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A NOVEL, SIMPLE METHOD FOR PURIFICATION OF ADULT PIG PANCREATIC ENDOCRINE CELLS

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Adult pig pancreatic endocrine cells were harvested by auto-digestion without added enzymes. The isolated, crude cells were purified by Mono-poly resolving medium (MPRM). The purity of the harvested cells was determined by dithizone staining and the number of pancreatic endocrine cells was counted. A large number of the cells were stained red with dithizone and showed high viability and a good insulin secretory response to glucose stimulation. The average number of cells purified by MPRM was $3.40 \pm 1.32 \times 10^5$ cells/g pancreas and the number of dithizone-stained cells was $2.81 \pm 1.09 \times 10^5$ cells/g pancreas. The insulin secretion from the pancreatic endocrine cells was maintained throughout a 40-day observation period and high glucose stimulation induced an increase in insulin secretion from the cultured cells. In the cells purified by MPRM, light and electron microscopic studies showed the cells to be typical pancreatic endocrine cells. The present purification method using MPRM allowed us to obtain quickly a large amount of adult pig pancreatic endocrine cells from the unpurified preparations. This is useful for transplantation and biochemical or biological studies of adult pig pancreatic endocrine cells.

Introduction

It is generally considered that purification is one of the most important procedures for successful pancreatic endocrine cell transplantation. There have been several reports of studies of islet purification from large mammalian pancreases[1)~3)]. In a recent study, we tried to isolate and purify single pancreatic endocrine cells from adult pig pancreas rather than isolate the whole islets[4)5)].

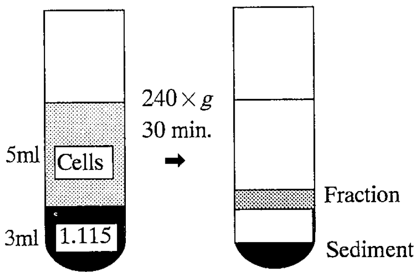
In this report, we describe a simple procedure for collecting a large number of purified adult pig pancreatic endocrine cells, using Mono-poly resolving medium (MPRM). The validity of the method was evaluated by morphological and functional studies of the high purified pancreatic endocrine cells.

Materials and Methods

1. Preparation and purification of the pancreatic endocrine cells

An adult pig (9～12 months) pancreas was obtained from a local slaughterhouse (Tokyo Shibaura Zouki, Tokyo, Japan), sterilized with 2% povidone-iodine solution as soon as possible, and transported to the laboratory in cold (4°C) RPMI 1640 medium (GIBCO Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO) and 5 mM D-glucose. Immediately after arrival, the pancreas was trimmed by careful dissection

of surrounding fat tissue, lymph nodes, vessels, and membranes on a ice-cold plate. Approximately 300 ml of cold (4°C) phosphate-buffered saline (PBS) (SIGMA, St. Louis, MO, USA) containing 3.0% newborn calf serum (NCS) (GIBCO) and 5.5 mM D-glucose was injected into the pancreatic duct. The pancreas was then divided into four portions. Each portion was minced with scissors into small pieces (1～2 mm in diameter), and the pieces were transferred into a steel filter-basket, with a mesh size of 0.7 mm, containing a small magnetic bar. The basket was placed in a beaker, and 40 ml of cold (4°C) PBS containing 3.0% NCS and 5.5 mM D-glucose was added. The minced tissue was stirred (130 rpm) with a magnetic stirrer (4-position magnetic stirrer. Model 1104. WA-KENYAKU, Tokyo, Japan) for 2 min, and the supernatant was discarded. This procedure was repeated twice. Then, PBS was added to the minced tissue and the mixture was gently stirred (130 rpm) for 10 min. Cells were colleted immediately from the beaker by centrifugation for 1 min at 1500 ×g. The resulting pellet of cells was resuspended in 30 ml of PBS and centrifuged at 170 ×g for 3 min. This procedure was repeated twice. The procedures from stirring for 10 min were repeated six times until most of the cells had been returned from the basket. All the cell pellets were collected. Finally, the harvest was purified by Mono-poly resolving medium (MPRM, Dainippon Pharmaceutical Co., Tokyo, Japan). MPRM is a sterile aqueous solution (a mixture of Ficoll and metrizoate[6], used as a supporting medium in centrifugation and flotation procedures) and is prepared at a density of 1.115 ± 0.002 g/ml. Three milliters of MPRM were placed in a tube (13 × 100 mm) and 5 ml of a mixture of the cells suspended in RPMI 1640 with 10% FBS, 5.5 mM D-glucose was layered onto the medium. This solution was centrifuged at 240 ×g for 30 min, and a fraction of dense cells which were floating in the medium were harvested with a Pasteur pipette (Fig. 1). These harvested cells were suspended in 45 ml of RPMI 1640 with 10% FBS, 5.5 mM D-glucose and centrifuged at 380 ×g for 3 min, followed by another wash and centrifugation at 170 ×g for 5 min. This procedure was repeated twice, and the harvested pellet was resuspended in RPMI 1640 with 10% FBS and 5.5 mM D-glucose. The purified cells were placed on individual Millicell-CMs (Millicell-HA; 0.45 μm culture plate insert, 12 mm diameter. Millipore Products Division, Bedford, MA, USA) in a plastic 12-well culture plate (CORNING, Boston, NY, USA) and cultured at 37°C, 5% CO_2 in an incubator. The procedure was repeated eight times.



