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• *RAPID COMMUNICATION* •

Small intestinal submucosa improves islet survival and function during *in vitro* culture

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Abstract

AIM: To evaluate the recovery and function of isolated rat pancreatic islets during *in vitro* culture with small intestinal submucosa (SIS).

METHODS: Pancreatic islets were isolated from Wistar rats by standard surgical procurement followed by intraductal collagenase distension, mechanical dissociation and Euroficoll purification. Purified islets were cultured in plates coated with multilayer SIS (SIS-treated group) or without multilayer SIS (standard cultured group) for 7 and 14 d in standard islet culture media of RPMI 1640. After isolation and culture, islets from both experimental groups were stained with dithizone and counted. Recovery of islets was determined by the ratio of counts after the culture to the yield of islets immediately following islet isolation. Viability of islets after the culture was assessed by the glucose challenge test with low (2.7 mmol/L) and high glucose (16.7 mmol/L) solution supplemented with 50 mmol/L 3-isobutyl-1-methylxanthine (IBMX) solution. Apoptosis of islet cells after the culture was measured by relative quantification of histone-complexed DNA fragments using ELISA.

RESULTS: After 7 or 14 d of *in vitro* tissue culture, the recovery of islets in SIS-treated group was significantly higher than that cultured in plates without SIS coating. The recovery of islets in SIS-treated group was about twice more than that of in the control group. In SIS-treated group, there was no significant difference in the recovery of islets between short- and long-term periods of culture (95.8±1.0% *vs* 90.8±1.5%, $P>0.05$). When incubated with high glucose (16.7 mmol/L) solution, insulin secretion in SIS-treated group showed a higher increase than that in control group after 14 d of culture (20.7±1.1 mU/L *vs* 11.8±1.1 mU/L, $P<0.05$). When islets were placed in high glucose solution containing IBMX, stimulated insulin secretion was higher in SIS-treated group than in control group. Calculated stimulation index of SIS-treated group was about 23 times of control group. In addition, the stimulation index of SIS-treated group remained constant regardless of short- and long-term periods of culture (9.5±0.2 *vs* 10.2±1.2, $P>0.05$). Much less apoptosis of islet cells occurred in SIS-treated group than in control group after the culture.

CONCLUSION: Co-culture of isolated rat islets with native sheet-like SIS might build an extracellular matrix for islets and provide possible biotrophic and growth factors that promote the recovery and subsequent function of islets.

Key words: Islet culture; Islet survival; Islet function; Small intestinal submucosa

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INTRODUCTION

In the last 10 years, both the prevalence and incidence of diabetes have increased sharply all over the world and this disease has already become one of the global health care problems. For example, there are 14 000 000 patients suffering from diabetes in USA and 1 000 000 of them suffer from type I diabetes mellitus[1]. Islet transplantation can control blood sugar effectively, reduce complications of diabetes and prevent hypoglycemia and insulin resistance caused by ectogenic insulin. Therefore, pancreatic islet cell transplantation may be an effective means for treating type I diabetes mellitus.

Human islet transplantation has been demonstrated to be a viable option for the treatment of type I diabetes mellitus[2]. However, there are still many difficulties hindering the transplantation alleviating complications of the disease. A major limitation for its use as a standard treatment is the lack of available viable islets for transplantation.

To maintain islets of Langerhans in tissue culture provides a chance for islet storage after being isolated from pan-

creas before clinical transplantation. The storage period can be utilized to assess the function of the islets and to confirm microbiologic sterility of the preparation. In addition, it can increase purity of the islet preparation and to reduce the immunogenicity of islets[3]. However, islet culture loses tissue mass over culturing time, which is one of its disadvantages[4]. Given the importance of transplanting sufficient islets to increase the chance of successful graft[5], any loss of islet tissue mass jeopardizes this possibility.

Both morphology and metabolic activities of cultured cells are affected by the composition of substrates on which they grow. Cultured cells may proliferate and/or perform their *in vivo* functions when cultured on substrates that closely mimic their natural environments. In fact, many commercially available matrices such as human extracellular matrix (ECM) derived from human placentas and matrigel (a soluble basement membrane extract from the Engelbreth-Holm-Swarm tumor) can be used in cell culture, which supports cell growth. Previous studies have showed that human islets cultured with bovine corneal endothelial cell matrix increase their ability to secrete insulin[6].

