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ORIGINAL ARTICLE

Rat islet culture in serum-free medium containing silk protein sericin

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Abstract
Background The development of islet cultures is desirable for successful clinical islet transplantation. Fetal bovine serum (FBS) has been used as a supplement in islet culture medium, but it may be an unsuitable supplement due recent animal health problems. We have evaluated the use of the silk protein, sericin, derived from *Bombyx mori* as a replacement for FBS in islet culture medium.
Methods Twenty rat islets were cultured in medium containing either sericin or FBS, or no supplement, for 14 days, during which time viable islets were counted in order to evaluate islet survival. Insulin secretion was measured in vitro by static incubation on days 3 and 7. In vivo function of cultured islets was tested by syngeneic transplantation. The islets were evaluated histologically and immunohistochemically after culture and transplantation.
Results Ninety-five percent of islets were viable after culture for 14 days in culture medium supplemented with either FBS or sericin, while no islets survived beyond 7 days in culture without supplement. No significant differences in stimulated insulin secretion were noted between two groups of islets grown on supplemented media. Following transplantation, islets cultured in FBS or sericin rapidly reversed hyperglycemia and maintained normal glycemic control. Histologically, islets cultured with sericin displayed a well-preserved structure and strong insulin staining before and after transplantation.
Conclusion Serum-free medium containing sericin appears to be useful for islet culture.

Keywords Islet culture · Islet transplantation · Sericin · Serum-free medium

Abbreviations
FBS Fetal bovine serum
HBSS Hank's balanced salt solution

Introduction

Islet transplantation represents an ideal treatment for insulin-dependent diabetes mellitus, offering a simpler and safer procedure than whole pancreas organ transplantation. Islet transplantation has been investigated since the 1970s, but until recently only 10% of patients who underwent pancreatic islet transplantation became insulin independent [1]. Recently, the Edmonton group introduced a steroid-free, rapamycin-based immunosuppressive protocol, with rapid infusion of islets immediately after isolation and use of two or more pancreas donors to supply sufficient numbers of islets. A study of 17 patients revealed that 14 patients achieved stable glucose levels following this protocol, with >80% of patients remaining insulin-independent at the 1-year follow-up [2].

The field of islet transplant still faces significant challenges even after success of the Edmonton group. Treatment requires high-quality facilities to prepare large volumes of islets for transplantation. Islet culture and/or

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cryopreservation are necessary for long-term storage and transport and are indispensable when establishing an islet bank. However, no special culture media have been formulated for islet culture, and commercially available media are still being used. These media need to be supplemented with fetal bovine serum (FBS), which must in turn be batch-tested. In addition, FBS is potentially unsuitable for use in clinical tissue transplantation in light of recent animal health problems, such as bovine spongiform encephalopathy (BSE). As prion disease may be transmitted through blood and blood preparations, the use of plasma preparations, such as FBS, for clinical use is not recommended [3, 4]. We have therefore focused on sericin as a potential replacement for FBS.

The protein sericin is the primary constituent of silk (20–30% of total cocoon weight), enveloping fibroin in successive sticky layers [5]. When cocoons are used for the production of silk textiles, sericin is generally removed from the cocoon and discarded. Sericin comprises five polypeptides with molecular weights varying from 8.0×10^4 to 3.09×10^5 Da [6]. Kato et al. [7] recently reported that sericin displays antioxidant properties and an inhibitory action against tyrosinase. These characteristics make the protein a valuable natural ingredient for use in the cosmetic and food industries [7]. Terada et al. reported that sericin also accelerates the proliferation of various cells [8].

In the study reported here, we evaluated the use of silk protein sericin produced from the silk gland of *Bombyx mori* as a replacement of FBS in islet cultures.

