Effects of human umbilical cord serum on proliferation and insulin content of human fetal islet-like cell clusters

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BACKGROUND: **Type 1 diabets is an autoimmune disease caused by the destruction of pancreatic β-cell with an increased incidence worldwide in the closing decades of the 20th century. This study was to investigate the effects of human umbilical cord serum (UCS) on the proliferation and function of human fetal islet-like cell clusters (ICCs) in vitro.**

METHODS: **Eight fresh pancreatic glands obtained after induction of labor with water bag were mildly exposed to collagenase V, and the digested cells were cultured in a RPMI-1640 medium plus 10% pooled UCS or fetal calf serum (FCS) to permit cells attachment and outgrowth of ICCs.**

RESULTS: **In 8 consecutively explanted glands, development and proliferation of ICCs were observed. In the presence of FCS, the outgrowth of ICC took place on the top of a fibroblast monocellular layer. UCS affected less growth of fibroblasts and increased the formation of ICCs about four-fold compared with explants from the same glands maintained in FCS. In both UCS and FCS, the insulin content of the medium was variable to a certain extent and progressively declined from day 2 to day 6. Dithizone-stained ICCs in UCS suggested that most cell clusters were islet cells (β-cells), and the purity of islets was estimated 80%-90%. The ultrastructure of the cultured cells showed a large number of granule-containing cells, most of which were identified as β-cells.**

CONCLUSION: **We conclude that in comparison with explants with FCS, the yield of ICCs and purification of islet cells are markedly increased by UCS and may facilitate the proliferation of pancreatic β-cells intended for islet transplantation.**

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KEY WORDS: **islet cell culture; islet-like cells clusters; umbilical cord serum; fetal calf serum**

Introduction

By 2010, the number of people with diabetes is expected to exceed 350 million.[1,2] Transplantation of insulin producing islet cells isolated in vitro from a donor pancreas could be a perspective cure for type 1 and some cases of type 2 diabetes.[3-5] Currently, however, lack of sufficient donor tissue limits its potential in clinical practice. Ways to overcome the problem include deriving islet cells from new sources and expanding existing islet cells in vitro. In this way, ex vivo expansion of human endocrine cells may provide an adequate source for β-cells and is vital for both biological studies and clinical treatments.[6,7]

The human fetal pancreas represents an important source of insulin-producing β-cells with a great potential for transplantation to diabetic patients.[8,9] Nevertheless, the relatively low number of endocrine cells (β-cells and non-β-cells) and the proliferation of human pancreatic islet cells have remained a challenge. It has previously been shown that such cells can be viably maintained in tissue culture media containing fetal calf serum (FCS) and human serum (HS) and that these explants continue to synthesize and release insulin.[10] This study was designed to evaluate the influence of human umbilical cord serum (UCS) on the proliferation and insulin content of human fetal islet-like cell clusters (ICCs) comparing with those of FCS.

Methods

Procurement and culture of the human fetal pancreas

Eight consecutively obtained human fetal pancreatic glands were collected in a period of 2 months at the Department of Obstetrics and Gynecology, the Affiliated Hospital of Luzhou Medical College, Sichuan, China after the approval of the Ethics Committee of the hospi-

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tal. The gestational age of these fetuses (3 were male and 5 female) varied from 14 to 32 weeks and all abortions were induced with water bag. The pancreas was usually dissected within 2 hours after abortion. All procedures were carried out under sterile conditions in a laminar flow cabinet according to the modified method.[11,12]

Blood samples were collected from umbilical cord vessels of 5 healthy and term-pregnancy delivered newborns, whose mothers had no history of any diseases at the time of birth. The samples were centrifuged to separate serum. The pooled serum or UCS was stored at −20 ℃ and kept inactivated and aseptic until use.[13]