Mono-poly resolving medium (MPRM)

Fig. 1 Schematical representation of layers for pancreatic endocrine cells purification using Mono-poly resolving medium (MPRM)

2. Morphological studies of pancreatic endocrine cells purified by MPRM

The cells purified by MPRM were stained with dithizone[7]. The total number of cells and the stained cells were counted. To identify the types of cells purified by MPRM, the cells were fixed with Bouin's fluid for light microscopic observation. The specimens were embedded in paraffin. Immunohistochemical staining was performed using the labelled strepto-avidin biotin method[8] with a LSAB kit (DAKO Japan, Kyoto, Japan) containing antibodies, including guinea pig anti-porcine insulin, rabbit anti-human glucagon, rabbit anti-human somatostain, and rabbit anti-human pancreatic polypeptide.

For electron microscopic observation, the cells were fixed with 2.5% glutaraldehyde for 1 hr, then rinsed, postfixed with 1% osmium

tetraoxide in 0.1 M phosphate buffer (pH 7.4) and embedded in Epon 812. Ultrathin sections were stained with 2% uranyl acetate and lead citrate, and examined with a H7000 Hitachi electron microscope at 75 kV.

3. Functional studies of pancreatic endocrine cells purified by MPRM

1) Function of fresh cells

Purified pancreatic endocrine cells were placed on individual Millicell-CMs (Millipore Products Division) in a plastic 12-well culture plate (CORNING). The cells were incubated overnight in 1 ml of RPMI 1640 containing 7.8 mM D-glucose and 10% FBS. The medium was then discarded and replaced with 1 ml of RPMI 1640 containing 3.3 mM D-glucose and 10% FBS. The cells were incubated for 90 min at 37°C, and the medium was replaced with either 1 ml of RPMI 1640 containing 3.3 mM D-glucose (Low glucose) or 16.7 mM D-glucose (High glucose) and 10% FBS. Following an incubation for 90 min at 37°C, the medium was collected and stored at −20°C for an immunoassay of insulin by the two-antibody system, as previously reported (^{125}I-Insulin; DAINABOT, Tokyo, Japan)[9].

2) Function of cultured cells

After one week of cell culture in RPMI 1640 containing 7.8 mM D-glucose, 10 mM nicotinamide and 10% FBS at 37°C, glucose-induced insulin secretion from the pancreatic endocrine cells was evaluated. The culture medium was replaced with 1 ml of RPMI 1640 containing 3.3 mM D-glucose and 10% FBS, and the cells were incubated at 37°C for at least 3 hr. The medium was then replaced with 1 ml

