Small Intestinal Submucosa Improves Islet Survival and Function in Vitro Culture

T. Xiaohui, X. Wujun, D. Xiaoming, P. Xinlu, T. Yan, T. Puxun, and F. Xinshun

ABSTRACT

Introduction. Most centers maintain isolated human islet preparations in tissue culture to improve the safety as well as the practicality of islet transplantation. However, maintaining viability and recovery of islets remains a challenge. Extracellular matrix (ECM) is one of the most important components of the islet microenvironment. Reconstruction of the cell-matrix relationship seems to be necessary to sustain the structure and function of differentiated islets. Small intestinal submucosa (SIS), a natural ECM, is well known to promote wound healing, tissue remodeling, and cell growth. The purpose of this study was to evaluate recovery and function of isolated rat pancreatic islets during in vitro culture with SIS.

Methods. Pancreatic islets isolated from Wistar rats following intraductal collagenase distension, mechanical dissociation, and EuroFicoll purification were cultured in plates coated with multilayer SIS (SIS-treated group) or without (standard cultured group) for 7 or 14 days in an islet culture media of RPMI 1640 (Gibco). The islets from both experimental groups were stained and counted with dithizone. Islet recovery following culture was determined by the ratio of counts after culture to the yield of islets immediately following islet isolation. The viability of the islets was assessed by a glucose challenge test with low glucose (2.7 mmol/L), high glucose (16.7 mmol/L), and high glucose solution supplemented with 50 μmol/L 3-isobutyl-1-methylxanthine solution. The apoptosis of islet cells was measured by relative quantification of histone-complexed DNA fragments by using enzyme-linked immunosorbent assay.

Results. After 7 or 14 days of in vitro tissue culture, the recovery in SIS-treated islets group was about double of that cultured in the plates without SIS coating. In the SIS-treated group, there was no significant difference between the short- and the long-term periods of culture (95.8% ± 1.0% vs 90.8% ± 1.5%, P > .05). Following incubation with high glucose (16.7 mmol/L) solution, the insulin secretion in the SIS-treated group showed a greater increase than the control group after 14 days of culture (20.7 ± 1.1 mU/L vs 11.8 ± 1.1 mU/L, P < .05). When islets were placed in the high glucose solution containing IBMX, the stimulated insulin secretion was more increased in the SIS-treated than in the control group despite the duration of the culture. The calculated stimulation index of SIS-treated group was about two to three times greater than the control group. In addition, the stimulation index of the SIS-treated group remained constant regardless of short-term
versus long-term culture (9.5 ± 0.2 vs 10.2 ± 1.2, P > .05). Much less apoptosis of islet cells occurred in the SIS-treated than in the control group.

Conclusion. Coculture of isolated rat islets with native sheetlike small intestinal submucosa seemed to build an ECM for islets providing possible biotrophic and growth factors that promote the recovery and subsequent function of islets.

In the last 10 years, both the prevalence and incidence of diabetes have escalated sharply in the countries all over the world. This disease has already become a global health concern. For example, there are 14 million patients suffering from diabetes in the United States among which 1 million patients suffered from type 1 diabetes mellitus. Islet transplantation can control blood sugar effectively, reduce the complication of the diabetes, and prevent the hypoglycemia and insulin resistance caused by exogenous insulin. So pancreatic islet cell transplantation has the potential to be an effective means to treat type 1 diabetes mellitus.

Human islet transplantation has been demonstrated to be a viable option for the treatment of type 1 diabetes mellitus. However, there are still many difficulties hindering progress, a major limitation of which is the lack of suitable quantities of viable islets for transplantation.

The ability to maintain islets of Langerhans in tissue culture provides a chance for islet storage after isolation from the pancreas before clinical transplantation. This storage period can be utilized to assess the function of the islets and to confirm microbiologic sterility of the preparation. In addition, it is also necessary to increase the purity of the islet preparation and to reduce the immunogenicity of the islets. However, one major disadvantage of islet cultures is the loss of tissue mass over culture time. Given the importance of transplanting a sufficient number of islets to increase the chance for a successful graft, any loss of islet tissue mass jeopardizes this possibility.

