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Selective cytotoxicity of MIA Pa Ca-2 conditioned medium to acinar cells: a novel approach to reduce acinar cell contaminants in isolated islet preparations from BALB/c mice

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Abstract Contamination of acinar cells in islet preparations has been shown to affect islet viability, functionality and yield adversely. Therefore, a strategy which would reduce acinar contamination in islet preparations is much sought. We here demonstrate selective cytotoxicity of conditioned medium (CM) of MIA Pa Ca-2 (human pancreatic carcinoma) cells to acinar cells and suitability of this approach as a simple method of obtaining a pure islet population without affecting their viability and yield. When isolated, islet preparations from BALB/c mice were exposed to conditioned media of MIA Pa Ca-2, acinar cells underwent extensive degeneration to yield 80–85 % pure islet population, and islets showed comparable viability to controls not exposed to conditioned media. They also maintained their normal morphology, as assessed by digital image analysis. Islets treated with a conditioned medium also preserved *in vitro* insulin secretion. Flow cytometric analysis of acinar cells treated with conditioned media revealed accelerated DNA damage (45 %), compared to controls (22 %). Results emphasize the role of factors secreted by MIA Pa Ca-2 cells in inducing selective toxicity to acinar cells.

Keywords MIA Pa Ca-2 · Conditioned medium · Acinar cell depletion · Islet purification

Abbreviations *CM* Conditioned medium · *DMEM* Dulbecco's modified Eagle's medium · *DTZ* Dithiozone · *FCS* Fetal calf serum · *STI* Soybean trypsin inhibitor · *KRBH* Kreb's Ringer bicarbonate buffer (pH 7.4) supplemented with 1 mg/ml BSA and 10 mmol/l HEPES

Introduction

Currently, type-I diabetes mellitus is treated with exogenous insulin administration, but traditional insulin therapy does not prevent long-term systemic complications, and therefore alternatives should be sought. Islet transplantation has been performed as a seemingly efficient alternative to daily insulin injection therapy. However, all these protocols need homogenous islet cell populations for transplantation. Contamination of pancreatic acinar cells in the islet cell preparation is the problem commonly encountered. Activation of acinar cell enzymes during the islet isolation procedure has been reported [5, 13]. Contaminating exocrine cells in the islet preparations secrete various activated proteolytic enzymes such as trypsin, neutral protease, carboxypeptidase that can damage the islet cells, adversely affect their viability, and significantly influence their yield [5, 14]. Acinar cell contamination has also been shown to induce a significant decrease in the stimulation index of insulin release from cultivated as well as encapsulated islets [7]. Inclusion of protease inhibitors in pancreatic digestion mixture has been shown to increase the islet yield [6, 11].

Handpicking of freshly isolated islets to obtain homogenous populations has been routinely practiced,

however, this method is tedious and time consuming. Many alternative approaches have been proposed. Immunological methods, such as purification of islets by selective lysis of the acinar cells and utilizing magnetic microspheres coated with acinar-cell-specific monoclonal antibodies for depletion of acinar cells, have been proposed [4]. Islet isolation using various density gradients have been reported [9, 11]. Flow cytometry [6] and automated procedures have also been reported [8]. Pancreatic islet isolation using the COBE 2991 cell processor in humans and in adult pigs have also been reported [2, 12]. Though many of these protocols generate around 80–96 % pure islets, they suffer from low cost effectiveness; low islet yield, and the laborious procedures involved [3] in obtaining homogenous islet populations free of acinar cells. Therefore, an easy, rapid, and cost effective method of generating pure islet cell populations without affecting their yield is desired.

We here report selective cytotoxicity of conditioned medium from 48 h culture (CM-48) of MIA Pa Ca-2 cells to pancreatic acinar cells and report its suitability as a simple and rapid method of obtaining pure islet cell populations without affecting their yield.

Materials and methods

Collection of CM of MIA Pa Ca-2

MIA Pa Ca-2 cell line (ATCC CRL-1420), was obtained from the National Center for Cell Science (NCCS) Cell repository and maintained at 37 °C in humidified 5 % CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM), (Gibco BRL, Rockville, Md., USA) supplemented with 10 % Fetal Calf Serum (FCS) (Trace Biosciences, made in Australia). CM was collected at 48 h, hereafter referred to as CM-48. It was stored at -20 °C until further use.

