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ISOLATION AND PURIFICATION OF RAT ISLET CELLS BY FLOW CYTOMETRY

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ABSTRACT

Flow cytometry has been employed as a method to study homogeneity of isolated islet subpopulations. After collagenase digestion of rat pancreas and elutriation of tissue fragments, islets were isolated and dissociated, and cells were analyzed and sorted according to their low forward angle light scattering properties by using automated flow cytometry. A standardized procedure was developed for the preparation of rat islet cell grafts for purification of islet cells. In this process, after collagenase digestion of pancreas, islets were isolated, dissociated, identification by dithizone method and then with enzymatic procedure by DNase and trypsin, the islet cells changed into single cells and beta cells were identified by immunofluorescence method and then assayed by flow cytometry. Methods have been developed for the preparation of suspension of viable rat pancreatic islet cells and their analysis and sorting in the fluorescence activated cell sorter (FACC IV, Becton Dickinson, Sunnyvale, Ca). Flow cytometry of these cells indicated that there were 91% of beta cells in cell suspension. Most of the exocrine particles were lost during digestion. Purified endocrine islet cell grafts were prepared by pure beta-cells, without endocrine non-beta cells. The purified aggregates were devoid of endocrine non-beta cells and damaged cells.

KEY WORDS

Islet cells, Isolation, Purification, Flow cytometry

INTRODUCTION

The endocrine (i.e. hormone-producing) cells of pancreas are grouped in the so-called islets of Langerhans. Discovered in the 1869 by the German pathological anatomist Paul Langerhans (1847-1888), the islet of Langerhans constitute approximately 1-2% of the mass of the pancreas. There are about one million islets in a healthy adult human pancreas, which are interspersed evenly throughout the organ and their combined weight is 1 to 1.5 gms. Each islet contains approximately one thousand cells and is 50-500 mm in diameter. Hormones produced in the islets of Langerhans are secreted directly into the blood flow by (at least) four different types of cells. Sixty five to eighty percent of the islet cells are insulin-producing beta cells. The second most abundant cell type is the glucagon-releasing alpha-cells (15-20%). Additionally, islets of Langerhans contain somatostatine producing delta-cells (3-10%) and pancreatic polypeptide containing pp-cells (1%). Histological, immunocytochemical, and ultrastructural studies have demonstrated that islets of Langerhans contain several different endocrine cell types (1). In rat islets, approximately 70-80% of the endocrine cells are insulin-containing beta cells, 15-20% is glucagons-containing alpha-cells or pancreatic polypeptide-containing pp cells, and the remaining 5-10% is somatostatin-containing delta-cells (2). The heterogeneity of islet cell types, their complex organization and the likelihood of cell-to-cell communications have limited characterization of the properties and functions of individual endocrine cell types (3, 4). Development of methods to separate islet cells into homogeneous population would permit examination of specific characteristic of the different islet endocrine cell types in the absence of influences of other cell-types (5, 6). In this study, we demonstrate that analysis of light scattering from single islet cells, by using flow cytometry, can be used to sort out rat islet cells into subpopulations enriched in beta, alpha and delta cells (7, 8).

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MATERIALS AND METHODS

Collagenase, crystalline trypsin, bovine pancreatic DNase, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-Ethan-sulfonic acid (HEPES) and silicon were supplied from Merck (Germany). Percol is a commercial solution containing silicon particles, coated with polyvinyl pyrolidone, pharmacia (Sweden). Ethylene glycol-bis (α-amino ethyl ether)-N, N, N', N',-tetra acetic acid (EGTA), bovine serum albumin fraction V, CMRL-1066 medium, anti-insulin antibody and fluorescein-labeled goat anti–guinea pig second antibody were supplied by Sigma-Aldrich Co.

Media preparation : All media were filter sterilized through a 0.22 mm filter. Other reagents used were sterilized by autoclave or sterile media and glassware were employed. Glassware used for collecting Langerhans islets cells was siliconized. The glassware was coated with poly-L-Lysine involved 30 minute incubation with a sterile 10 mg/ml silicon solution followed by washing with distilled water. Separation of the Langerhans islets and the cellular purification were carried out in EHPES-buffered Earle's-Hepes medium (EH) with following composition: 124 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1.0 mM NaH_2PO_4, 14.3 mM $NaHCO_3$, 2.8 mM Glucose, and 10 mM HEPES. The medium was supplemented with 2.5% (w/v) bovine serum albumin, equilibrated with 5% CO_2 and adjusted to pH 7.30 at room temperature. The islets were isolated from male adult Wistar rats' pancreas (200-250 grams body weight) through collagenase digestion by the method of Lacy and Kostianovskt (9) which was modified to increase the yield.