Both the morphology and the metabolic activity of cultured cells are affected by the composition of the substrates on which they grow. Cultured cells may proliferate or perform their in vivo functions when cultured on substrates that closely mimic their natural environments. In fact, there are many commercially available matrices for cell culture, which have been shown to support cell growth, such as human extracellular matrix, derived from human placenta, and Matrigel, a soluble basement membrane extract from the Engelbreth-Holm-Swarm tumor. Previous studies involving human islet cultures with bovine corneal endothelial cell matrix showed an enhanced ability to secrete insulin.

Small intestinal submucosa (SIS) is a relatively acellular collagen-based matrix derived from porcine small intestine by mechanical removal of the mucosal and smooth muscle layers. The resulting cell-free translucent sheets are about 100 μm thick. The collagen-based matrix is comprised of highly conserved collagens, glycoproteins, proteoglycans, and glycoaminoglycans in their native configuration and concentrations. In addition, SIS includes various growth factors, such as fibroblast growth factor-2 (FGF-2), transforming growth factor-β (TGF-β), and vascular endothelial cell growth factor (VEGF), which promote cell growth.

In 1966, Matsumoto et al described the use of an inverted small intestine to replace large veins in dogs. Improved processing of this biomaterial (SIS) has made it useful for tissue-engineering studies. Extensive in situ tissue remodeling from SIS has been shown in both rat and canine models. The SIS has been used as a scaffold for proliferation, remodeling, and regeneration of a variety of host tissues, including blood vessels, dura mater, urinary bladder, abdominal wall, and tendons. Grossly and microscopically, the remodeled tissue resembles the native tissue. Recently, SIS was evaluated for potential in vivo use in hepatocyte transplantation. For these reasons, in this study we investigated whether the use of SIS in coculture with rat islets seeking to imitate the natural growth environment of islets improved islet survival and their in vitro function.

Materials and Methods

Animals

Male Wistar rats (250 to 400 g body weight, Animal Lab 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 the Study

Islets were isolated by using collagenase and purified by discontinuous Euroficoll gradients. The purified islets were separated into two experimental groups: the study group were islets cultured in plates coated with SIS, and the control group were cultured under standard conditions without SIS coating. The number of islets was counted after isolation and after culture. Recovery of islets following culture was determined by the ratio of counts after culture to the yield of islets immediately following islet isolation. Islet function was determined by a static glucose challenge test after a short (7 days) or a long (14 days) period of culture. The glucose challenge test was performed both in the absence and in the presence of 3-isobutyl-1-methylxanthine (IBMX). Apoptosis of islet cells was measured by relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) out of the cytoplasm of cells after apoptosis or when released from necrotic cells.

Preparation of Soluble SIS Supplement

The SIS preparation followed standard procedures described by Badylak’s lab. In brief, freshly harvested porcine jejunum was obtained from a local slaughterhouse. The tube of intestinal...
material was rinsed until free of contents and inverted. The superficial layers of the tunica mucosa were removed by scraping with a knife handle. The tissue was then reverted to its original orientation and the serosa and tunica muscularis were removed. The resulting membrane was approximately 80 to 100 μm thick, and consisting of the tunica submucosa and the basilar portion of the tunica mucosa. The prepared intestinal submucosa tube split open longitudinally was rinsed extensively in water to rid it of any cells associated with the matrix eliminating cell degradation products. The SIS sheets were sterilized by exposure to 1 g/L peracetic acid. To make multilayered SIS, five sheets of SIS mechanically compressed by vacuum pressing were allowed to dry. The multilayer SIS was then terminally sterilized with ethylene oxide and kept until ready to use. Resoaking in saline prior to use made the SIS sheets pliable and soft.