After removal, the intact gland was maintained in Ca^{2+}- and Mg^{2+}-free D-Hank's solution at 4 ℃ for further processing. For tissue culture, the gland was washed repeatedly in 5 ml D-Hank's solution with 100 U/ml penicillin and 100 μg/ml streptomycin in glass dishes. Nonpancreatic tissues and loose connective tissue capsules surrounding the gland were also dissected free. The pancreas was successively minced with scissors to make segments of about 1 mm^3, which were then transferred to glass vials containing 5 ml D-Hank's solution with 2.5 mg/ml V-type collagenase (528 U/mg, pH 7.2, Sigma, USA). The fragments were shaken rapidly in a water bath at 37 ℃, and incubated in D-Hank's solution at 4 ℃ for 5-10 minutes when they were almost disintegrated and noticed by naked eyes. The tissue suspension was washed twice in 10 ml D-Hank's solution and centrifuged (500 ×g for 6 minutes). The pellet was subsequently transferred to a 5 ml serum-free RPMI-1640 medium (Sigma, USA) and filtered through a 108-μm-pore sized nylon mesh. The resulting pellet was resuspended in a 2 ml RPMI-1640 medium and divided into two culture dishes, each containing 3 ml RPMI-1640 (11.1 mmol/L glucose). Finally, 1 ml 10% FCS (Sigma) or 1 ml 10% (vol/vol) UCS was added to each dish. Cultures were performed in incubators at 37 ℃ with 95% O_2/5% CO_2. The culture medium was changed after 24 hours and subsequently at 2 days intervals until day 6. Samples of the medium were collected every second day for analysis of insulin release and content, and inverted phase contrast microscopy was also performed serially.

Light and electron microscopy

After being washed in D-Hank's solution, the tissue samples were fixed in Bouin's fluid for light microscopy. Sections, 5-μm thick, were cut and stained with hematoxylin-eosin. To demonstrate and detect β-cells, the sections were stained with dithizone (DTZ, Sigma, USA).[14] DTZ is a zinc-chelating agent known to selectively stain pancreatic β-cells because of their high content of zinc. Other tissue samples intended for electron microscopy were fixed in 2.5% (vol/vol) glutaraldehyde dissolved in phosphate buffer (pH 7.2). After dehydration, they were embedded in plastic resin. Ultrathin sections or semithin sections were prepared, mounted, and observed under a transmission electron microscope (TEM) for ultrastructural analysis.

Number of ICCs formed in culture

At the end of the culture, the approximate number of ICCs stained with DTZ was observed under a stereomicroscope in different culture dishes containing explants immediately before harvest.

Insulin release and insulin content

On day 2, 4 and 6 of incubation, the upper layer of the culture media was extracted and insulin secretion was analyzed by radioimmunoassay. After the last incubation, all ICCs were transferred into tubes containing Hank's solution and fragmentated thoroughly. Subsequently, the homogenates were mixed with acidic ethanol (0.18 mol HCl in 70% anhydrous ethanol) and extracted twice in succession. The insulin content of the extracts was measured radioimmunologically.

Statistical analysis

The data were expressed as mean ± standard deviation and compared by Student's *t* test for paired samples. And the statistical significance of the differences between mean values was determined by the P value less than 0.05.

Results

Culture characteristics of explants in tissue culture

A 4- to 5-day culture period was required for complete formation of ICCs released from the collagenase-digested pancreas. During the days of initial culture, there were outgrowth of fibroblast-like cells and rudimental formation of ICCs on the top of the bottom layer. To get rid of fibroblasts, ICCs were later transferred to another culture medium easily by gentle suction through a pipette. In general, ICCs' growth activity reached a plateau and formed a well-distributed monolayer on day 4 or 5, when no further proliferation of ICCs took place. Therefore explants maintained in a culture medium containing UCS showed a growth pattern distinctly different from those kept in FCS. In the latter medium there was a vigorous outgrowth of a monolayer of fibroblasts and a rare formation of spherical ICCs loosely attached to the monolayer. By contrast, in UCS there was only a minor growth of fibroblasts but a rapid formation of ICCs, which were found in small, numerous and macroscopic clumps attached to the surface of the culture dish, and most kept floating freely.