Animals : Donor tissues were prepared from male adult Wistar rats (200-250 grams body weight)

Separation of Langerhans islet cells : For identifying the pancreas, two hours before dissection, 32 male Wistar rats (200-250 grams body weight) were injected interaperitoneally with pilocarpine (0.2 ml from 0.2% solution) (10-11). To carry out dissection, first the animals were anesthetized in an appropriate desicator. Then, by opening the rat's abdomen and closing the pancreatic canal, pancreas was distended by injection of 10 ml Earle's-Hepes containing 1.5 mg/ml collagenase. The gland was removed, cleaned from lymphonodes and fat tissue and minced. After 15 sec. sedimentation, the supernatant fluid was discarded and the tissue suspension diluted with an equal volume of Earle's-Hepes containing 4 mg/ml of collagenase. The tissue was digested at 37°C for 10 min. under continuous shaking (300 strokes/min) and then dispersed by gentle pipetting at room temperature for 3 min. The digest was filtered through a 500-mm nylon screen and the filtrate washed by three successive centrifugations and resuspensions in Earle's-Hepes. The filter residue was resuspended in EH without collagenase and further dispersed at 37°C for 4 minute in a shaking incubator (300 strokes/min). The second digest was finally washed in Earle's-Hepes buffer. The washed filtrate and residue fractions were examined under a dissecting microscope and clean islets were aspirated from the preparation. Using this procedure, 7000-13000 islets were routinely isolated from 32 rat pancreas within 2 h after beginning of the dissection (4).

Purification of Langerhans Islets Cells : The isolated islets were washed by three sedimentations in calcium-free EH and then resuspended in calcium-free EH containing 1 mM Ethylene glycol-bis (β-amino ethyl ether)-N, N, N', N',-tetra acetic acid (EGTA) (5 ml/1000 islets). This suspension was first maintained for 8 min at room temperature, continuously aspirated through a siliconized Pasteur pipette (9-inch), and then supplemented with trypsin (final concentration 25mg/ml) and DNase (final concentration 2mg/ml) before the aspiration was continued at 30°C. The degree of enzymatic dissociation was regularly checked under phase contrast microscope and stopped when 60-70% of the cells occurred as single units this was usually the case after 10 min. The islet cell suspension was then immediately diluted with 40 ml ice-cold calcium-free EH and filtered through a 63-mm nylon screen to remove occasional large cell clumps and undigested material. An isotonic Percoll solution with density 1.040g/ml was layered underneath the filtrate in order to remove debris and dead cells during a subsequent centrifugation at 300×g for 6 min (10). The percoll pellet was collected, suspended in 50 ml CMRL-1066 medium containing 2 mM glutamine and 0.2% BSA and incubated for 20 min at 37°C under 7.5% CO_2. At the end of this incubation, the cells were resuspended and centrifuged at 100 ×g for 1 min. The pellet, which contained mostly small cell clump which had not been completely dissociated, was resubmitted to gentle pipeting in 10 ml calcium-free EH containing EGTA. This mechanical dispersion was done for 8 minutes, after which the preparation was filtered through a percoll layer (300×g, 5 min). Meanwhile, the initial supernatant, containing most single beta cell, was centrifuged at 300×g for 6 min. The pellets collected after the two latter centrifugations were combined, washed once in Earle's-Hepes, and resuspended in Earle's-Hepes. The final cell suspension contained 7.5×10^6-10^7 cells obtained from 32 rat pancreas. This method should be carried out appropriately, carefully and rapidly so that the islets cells are less damaged (4, 11).

