset of CFDM. The third patient, a 29-year-old male who developed IGT at an age of 20 years and CFDM one year later (Figure 3C), showed an increase of 49.8% in the one-year period before the onset of IGT.

In a longitudinal study, 357 serum samples of which 246 were prediabetic samples from 32 CF patients were examined for hsp60 autoantibodies. The patients were divided into two groups of 15 and 17 each. Group 1 was composed of patients in whom IGT was not detected before the onset of CFDM. Group 2 was composed of patients in whom IGT was detected prior to the onset of CFDM. In group 1, we found a significant increase of 42.5% ($P=0.0109$) in the hsp60 antibody in the one-year period prior to the onset of CFDM (Figure 4A). In group 2, the increase in hsp60 antibodies occurred prior to the onset of IGT rather than before CFDM (Figure 4B and 4C). Figure 5 shows the combined results of sera from all 32 patients. We found a significant increase of 27.0% ($P=0.0051$) of the hsp60 autoantibody during the one-year period before glucose intolerance.

Discussion

In the present study, prediabetic serum samples from patients with CF were investigated for hsp60 autoantibodies. This was done using an immunostaining technique in which the serum samples were preabsorbed with 60-kDa GroEL of $P. aeruginosa$ to avoid interference in the assay produced by bacterial-specific antibodies. The study revealed an increase of the hsp60 autoantibody in the one-year period prior to the onset of IGT rather than before CFDM (Figure 4A). In group 2, the increase in hsp60 antibodies occurred prior to the onset of IGT rather than before CFDM (Figure 4B and 4C). Figure 5 shows the combined results of sera from all 32 patients. We found a significant increase of 27.0% ($P=0.0051$) of the hsp60 autoantibody during the one-year period before glucose intolerance.

In the non-obese diabetic (NOD) mouse model of spontaneous diabetes, hsp60 autoantibody also
precedes the onset of diabetes [8]. In both the mouse model and during glucose intolerance in CF, the amount of hsp60 autoantibodies increases and subsequently decreases as the disease becomes clinical. These observations suggest that the development of glucose intolerance in CF might be autoimmune and not merely due to ‘plugging’ of the exocrine pancreas and physical damage to the β-cells.

The recombinant pancreatic hsp60 protein produced in the present study is very like the mitochondrial hsp60 (Accession no. M34664) but with minor differences; two cysteine residues are found in the pancreatic hsp60 whereas only one is present in the mitochondrial hsp60. The pancreatic hsp60 was sequenced in two directions on each DNA strand in order to limit the occurrence of sequencing artifacts. However, the 557–560 GMGG deletion at the C-terminal end of the pancreatic hsp60 could well be due to a cloning artifact, since the 3′ end primer used for cloning recognises the repeated ATGGGCGGC sequences corresponding to the repeated amino acids sequence MGG. We found no differences between the pancreatic and the mitochondrial hsp60 within the immunological important p277 sequence (437–460); no differences were found in a region spanning 395–506. Thus, sequence differences in the area of p277 cannot account for the tissue specificity of hsp60 autoimmunity in IDDM. Whether other differences between the pancreatic and mitochondrial hsp60 play any role immunologically is not known.

In human cells, several hsp60-like genes have been identified; most of them pseudo-genes (Acc. no. M34660–63), but two, mitochondrial hsp60 (Acc. no. M34664) and pancreatic hsp60 (this study) encode expressed genes. It is possible that mammalian cells may activate several genes encoding proteins of the 60–65 kDa hsp family, but that each gene may be active at different stages in cell development or in different tissues.

Figure 3. Three prediabetic CF patients (A–C) evaluated over a 3–4 year period during onset of glucose intolerance. The patients were evaluated for glucose tolerance by OGTT and hsp60-specific IgG antibodies by immunostaining (top panel). (A) A 20-year-old CF girl who developed CFDM at an age of 13 years; lanes are sera collected on: (1) 8/7–87, (2) 15/8–88, (3) 3/5–90, (4) 8/6–90, (5) 4/12–90, (6) 28/1–91, (7) 14/3–91, (8) 16/4–91 and (9) 2/7–91. (B) A 19-year-old CF girl, who developed CFDM at the age of 11 years; lanes are: (1) 22/9–87, (2) 12/1–88, (3) 27/4–88, (4) 28/2–89, (5) 9/2–90, (6) 23/4–90, (7) 8/6–90, (8) 35th week–90, (9) 19/4–91 and (10) 24/5–91. (C) A 29-year-old CF male who developed IGT at the age of 20 years and CFDM one year later (C); lanes are: (1) 25/8–87, (2) 23/10–87, (3) 4/1–88, (4) 12/4–88, (5) 11/7–88, (6) 4/1–89, (7) 6/3–89, (8) 9/5–89, (9) 11/7–89, (10) 11/9–89 and (11) 6/11–89. The patients developed IGT or CFDM one year later (C); lanes are: (1) 22/3–91, (2) 27/8–90 (B) and 22/3–89 (C) with a 2 h blood glucose level of 14.6 mM, 16.6 mM and 11.6 mM, respectively. An immunoblot scanning assay revealed an increase of the hsp60 antibody of 37.1% and 25.8% in a 3–4 month period, and an increase of 63.4% and 63.7% in a one-year and 1.5-year period prior to onset of CFDM (A and B, respectively) and an increase of 49.8% in a one-year period prior to onset of IGT (C). All lanes were loaded with human hsp60 (1 µg/lane) and all patient samples were preabsorbed with 60-kDa P. aeruginosa GroEL (4 mg GroEL/ml sera).
A study of 215 CF patients, of which 211 had the Δ508 mutation, showed no difference in pancreatic function between patients homozygous or heterozygous for the Δ508 mutation. Endocrine pancreatic function was normal in 72.5%, impaired in 12.3%, and diabetic in 15.2% of the patients, with no difference between CF patients homozygous or not for Δ508 [12].