Islet Isolation and Culture

Islets were isolated and purified from rat pancreata according to a previously described procedure. Briefly, rats were anesthetized with intraperitoneal pentobarbital. Pancreata were 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 minutes at 37°C. Islets were purified on a discontinuous Euroficoll gradient (Sigma), handpicked under an inverted light microscope, pooled, and then separated into two experimental groups: the study group islets were cultured in plates that were coated by suitable-size multilayer SIS, and the control group without SIS coating. 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°C in a humidified 50% CO2 atmosphere for a period of 7 or 14 days.

Islet Quantification and Recovery

The islets from both experimental groups stained with dithizone were 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 IE (islet equivalent, number of islets of 150-μm diameter equal in volume to the sample). Recovery of islets following culture was determined by the ratio of counts after culture to the yield of islets immediately following islet isolation.

Islet Viability

For each group, six separate samples of 20 islets each were tested simultaneously for their stimulated insulin secretion. Islets (150 ± 50 μm in diameter) were first preincubated for 45 to 50 minutes in 2 mL RPMI 1640 solution containing 2.7 mmol/L glucose. Insulin secretion was then assessed by three incubations with RPMI 1640 solutions containing (1) 2.7 mmol/L glucose, (2) 16.7 mmol/L glucose, or (3) 16.7 mmol/L glucose with Ig/LIBMX (Sigma), each for 45 minutes. At the end of incubation with each solution, the supernate was completely removed for insulin secretion radioimmunoassay (Department of Isotope, China Institute of Atomic Energy). Insulin secretory responses of 20 islets to glucose stimulation were expressed as mU/L. To eliminate variations in islet size, causing differences in insulin response, the stimulation index for each group of islets was determined by the ratio of the insulin secretion stimulated by high glucose solution containing IBMX to the basal insulin secretion stimulated by low glucose.

Evaluation of Apoptosis of Islet Cells

Apoptosis of islet cells was measured by relative quantification of DNA/histone fragmentation (mono- and oligonucleosomes) in culture supernates. The supernatants media of 7- or 14-day cultures were collected from every well of the two groups for analysis by the enzyme-linked immunosorbent assay (ELISA) kit system of Cell Death Detection (no. 1774425; Roche) measuring cytoplasmic histone-associated DNA fragments generated by cell death based on the reactions of 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, antihistide (biotin-labeled), and anti-DNA (peroxidase-conjugated) in the well coated with streptavidin of a 96-well plate. Antibody-nucleosome complexes are bound to the microplate by the streptavidin. The solution mixture was incubated for 2 hours at room temperature. The solution was then collected and the well completely rinsed three times with 150 μL of an incubation buffer and 100 μL of premixed 2.2’-azino-di-[3-ethylbenzthiazoline sulfonate] substrate solution added to each well. The mixed solution was agitated on a plate shaker for 20 minutes at room temperature prior to absorbance measurement at a wavelength of 405 nm. The enrichment of nucleosomes due to the apoptosis of islet cells were calculated from the absorbance values by using the following formula: enrichment factor of nucleosomes = (values of the sample – the background values)/values of the negative control. The assay was done independently three times for each experimental group. This method permits us to specifically determine mono- and oligonucleosomes released into culture supernates that accompany apoptosis and necrosis of islet cells. The accumulation of mono- and oligonucleosomes in the cytoplasm of apoptotic and necrotic cells causes the DNA degradation, which is known to occur several hours before the breakdown of the plasma membrane. Thus, it is impossible to detect the apoptotic and necrotic changes of cultured islets much earlier than other evaluation methods.

Statistical Analysis

Results are expressed as mean values ± standard error of the mean (X ± Sx). Differences between the experimental groups were analyzed by student t tests using SPSS10.0. A P value < .05 was considered statistically significant.