Materials and methods

Experimental animals

Male inbred Lewis rats (Charles River Japan, Yokohama, Japan), 8–10 weeks old, served as islet donors and recipients. Diabetes was induced by intravenous injection of streptozotocin (60 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) through the caudal vein 7 days before transplantation. Rats showing non-fasting blood glucose levels of >350 mg/dl were used as transplant recipients. Blood glucose levels were determined after 6 h of fasting using the glucose oxide method on a Medisafe automatic analyzer (Terumo, Tokyo, Japan). All study protocols were approved by the Institute of Experimental Animals at Fukui Medical University, Japan.

Islet isolation

Laparotomy was performed under anesthesia by intraperitoneal injection of 40 mg/kg Nembutal. After clamping the distal end of the common bile duct, cold Hank's balanced salt solution (HBSS; Sigma-Aldrich) containing 2.0 mg/ml collagenase S-1 (Nitta Zeratin, Osaka, Japan) was injected into the duct at the hilum to achieve full distension of pancreatic tissue. The pancreas was removed and incubated in a 50-ml centrifuge tube (Sumitomo Bakelite, Tokyo, Japan) for 25 min in a 37°C water bath. After three washes in cold HBSS, digested pancreatic tissue was filtered through a 500-μm mesh, followed by two washes of the filtered tissue as previously described [9]. After the digested tissue was suspended in 20 ml of Histopaque (Sigma-Aldrich), 20 ml HBSS was slowly layered onto the Histopaque layer. Following centrifugation at 2500 rpm for 20 min, islets at the interface were collected [10]. The islets were washed twice in cold HBSS, and isolated islets were placed into culture dishes.

Islet culture and survival of cultured islets

Twenty islets picked from a pool of freshly isolated islets were cultured in RPMI1640 medium (Sigma-Aldrich) supplemented with 10 m*M* nicotinamide (Wako Pure Chemical Industries, Osaka, Japan) [11] and penicillin–streptomycin. The culture media were additionally supplemented with FBS (10%) or various concentrations (0.025, 0.05, 0.1 or 1%) of sericin (Seiren, Fukui, Japan), or not supplemented with either. The islets were cultured at 37°C in humidified air (5% CO_2, 95% air). Culture media were changed on culture days 1, 3, 7, 10 and 14. The numbers of surviving islets cultured with different supplements were counted separately after a 14-day culture and compared. Results obtained from six separate experiments were expressed as the mean ± standard deviation.

Islet insulin release assay

An insulin release assay was performed on culture days 3 and 7 using a static incubation test. Islets from the FBS and 0.1% sericin groups ($n = 6$ each) were pre-incubated for 1 h at 37°C in low-glucose medium (2 ml RPMI1640; Gibco, Auckland, New Zealand) supplemented with 3.3 m*M* glucose and 0.1% FBS. After preincubation, the islets were sequentially incubated in 2 ml of low-glucose medium, high-glucose medium (20 m*M* glucose and 0.1% FBS) and low-glucose medium, for 1 h in each medium [12]. A radioimmunoassay was used to determine the amount of secreted insulin in the culture media following each incubation.

Islet transplantation

Diabetic recipient rats were anesthetized using Nembutal. Approximately 800 islets that had been cultured for 7 days

with either FBS or 0.1% sericin were transplanted into the left renal subcapsular space of a diabetic recipient through a micropipette (200 µl) [9]. As all cultured islets were derived from a common pool of freshly isolated islets, islets transplanted into each group were equivalent. Non-fasting blood glucose levels in transplanted rats were monitored for 28 days when transplants were removed by nephrectomy. Following nephrectomy, increases in non-fasting blood glucose levels were monitored.

Morphology

Islets cultured with FBS and sericin were examined for their morphology under an inverted microscopy daily during the 14 days of culture. For histological examination, cultured or transplanted islets were placed in 10% formalin and then fixed in paraffin. Sections (4 µm thick) were stained with hematoxylin and eosin. Islet grafts were also examined immunohistochemically with anti-insulin antibody (Biogenesis, England, UK) by the EnVision method (Dako, Kyoto, Japan).