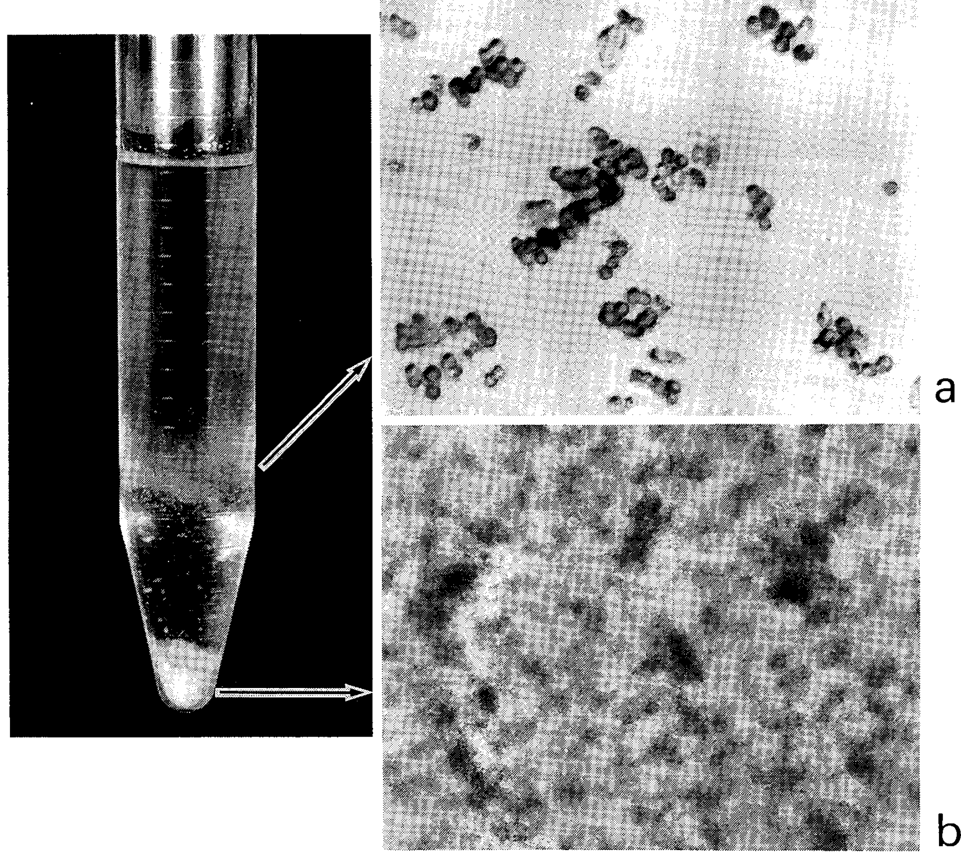


Fig. 2 Photomicrographs of purified pancreatic endocrine cells using MPRM
(a) Most of the purified cells are positively stained with dithizone, thus contamination by exocrine tissue is minimum.
(b) The sediment consists of small fragments of acinar and ductal tissue that are not stained with dithizone.

of RPMI 1640 containing 3.3 mM D-glucose (Low glucose) or 16.7 mM D-glucose (High glucose) and 10% FBS. The incubation media were collected 90 min later and stored at −20°C for the insulin assay.

Other purified cells were kept for 40 days in RPMI 1640 containing 7.8 mM D-glucose, 10 mM nicotinamide and 10% FBS at 37°C. The medium was replaced every 3 days. Insulin concentrations in each medium collected were subsequently determined.

4. Statistical analysis

Results are expressed as mean ± S.D. Unpaired Student's t test was used for statistical analysis of data. Significance in differences was defined when $p<0.05$.

Results

The cells purified by MPRM always formed a clearly demarcated cell fraction that was easy to collect with a Pasteur pipette (Fig. 1, 2). Immediately after collection of the adult pig pancreatic endocrine cells, microscopic examination showed a mixture of single cells and a few cellular aggregates. The average number of cells purified by MPRM was $3.40 \pm 1.32 \times 10^5$ cells/g pancreas and that of dithizone-stained cells was $2.81 \pm 1.09 \times 10^5$ cells/g pancreas. The proportion of dithizone-stained cells among the purified cells was 82.6 ± 2.5%. Most of the cells purified by MPRM stained with dithizone, indicating that these cells were rich in B cells. The sediment included small fragments of acinar and ductal tissue that were not stained with dithizone (Fig. 2). The number of pancreatic endocrine cells purified by MPRM and dithizone-stanined cells are shown in Table 1.

By immunohistochemical staining, cells purified by MPRM were positive for anti-insulin, anti-glucagon, anti-somatostatin and anti-pancreatic polypeptide antibodies. These purified cells consisted of B cells, A cells, D cells and PP cells (Fig. 3). Proportion of cell types purified by MPRM were summarized in Table 2.

