Beta-cell markers and autoantigen expression by a human insulinoma cell line: similarities to native beta cells

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Abstract

In the present study we have evaluated the expression of different beta-cell markers, islet molecules and auto-antigens relevant in diabetes autoimmunity by a human insulinoma cell line (CM) in order to define its similarities with native beta cells and to discover whether it could be considered as a model for studies on immunological aspects of Type 1 diabetes.

First, the positivity of the CM cell line for known markers of neuroendocrine derivation was determined by means of immunocytochemical analysis using different anti-islet monoclonal antibodies including A2B5 and 3G5 reacting with islet gangliosides, and HISL19 binding to an islet glycoprotein. Secondly, the expression and characteristics of glutamic acid decarboxylase (GAD) and of GM2–1 ganglioside, both known to be islet autoantigens in diabetes autoimmunity and expressed by human native beta cells, were investigated in the CM cell line. The pattern of ganglioside expression in comparison to that of native beta cells was also evaluated. Thirdly, the binding of diabetic sera to CM cells reacting with islet cytoplasmic antigens (ICA) was studied by immunohistochemistry.

The results of this study showed that beta cell markers identified by anti-islet monoclonal antibodies A2B5, 3G5 and HISL-19 are expressed by CM cells; similarly, islet molecules such as GAD and GM2–1 ganglioside are present and possess similar characteristics to those found in native beta cells; the pattern of expression of other gangliosides by CM cells is also identical to human pancreatic islets; beta cell autoantigen(s) reacting with antibodies present in islet cell antibodies (ICA) positive diabetic sera identified by ICA binding are also detectable in this insulinoma cell line.

We conclude that CM cells show close similarities to native beta cells with respect to the expression of neuroendocrine markers, relevant beta cell autoantigens in Type 1 diabetes (GAD, GM2–1, ICA antigen), and other gangliosides. Therefore, this insulinoma cell line may be considered as an ideal model for studies aimed at investigating autoimmune phenomena occurring in Type 1 diabetes.

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Introduction

The difficulty in obtaining viable human pancreatic beta cells in large amounts is still a major problem in many areas of research into the pathophysiology of endocrine pancreas and in particular for studies on the autoimmune pathogenesis of Type 1 diabetes. So far, attempts to isolate human insulinomas in long-term culture have been mostly unsuccessful (Chick et al. 1973, Yip & Schimer 1973, Adcock et al. 1975) and the only alternatives to human pancreas-derived beta cells are represented by animal-derived insulinomas such as the rat insulinoma cell line RIN (Gazdar et al. 1980), the hamster insulinoma HIT (Senterre et al. 1981) and also by the mouse transfected cell line beta TC (Efrat et al. 1988).

A human insulinoma cell line denominated CM was isolated from tumour cells obtained from ascitic fluid of a patient with pancreatic insulinoma (Gueli et al. 1987). This cell line grows spontaneously in vitro as an adherent monolayer with a tendency to aggregate in structures resembling pancreatic islets. Electron microscopic analysis revealed the presence of cytoplasmic secretory type granules delimited by a membrane with a diameter between 110 and 160 nm containing an electron-dense core (Gueli et al. 1987). Synthesis of insulin and C-peptide in the presence of 11 nm glucose was demonstrated using a radioimmunoassay and the production of both was inhibited by incubating the cells with somatostatin (Cavallo et al. 1992). Production of glucagon, somatostatin, gastrin and pancreatic polypeptide
was not detected in CM cells (Gueli et al. 1987) by cytochemistry.

All these features make this cell line a potentially ideal model for metabolic studies on pancreatic beta cells in vitro.

