

Successful Human Islet Isolation Utilizing Recombinant Collagenase

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The enzymatic dissociation of acinar tissue by collagenase is a substantial step in the isolation of pancreatic islets. Although essential collagenase components have been purified, the variability in the activity of different batches limits long-term reproducibility of isolation success. The utilization of purified recombinant proteases would solve this problem. In the present study, pancreases from multiorgan donors were dissociated by means of digestion-filtration using either Liberase HI ($n = 51$) or a recombinant collagenase blend ($n = 25$). No significant differences were found regarding islet yield before and after purification, the percent of exocrine-attached islets, and final purity. However, the ratio between islet equivalents and islet numbers indicated a lesser fragmentation in islets isolated with recombinant collagenase ($P < 0.01$). In contrast, viability was slightly higher in islets isolated with Liberase (92.3 ± 0.8 vs. $85.6 \pm 2.9\%$; $P < 0.05$). Insulin release during static glucose incubation was not different between experimental groups. Islet transplantation into diabetic nude mice resulted in sustained normoglycemia in either group until the graft was removed. These results demonstrated that viable human islets can be isolated using recombinant collagenase. Final optimization of this enzyme blend would offer continuous reproducibility of isolation success. *Diabetes* 52:1143–1146, 2003

Isolating significant amounts of pancreatic islets from pancreas tissue requires complex separation techniques if the structural and functional integrity of the islets as microorgans is to be preserved. The empirical evolution of islet isolation from the pancreases of large animals has led to the development of methods that combine both mechanical and enzymatic dissociation of the pancreatic tissue (1). Within the discontinuous process, collagenase administration into the pancreas represents a substantial step. This crude enzyme mixture, consisting of at least 12 different proteases, is produced by *Clostridium histolyticum* during a fermentation process

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HBSS, Hank's balanced salt solution; HTK, histidine tryptophane ketoglutarate; IEQ, islet equivalent number; UW, University of Wisconsin.

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(2). The variability between the activity of different collagenase batches was first described over 20 years ago (3) and still represents one of the strongest predictors for the success or failure of human islet isolations (4). Because of the complexity of interactions between different collagenase enzyme activities and intrinsic activities of the pancreas, prescreening of different collagenase lots in the human pancreas is an essential prerequisite for successful mass isolation of viable human islets (5). Although significant improvements have been made in the purification of essential collagenase components (6), including the substantial reduction of endotoxin (7) and toxicity testing of enzyme blends (8), batch-to-batch variability has not been completely eliminated (9). The production of essential enzymes as highly purified recombinant proteins would help to solve this problem (10). The present study was the first to evaluate the efficacy of a recombinant enzyme blend produced for the isolation of islets from the human pancreas in comparison to Liberase HI, a widely used product in the field of clinical islet isolation. The results of our study suggest that this blend of recombinant proteases is as efficient and suitable for the mass isolation of viable human islets as is Liberase HI.

RESEARCH DESIGN AND METHODS

Donors. After receiving legal consent, human pancreases were obtained through the Eurotransplant International Foundation and procured from multiorgan donors fulfilling our standard selection criteria (11). The organs were perfused in situ via the abdominal aorta with cold histidine tryptophane ketoglutarate (HTK) or University of Wisconsin (UW) solution. As a rule, the organs were dissected in situ by the liver team with or without the duodenum after the removal of the heart, lung, and liver and sometimes the kidney. The time interval between termination of hypothermic perfusion and termination of pancreatectomy during in situ procurement was defined as the secondary pancreatic warm ischemia time. Within 12 h, the pancreases were shipped in HTK or UW solution (4°C) from the donor centers to the isolation laboratory.

Enzymes. Pancreas dissociation was performed by four prescreened lots of Liberase HI (Roche, Indianapolis, IN) or three different lots of a recombinant enzyme blend (Roche, Penzberg, Germany) produced in an experimental setting (10). Administration of Liberase or the recombinant collagenase was performed by randomization in an alternate sequence, except for the organs committed for clinical islet transplantation. As described for Liberase (12), the recombinant enzyme mixture was comprised of class I and class II collagenase and neutral protease (13,14) and was blended in correspondence to the specific collagenase activity of efficient Liberase lots (84614220: 2218; 85176120: 2433; 85210020: 2228; and 90272420: 2523 Wunsch units/vial). The Wunsch activity of each recombinant lot was 2,500 units/vial. Neutral protease activity, as determined in a casein-resorufin assay (Roche, Penzberg, Germany) varied from 4,000 to 10,000 units/vial, which was within the relative range of the Liberase lots used (84614220: 62642; 85176120: 82607; 85210020: 73451; and 90272420: 153857 units/vial). The endotoxin level was <24 EU/mg, as measured in a gel-clot assay (Charles River Endosafe, Wilmington, MA). In contrast to Liberase, which is purified from a crude enzyme mixture produced by *Clostridium histolyticum* (5), recombinant collagenase of class I and class II was separately produced by genetically modified *E. coli* bacteria transfected

with the genes of class I or class II collagenase isolated from *Clostridium histolyticum* (10).