Table 1 Number of adult pig pancreatic endocrine cells purified by MPRM

Experiments	Purified cells	Dithizone-stained cells
1	2.06×10^5	1.65×10^5
2	1.98×10^5	1.64×10^5
3	2.80×10^5	2.40×10^5
4	3.92×10^5	3.15×10^5
5	6.12×10^5	5.10×10^5
6	3.16×10^5	2.71×10^5
7	3.94×10^5	3.16×10^5
8	3.24×10^5	2.69×10^5
Average	$3.40\pm1.32\times10^5$	$2.81\pm1.09\times10^5$

Numbers (cells/g pancreas) obtained from 8 expreiments are given separately. At bottom, the overall data are expressed as mean±S.D.

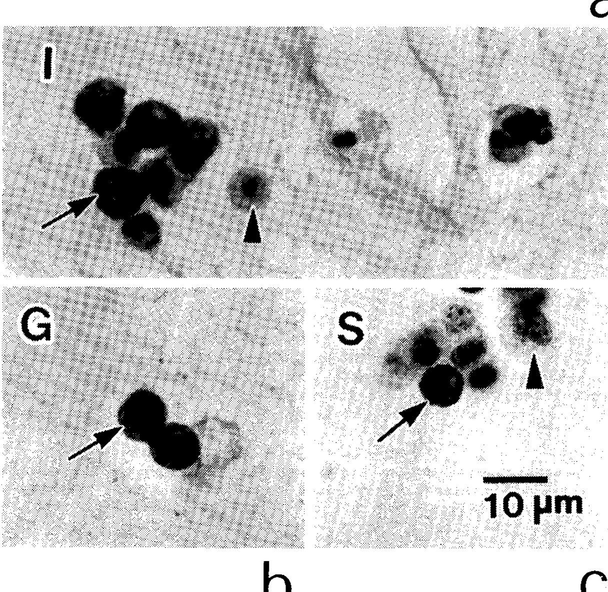


Fig. 3 Immunohistochemical staining (labelled strepto-avidin biotin method) of adult pig pancreatic endocrine cells purified by MPRM
These purified cells included cells stained by (a) anti-insulin [I], (b) anti-glucagon [G], and (c) anti-somatostatin [S] antibodies. (×132)
↑; typically stained cell, ▲; unstained cell.

By electron microscopic observation, at least two types of cells were found in a cell cluster (Fig. 4a). The B cells contained secretory granules showing a special crystalline pattern (Fig. 4b). The A cells contained dense secretory granules with a homogenous content (Fig. 4c). Both the A and B cells had a spherical nucleus,

Table 2 Proportion of pancreatic endocrine cell types purified by MPRM

Intensity of staining	+	±	−	Total number of cells
B-Cell	60.0% (1,592)	5.7% (151)	34.3% (908)	(2,651)
A-Cell	45.8% (972)	6.5% (138)	47.4% (1,013)	(2,123)
D-Cell	6.9% (85)	2.9% (35)	90.2% (1,108)	(1,228)
PP-Cell	1.1% (8)	0% (0)	98.9% (741)	(749)

Numbers in parenthesis are those of counted cells. Intensity of staining was expressed by + ; stained, ± ; weakly stained, − ; not stained.

Golgi apparatus, mitochondria, rough endoplasmic reticulum and ribosomes in the perinuclear cytoplasm.

In the cells purified by MPRM, the secretion of insulin in the freshly purified cells and in those cultured for a week is shown in Fig. 5a. High glucose stimulation resulted in a good response of insulin secretion in cells cultured for a week but not in the fresh cells, although the difference was not significant. Spontaneous insulin release (insulin accumulation) from cells purified by MPRM was maintained over 40 days of culture (Fig. 5b).

Discussion

MPRM has been used for the generation of density gradients for the isolation of leukocytes in one centrifugation step, due to its lower viscosity and easily adjustable osmolarity[10]. The diameters of neutrophils, lymphocytes and adult pig pancreatic endocrine cells are very similar: neutrophils, 10～16 μm; lymphocytes, 7～16 μm; and adult pig endocrine cells, about 10 μm (Fig. 4). This similarity is thought to be useful for purification of adult pig pancreatic endocrine cells using MPRM. That is, the cells are separated primarily on the basis of differences in cell diameter and to a lesser extent on differences in density ($dr/dt = a^2(Dc - Dm)\omega^2 r/\kappa\eta$; r: distance of the cell from the center of revolution, t: time, a: diameter of the cell, Dc: density of the cell, Dm: density of the gradient at the location of the cell, ω: angular velocity, η: viscosity of the gradient at the location of the cell, κ: constant)[11].

