Effects of glucose concentration on differentiation of rat pancreatic duct stem cells★

Chen Hao-qiang[1], Shi Guang-jun[2], Xu Ping[2], Wu Xiao-ping[3]

Abstract

BACKGROUND: Glucose is an important factor on differentiation of pancreatic duct stem cells, it relates to the quantity and secretion function of insulin-producing cells after differentiation.

OBJECTIVE: To compare the insulin secretion capacity of the differentiated rat pancreatic stem cells induced by various glucose concentrations.

METHODS: Rat stem cells were isolated and purified from pancreatic duct cells using collagenase V and Ficoll-400. These stem cells were randomly divided into 10 groups. Every group was induced to culture, proliferate, differentiate and form insulin-producing cells in vitro. The differentiation of all groups was performed in medium with different concentrations of glucose. The immunofluorescence staining was used to identify the pancreatic duct stem cells. The electrochemical luminescence method was used to detect the insulin release from stem cell differentiated islets.

RESULTS AND CONCLUSION: The stimulation index of glucose 20.6, 25.6, 30.6 mmol/L groups was higher than that in other groups ($P < 0.05$), but there was no difference between each two groups among these three groups ($P > 0.05$). The insulin releasing of glucose 15.6, 20.6, 25.6 groups was higher than that in other groups ($P < 0.05$), but there was no difference between each two groups among these three groups ($P > 0.05$). The best insulin secretion capacity of insulin-producing cells can be gained by controlling concentration of glucose as 20.6−25.6 mmol/L when pancreatic duct stem cells differentiated into insulin-producing cells.

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INTRODUCTION

Islet transplantation is a cell alternative therapy for type 1 diabetes mellitus[1]. In contrast to pancreas transplantation, it has some superiorities such as simple surgery procedures, small surgical trauma and fewer complication[2]. However, islet transplantation have limitations including the short supply of donor pancreas, severe immune rejection and side effects of immunosuppressants. These problems may be solved by the differentiation of stem cells for insulin-producing cells[3-4]: (1) A large number of islet cells candidate for islet transplantation can be supplied. (2) Islet cells differentiated from autologous stem cells could avoid allograft-caused immune rejection and reduce the use of immunosuppressants. Pancreatic duct stem cells are currently considered one of the most valuable stem cells. Most previous studies use a combination of multiple factors for the differentiation of pancreatic duct stem cells, but few experiments research the optimal concentration range of each factor. Glucose is proved as the most influencing factor for the stem cells differentiation[5]. This study aimed to analyze the effects of glucose concentration on differentiation of rat pancreatic duct stem cells, and to help determine a standardized experimental scheme for the differentiation of pancreatic stem cells.

MATERIALS AND METHODS

Design

A randomized controlled observation.

Time and setting

The experiment was carried out in the Laboratory of Cell and Hepato-Pancreato-Biliary at Qingdao Municipal Hospital, China between November 2009 and May 2010.

Materials

Five Wistar male rats (SPF class, aged 8−10 weeks, weighing 250−300 g) were purchased from the Animal Breeding Centre of Shandong Lukang Pharmaceutical Co., Ltd., China, with the license number of SCXK2008-0002. All the processes obey the ethical standard of animals.

Main reagents and antibody are as follows:

Reagent and antibody	Source
V collagenase, FicollDL-400, dithizone, insulin-transferring-selenium	Sigma, USA
Fetal calf serum, low-glucose DMEM/F12 culture medium	Hyclone
Bovine serum albumin	Amresco
RPMI1640 culture medium	Gibco
Epidermal growth factor and basic fibroblast growth factor	Chemicon
Nicotinamide	Merck
Rabbit-anti-rat cytokeratin 19 polyclonal antibody, rabbit-anti-rat pancreatic duodenal homeobox-1, rabbit-anti-rat Nestin monoclonal antibody, goat-anti-rabbit FITC-conjugated IgG	Boster, Wuhan, China