This study indicates that there is no correlation between the Δ508 mutation and the development of CFDM.

The genotypes represented by the class II molecules HLA-DR3, DR4 and DR3/4, which classically confer susceptibility to IDDM, and of HLA-DR2, which classically confers resistance to IDDM, were not different in diabetic compared to non-diabetic CF patients. Also, islet cell cytoplasmic antibodies (ICA) could only be detected in two of 236 (0.8%) serum samples. This argues against the importance of these genetic factors in the induction of CFDM. In a recent study, however, both GAD autoantibodies and IA-2 autoantibodies have been shown to be present in CF using quantitative ELISA [14]. These studies clearly show that both the GAD autoantibody and the IA-2 autoantibody are present in CF with 50% and 40%, respectively. In addition, it has been reported that CFDM may be related to the HLA-DQB1 genotype [15] and suggested a possible link between genotype and diabetes,
implicating an immunological complicity in the disease process.

In type I IDDM, diabetes is caused by a T cell-dependent autoimmune destruction of the pancreatic insulin-producing β-cells leading to a complete dependence on exogenous insulin [16–17]. The pancreatic β-cell destruction depends on the activation of CD4+ T cells following presentation of antigen associated with the HLA class-II molecule on macrophages, B lymphocytes and activated T lymphocytes [18]. In CFDM, β-cell destruction similarly may be mediated by CD4+ T cells. If CFDM is an autoimmune disease, antibody-mediated autoimmunity due to the presence of autoantibodies directly involved in β-cell killing seems not likely, in that, no CF infant born to a CF diabetic mother has been reported to have acquired diabetes in utero, even though maternal antibodies do pass through the placenta to the foetus. Hence, it is more likely that CFDM could be a T cell-mediated autoimmune reaction. The hsp60 autoantibodies found in preCFDM are likely therefore to be T-cell dependant, and, as such, may serve as a marker for the pathological process.

Several studies have investigated the prevalence of hsp60 autoantibody in type I IDDM patients [3, 19–21]. Several of these papers failed to find a correlation between disease and the hsp60 autoantibody. A study using human islet cell extracts reported a failure to find serum antibodies to islet hsp60 in human IDDM patients [19]. Moreover, serum antibodies in IDDM patients were reported not to bind native or trypsinized islet hsp60 [21]. However, these studies included only a very small number of patient samples (6–7 samples) which they assayed using immunoprecipitation. Moreover, these studies did not include samples from prediabetic patients and made no attempt to eliminate interference produced by antibodies to bacterial GroELs. The absorption technique we described here appeared to be effective in this regard.

In a later study, the level of serum hsp60 antibodies using a recombinant murine insulinoma hsp60 was found to be significantly higher in IDDM patients than in healthy subjects [20]. This study used a solid phase assay (ELISA) and included 84 IDDM patients in which 15.5% were positive for the hsp60 autoantibody compared to only 1.2% in healthy persons. One may conclude that the method used for the detection of the autoantibody is important as is the number of subjects included in the study. The method for detection of the hsp60 autoantibody may have a unique problem because of interfering bacterial specific antibodies. Detection of other autoantibodies may not have this problem, but the choice of method may still be critical. For example, autoantibodies against human insulin correlate better with onset of IDDM using liquid phase than solid phase assay [22].

Other studies have investigated the hsp60 antibody in type I IDDM, but used bacterial GroEL proteins in their assays [23–25]. One of these studies examined 52 IDDM sera and found 32% and 70% to be positive to Mycobacterium leprae hsp65 using ELISA and immunostaining, respectively, whereas all healthy controls were negative [23]. This study indicates that immunostaining may be a better and perhaps more specific technique to evaluate hsp60 autoantibody. Another study examined prediabetic IDDM and newly diagnosed IDDM, but failed using ELISA to find differences between prediabetic twins and control subjects. This failure could, however, be explained by the use of Mycobacterial tuberculosis hsp65, since some controls may have upregulated their response to this protein. It is interesting, however, that study found that three out of four prediabetic twins showed increased levels of mycobacterial hsp65 antibodies prior to the onset of IDDM or at the time of diagnosis [23]. Unfortunately, this study included only 12 serum samples from four IDDM patients. In a recent study of patients with CF, relative increase in the bacterial hsp60 antibody, using P. aeruginosa GroEL and ELISA, could be detected in a one-year period prior to the onset of diabetes [5]. This study included 320 serum samples collected from 29 CF patients, of which 167 were prediabetic serum samples.

In a recent study, upregulated immunity to hsp60 was found in newly diagnosed type I diabetic patients, whereas type II diabetics and healthy donors showed significantly fewer responses [10]. In CF, chronic infection may upregulate self-hsp60 expression in the context of accessory ‘danger’ signals, and autoimmunity may thus get activated. The fact that hsp60 autoantibodies mark the time of onset of glucose intolerance in CF supports the hypothesis that of hsp60 autoimmunity may play a role in CF. In the NOD mouse, diabetes may be transient [9] a phenomenon also observed in standard mice autoimmunised to hsp60 [26]. The transient appearance of IGT is common in CF, and most patients revert to normal glucose tolerance. However, some CF patients may acquire IGT repeatedly and subsequently develop CFDM. Thus, IGT in CF may be a phenomenon similar to transient diabetes in the NOD mouse model; it is conceivable, therefore, that glucose intolerance in CF may be a result of an autoimmune reaction. One might consider a possible vaccination strategy using an hsp60 peptide for downregulating hsp60 autoimmunity, as has been done successfully in mice models of IDDM [27] and which is now in clinical trials in newly diagnosed type I patients.

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