Statistical analysis

Student's t test was used to evaluate differences in insulin secretion and stimulation index between FBS and Sericin groups. Values of $P < 0.05$ were considered statistically significant.

Results

Survival rate of cultured islets

We first determined the optimal sericin concentration for islet culture. Assessment of the culture of islets with various concentrations of sericin for 14 days revealed that 0.1% sericin was the optimal concentration for islet culture. Islet survival rate on day 14 was 96.7 ± 4.08% with 0.1% sericin, but significantly lower with the other concentrations (0.025%, 43.3 ± 25.4%; 0.05%, 50.0 ± 28.8%; 0.5%, 42.5 ± 33.6%; 1%, 22.5 ± 31.3%, $n = 6$) (Fig. 1a). When 0.1% sericin and FBS were compared for their ability to support islet survival in culture, both maintained ≥95% viability after 14 days of culture (95.0 ± 5.3 and 95.5 ± 4.4%, respectively; $n = 10$). Few islets were alive after 7 days in culture in medium not containing a FBS or sericin supplement (1.5 ± 4.8%, $n = 10$) (Fig. 1b).

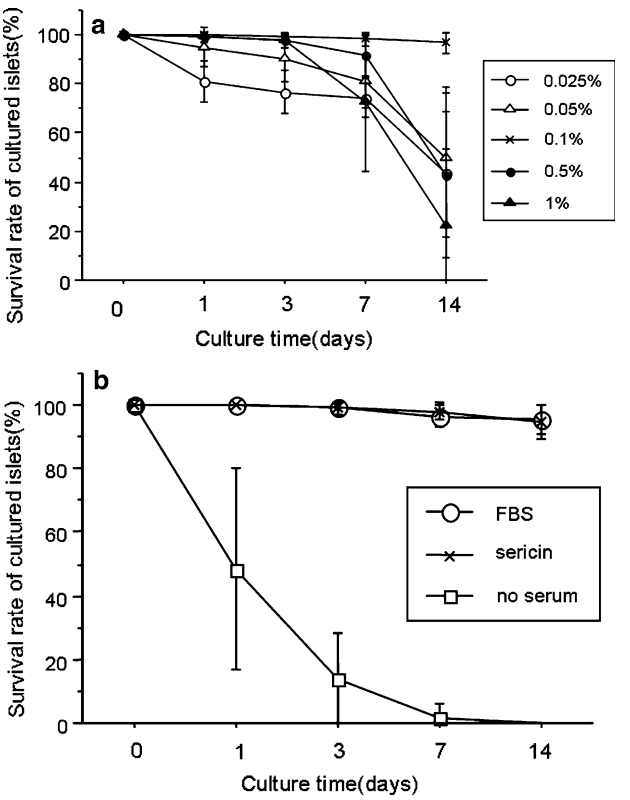

Fig. 1 **a** Twenty islets were cultured and counted for 14 days in medium supplemented with different concentrations of sericin ($n = 6$). The best sericin concentration for islet culture was 0.1%. **b** Twenty islets were cultured and counted for 14 days in medium supplemented with 10% fetal blood serum (*FBS*) or 0.1% sericin, or not supplemented with either (no serum; $n = 10$). In both the sericin and FBS groups, 95% of islets remained viable by culture day 14, while islets disappeared within 7 days in the no-serum group

Insulin release assay for islets

On culture day 3, there were no significant differences in insulin secretion stimulated at 20 mM (high) glucose between the FBS and sericin groups ($P = 0.95$). On culture day 7, stimulated insulin secretion tended to be lower in the sericin group than in the FBS group, although the difference was not statistically significant, ($P = 0.24$). The stimulation index did not differ significantly between the two groups on day 3 ($P = 0.96$), but it did tend to be slightly higher on day 7 in the sericin group compared to the FBS group ($P = 0.07$). Table 1 provides the data on the insulin release assay.