Islet isolation

Principles of laboratory animal care (NIH publication No. 86–23, revised 1985) were followed in all the animal experiments carried out. Islets were isolated by following the protocol reported earlier by the authors [10]. In short, BALB/c mice (8–10 weeks) were killed by cervical dislocation, and the pancreases were dissected. They were finely minced and digested in DMEM containing 1 % collagenase Type V, (Sigma Chemical , St. Louis, Mo., USA), 2 % BSA, Fraction V, (Sigma) and 2 mg/ml Soybean Trypsin Inhibitor (STI) (Sigma). Digestion was stopped by adding chilled DMEM with 10 % FCS. This mixture was then washed twice with RPMI 1640 by centrifugation at 1000 rpm for 10 min. Then it was vortexed, and pellets were sown in 25 cm^3 culture flasks (Nunc, Roskilde, Denmark) containing RPMI-1640 (Gibco BRL, Rockville, Md.,USA) pH 7.2 supplemented with 10 % volume/volume FCS (Gibco). These flasks were incubated at 37 °C in a CO_2 incubator (Forma, Marietta, Ohio, USA) gassed with 5 % CO_2 in air. Islets were observed after 6 h of incubation with an inverted microscope (Olympus, Tokyo, Japan).

Treatment of islets preparations with CM-48

An equal volume of CM-48 was added to the Islets cultured in RPMI-1640 and incubated for 6 h. Cultures without CM were treated as controls. Cultures were observed with a phase contrast microscope (Olympus, Tokyo, Japan) to monitor islet- and acinar cell morphology. After 6 h, acinar cell viability was taken by trypan blue dye exclusion (0.04 % trypan blue). Islets were washed with RPMI-1640 and resuspended in fresh RPMI-1640 with 10 % FCS. Islet cell viability was assessed after 24 h by trypan blue dye exclusion. Cells that turned blue were scored as dead, while viable cells did not take up the dye. Specificity of islets was determined by dithiozone (DTZ) staining (Sigma, St. Louis, Mo., USA). Diathiazone stock solution (39 mmol/l was prepared by dissolving 100 mg of DTZ in 10 ml DMSO, filtered, aliquoted, and stored at -15 °C) [10]. Routine staining was carried out by adding 10 µl DTZ stock to islets suspended in 1 ml Krebs-Ringer bicarbonate buffer (pH 7.4) with HEPES (10 mmole/l) and incubated at 37 °C for 10–15 min. The stained islets were counted under an inverted microscope (Olympus, Tokyo, Japan).

Islet morphometry analysis by digital image analysis

Morphometry analysis was performed with a digital image analysis system (Kontron Elektronik, Munich, Germany) connected to a Zeiss Axioplan 2 microscope. Islets were observed, images were captured with a VarioCam PCO CCD imaging camera and processed to obtain binary images. Binary images were taken in further computations, using image analysis software (KS400 ver. 2.0) to calculate area and diameter of CM-48 exposed and control islets.

In vitro islet functionality determination

Triplicate groups of 10 islets each CM treated plus controls were handpicked and placed in a single well of a 24-well plate, (Nunc, Roskilde, Denmark) each containing 1 ml of Kreb's Ringer bicarbonate buffer (pH 7.4) supplemented with 1 mg/ml BSA (Sigma Chemical, St. Louis, Mo., USA) and 10 mmol/l HEPES (hereafter referred to as KRBH) supplemented with 5.5 mmole/l glucose. The plates were incubated at 37 °C in 5 % CO_2 atmosphere for 1 h. The supernatant was collected and stored at -20 °C and assayed for basal insulin level. The islets were then challenged with KRBH supplemented with 16 mmole/l glucose and incubated for a period of 1 h. At the end of incubation, the supernatant was collected and stored at -20 °C. The immunoreactive insulin concentration of all the stored samples was assayed with the RIA kit (DPC, L. A. Calif.,USA).

Assessment of islet yield

Islet yield was assessed by a single observer "blinded" to the islet treatment.Only intact islets of a diameter larger than 100 µm were included. Islets were handpicked and counted under a binocular dissecting microscope (× 25) with background green illumination and white side lighting.The number of islets was determined by counting islets in three aliquots of 10 % of islet pellets and multiplying the mean number of islets in these aliquots by 10 ($n = 5$).