Purity determination of islet cells by Flow Cytometery : The freshly dissociated islet cells were submitted to auto fluorescence-activated cell sorting using a (FACC IV, Becton Dickinson, Sunnyvale, Ca) equipped with two argon lasers (Argon 164-06 and UV-argon 171-17, Spectra-physics, Mountain view, Ca) (12), illuminating the cells at 488 nm, so that the emission at 510-550 nm could be taken as a parameter for their flavin adenine dinucleotide (FAD) content (13). At 2.8 mM glucose, single β-cell displayed a 3-fold higher FAD fluorescence than single non-β cell, while its light scatter activity was also 50% larger than non-β cell. Selection of appropriate windows allowed the simultaneous isolation of single beta and single non- beta islet cells (14). The Langerhans islets cells purified with the collagenase method were prepared with 91% of beta cells in the cellular suspension (Figs 1 and 2).

Islet and β-cell identification : Islets were specifically stained by dithizone. Ten mg dithizone was dissolved in absolute ethyl alcohol then 50 ml concentrated NH_4OH was added and supplemented with 12 ml Hank's solution [45mM Na_2HPO_4, 2.5mM citric acid, 0.1% Triton X-100], before using, the solution was diluted with Hank's solution (pH 7.8) by 1 to 100, passed through a 0.22 μm filter membrane. Islet suspension was mixed with dithizone and placed 10 min and identified by light microscope.

Identification of β -cells by Immunofluorescence methods : β -Cells were fixed by using Bouin's solution [71.4% picric acid solution (1.2% w/v), 23.8% formalin, and 4.8% glacial acetic acid]. After 24 h, cells were rinsed three times with Phosphate Buffer Solution (PBS), dehydrated and permeabilized with graded concentrations of ethanol, and incubated for 2 h at room temperature with an anti-insulin antibody diluted 1:1,000 in PBS. After rinsing, slides were incubated for 1 h at room temperature with a fluorescein-labeled goat anti–guinea pig second antibody (1:400). After rinsing in PBS, slides were covered with 0.02% p-phenylenediamine in PBS-glycerol (1:2, V/V) and screened by fluorescence microscopy.

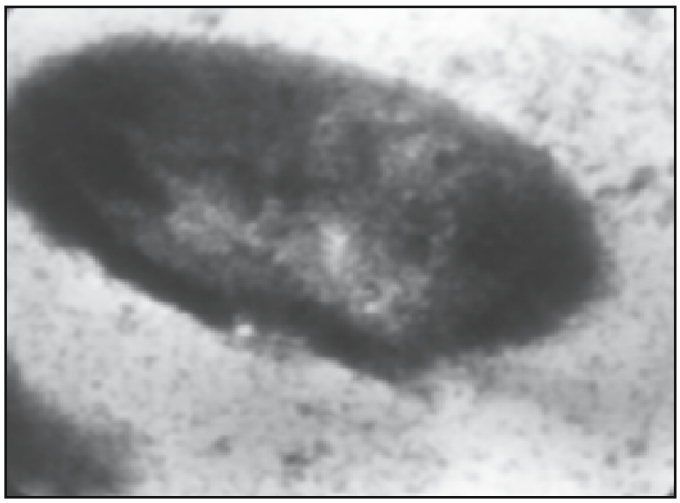

Fig 1 : The electronic micrograph of purified β- cell of Langerhans islets at the cell suspension stained by Osmium tetraoxide The cell suspension made by using 32 pancreas contained 7.5×10^6-10^7 cells.