In order to establish an in vitro model for studies on the autoimmune pathogenesis of Type 1 diabetes, a number of characteristics need to be taken into account, including the immunological properties and the expression of relevant antigenic structures. So far, few beta cell molecules have been proposed as possible beta cell autoantigens inducing humoral and cellular responses of the immune system in Type 1 diabetes. These molecules include the glutamic acid decarboxylase (GAD) which is expressed in considerable amounts by native beta cells, the islet cell ganglioside GM2-1, the recently identified 37/40 kDa tyrosine phosphatase (Hatfield et al. 1995), insulin, pro-insulin, carboxypeptidase H and others (Atkinson & Maclaren 1994).

The aim of the present study was to characterize the CM line with respect to its pattern of beta-cell antigen expression in order to determine to what extent this cell line is representative of native beta cells and suitable for use in studies on the autoimmune pathogenesis of Type 1 diabetes.

Specifically, in order to assess the positivity of the CM line for known markers of human beta cells, a panel of anti-islet monoclonal antibodies was used: monoclonal antibody A2B5 (Eisenbarth et al. 1979, 1982, Kundu et al. 1983, Fredman et al. 1984) and 3G5 (Powers et al. 1984, Nayak et al. 1987) whose antigens have properties of gangliosides; monoclonal antibody HISL-19 directed towards a glycoproteic antigen (Krish et al. 1986, Srikanta et al. 1986). Furthermore, the expression of cytokeratin was also investigated to confirm the ectodermal derivation of the cell line.

In addition, the expression by the cell line of the main candidate autoantigens in Type 1 diabetes was studied. The presence of GAD, which is expressed by human beta cells and is one of the main targets for the autoantibody and T-cell responses in Type 1 diabetes (Atkinson et al. 1993) was evaluated. Furthermore, since a number of gangliosides have been shown to be islet specific within the pancreas and one of them (GM2-1) (Dotta et al. 1995) appears to act as an autoantigen in beta cell autoimmunity, the pattern of ganglioside expression by the cell line was determined.

Finally, we evaluated the reactivity of CM cells with islet cell antibodies (ICA) positive sera to determine whether ICA antigens are expressed by these cells.

Materials and Methods

Cell line

The CM line was isolated from tumour cells obtained from ascitic fluid of a patient with pancreatic insulinoma (Gueli et al. 1987). Cells were grown in a humified incubator with 5% CO₂ at 37 °C in RPMI 1640 culture medium supplemented with 1% glutamine, 1% streptomycin-penicillin (10 mg/ml and 10 000 U/ml respectively) and 5% fetal calf serum (FCS). Cells used for the present study were obtained from the 70th passage and we cultured the CM cell line up to approximately the 100th passage. Cells in culture showed typical features of an adherent semi-confluent monolayer, with a tendency to aggregate in structures similar to pancreatic islets (Fig. 1).

By molecular typing it has been shown that CM cells possess the DQA52 arg/non arg and DQB 57 non asp/non asp HLA genotype encoding two insulin-dependent diabetes mellitus (IDDM) susceptibility heterodimers.

Sera tested

Sera were collected from five high titre ICA positive and three ICA negative new onset IDDM patients and from three ICA negative control subjects matched for age and sex. Positivity for ICA was determined by the classical indirect immunofluorescence technique on sections of human pancreas as described elsewhere (Bonifacio et al. 1990).

Sera were separated and stored at −20 °C until used for immunohistochemical analysis to detect reactivity with CM cells.

Characterization of beta cell markers

Immunofluorescence staining by monoclonal antibodies

CM cells were grown to confluence onto glass coverslips placed on the bottom of 6-well polystyrene plates (Falcon, Becton-Dickinson, Oxford, UK), each well containing 2 ml of a 10⁶ cells/ml suspension. The slides were then fixed and permeabilized in acetone for approximately 1 min and used for staining with monoclonal antibodies and sera.

For the determination of beta cell markers the following anti-islet monoclonal antibodies were used:

Monoclonal antibody A2B5, which reacts with different tissues of neural derivation as well as with pancreatic islet cells (Eisenbarth et al. 1979, 1982); the A2B5 antigen has properties of a polysialo-ganglioside (Kundu et al. 1983, Fredman et al. 1984).