RESULTS

After 7 or 14 days of in vitro tissue culture, the group of islets cultured with SIS showed a twofold greater (P < .05) recovery than the control group (Table 1). In the SIS-treated group, there was no statistical difference between the short and the long periods of culture (95.8% ± 1.0% vs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery</td>
<td>Standard</td>
<td>67.1%±2.6%</td>
<td>42.2%±1.5%</td>
</tr>
<tr>
<td></td>
<td>SIS</td>
<td>95.8%±1.0%*</td>
<td>90.8%±1.5%*</td>
</tr>
<tr>
<td>Enrichment factor</td>
<td>Standard</td>
<td>1.98±0.06</td>
<td>2.90±0.23%</td>
</tr>
<tr>
<td></td>
<td>SIS</td>
<td>1.08±0.02*</td>
<td>1.12±0.09%*</td>
</tr>
</tbody>
</table>

Enrichment factor of nucleosomes = (values of the sample – the background values)/values of the negative control.

*P < .05 vs standard culture.
Most islets cultured in the control group lost their initial morphology, becoming “loose” in appearance at the end of the culture, whereas islets cultured with SIS exhibited excellent morphology (Figs 1 and 2).

In vitro viability of the islets was assessed using a glucose challenge test. Islets cultured under standard conditions showed a decrease in glucose-stimulated insulin secretion (Fig. 1), whereas islets cultured with SIS maintained their insulin secretion capacity (Fig. 2).

**Fig 1.** Islet cultured for 14 days under standard conditions became separated in appearance (original magnification, 10×40).

**Fig 2.** Islet cultured for 14 days on the SIS exhibited excellent morphology (original magnification, 10×40).
challenge test. There was no statistical difference in the basal level of insulin secretion between the SIS-treated and the control group in the short versus long period of culture (9.1 ± 0.3 vs 7.8 ± 0.6 mU/L from 7 days culture; 5.9 ± 0.4 vs 7.1 ± 0.4 mU/L from 14 days culture, respectively; \( P > .05 \)). There was a two- to threefold increase in insulin secretion from both groups upon stimulation by high glucose (16.7 mmol/L). After 14 days of culture, the SIS-treated group showed a significantly greater increase than the control group (20.7 ± 1.1 mU/L vs 11.8 ± 1.1 mU/L, \( P < .05 \)). When islets were placed in a high glucose solution containing IBMX, the stimulated insulin secretion was significantly higher in the SIS-treated group than that in the control group regardless of short or long culture periods. The same result occurred when using the stimulation index as a reference, the ratio of insulin secreted upon stimulation by high glucose plus IBMX to basal insulin secreted. The stimulation index from the SIS-treated group was about two to three times that of the control group. In addition, the stimulation indices of the SIS-treated group were equivalent in the short- and long-term culture groups (9.5 ± 0.2 vs 10.2 ± 1.2, \( P > .05 \); Table 2).

Apoptosis of islet cells was measured by relative quantification of DNA/histone fragmentation (mono- and oligonucleosomes) using ELISA in culture supernates. The enrichment factor of nucleosomes was significantly lower in the SIS-treated group than that in the control group regardless of short or long culture periods. The same result occurred when using the stimulation index as a reference, the ratio of insulin secreted upon stimulation by high glucose plus IBMX to basal insulin secreted. The stimulation index from the SIS-treated group was about two to three times that of the control group. In addition, the stimulation indices of the SIS-treated group were equivalent in the short- and long-term culture groups (9.5 ± 0.2 vs 10.2 ± 1.2, \( P > .05 \); Table 2).