Methods

Isolation, purification and proliferation of pancreatic duct stem cells

The steps of stem cells isolation and proliferation referred to the method of Bonner-Weir *et al* [6-7]. The pancreatic tissue was obtained by retrograde puncture of bile duct. The pancreatic tissue was

isolated and digested with V collagenase, then were purified by Ficoll discontinuous density gradient centrifugation. Pancreatic duct epithelium cells were concentrated on the top and middle layers. These cells were cultured in RPMI-1640 medium containing 10% fetal calf serum, 10^5 U/L penicillin, and 100 mg/L streptomycin at 37 ℃, 5% CO_2. At 2−4 days, the non-adherent tissue (both viable and dead) was removed with a media replenished, while the adherent or residual cells were expanded for up to 1 week with additional media changed every 2−3 days. At about 1 week, the media was changed to serum-free DMEM/F12 medium (containing 5.6 mmol/L glucose) with 1 g/L supplement (5 mg/L insulin + 5 mg/L transferring + 5 mg/L selenium), 2 g/L bovine serum albumin, 20 μg/L basic fibroblast growth factor, 100 μg/L epidermal growth factor, 10^5 U/L penicillin, and 100 mg/L streptomycin. These cells were expanded for 1−2 weeks with additional media changes every 2−3 days until reaching near confluence or forming substantial plaques of epithelial cells.

Immunofluorescence staining of pancreatic duct stem cells immune markers

Cell smears were fixed for 15 minutes in 10% methanol, and then rinsed in the PBS for three times, each 3 minutes. Sections were dropped into serum blocking liquid and placed at 37 ℃ for 30 minutes. Then rabbit-anti-rat cytokeratin 19, rabbit-anti-rat pancreatic duodenal homeobox-1, rabbit-anti-rat Nestin (1: 100) antibodies were added to incubate cells at room temperature for 1 hour. Then second antibody at 1: 100 dilution was added to incubate the cells for another 30 minutes at 37 ℃. After the sections were rinsed three times with PBS, each 3 minutes, fluorescence-labeled antibody (0.01 mol PBS 1: 10 diluted SABC-FITC) was added to incubate for another 30 minutes at 37 ℃. Then sections were rinsed four times with PBS solution, each 5 minutes, fluorescence microscope was used to observe the results[8-9].

Differentiation of pancreatic duct stem cells to islet cells in different concentrations of glucose

Obtained cells were randomly divided into ten groups and cultured in ten T25 flasks. These cells were induced with serum-free DMEM/F12 medium containing 1 g/L insulin-transferring-selenium, 2 g/L bovine serum albumin, 10 mmol/L nicotinamide, 10^5 U/L penicillin,100 mg/L streptomycin, and different concentrations of glucose. Group 1 was control group and its concentration of glucose was 5.6 mmol/L. The glucose concentration of other experimental groups was respectively 10.6, 15.6, 20.6, 25.6, 30.6, 35.6, 45.6, 55.6, 105.6 mmol/L. All groups were induced for 2 weeks.

Release of insulin induced by glucose

The stem cell differentiated islets were cultured on dextrose solutions at low glucose concentration (2.7 mmol/L) in RPMI-1640 medium of 96-well plate at 37 ℃. Every group had 5 plates. After 2 hours, the media was changed to RPMI-1640 medium with high glucose concentration (16.7 mmol/L) for another 2 hours. After stimulation, the supernatant was collected and preserved at −20 ℃. Then all cells were cultured in RPMI-1640 medium containing 11.1 mmol/L glucose for 3 days. The supernatant was collected and preserved at −20 ℃. The insulin content was determined by electrochemical luminescence method.

Main outcome measures

Pancreatic duct stem cells were observed under microscope. Insulin-secreting cells were dyed by dithizone[10]. Pancreatic duct stem cells immune markers were observed by fluorescence microscope. The insulin release and stimulation index of all groups were determined. Stimulation index = insulin content (stimulated by low glucose concentration) / insulin content (stimulated by high glucose concentration)[11]. The higher the stimulation index, the stronger the insulin secretion capacity.