The number of ICCs formed and their insulin con-

Table. Characterization of ICCs formed in vitro during culture of human fetal pancreatic explants in a RPMI-1640 medium supplemented with either 10% FCS or 10% UCS ($n=8$, means±SD)

	Culture supplement	
	FCS	UCS
Number of ICCs formed	186.00±55.00	736.00±76.00
Insulin secretion (ng/well) on day 2	757.17±80.22	983.80±74.73 *
Insulin secretion (ng/well) on day 4	658.33±64.18	845.67±35.52 *
Insulin secretion (ng/well) on day 6	338.17±57.21	607.67±36.99 *
Total insulin content (ng/well)	405.33±19.15	1049.17±26.82 *

*: $P<0.05$, compared with FCS group.

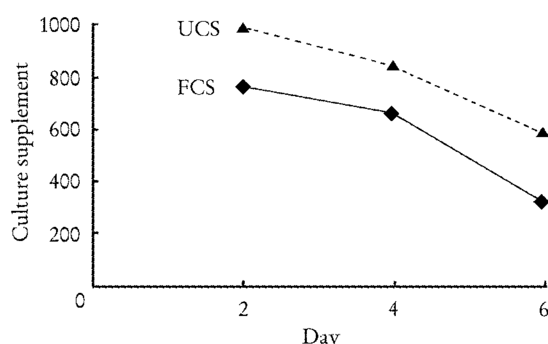

Fig. Medium insulin content of fetal pancreatic explants from 8 consecutively cultured glands. A group of collagenase-treated pancreas cultured in RPMI-1640 medium plus 10% FCS (solid line), and the other group cultured with addition of 10% UCS (dashed line). Values are expressed as means±SD.

tent are shown in Table. In the 8 consecutively cultured glands, the number of ICCs in a UCS-containing medium was about four-fold higher than the number formed during culture in a FCS-supplemented medium.

Insulin release and insulin content

Insulin release into the culture medium varied greatly among different glands. Explants from the 8 cases showed a progressive decline in the insulin secretion from day 2 to 6, regardless of the source of serum supplement (Fig.). The rate of insulin release on day 6 and 2 was 60% (UCS) and 45% (FCS), respectively. ICCs harvested on day 6 contained comparable amounts of insulin and the insulin content was 607.67±36.99 μg in a medium with UCS and 338.17±57.21 μg in a medium with FCS, respectively. UCS increased the formation of ICCs about four-fold compared with explants from the same glands maintained in FCS.

Light and electron microscopy

Growth of ICCs as spherical bodies on a basement of fibroblasts in FCS or free floating in UCS was observed under an inverted phase contrast microscope. Generally, ICCs formed in FCS were larger and more irregular in shape than those developed in UCS. There were no apparent differences in the microscopic or ultrastructural appearance between explants grown in FCS and UCS. In both groups of ICCs, duct-like structures were occasionally observed. Within the individual ICCs, distinct DTZ-positive cell clusters, being stained crimson, existed singly or in groups with minor nonstained cell clusters. We reckoned that the rate of DTZ-positive cells was about 80%-90%. Electron microscopy revealed a well-preserved ultrastructure in most preparations, but also a heterogeneous cell population composed of a mixture of most granulated endocrine-like cells and some cell profiles showing only a few or no granules, even duct-like structures. This kind of non-granular cells with less-organelles proved probably to be pancreatic stem cells in 2000.[15,16]

Discussion

In recent years, dramatic advances in islet transplantation have been made through research using dead human fetal tissues.[17] Early fetal donor cells have an advantage because they engraft readily and do not cause graft-versus-host disease. Similarly, the fetus is an ideal recipient of allogenic fetal cells as it is incapable of rejecting them early in gestation. However, this research has led to controversy over the ethics of using human fetal tissues, particularly those from induced abortions. Although legalized abortion remains a hotly debated issue in the USA, China and some other countries, a consensus has been reached in the scientific community on the ethical use of fetal tissues in research and clinical transplantation.[18,19]