Islet transplantation

A total of 800 islets cultured for 7 days with either FBS or 0.1% sericin were transplanted into syngeneic streptozotocin-induced diabetic rats. The experiments were repeated six times. Blood glucose levels normalized within 7 days after transplantation and subsequently remained normal in both groups of recipients transplanted with FBS- or sericin-cultured islets (Fig. 2). All recipient rats reverted to the diabetic state after nephrectomy.

Table 1 Insulin release assay

Experimental groups[a]	Stimulant (glucose)			Stimulation index
	3.3 mM	20 mM	3.3 mM	
FBS group (3 days)	1.50 ± 0.40	4.02 ± 1.07	1.95 ± 0.60	2.92 ± 1.21
Sericin group (3 days)	1.57 ± 0.42	4.07 ± 1.77	1.30 ± 0.54	2.97 ± 2.19
FBS group (7 days)	2.28 ± 0.90	4.83 ± 1.44	2.53 ± 0.95	2.12 ± 0.55
Sericin (7 days)	1.47 ± 1.02	3.67 ± 1.75	1.67 ± 0.72	2.82 ± 0.68

Insulin secretion (ng/ml/h per ten islets). Insulin secretion in FBS group ($n = 6$) and sericin group ($n = 6$) on culture days 3 and 7. Islets were exposed for 1 h to different concentrations of glucose (3.3, 20 or 3.3 mM), as indicated. Values represent the mean ± SD

[a] FBS group, Islets supplemented with 10% FBS; sericin group, islets supplemented with 0.1% sericin

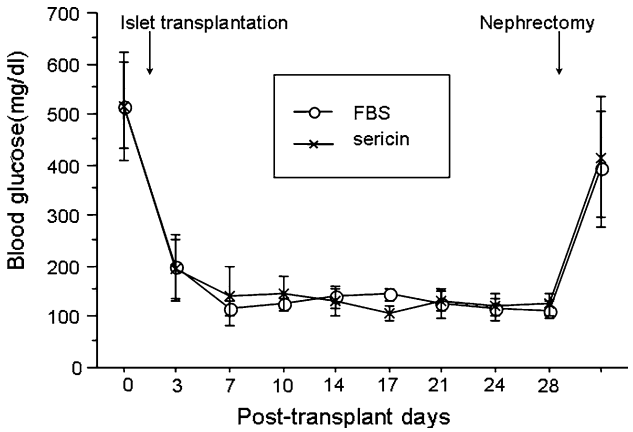

Fig. 2 Non-fasting blood glucose levels in rats after transplantation of 800 islets cultured in FBS ($n = 6$) or sericin ($n = 6$). No significant differences were noted between the two groups

Histology

No significant differences in morphology were identified between the islets incubated with 0.1% sericin and those incubated with 10% FBS during the 14-day culture. Histologically, islets cultured for 7 days with either 0.1% sericin or FBS displayed a well-preserved structure without significant morphological differences (Fig. 3). Similarly, islet grafts removed at 28 days after transplantation showed a good structure and numerous insulin-positive cells; there were no significant differences between the two groups (Fig. 4).

Discussion

While islet transplantation is currently considered to be an effective treatment for type I diabetes, the issue of islet culture needs to be resolved before this therapy can be widely applied. Several studies have reported the utility of serum-free culture media for islet cultures. Rush et al. [13] achieved long-term islet storage using a Memphis serum-free medium containing insulin, selenious acid, transferrin, linoleic acid and bovine serum albumin. Clayton et al. [14] employed serum-free medium containing hormones, albumin, ethanolamine, phosphoethanolamine and prostaglandins, while Clark and Chick [15] used serum-free medium containing proteose peptone, transferrin, insulin-like growth factor I, ethanolamine, the insulinotrophic fragment of human growth hormone, phosphoethanolamine and human serum albumin. However, these serum-free media contain albumin, including bovine albumin, and thus the risk of diseases such as BSE cannot be completely eliminated. As an alternative, human serum albumin can be used without a risk of BSE, but this protein is expensive when culturing large volumes of islets. In this context, sericin is much more cost effective: when the use of 10% FBS and 0.1% sericin in islet culture is compared, the cost of sericin is about 1/200 that of FBS. Nakano et al. [16] demonstrated the superiority of FBS to human serum albumin in human islet transplantation to diabetic nude mice**.** They proposed that the various components of FBS, other than albumin, such as growth factors, may help human islets to recover from the damage caused by preservation and isolation**.** The results of this study, in which we used sericin, a silkworm protein, for islet culture instead of FBS, reveal that sericin can be as effective as FBS in supporting islet viability during culture.