SIS is a relatively acellular collagen-based matrix derived from porcine small intestine by mechanical removal of the mucosal and smooth muscle layers[7]. The resulting cell-free translucent sheets are about 100 mm thick. The collagen-based matrix comprised highly conserved collagen, glycoprotein, proteoglycan, and glycoaminoglycan in their native configuration and concentration. In addition, SIS includes various growth factors such as fibroblast growth factor-2 (FGF-2), TGF-β and vascular endothelial cell growth factor (VEGF), which promote cell growth[8].

In 1966, Matsumoto *et al*[9] reported that inverted small intestine could replace large veins in dogs. Improved processing of this biomaterial (SIS) has made it readily available for tissue engineering studies. Extensive *in situ* tissue remodeling from SIS has been shown in both rat and canine models. SIS has been used as a scaffold for proliferation, remodeling, and regeneration of a variety of host tissues including blood vessels[10], dura mater[11], urinary bladder[12], abdominal wall[13], and tendons[14]. Grossly and microscopically the remodeled tissue resembles the native tissue. Recently, SIS has been evaluated for its potential *in vivo* use in hepatocyte transplantation[15]. For these reasons, in this study we have investigated whether the use of SIS in co-culture with rat islets could improve islet survival and their *in vitro* function.

MATERIALS AND METHODS

Animals

Male Wistar rats (250-400 g body weight, Animal Laboratory of Xi'an Jiaotong University, China) were used for islet isolation. All animals received care in compliance with the guidelines of the local Animal Care and Use Committee following National Institutes of Health guidelines.

Design of study

Islets were isolated using a collagenase and purified by discontinuous Euroficoll gradient. The purified islets were separated into study group and control group. In study group, islets were cultured in plates coated with SIS. In control group, islets were cultured in plates not coated with SIS. The number of islets was counted after isolation and culture. Recovery of islets was determined by the ratio of counts after the culture to the yield of islets immediately following islet isolation. Islet function was determined by a static glucose challenge test after a short period of culture for 7 d and a long period of culture for 14 d. Glucose challenge test was performed in the absence and presence of IBMX. Apoptosis of islet cells following culture was measured by relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) out of the cytoplasm of cells by ELISA.

Preparation of soluble SIS supplement

SIS was prepared as previously described[10]. In brief, freshly harvested porcine jejunum was obtained from a local slaughterhouse. The tube of intestinal material was rinsed, until it was free of contents and inverted. Superficial layers of the tunica mucosa were removed by scraping with a knife handle. The tissue was then reverted to its original direction, serosa and tunica muscularis were removed. The resulting membrane was approximately 80-100 μm thick consisting of tunica submucosa and basilar portion of the tunica mucosa. The prepared intestinal submucosa tube was split open longitudinally and rinsed extensively in water to get rid of any of the cells associated with the matrix to eliminate cell degradation products. The sheets of SIS were sterilized by exposure to 1 g/L per acetic acid. To make multilayered SIS, five sheets of SIS were mechanically compressed by vacuum pressing and dried. The multilayered SIS was then terminally sterilized with ethylene oxide and kept until ready to use. Re-soaking in saline prior to use made the SIS sheets pliable and soft.

Islet isolation and culture

Islets were isolated and purified from the rat pancreas as previously described[16]. Briefly, rats were anesthetized with intraperitoneal pentobarbital. Pancreas was infused via the common bile duct with Hanks' balanced salt solution, set apart, and minced on ice. Digestion was performed with type V collagenase (7.5 g/L; Sigma) for 25 min at 37 ℃. Islets were purified on a discontinuous Euroficoll gradient (Sigma), handpicked under an inverted light microscope, pooled and then separated into study group and control group. In study group, islets were cultured in plates coated with suitable size multilayer SIS. In control group, islets were cultured in plates not coated with SIS. Islets were cultured in RPMI 1640 medium (Gibco) supplemented with 100 mL/L fetal calf serum (Gibco), 200 kU/L penicillin, 100 mg/L streptomycin, and 2 mmol/L L-glutamine (Gibco) at 37 ℃ in a humidified 50 mL/L CO_2 atmosphere for a period of 7 or 14 d.

Islet quantification and recovery

Islets from both groups were stained with dithizone and counted in accordance with the criteria established at

the 1989 International Workshop on Islet Assessment after isolation and culture. The number of islets was determined using an optical graticule attached to the eyepiece of a dissecting microscope and converted to the standard of islet equivalent (IE). Recovery of islets following culture was determined by the ratio of counts after the culture to the yield of islets immediately following islet isolation.