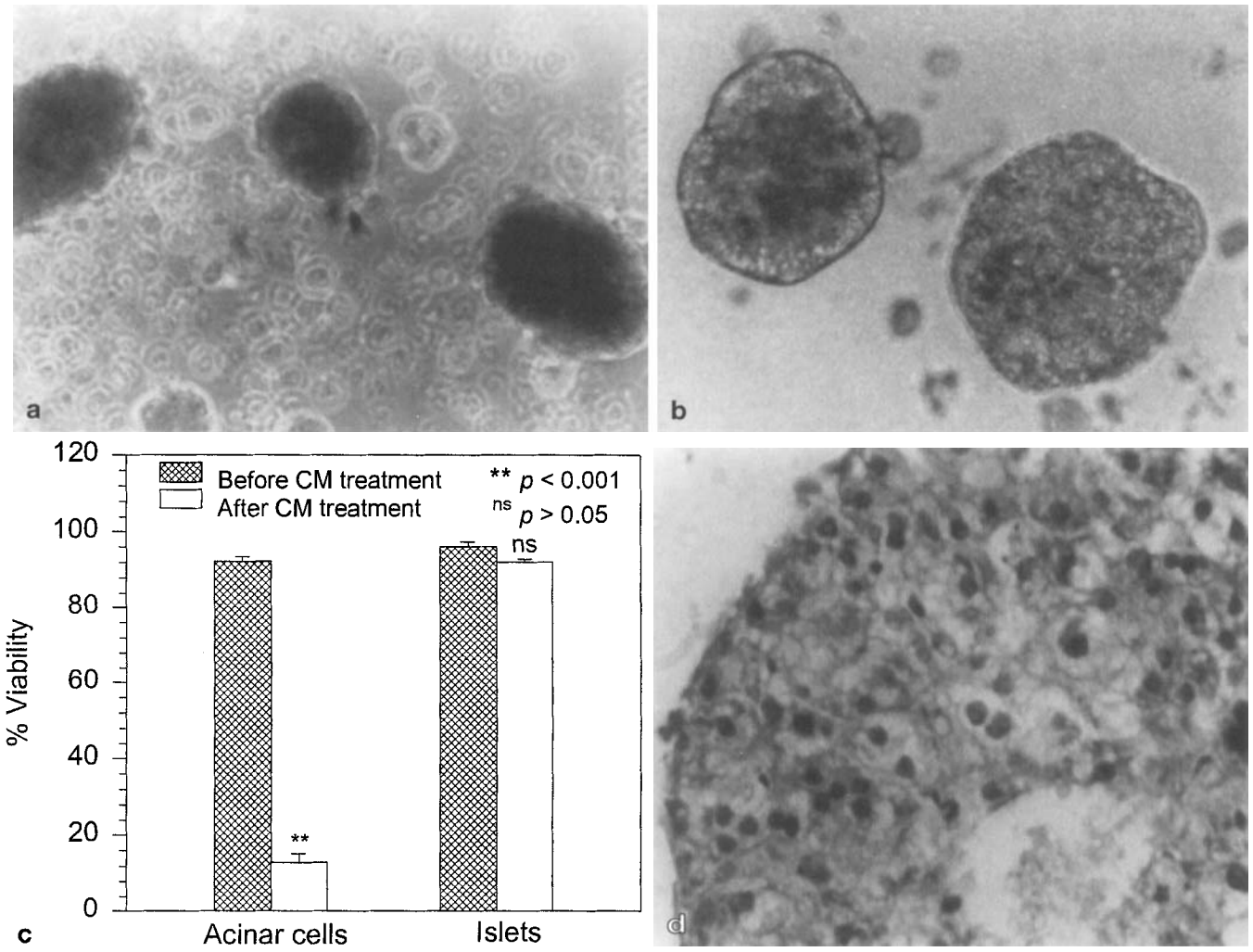

Fig. 1 a Control islets after isolation. Note the presence of acinar cells around islets (× 200). **b** Islets after treatment with CM-48, note the significant decrease in acinar cell number (× 200). **c** Viability of acinar cells and islets assessed by trypan blue dye exclusion after 6 h and 24 h of CM-48 treatment respectively. **d** CM-48 treated islet stained with H&E. Note the good preservation of islet cells (× 150)

Histological procedures

Islet control samples ($n = 5$), and samples treated with CM-48 ($n = 5$), were fixed with 10% buffered formaldehyde for 4–6 h. Sections of 4 μm- thickness were taken on a rotary microtome (Reichert-Jung 2030 Biocut, Cambridge instruments, Heidelberg) and stained with Haematoxylin & Eosin.