Statistical analysis

The statistical analysis was carried out by the first author. The analysis was conducted with SPSS 17.0 software, and measurement data were expressed as Mean ± SD.

RESULTS

Isolation, purification, proliferation and differentiation of pancreatic duct stem cells

The pancreatic tissue was obtained by the retrograde puncture of bile duct (Figure 1).

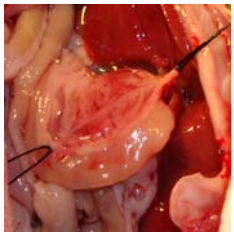


Figure 1 Obtaining the pancreatic tissue

Pancreatic duct cells following Ficoll purification were mainly distributed in 1.037/1.069 and 1.069/1.096 interfaces (Figure 2).

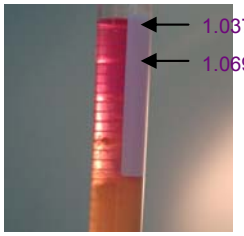


Figure 2 Purifying the pancreatic duct stem cells

After cultivation, these cells were expanded until reaching near confluence (Figure 3).

Figure 3 Reaching near confluence of the pancreatic duct stem cells (×200)

When the cells grew to reach 70% confluence, they were induced to differentiate to insulin-producing cells (Figure 4).

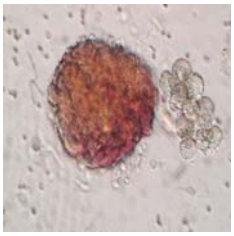
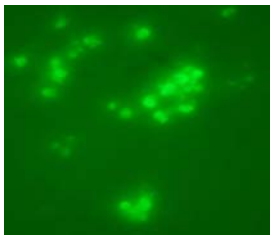


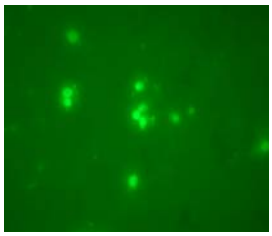
Figure 4 Insulin-secreting cells (Dithizone staining, ×400)

Immunofluorescence staining of pancreatic stem cells immune markers

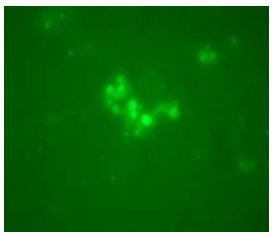
The sections of pancreatic stem cells were positive for all immune markers (rabbit-anti-rat cytokeratin 19, rabbit-anti-rat pancreatic duodenal homeobox-1, rabbit-anti-rat Nestin; Figure 5).



a: Immunofluorescence staining of CK-19



b: Immunofluorescence staining of Pdx-1



c: Immunofluorescence staining of Nestin

Figure 5 Immunofluorescence staining of pancreatic stem cells immune markers (×200)

Release of insulin induced by glucose (Tables 1−2)

Table 1 Comparison of stimulation index of all groups ($\bar{x}$±s, n=5)

Group	Glucose (mmol/L)	Stimulation index
1	5.6	1.030±0.013
2	10.6	1.047±0.007
3	15.6	1.024±0.005
4	20.6	1.149±0.014[ab]
5	25.6	1.157±0.007[ab]
6	30.6	1.132±0.012[ab]
7	35.6	1.067±0.004[a]
8	45.6	1.044±0.013
9	55.6	1.036±0.009
10	105.6	1.015±0.012

[a]P < 0.05, *vs.* 1 group; [b]P < 0.05, *vs.* 7 group

Table 2 Comparison of insulin releasing of all groups ($\bar{x}$±s, n=5)

Group	Glucose (mmol/L)	Insulin releasing (μg/L)
1	5.6	0.388±0.011
2	10.6	0.390±0.012
3	15.6	0.404±0.019[ab]
4	20.6	0.405±0.017[ab]
5	25.6	0.405±0.016[ab]
6	30.6	0.393±0.007[a]
7	35.6	0.391±0.009
8	45.6	0.385±0.006
9	55.6	0.384±0.002
10	105.6	0.379±0.007

Results indicated all differentiated cells have the ability to secrete insulin. Both stimulation index and insulin secretion were higher than other groups when glucose concentrations were 20.6 and 25.6 mmol/L.