Islet viability

For each group, 6 separate samples of 20 islets each were tested simultaneously for their stimulated insulin secretion. Islets (150±50 μm in diameter) were first pre-incubated for 45-50 min in 2 mL RPMI 1640 solution containing 2.7 mmol/L glucose. Insulin secretion was then assessed by three consecutive incubations with RPMI 1640 solution containing 2.7, 16.7, and 16.7 mmol/L glucose with 1 g/L IBMX (Sigma), each for 45 min. At the end of incubation with each solution, the supernatant was completely removed and used to test the insulin secretion level by radioimmunoassay (Department of Isotope, China Institute of Atomic Energy). Insulin secretory responses of 20 islets to glucose stimulation were expressed as milliunit per liter. To eliminate variation of islet size causing differences of insulin response, the stimulation index for each group of islets was determined by the ratio of insulin secretion stimulated by high glucose solution containing IBMX to the basal insulin secretion stimulated by low glucose solution.

Evaluation of apoptosis of islet cells

Apoptosis of islet cells was measured by relative quantification of DNA/histone fragmentation (mono- and oligonucleosomes) in culture supernatants. After 7-14 d of incubation, culture medium was collected from each well of two groups. The supernatants were analyzed to measure cytoplasmic histone-associated DNA fragments generated by cell death based on mouse monoclonal antibodies against DNA and histones, respectively. The cultured supernatant sample (20 μL) was mixed with 80 μL of immunoreagent containing two monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated) in the well coated with streptavidin of a 96-well plate. Antibody–nucleosome complexes were bound to the microplate by streptavidin. The solution mixture was incubated by gently shaking (300 r/min) for 2 h at room temperature. The solution was then collected and the well was completely rinsed thrice with 150 μL of an incubation buffer and 100 μL of premixed 2, 2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) substrate solution was added to each well. The mixed solution was agitated on a plate shaker for 20 min at room temperature and the solution absorbance was measured at a wavelength of 405 nm. The enrichment of nucleosomes due to apoptosis of islet cells was calculated from these absorbance values by the following formula: enrichment factor of nucleosomes=(values of the sample-the background values)/values of the negative control. The assay was done independently thrice for each experimental group. This method could permit us to specifically determine mono- and oligonucleosomes released into culture supernatants accompanying apoptosis and necrosis of islet cells. Accumulation of mono- and oligonucleosomes in the cytoplasm of apoptotic and necrotic cells caused DNA degradation, occurring several hours before the breakdown of plasma membrane.

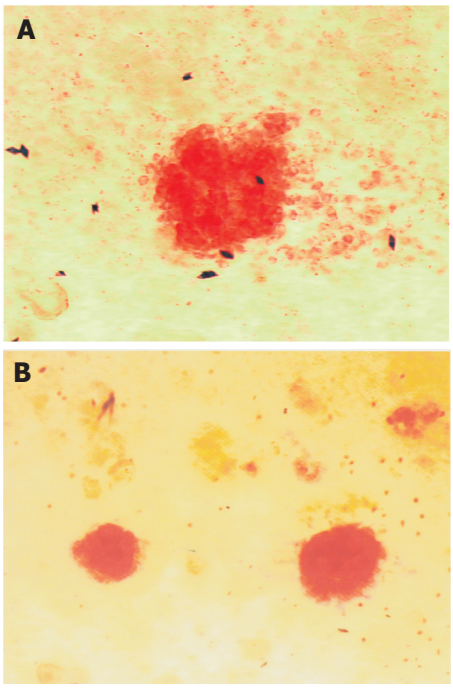

Figure 1 Morphology of islets after culture. **A:** Loose appearance of islets after 14 d of culture under standard condition; **B:** excellent morphology of islets after 14 d of culture on SIS (×400).

Statistical analysis

Results were expressed as mean±SE. Differences between the two groups were analyzed by *t*-test using SPSS10.0. $P<0.05$ was considered statistically significant.

RESULTS

After 7 and 14 d of *in vitro* tissue culture, SIS-treated group indicated a significantly higher ($P<0.05$) recovery of islets than control group (Table 1). The recovery in SIS-treated group was about twice more than that in control group. In SIS-treated group, there was no statistical difference in the recovery between the short and the long periods of culture (95.8±1.0% *vs* 90.8±1.5%, $P<0.05$). Most islets cultured in control group lost their initial morphology, becoming "loose" in appearance at the end of culture. Islets cultured with SIS exhibited excellent morphology (Figure 1).