Sericin is a silkworm protein that is isolated during silk production. The fiber made by silkworms comprises fibroin, which forms the fiber core, and sericin, a water-soluble protein surrounding the fibroin. Most of the sericin is discarded during commercial silk production, as fibroin is the primary component utilized. The amino acid composition of sericin is unique, with about 30% serine [17]. Since sericin accounts for about 25% of the fiber made by silkworms, its potential use has long been under investigation. The moisture retention and antioxidant properties of sericin have been recognized in recent years, and research into cosmetic and fabric processing applications is being actively pursued. Terada et al. [8] reported that sericin is just as effective as FBS in facilitating the proliferation of four mammalian cell lines (murine hybridoma 2E-O,

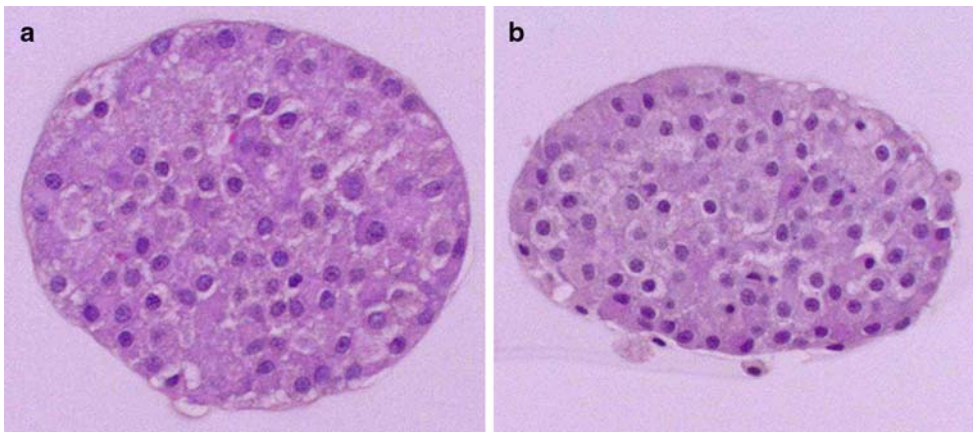

Fig. 3 Light micrography of islets after culture day 7 in medium containing: **a** 10% FBS, **b** 0.1% sericin. Stain: H&E, ×200