Overlapping of densities and diameters of a variable fraction of exocrine and endocrine tissue is contributory in impure preparations. Damaged exocrine tissues release proteolytic enzymes that provoke cell aggregation, which alters tissue density and greatly reduces the efficiency of the density gradient centrifugation[11]. The purification method using MPRM was capable of enriching pancreatic endocrine cells to above 80% in a final preparation.

In the present study, a large number of cells purified by MPRM were stained with dithizone[7]. The prevalence of the purified endocrine cells was quite similar to that found in the native islets. The fine structure of the purified cells showed characteristics of B and A cells from the intact pancreas and the structure of the purified cells was maintained. Watari[12] reported that the A cells of fish through mammals commonly have round, dark, secretory granules covered with a clear, limiting membrane. B cells show a core of dark secretory granules surrounded by a broad space and a limiting membrane. The core of the secretory granules of B cells is less electron-dense than that of A cells, and the shape of the core differs markedly between animals. The rectangular-shaped core pattern of these adult pig B cells resemble that seen in human B cells.

The purity of the pancreatic endocrine cells is crucial for successful transplantation. The advantages of transplanting highly purified pancreatic endorine cell preparations include increased safety, reduced immunogenicity of the graft, and overall improvement in the outcome of pancreatic endocrine cell transplantation. There have been several reports of severe portal hypertension[13], and cases of death resulting from intraportal auto-transplantation of unpurified pancreatic grafts[14]. Gray et al[15] reported that exocrine contamination inhibited implantation of pancreatic endocrine cells