In vitro viability of the islets was assessed using the glucose challenge test. There was no statistical difference in the basal level of insulin secretion between SIS treated group and control group (9.1±0.3 mU/L *vs* 7.8±0.6 mU/L after 7 d of culture, 5.9±0.4 mU/L *vs* 7.1±0.4 mU/L after

Table 1 Recovery of rat islets and enrichment factor of nucleosomes after being cultured for 7 and 14 d (n = 6, mean±SE)

Parameter	Group	7 d (%)	14 d (%)
Recovery			
	Standard culture	67.1±2.6	42.2±1.5
	SIS culture	95.8±1.0[a]	90.8±1.5[a]
Enrichment factor			
	Standard culture	1.98±0.06	2.90±0.23
	SIS culture	1.08±0.02[a]	1.12±0.09[a]

[a]P<0.05 *vs* standard culture.

Table 2 Stimulated insulin secretion of rat islets cultured for 7 and 14 d during static incubation assay (n = 6, mean±SD)

Group	T (d)	Low glucose solution (2.7 mmol/L)	High glucose solution (16.7 mmol/L)	High glucose solution +50μmol/L IBMX	SI[1]
Standard culture	7	9.1±0.3	18.3±1.1	51.3±3.4	5.7±0.4
SIS culture	7	7.8±0.6	21.8±1.9	75.8±2.1[a]	10.2±1.2[a]
Standard culture	14	5.9±0.4	11.8±1.1	20.5±1.8	3.6±0.4
SIS culture	14	7.1±0.4	20.7±1.1[a]	66.9±3.7[a]	9.5±0.2[a]

[1]SI (stimulation index)=insulin secretion during incubation with 16.7 mmol/L high glucose solution+50 μmol/L IBMX/insulin secretion during incubation with 2.7 mmol/L low glucose solution. [a]P<0.05 *vs* standard culture.

14 d of culture, P>0.05). There was a two- to threefold increase in insulin secretion in both groups upon stimulation by high glucose solution (16.7 mmol/L). After 14 d of culture, SIS-treated group showed a significantly higher increase in insulin secretion than control group (20.7±1.1 mU/L *vs* 11.8±1.1 mU/L, P<0.05). When islets were placed in the high glucose solution containing IBMX, the stimulated insulin secretion was significantly higher in SIS-treated group than in control group. Similar results occurred when the stimulation index was used as a reference (a ratio of insulin secreted upon stimulation by high glucose solution plus IBMX to basal insulin secreted). The stimulation index of SIS-treated group was about 2-3 times than that of the control group. In addition, the stimulation index of SIS-treated group were statistically equivalent to that of the group cultured for 7 and 14 d group (9.5±0.2 *vs* 10.2±1.2, P>0.05) (Table 2).

Apoptosis of islet cells was measured by relative quantification of DNA/histone fragmentation (mono- and oligonucleosomes) in culture supernatants using ELISA. The enrichment factor of nucleosomes was significantly lower in SIS-treated group than in control group, suggesting that much less apoptosis of islet cells occurred in SIS-treated group (Table 1).

DISCUSSION

Islet transplantation is a viable option of treatment for insulin-dependent diabetes mellitus. Maintaining islets in culture is important to increase the safety, practicality and efficacy of successful clinical results. In addition to reducing the immunogenicity of islets[3,17], islet culture simplifies the procedure of transplantation, which can be scheduled during regular hospital hours, and the patient is not necessary to move from a distant location to the transplantation center before a donor is available. Culture may facilitate novel immunosuppressive techniques[18]. Moreover, improved methods and supplementation of islet culture provide a unique opportunity to optimize recovery of islet mass for transplantation. Islets do not form monolayers, fail to replicate and contain multiple cell types, making them similar to a tiny organ. All these are the unique challenges for maintaining islets in culture. Procedures related to isolation and *in vitro* culture can lead to a considerable loss of islet tissue. Apoptosis and anoikis may be the mechanisms involved in tissue loss[19,20].

It has been reported that binding of ECM to integrin, a protein located on cell membrane, stimulates intracellular signaling pathways to prevent cells from their entry into the cascade of apoptosis[21], suggesting that interaction between cells and ECM plays an important role in the subsequent cell behaviors. It is possible that cell–ECM interaction is disrupted by the procedures related to islet isolation and purification. As a consequence, functions of isolated islets could be damaged. A recent study showed that the viability of porcine islet cells embedded in fibrous skeleton of pancreas is higher viability which may be due to cellular adhesion and ligation of ECM to integrins as opposed to their isolated counterparts. Further studies of these embedded cells demonstrated that the viability of the cells is sustained for 30 days or more and islet function is excellent 30 days after the isolation[22].