**DISCUSSION**

Islet transplantation is now a viable option for the treatment of insulin-dependent diabetes mellitus. Maintaining islets in culture is important to increase the safety, practicality, and efficacy imperative for successful clinical results. In addition to reducing the immunogenicity of islets,3,17 culture simplifies the logistics of transplantation—the procedure can be scheduled during regular hospital hours, and the patient does not have to move from a distant location to the transplantation center before a donor becomes available. Culture may facilitate novel immunosuppressive techniques.18 Moreover, improving methods and supplementation of islet culture provide a unique opportunity to optimize recovery of islet mass for transplantation in order to increase success with single donor transplants. Nevertheless, islet culture remains problematic. Islets do not form monolayers, fail to replicate, and contain multiple cell types, making them similar to a tiny organ. All of the above create unique challenges for maintaining islets in culture. The procedures related to isolation and in vitro culture can lead to considerable losses of islet tissue. Recently, the mechanisms involved in the tissue loss have been recognized as apoptosis and anoikis.19,20

It has been reported that binding of the extracellular matrix (ECM) to integrin, a protein located in the cell membrane, stimulates intracellular signaling pathways to prevent cells from entry into an apoptosis cascade.21 Thus, there is no doubt that an interaction with the ECM plays an important role in subsequent cell behavior. It is possible that the cell-ECM interaction is disrupted by procedures related to islet isolation and purification. As a consequence, the functions of isolated islets could be damaged. Indeed, a recent study indicated that porcine islet cells embedded in the fibrous skeleton of the pancreas had a higher rate of viability, apparently due to cellular adhesion and ligation of the ECM to the integrins as opposed to their isolated counterparts. Further studies of these embedded cells have demonstrated that viability of the cells was sustained for 30 days or more and excellent islet function in perfusion studies performed over 30 days after isolation.22

A second important mechanism is anoikis, which means apoptosis when cells are separated from their ECM. This concept was described by Frisch et al23 and further developed by Thomas et al.24 Now it is well known that the anoikis is due to a death signal induced by the MEKK-1 group of caspases.23,24 As a critical safeguard of nature, anoikis can prevent cells from taking up residence and growing in ectopic positions, as in tumor metastases. Thomas et al reported that the apoptotic rate was markedly reduced when cells attached to an ECM, suggesting that cell-ECM connections attenuate the anoikis death signal.22 So the ECM plays an essential role in maintenance of differentiated cells. The preparation of islet isolation—in particular, enzymatic digestion of the pancreas—results in loss of the peripheral basement membrane or interstitial membrane of the islet. It has been suggested that purity is not essential for engraftment to be achieved. The presence of other cellular elements may be critical for engraftment and long-term maintenance of graft function and survival.25 Additional support for this hypothesis is that the transplan-

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**Table 2. Stimulated Insulin Secretion of Rat Islets Cultured for 7 and 14 Days During Static Incubation Assay \( n = 6, \bar{X} \pm S_x \text{ mU/L} \)**

<table>
<thead>
<tr>
<th>Group</th>
<th>t/d</th>
<th>Low Glucose (2.7 mmol/L)</th>
<th>High Glucose (16.7 mmol/L)</th>
<th>High Glucose + 50 μmol/L IBMX</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard culture</td>
<td>7</td>
<td>9.1 ± 0.3</td>
<td>18.3 ± 1.1</td>
<td>51.3 ± 3.4</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>SIS culture</td>
<td>7</td>
<td>7.8 ± 0.6</td>
<td>21.8 ± 1.9</td>
<td>75.8 ± 2.1*</td>
<td>10.2 ± 1.2*</td>
</tr>
<tr>
<td>Standard culture</td>
<td>14</td>
<td>5.9 ± 0.4</td>
<td>11.8 ± 1.1</td>
<td>20.5 ± 1.8</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>SIS culture</td>
<td>14</td>
<td>7.1 ± 0.4</td>
<td>20.7 ± 1.1*</td>
<td>66.9 ± 3.7*</td>
<td>9.5 ± 2*</td>
</tr>
</tbody>
</table>

SI (stimulation index) = insulin secretion during incubation with 16.7 mmol/L glucose + 50 μmol/L IBMX/insulin secretion during incubation with 2.7 mmol/L glucose.