It was shown early that the human fetal pancreas tissue in a RPMI-1640 culture medium supplemented with 10% human serum (HS) leads to a proliferation of ICCs.[20-22] This experiment including 6-day culture in RPMI-1640 (plus 11.1 mmol/L glucose) with different serum additions (UCS or FCS) was performed to compare between the different groups of ICCs based on their morphologic changes and insulin contents. And a comparison between different culture media and different glucose concentrations showed that islets cultured in RPMI-1640 with 11.1 mmol/L glucose had the highest insulin production.[23]

Despite some differences in the fetal age of human pancreatic materials, there was still a consistent pattern of development, without obvious age-related variations in the proportion of insulin-containing β-cells.[24] A recent study indicated that all pancreatic glands should be obtained at a fairly late stage (>28-36 weeks) of fetal development for the best proliferation of endocrine cells in vitro.[25] The fetal pancreatic tissues of the aborted fetus obtained after induction of labor with water bag showed a better survival in vitro than those obtained after abortifacient agent-induced abortion. This may be

resulted from the elimination of direct injurious effect of abortifacient agents and saving of the procedural time. And the time between completion of the abortion and dissection of the fetal pancreas also appears to be crucially important to the viability of the explants and should be limited to 1.5-2 hours.

In promoting the formation of ICCs in vitro UCS seemed to be better than FCS. It is known that some special secretory granules appear in islet cells and β-cells begin to have secretory activity in the 15th week, when insulin and insulin-like growth factor-I (IGF-I) can be detected in cord blood. Moreover, insulin and IGF-I have been shown to stimulate proliferation and insulin production in islets of Langerhans.[26] An effect very similar to that of UCS can be observed when HS is used in the culture medium instead of FCS. And ICCs remain free-floating vigorously and fibroblasts develop poorly in the culture medium when either UCS or HS is added. Goldman and Colle[20] reported that they could isolate islets from the human fetal pancreas and that these islets remained intact, free-floating, and functional for 2 to 5 months in the media supplemented with serum from human umbilical cord or adult donors. They also noted that substitution of fetal calf serum for HS usually resulted in attachment of islets and relatively early (3 weeks) functional arrest. Thus, addition of human-specific supplementation including UCS and HS to the culture medium seems to be essential for optimal proliferation of ICCs.[27-30]

An initial decrease in the insulin concentration, especially in the first 4-6 days during culture, in the presence of either HS or FCS has also been reported by some scientific teams.[31,32] This is probably due to a gradually decreased leakage of hormones for nonviable cells and marked hormone degradation in the culture medium, suggesting that the mechanism of insulin secretion by fetal β-cells at a fairly early stage is not yet fully developed.[33]

The proliferative activity of ICCs with different serum supplementation is assessed by calculating the percentages of DTZ-positive cell clusters that should also be positive for insulin. DTZ staining is usually recognized as a valuable part of a possible approach to identify human pancreatic islets. Shiroi and his coworkers[34] demonstrated that DTZ-stained clusters contained cellular components secreting insulin. ICCs developed in culture medium supplemented with UCS, compared with those in FCS, contained huge amounts of β-cells, in agreement with the light microscopic findings of most DTZ-negative cell clusters scattered among occasional DTZ-positive cell clusters. Thus, culture in medium containing UCS produced higher number of β-cells to such an extent that the total yield of ICCs exceeded that observed in FCS addition.[35]

Because the availability of fetal pancreatic material is very limited, the increased yield of ICCs and purification of islet cells after in vitro culture in the presence of UCS is of great significance. The most pronounced effect exerted by UCS would provide a powerful backing on the proliferation and endocrine function of ICCs in vitro and allow patients with diabetes to benefit greatly from this development.[18]

Competing interest

The author or authors do not choose to response to the statements listed in Instructions for Authors.

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