Frisch *et al*[23] and Thomas *et al*[22] have shown that anoikis resulted from a death signal induced by MEKK-1 group of caspases[23,24]. As a critical safeguard of nature, anoikis can prevent cells from taking up residence and growth in ectopic positions as in tumor metastases. Thomas *et al*[22] reported that the apoptotic rate is markedly reduced when cells are attached to ECM, suggesting that ECM plays an essential role in the maintenance of differentiated cells. Preparation of islet isolation especially enzymatic digestion of pancreas can result in the loss of peripheral basement membrane or interstitial membrane of islets, suggesting that purity is not essential to achieve engraftment. The presence of other cellular elements may be critical for engraftment and long-term maintenance of graft function and survival[25]. The fact that transplantation of purified preparations of autologous islets loses islet function in early periods also supports this hypothesis[26].

A number of novel approaches have been developed to create a favorable physiological microenvironment for islets in culture to limit anoikis. The major method is to use ECM. Studies indicate that ECM can increase islet survival time and/or insulin secretion[21,27,28]. As a natural ECM, SIS is obtained from the intestine using a process that retains the natural composition and configuration of matrix molecules such as collagen (Types I, III and VI), glycosaminoglycans (hyaluronic acid, chondroitin sulfate A and B, heparin and heparin sulfate), proteoglycans,

glycoproteins (fibronectin), and growth factors (FGF-2, TGF-β, and VEGF), which play an important role in host tissue repair, remodel, and cell growth[8]. Mapping of the distribution of significant proteins and proteoglycans in small intestinal submucosa by fluorescence microscopy indicates that heparan sulfate proteoglycans (HSPG) is extensively distributed but concentrated in vessels. FGF-2 is diffusely distributed and associated with fibrous structures. VEGF is distributed mainly around the vessels. Collagen fibrils are distinctly present in the background. This anatomic structure of SIS is likely to play an important role in the regeneration of tissues and factors in remnant vessels may facilitate penetration of the matrix along these avenues[29].

SIS has excellent mechanical properties (high compliance, a high burst pressure point, and an effective porosity index), satisfactory histocompatibility, non-immunogenicity and safety for human use, allowing it to be used for vascular and connective tissue graft construct[7,10-14]. The porous nature and three-dimensional microarchitecture of SIS allow diffusion of cell nutrients and induce proliferation, remodeling and regeneration of host tissues when implanted in microenvironments *in vivo* (e.g. artery, tendon, bone, and articular cartilage)[10,14,30,31]. The same situation occurs in the cells during in vitro culture. In a recent study, several cell lines, including NIH Swiss mouse 3T3 fibroblasts, NIH 3T3/J2 fibroblasts, primary human fibroblasts, primary human keratinocytes, human microvascular endothelial cells (HMECs) and rat osteosarcoma (ROS) cells were cultured in the presence of sheet form SIS. All cell types showed the ability to attach and proliferate. All fibroblast cell lines and keratinocytes proliferated and/or migrated into the three-dimensional scaffold of SIS matrix. Co-culturing of NIH 3T3/J2 fibroblasts and primary human keratinocytes with SIS resulted in a distinctive spatial orientation of the two cell types. Fibroblasts populated the mid-substance of the three-dimensional matrix and keratinocytes formed an epidermal structure with rete ridge-like formation and stratification, when the composite was lifted to an air liquid interface in culture[32]. For these reasons, we hypothesize that SIS with native sheet-like configuration can imitate the natural growth environments of islets and improve islet survival and function *in vitro*, thus decreasing loss of tissue mass during culture.

We observed a higher recovery and stimulation index of islets and a lower enrichment factor of nucleosomes in SIS-treated group than in control group. These results may prove our hypothesis and are comparable to previous studies in different cell lines[32] and islets[6]. In pancreas, ECM is composed of interstitial matrix and basement membrane, the latter is composed of fibronectin, laminin and collagens IV and V[33]. These submucosal cell growth substrates provide islet cells with a collagenous matrix environment *in vitro*, resembling that in *in vivo*. We think that the unique configuration and composition of SIS may contribute to such improvements for islet culture. Firstly, the porous nature of SIS allows diffusion of cell nutrients, thus decreasing the central cell damage of isolated islets of Langerhans[34]. Secondly, SIS provides a substratum with a three-dimensional scaffold allowing for cell migration and spatial organization[35]. Thirdly, abundant collagen and various growth factors activate the signaling pathways, which induce attachment, proliferation, and differentiation of the cells[36].

In conclusion, co-culture of isolated rat islets with native sheet-like SIS can provide an ECM and possible biotrophic and growth factors, which promote the recovery and subsequent function of islets during *in vitro* tissue culture. In view of the rapid degradation of SIS *in vivo*[37], whether SIS improves the recovery and subsequent function of islets *in vitro* and the effect of SIS on islets *in vivo* needs to be further studied.

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