\(^{*} P < .05\) vs standard culture.
tation of purified preparations of autologous islets have lost islet function in early periods. A number of novel approaches have been developed recently including the creation of a more physiological microenvironment for the islet in culture to limit anoikis. The major method is the use of ECM. Numerous studies have described increased islet survival and/or insulin secretion by the use of ECM owing to a beneficial effect of ECMs on islet survival in vitro to integrin-matrix interactions. As a natural ECM, SIS is obtained from the intestine by using a process that retains the natural composition and configuration of matrix molecules, such as collagen (types I, III, VI), glycosaminoglycans (hyaluronic acid, chondroitin sulfate A and B, heparin, and heparan sulfate), proteoglycans, glycoproteins (fibronectin), and growth factors (FGF-2, TGF-β, and VEGF), which are known to have important roles in host tissue repair, remodelings and cell growth. Mapping of the distribution of significant proteins and proteoglycans in small intestinal submucosa by fluorescence microscopy indicated that heparan sulfate proteoglycans were widely distributed but concentrated in vessels. FGF-2 was distributed diffusely and associated with fibrous structures. VEGF was distributed mainly around vessels. Collagen fibrils were distinctly present in the background. The anatomic structure of SIS is likely to play an important role in the regeneration of tissues, factors in remnant vessels may facilitate penetration of the matrix along these avenues.

SIS has excellent mechanical properties, namely, one high compliance, a high burst pressure point, and an effective porosity index as well as satisfactory histocompatibility due to its lack of immunogenicity and safety for human use, for vascular and connective tissue constructs. The porous nature and three-dimensional microarchitecture of SIS allow diffusion of cell nutrients inducing proliferation, remodeling, and regeneration of host tissues when implanted in a number of microenvironments in vivo, for example, artery, tendon, bone, and articular cartilage. The same situation applies to cells during in vitro culture. In a recent study, several cell lines were cultured in the presence of the shear from SIS, including NIH Swiss mouse 3T3 fibroblasts, NIH 3T3/2 fibroblasts, primary human fibroblasts, primary human keratinocytes, human microvascular endothelial cells, and rat osteosarcoma cells. All cell types showed the ability to attach and proliferate. The fibroblast cell lines and the keratinocytes proliferated or migrated into the three-dimensional scaffold of the SIS matrix. Coculture of NIH 3T3/2 fibroblasts and primary human keratinocytes with SIS yielded a distinctive spatial orientation of the two cell types. The fibroblasts populated the midsubstance of the three-dimensional matrix and the keratinocytes formed an epidermal structure with rete ridgelike formation and stratification when the composite was lifted to an air-liquid interface in culture. For these reasons, we hypothesized that SIS with native sheetlike configuration could imitate the natural growth environments of islets, improving their survival and function in vitro, thus decreasing the loss of tissue mass during culture.

In our study we observed a higher recovery and stimulation index and a lower enrichment factor of nucleosomes from the group of islets cultured with SIS when compared with the control group. These results prove our hypothesis. They are comparable to previous studies with different cell lines and with islets cultured with endothelial matrix. In the pancreas, the extracellular matrix is composed of interstitial matrix and basement membrane, the latter of which is composed of fibronectin, laminin, and collagens IV and V. The submucosal cell growth substrates provided islet cells with a collagenous matrix environment in vitro, resembling that found in vivo. We think that the unique configuration and composition characteristic of SIS improve islet culture. First, the porous nature of SIS allows diffusion of cell nutrients, thus decreasing the central cell damage of isolated islets of Langerhans. Second, SIS provides a substratum with a three-dimensional scaffold that allows for cell migration and spatial organization. Third, abundant collagen and various growth factors activate signaling pathways, which induce attachment, proliferation, and differentiation of cells.

The results of the present study suggested that coculture of isolated rat islets with native sheetlike SIS provided an ECM as well as possible biotrophic and growth factors, which promote the recovery and subsequent function of islets during in vitro tissue culture. In view of results of rapid degradation of SIS in vivo, future studies must investigate whether SIS would improve the recovery and subsequent function of islets in vitro for longer culture periods as well as affect them in vivo.

REFERENCES