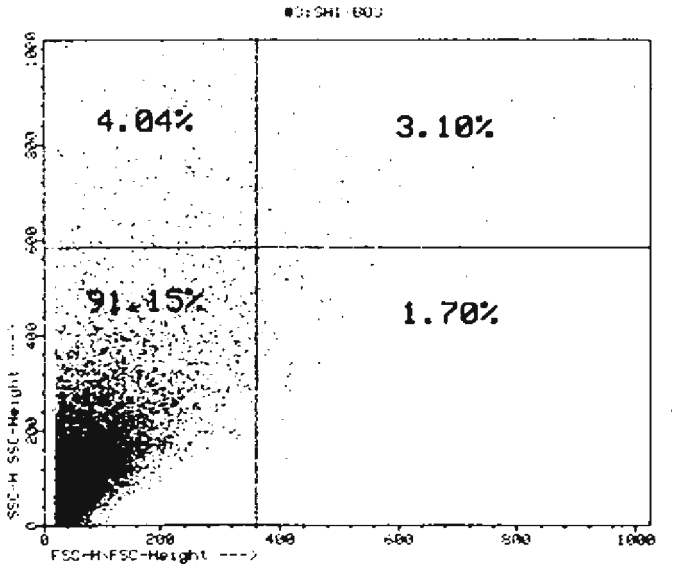

Fig 2 : Superficial distribution curve of Langerhans islets cells suspension obtained from flow cytometry, in the flow cytogram of a homogenous bulk of cells with purity of 91% which belongs to the cells with fewer granularities among the langerhans islets cells, i.e. beta cells. Single cell analysis of β-cell in graph 1 quadrant (3) on the x-axis sideward scatter and on the y-axis forward scatter is plotted. In quadrant (3), the total population is scatter " Peacock tail" that most of the B-cells are depolarized in quadrant (3), and 4.04% cells are classified as live in quadrant (1) and 3.10% classified as live in quadrant (2) and 1.70% cells are classified as live in quadrant (2).

RESULTS AND DISCUSSION

Flow cytometry is a technique by which the physicochemical specifications of the cells or any biological component are recorded individually when they pass against laser beam. The individuality and solution nature of the cells are important in flow cytometry. The sample must be a solution from the outset or be made into a solution with enzymatic methods, in which each tissue is prepared with special method of its own. Measurement of parameters such as size, form, DNA content, surface cell receptors, enzymatic activity, membrane permeability and calcium pump are possible with this method. Our goal in flow cytometry is to find out information on the homogeneity of beta cells and the percentage of homogeneity

of these cells in cellular suspension obtained at the end of purification of the Langerhans islets cells so as to determine the percentage of beta cells in the suspension. In view of the considerable difference in the sizes of types of Langerhans islets cells, a sample cellular suspension solution can be injected into the flow cytometry system, model (FACC IV, Bacton Dickinson, Sunnyvale, Ca) and obtain the appropriate graph, which indicates the types of cells and their percentage in the suspension. The Langerhans islets cells purified with the collagenase method were prepared with 91% of beta cells in the cellular suspension (15) (Fig 1). As the suspension of insulin-secreting beta cells is supplied by the pancreas of healthy rats with the collagenase-digestion method as modified by us. Supply of pure Langerhans islets cells of rats requires combination of mechanical action (cutting the pancreas into pieces) and simultaneous use of the enzymes (trypsin and DNase). This method depends on conditions of dissociation of pancreatic cells from other tissues. Addition of DNase reduces the dissociated, slicked pancreatic cells and trypsin prevents formlessness of the dissociated cells. Moving to another container eliminates the smaller pieces and increases the concentration of larger particles of the islets. Filtration and incubation separate the purified cells from the slicked cells.The four quadrant of the cytogram shows that on the surface distribution curve, the Langerhans islets cells suspension is homogenous with the purity of 91% that belongs to the cells with fewer granularities among the Langerhans islets cells i.e. beta cells (Fig 1).There are many reports indicating the purification of islet cells from pancreas but none has shown the single pure β- cell as we have done (2, 5, 9, 12). In addition, identification of two cell populations of the islets that were different in terms of size conformed to the small-sized pp-cells, delta-cells and alpha-cells and the bigger-sized beta-cells for identifying the insulin-secreting cells in the cellular suspension (16). Pancreatic islets were obtained from adult rats using the collagenase digestion optimized/modified method of Lacy and Kostianovsky (8). Isolated islets were dissociated in calcium- free medium containing trypsin and DNase. The islet cell suspension was cleared from debris and dead cells via centrifugation through a percoll layer of density 1.040 g/ml (17). After preincubating the cells for 10 min in ice bath, they were centrifuged at 800g for 5 min. and resuspended in physiological serum. The cells were analyzed and separated in a fluorescence- activated cell sorter (FACS can) and obtained appropriate graph, which indicates the types of cells and their percentage in the suspension. The Langerhans islets cells purified with the collagenase method were prepared with 91% of beta cells in the cellular suspension. Single cell analysis of β-cell in graph 1 quadrant (3) on the x-axis sideward scatter and on the y-axis forward scatter is plotted. In quadrant (3), the total population is scatter " Peacock tail" that most of the β-cells are depolarized in quadrant (3), and few cells are classified as live in quadrant (1) and only a few cells classified as live in quadrant (2) and small amount cells are classified as live in quadrant (Fig 2).

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