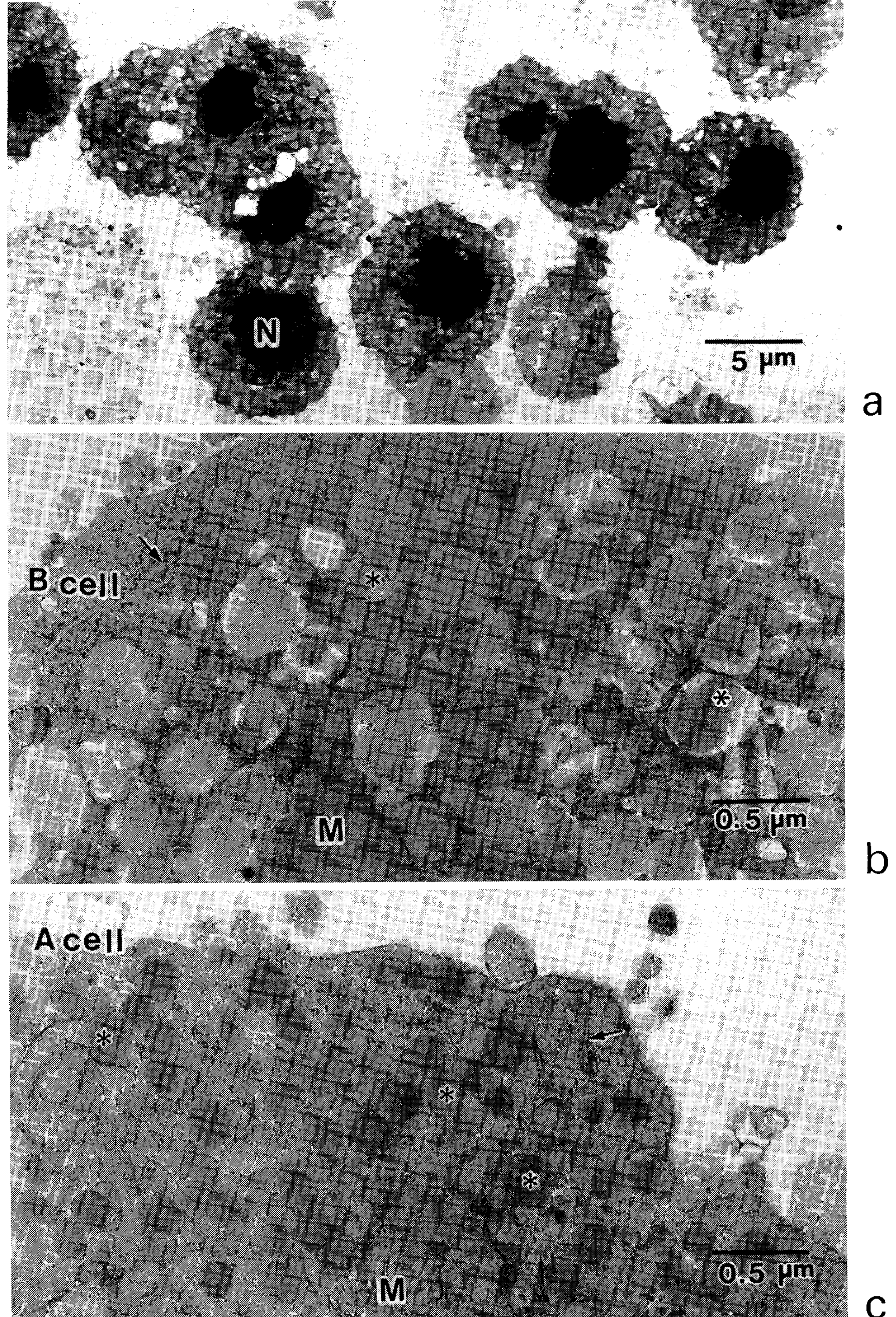


Fig. 4 Electron micrographs of adult pig pancreatic endocrine cells purified by MPRM
(a) Low power electron micrograph of pancreatic cells (×3,000)
(b) Electron micrograph of B cell (×30,000)
(c) Electron micrograph of A cell (×30,000)
The ultrastructure of B and A cells purified by MPRM was well-preserved.
M; mitochondrion, N; nucleus, *; secretory granule, ↑; rough endoplasmic reticulum.