Monoclonal antibody 3G5 which reacts with all cells from pancreatic islets in different species including human, mouse and rat (Powers et al. 1984) and is also reactive with the rat insulinoma cell line RIN m5F; the 3G5 antigen appears to have properties of a disialo-ganglioside (Nayak et al. 1987).

Monoclonal antibody HISL-19 which reacts with human, porcine, bovine but not with rodent islet cells and with other endocrine cell types as well as with cells of neural derivation (Krish et al. 1986, Srikanta et al. 1986).
There is evidence that the antigen recognized by this antibody is a glycoprotein (Srikanta et al. 1986).

Briefly, 50 µl aliquots of each tested antibody (at a dilution of 1:100 in PBS/BSA) were added to fixed cells. After 60 min incubation at room temperature and two washes with PBS containing 1% BSA, cells were incubated at room temperature with 50 µl of a fluorescein isothiocyanate (FITC)-conjugated goat antiserum to IgG, 1:100 (Sigma, St Louis, MO, USA). Finally, after five washes, cells were mounted on a glass slide and visualized under a fluorescence microscope (Zeiss, Germany).

The expression of cytokeratin was determined by direct immunofluorescence using FITC-conjugated monoclonal antibody to human cytokeratin; non-conjugated anti-cytokeratin monoclonal antibody was used as a control to inhibit the binding. Both antibodies were purchased from Sigma.

Detection of islet molecules with properties of autoantigen

GAD analysis by immunoblotting Detection of affinity purified GAD by Western blot was carried out as follows: CM cells were homogenized in extraction buffer containing 0.2 mM phenylmethylsulphonylfluoride, 1% NP-40, pH 8.0, 0.2 mM 2-aminoethylisothiouronium bromide (AET), 0.2 mM pyridoxal-5-phosphate (PLP), 1 mM EDTA and 4 µg/ml each of pepstatatin A, leupeptin, antipapain and aprotinin (Sigma).

The samples were centrifuged at 10,000 r.p.m. for 30 min at 4 °C and the supernatants stored at −20 °C. The protein extract was then subjected to affinity chromatography purification of GAD using GAD-6 monoclonal antibody coupled to protein A-Sepharose CL-4B and followed by elution of bound material with Tris-buffered saline pH 11. Eluted proteins were separated according to their molecular weight by discontinuous sodium dodecyl-sulphate SDS-PAGE, as described elsewhere (Laemmli 1970). The gels were stained with Coomassie Blue. Proteins were electrophoretically transferred from polyacrylamide to nitrocellulose filters and then immunostained with monoclonal antibody GAD-6, a mouse monoclonal reacting with the 65 kDa isofrom of human GAD. After incubation with peroxidase-conjugated anti-mouse IgG (Sigma), positive bands were visualized by the addition of 4-chloro-1-naphtol and H2O2.

Ganglioside analysis by HPTLC Gangliosides have been isolated using the method of Svennerholm & Fredman (1980) with minor modifications. Briefly, after extraction in chloroform:methanol:water (4:8:3, v:v:v) followed by Folch partition, acidic glycolipids (gangliosides) were purified by Sep-Pak C18 column chromatography and by ion exchange chromatography with DEAE Sephadex A25 as packing matrix.

Ganglioside analysis was performed on silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) in chloroform:methanol:0.25% aqueous KCl (5:4:1, v:v:v) followed by visualization of sialic acid containing material (gangliosides) using resorcinol spray reagent. Quantitative determination of ganglioside expression was achieved by scanning densitometric analysis (scanning densitometer model GS365, Hoefer Inc., San Francisco, CA, USA) of resorcinol stained bands.