Acinar cell isolation and flow cytometry (FACS) analysis

Acinar cells were isolated by following the method reported earlier, [1] with some modifications. Briefly, pancreatic tissue from 2 BALB/c mice was dissected, minced, and incubated with 20 ml of 0.02% Trypsin-0.25% EDTA for 5 min at 37 °C while shaking at 120–140 cycles per min. It was centrifuged at 500 g for 2 min and then rinsed with DMEM containing STI 0.1 mg/ml. The tissue was then resuspended in 20 ml of digestion solution containing 1 mg/ml collagenase P (Boehringer Mannheim, Germany), 20% FCS and kept for 15 min at 37 °C with shaking. It was rinsed, resuspended in DMEM, and layered on to 6% Ficoll with 5% FCS. Acinar cells were selectively sedimented by centrifugation at 100 g for 10 min then rinsed twice with the medium and suspended in 10 ml DMEM. Freshly isolated cells were 90% viable (trypan blue staining, 0.04%). Acinar cells were exposed to CM-48 for 6 h in 24-well plates, with unexposed cells taken as controls. Cells were then labeled with Propidium Iodide and analyzed for DNA damage with a FACS workstation (Becton Dickinson, Franklin Lakes, N.J. USA) equipped with a 488 nm Argon laser.

Statistical analysis

Computations were performed using the Jandel Scientific Sigma Stat statistical package (version 4 for windows 95). Results are expressed as mean ± (SEM) for normally distributed data. The statistical significance of differences among groups was analyzed using Student's *t* test.

Table 1 Morphometry and in vitro functionality of CM- treated and untreated control islets after 24 h in culture

	5.5 mM glucose Insulin (μU/10islets)	16.6 mM glucose	Diameter (μm)	Area (μm²)
Control	84.3	214.35	94.42 ± 3.57	10163.72 ± 1022
CM treated	81.7	208.46	93.6 ± 3.32	9892.34 ± 1014

Results and discussion

When MIA Pa Ca-2 CM-48 was added to cultured islets from BALB/c mice, it was observed that at around 6 h of exposure, acinar cells underwent extensive degeneration $P < 0.001$ (Fig. 1 a, 1 b, 1 c) resulting in approximately 85–90% pure islet population. However, islet preparations unexposed to CM-48 contained intact acinar cells (Fig. 1 a). CM-48 did not show any adverse effects on the viability of islet cells, as viability of islets incubated with CM-48 was comparable to the control preparations when taken after 24 h of treatment (Fig. 1 c) $P > 0.05$. The islet yield was found to be in the range of 1264 ± 118 per mouse pancreas for the controls ($n = 8$), and 1360 ± 126 for the CM-48 treated ($n = 8$) $P < 0.001$. This suggests that CM-48 is selectively toxic to acinar cells, while it maintains the viability and yield of islet cells. The islets were also stained with DTZ, indicating their specificity and identity. CM-treated islets were also seen to have normal architecture and morphology, (Fig. 1 b) indicated by absence of islet-peripheral cell loss. The digital image analysis also supports this observation, as diameter and area of CM-48 treated islets was seen to be maintained (Table 1). CM-48–treated islets also showed normal profiles for basal and stimulated insulin secretion (Table 1). This indicates the maintenance of their ability to respond to changes in environmental glucose concentration. CM-48 thus permitted maintenance of structural and functional integrity as well as viability of islets suggesting its specific and selective role in depleting pancreatic acinar cells. This is further confirmed by the Heamatoxylin-Eosin staining, which showed islet morphology in the CM-48 treated group as being well preserved (Fig. 1 d).

To understand the probable mechanism of this toxicity to acinar cells, we checked for the DNA damage induced to acinar cells of BALB/c mice by CM-48. FACS analysis showed that 45% of acinar cells exposed to CM-48 suffered DNA damage, compared to 22% of cells unexposed to CM-48. Thus accelerated, DNA damage and subsequent degeneration seems to be one of the probable causes of acinar cell depletion in the islet preparations. All these results suggest that soluble factors released in MIA Pa Ca-2 conditioned medium play a pivotal role in inducing selective degeneration of acinar cells. However, the exact pathways leading to DNA damage of acinar cells need to be elucidated.

The earlier reported methods of obtaining pure islet cells rely on a sophisticated setup and the use of expensive chemical reagents [6, 8]. These isolation procedures also suffer from drawbacks such as low yield and laborious procedure [3]. The present study conclusively demonstrates selective toxicity of MIA Pa Ca-2 Conditioned Medium to pancreatic acinar cells and its possible application as an easy alternative to generate pure, viable islets free of acinar cell contaminants in a short time span without decreasing their yield.

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