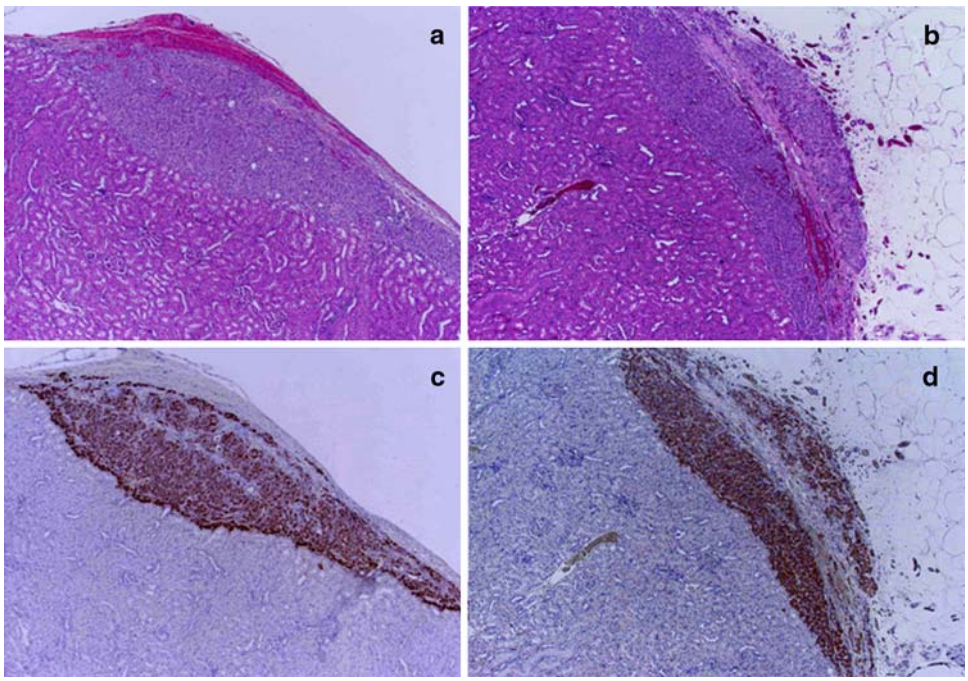

Fig. 4 The graft-bearing kidney was removed at 28 days after islet transplantation and the graft examined histologically (H&E: **a** FBS, **b** sericin) and immunohistochemically (insulin stain: **c** FBS, **d** sericin); ×40

human hepatoblastoma HepG2, human epithelial HeLa and human embryonal kidney 293 cells). The results of our study confirm the usefulness of sericin in islet cultures.

Sericin is highly stable against heat and is water soluble. High-pressure heat sterilization and mechanical sterilization are thus possible. Although the effects of sericin on the human body have not been fully investigated, silk sutures that contain low levels of sericin have been long been used in surgical operations without adverse reactions, such as sensitization. Thus, sericin would appear to be safe for human use.

Is sericin a complete substitute for FBS? When islets were cultured with 10% FBS, they remained viable for >1 month, whereas most islets cultured with 0.1% sericin died after 1 month. The advantages of FBS are multiple in that it supplies the islets with proteins with specific functions, such as transferrin, with substances bound to serum proteins, such as fatty acids bound to albumin, with attachment factors, such as fibronectin, with hormones, such as insulin, and with growth hormone, hydrocortisone and probably other factors not yet identified, such as "transferrin-like growth factor" [18]. According to reports by Rush et al. [13], Clayton et al. [14] and Clark and Chick [15], successful long-term islet culture in serum-free media requires a variety of compounds, such as various hormones, transferrin and others, in addition to serum albumin. While sericin has been shown to be resistant to antioxidation and protease [19], the exact function of sericin and the mechanism of the beneficial effect of sericin in the islet culture system have yet to be fully elucidated. The possible mechanisms underlying the beneficial effects of sericin, such as its effect on cell apoptosis and cell replication, and

its intercellular signaling pathway should be further explored since there is little information available on the mechanism mediated by sericin. As serum-free medium containing sericin supports the survival of islets for 14 days, further supplementation of sericin-containing medium with various hormones and growth factors may enable the long-term survival of islets comparable to that supported by FBS-containing medium.

In the study reported here we investigated the use of serum-free media containing sericin in culturing islets. While islets did not survive for 7 days in non-supplemented, serum-free medium, islet survival was supported equally well for 14 days by serum-free medium containing sericin or FBS, with comparable numbers of surviving islets, islet morphology, insulin secretion in vitro and glycemic control after transplantation into syngeneic recipients. These findings suggest that islets can be cultured using serum-free medium containing sericin for 14 days, and maybe longer when the medium is further supplemented with various hormones and growth factors.

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