Figure 1 Haematoxylin staining on adherent semiconfluent monolayer of CM cells showing their characteristic tendency to aggregate in structures similar to pancreatic islets.
Figure 2 (a) Indirect immunofluorescence of CM cells with monoclonal antibody A2B5 (1:100 dilution) binding to islet polysialoganglioside antigen. (b) Indirect immunofluorescence of CM cells with monoclonal antibody 3G5 (1:100 dilution) binding to islet disialoganglioside antigen. (c) Indirect immunofluorescence of CM cells with monoclonal antibody HISL-19 (1:100 dilution) recognizing an islet-associated glycoprotein. (d) Indirect immunofluorescence of CM cells with non-specific control monoclonal antibody P3X63 (1:100 dilution).

**Binding of diabetic sera to insulinoma cells**

The reactivity of CM cells with selected sera was evaluated in order to define whether the cell line is a suitable substrate for the detection of autoantibodies in sera from patients with Type 1 diabetes.

Briefly, 50 µl aliquots of each serum (at dilutions ranging between 1:4 and 1:64 in PBS/BSA) were added to fixed cells. After 60 min incubation at room temperature and two washes with PBS containing 1% BSA, cells were incubated at room temperature with 50 µl of an FITC-conjugated sheep anti-human IgG (1:50; Wellcome, Beckenham, Kent, UK) and after five washes mounted on a glass slide and visualized under a fluorescence microscope (Zeiss, Germany).

**Results**

**Expression of beta cell markers**

**Monoclonal antibodies defined antigens** The immunohistochemical analysis of antigen expression by the CM cell line showed the presence of a specific immunoreactivity of CM cells with monoclonal antibodies A2B5 (Fig. 2a) and 3G5 (Fig. 2b) both binding to islet gangliosides, and with HISL-19 (Fig. 2c) recognizing an islet-associated protein, all considered to represent neuroendocrine and islet beta cell markers. No reactivity was observed when a monoclonal antibody (P3X63) with irrelevant specificity was used (Fig. 2d). Finally, intracellular cytokeratin was detected in CM cells.

**Detection of islet molecules with autoantigenic properties**

**GAD expression** The expression of the 65 kDa isoform of GAD by CM cells was shown by immunofluorescence staining with monoclonal antibody GAD-6 (Fig. 3a) and also confirmed by Western blot analysis of affinity purified GAD from CM cells showing a specific reactivity with a band of approximate molecular mass 65 kDa (Fig. 3b). By using a rabbit anti-GAD polyclonal antiserum which recognizes both isoforms of GAD we showed that CM
cells express only the 65 kDa isoform seen as a single band on Western blot (not shown).

Ganglioside expression
The biochemical analysis of ganglioside expression by the cell line showed the presence of the following pattern: three components were identified, one co-migrating with GM3 standard, another with GD3 standard and a third with a migration position between GM2 and GM1 standard (GM2–1) (Fig. 4). Quantitative determination of ganglioside expression, achieved by scanning densitometric analysis of resorcinol stained bands showed that GM3 represented 50.5%, GM2–1 41% and GD3 8.5% of total ganglioside content.

Binding of sera to CM cells
Positive immunoreactivity was found when CM cells were incubated with all ICA positive but not with ICA negative sera, as shown in Fig. 5a,b,c. Within the positive samples, differences in the pattern of staining were observed and at least two types of staining were detected: 2 of the 5 positive sera tested were characterized by a patchy distribution of the specific staining (Fig. 5a) whilst 3 sera showed a more diffused distribution of the fluorescence (Fig. 5b).

Discussion
Here we describe the analysis of beta cell markers and antigens expressed by the CM human insulinoma cell line. In virtue of its property this cell line is an ideal tool to study the mechanism of beta cell destruction occurring in Type 1 diabetes.