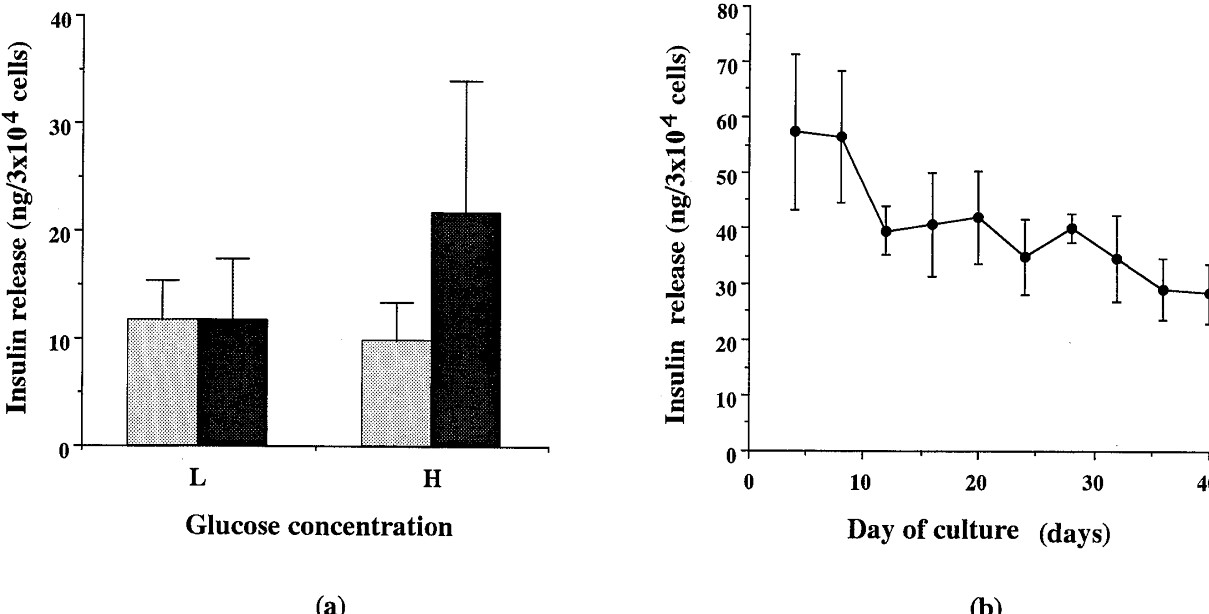


Fig. 5 Secretion of insulin by purifed adult pig pancreatic endocrine cells (Data are expressed as mean ± S.D.)
(a) Purified cells (freshly purified or cultured for one week) were incubated with either RPMI 1640 containing low glucose (3.3 mM D-glucose) or high glucose (16.7 mM D-glucose). Following incubation, the medium was collected for insulin assay. Cells cultured for one week with high glucose showed enhanced insulin secretion.
L; low glucose (3.3 mM), H; high glucose (16.7 mM), ▒: freshly purified cells, ■: one-week cultured cells.
(b) Other purified cells were cultured up to 40 days. The insulin concentration of the medium was determined every 3 days when the medium was changed. Spontaneous insulin release was maintained throughout the observation period.

transplanted beneath the kidney capsule due to tissue necrosis and fibrosis. Ulrichs and Muller-Rucholtz[16] showed that human exocrine tissue may express MHC class II antigen because preparations of human crude islets provoked a greater response in a mixed islet lymphocyte culture than did those of purified islets. Furthermore, Lacy[17] mentioned that lymphoid antigen-presenting cells in the islets (passenger leucocytes) are responsible for initiation of rejection of islet allografts. Therefore, highly purified adult pig pancreatic endocrine cells using MPRM may be useful for prevention of allograft rejection.

Though glucose-induced insulin secretion in freshly purified cells was impaired in the present study, the function recovered after one week of culture. The reasons for the poor insulin response of freshly purified pancreatic endocrine cells to glucose stimulation is presently unknown. However, development of B cell function in culture, especially in the presence of nicotinamide has been reported[18]. Okamoto[19] reported that islet damage causes free radical production as a final common pathway in B-cell damage. Superoxide production at the inflammatory site would be the source of islet toxicity and it can be converted to the reactive hydroxyl radical within the cell. This radical is extremely toxic; it can cause DNA breakage, and DNA damage then activates the repair enzyme poly-(ADP-ribose)-synthetase which depletes the cell's nicotine adenine dinucleotide (NAD). Nicotinamide would be protective by inhibiting poly-(ADP-ribose)-synthetase and maintaining cellular NAD levels that protect the cell from radical damage. Therefore, we use nicotinamide in the culture medium during the

incubation period.

The present purification method, using a single density layer of MPRMA is a simple and effective procedure to obtain a large amount of pure pancreatic endocrine cells. It is thought to be useful for transplantation of pancreatic endocrine cells or studies of their biochemical or biological properties.

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簡便な成熟ブタ膵内分泌細胞分離法の検討

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膵細胞移植に際しては，大量かつ高純度の膵内分泌細胞を得ることが重要である．本研究では，リンパ球分離溶液を用いた簡便な成熟ブタ膵内分泌細胞の分離法を検討した．屠殺場より入手した成熟ブタ膵を細切・攪拌した後，細胞懸濁液を遠心分離し単離膵細胞を収集した．これをリンパ球分離溶液であモノポリ分離溶液（mono-poly resolving medium: MPRM）を用いて，膵内分泌細胞を外分泌細胞および血管内皮細胞，血液細胞などより分離・精製した．得られた膵分泌細胞数を計測し，形態学的・機能的観察を行った．その結果，①得られた膵内分泌細胞数:$3.40\pm1.32\times10^5$/gのうち，ジチゾン染色陽性細胞数は$2.81\pm1.09\times10^5$/gで，純度は82.6 ± 2.5%であった．②免疫組織化学染色では，60%がB細胞であった．③電顕では，典型的な分泌顆粒を有するB細胞，A細胞が認められた．④グルコース負荷試験では，分離直後のインスリン分泌能低下は1週間の培養で改善し，また40日間の培養中インスリンの分泌が保たれた．成熟ブタ膵内分泌細胞径は，ヒトリンパ球径に類似しており，MPRMを用いた1回の遠心操作で，膵分泌細胞は明瞭に分離され安定した細胞数が得られた．また，膵内分泌細胞の構成は膵島におけるそれと類似しており，超微細構造も保たれていた．

以上より，MPRMを用いた膵内分泌細胞の分離法は，簡便で安定した細胞数が得られ，その形態・機能とも良好で有用な方法であると考えられた．