DISCUSSION

The critical factors of the proliferation and differentiation of pancreatic duct stem cells include: (1) culture medium and serum; (2) nutrients including glucose, nicotinamide and all-trans retinoic acid[12-13]; (3) sodium butyrate; (4) glucagon-like peptide 1[14-15]; (5) growth factors including epidermal growth factor, basic fibroblast growth factor, betacellulin, hepatocyte growth factor and Activin A[16-21]; (6) synthetic drug LY294002[22-23]; and (7) extracellular matrix. Among them, epidermal growth factor and basic fibroblast growth factor have been proved to induce pancreatic duct stem cells proliferation, but has no differentiation effect. Other growth factors all have differentiation effect, but glucose is considered as the main factor for differentiation of pancreatic duct stem cells.

Most of previous experiments utilized a combination of multiple factors on differentiation of pancreatic duct stem cells. The main purpose of these experiments is to prove that pancreas duct stem cells could differentiate into insulin-producing cells. However, few experiments focus on specific parameters of every factor such as glucose concentration. The present experiment aimed to study the effects of glucose concentration on differentiation of pancreatic duct stem cells.

What's the relationship between glucose concentration and differentiation of pancreatic duct stem cells? Whether the higher glucose concentration indicates the stronger effect of differentiation? Studies have proved that hyperglycemia decreases the insulin secretion of islet cells. Therefore it is necessary to find a correct glucose concentration range. The glucose concentration must be able to get not only more insulin-producing cells but also higher insulin secretion. According to the result of this experiment, the stimulation index of glucose 20.6, 25.6, 30.6 mmol/L groups were higher than that in other groups (P < 0.05), but there was no difference compared with each other in these groups (P > 0.05). The insulin releasing of glucose 15.6, 20.6, 25.6 mmol/L groups were higher than that in other groups (P < 0.05), but there was no difference compared with each other in these groups (P > 0.05). Therefore, 20.6−25.6 mmol/L may be the best range of glucose concentration.

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葡萄糖浓度与大鼠胰腺导管干细胞的诱导分化*

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摘要

背景：葡萄糖是胰腺导管干细胞分化的重要因素之一，与分化后胰岛素分泌细胞数量及分泌能力相关。

目的：对比不同浓度葡萄糖诱导下，胰腺导管干细胞分化后细胞的胰岛素分泌能力。

方法：使用胶原酶Ⅴ及Ficoll-400分离及纯化Wistar大鼠胰腺上皮细胞，获取胰腺导管干细胞，将干细胞分为10组，体外培养、增殖及分化形成胰岛素分泌细胞。各组在含有不同浓度葡萄糖的培养基中进行分化。采用免疫荧光染色法鉴定胰腺导管干细胞，光化学发光法检测分化出的胰岛素分泌细胞的胰岛素分泌量。

结果与结论：葡萄糖浓度为20.6，25.6，30.6 mmol/L组细胞的刺激指数高于其他组($P < 0.05$)，但这3组两两比较差异无显著性意义($P > 0.05$)。葡萄糖浓度为15.6，20.6，25.6 mmol/L组细胞胰岛素分泌量高于其他组($P < 0.05$)，但这3组两两比较差异无显著性意义($P > 0.05$)。提示胰腺导管干细胞分化为胰岛素分泌细胞实验中，当分化时培养液所含葡萄糖浓度为20.6~25.6 mmol/L时，所得细胞胰岛素分泌能力最强。

关键词：胰岛移植；胰腺导管干细胞；葡萄糖浓度；胰岛素；分泌量
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