The establishment of a human insulinoma line in long-term culture is an extremely important achievement for research in the area of pancreatic beta cell immunopathology. The main source of human beta cells was represented by pancreatic islets freshly isolated by collagenase digestion of human pancreas followed by trypsin digestion into single islet cells, from which beta cells are separated by cell sorting. This procedure, in addition to being time consuming, only yields small amounts of tissue. To overcome the limited availability of beta cell material, insulin-producing rat (RIN; Gazdar et al. 1980) and hamster (HIT; Senterre et al. 1981) islet beta cell lines have been used in various immunological studies in vitro. However, although these beta cell lines provide suitable models for beta cell research, they may not be representative of human beta cells.
The results obtained in this study demonstrate that the human insulinoma cell line CM expresses known markers of beta cell differentiation such as those defined by monoclonal antibodies A2B5, 3G5 and HISL-19. In addition, islet molecules known to be autoantigens associated with the autoimmune response in Type 1 diabetes have been detected in CM cells including the 65 kDa isoform of the enzyme GAD which represents the main target of anti-GAD humoral and cellular immune response in Type 1 diabetes (Diaz et al. 1992, Hagopian et al. 1993, Tisch et al. 1993) and the islet autoantigen GM2–1 which is expressed in large amounts by CM cells. Interestingly, the pattern of ganglioside expression displayed by CM cells (both qualitatively and quantitatively) is virtually identical to that of native pancreatic islets (Dotta et al. 1995) unlike that displayed by the majority of transformed cell lines described so far, which were shown to have a more complex glycolipid pattern compared with their untransformed counterpart (Gillard et al. 1989).

The autoantigens involved in the process of beta cell destruction in Type 1 diabetes have only been characterized in part and few observations focus on the role of glycolipids (mainly gangliosides) besides protein antigens. The CM cell line could provide a useful tool for studying the nature of target autoantigens in Type 1 diabetes since it is an unlimited source of beta cell material and continues to express the main antigenic characteristics typical of native beta cells even after several passages. This is of relevance since it has been demonstrated that other insulinoma cell lines such as RIN and subclones of RIN cells can lose the antigenicity in regard to changes in glycolipid, principally ganglioside, expression on the cell surface (Thomas et al. 1987). One further aspect in this respect which deserves some comment is the presence in CM cells of two HLA DQ genes encoding susceptibility heterodimers Arg 52 in the DQA1 gene and non Asp 57 in the DQB1 gene (Khalil et al. 1990, Buzzetti et al. 1993). The presence of such heterodimers may be important in studies aimed to investigate in vitro autoantigen presentation being the putative antigen(s) presented ideally by the CM cells to mononuclear cells. Whether the occurrence of these heterodimers is part of the process leading to beta cell destruction may perhaps be clarified using CM cells. For example, hyper expression of HLA Class II antigens can be induced on CM cells in vitro by cytokines and proliferation of HLA-compatible lymphocytes can be tested in the presence of selected autoantigens.

CM cells also showed a specific reactivity with diabetic ICA positive sera but not with ICA negative sera when analysed by means of indirect immunofluorescence. The ICA positive sera have been tested on different passages of the CM cells and appeared to maintain the positive reaction compared with the ICA negative sera. The differences observed in the pattern of staining between positive sera probably reflect the heterogeneity of the autoantibodies present in sera of patients with recent onset Type 1 diabetes which bind to different beta cell structures (e.g. GAD, gangliosides or others) expressed by CM cells.

The expression of GAD65 has also been tested on different passages (from the 70th to approximately the 100th) and appeared to be positive in all tests performed.

In conclusion, the CM cell line shows profound similarities with native beta cells with respect to the pattern of antigen expression, in addition to morphological and endocrine properties (Gueli et al. 1987, Cavallo et al. 1992). Thus, it may represent an extremely useful model for the in vitro study of metabolic, endocrine and immunological properties of beta cells.

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Figure 5 (a and b) Indirect immunofluorescence of CM cells with ICA positive sera (1:16 dilution) showing two characteristic patterns of staining. In (a) CM cells were characterized by a ‘patchy’ distribution of the specific staining. In (b) CM cells show a more diffuse distribution of the fluorescence. (c) Indirect immunofluorescence of CM cells with ICA negative serum (1:16 dilution).
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