Isolation procedure. Isolation and purification of islets were performed as previously described in detail (15). Briefly, enzymes were reconstituted and dissolved in cold (8°C) Hank's balanced salt solution (HBSS; Biochrom, Berlin, Germany) at a concentration of 3 mg/ml. Before being digested in a modified continuous digestion-filtrations device (1), trimmed pancreata were intraductally distended by hand with the enzyme solutions at a ratio of 2 ml/g pancreatic tissue. The entire system was filled with warmed HBSS recirculating continuously to maintain a digestion temperature of 32–37°C while the digestion chamber was vertically agitated with 300 oscillations per min. During recirculation, samples were taken to monitor the dissociation of the tissue. As soon as significant numbers of cleaved islets appeared in the biopsies, the system was diluted with fresh HBSS either used at room temperature or at 8°C (depending on islet cleavage and total amount of tissue) to collect digested tissue in cold UW solution. Subsequently, islets were purified on a Cobe 2991 (16) using a continuous HBSS-Ficoll (Biochrom) gradient. Purified islet fractions were pooled in supplemented CMRL 1066 (Biochrom) for subsequent culture at 37 or 22°C.

Isolation outcome. Islet yield was evaluated before and after purification by converting islets to islet equivalent number (IEQ), with an average diameter of 150 µm (17). The ratio of IEQ to islet numbers was calculated as isolation index. The percent of islets still mantled or attached with exocrine tissue and final purity obtained after purification was estimated by visual control of defined samples under the microscope.

Islet quality control. After islets were cultured at 37°C for 18–36 h, the insulin content and glucose-stimulated insulin release were measured during 90 min of static incubation of hand-selected islets in TCM 199 containing either 30 or 300 mg/dl glucose using an enzyme immunoassay specific for human insulin (Sigma, Deisenhofen, Germany). The relative insulin release was expressed as a stimulation index and calculated as the ratio of stimulated to basal insulin secretion (17). Simultaneously, the viability of islets was examined by a trypan blue exclusion assay (18).

In vivo function was assessed in NMRI nude mice (Harlan, Hannover, Germany) rendered diabetic by a single intravenous injection of 220 mg/kg streptozotocin (Sigma, Deisenhofen, Germany) 3–4 days before transplantation. Initial aliquots of 1,500 IEQ were cultured at 22°C until hyperglycemia in nude mice was established. Only animals with nonfasting serum glucose levels >350 mg/dl were considered for islet transplantation beneath the kidney capsule. Blood samples from the tail vein of recipients were taken daily for the first 8 days of observation and every 2nd day for the next 32 days of monitoring. Postprandial serum glucose levels were determined using a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). Nonfasting serum glucose levels <200 mg/dl were defined as normoglycemia and considered as graft function. After 34 days of observation, nephrectomy of graft-bearing kidneys was performed to demonstrate immediate return of hyperglycemia.

Data analysis. All values are expressed as means ± SE. Comparisons of data between experimental groups were carried out by the nonparametric Mann-Whitney test. Analysis of categorical donor characteristics (gender and pancreas appearance) were performed by Pearson's χ^2 test. Significance was determined at $P < 0.05$; $P > 0.05$ was considered NS.

RESULTS

Donor characteristics. The differences between donor characteristics of pancreata digested with recombinant collagenase ($n = 25$) or Liberase HI ($n = 51$) regarding age (range 20–62 years), BMI (21.5–45.2 kg/m²), perfusion volume (1–10 l), secondary pancreatic warm ischemia (14–75 min), cold ischemia (4.0–12.2 h), and pancreas weight (35–155 g) were not statistically significant (Table 1). The proportion of male donors in the recombinant group (64.0%) did not vary significantly from that of the Liberase HI group (52.9%; NS by Pearson's χ^2 test). Nearly half of all pancreata used for digestion by recombinant collagenase (44.0%) or Liberase HI (39.2%; NS) showed fatty degeneration. The proportion of fibrosis observed in pancreata digested with recombinant collagenase (48.0%) was higher than that in pancreata treated with Liberase HI (25.5%; $P < 0.05$). Likewise, pancreata simultaneously expressing signs of fibrosis and fatty degeneration were used in a higher percentage for digestion with recombi-

TABLE 1

Variables of pancreas donors selected for subsequent pancreas digestion using recombinant collagenase or Liberase HI

	Recombinant collagenase	Liberase HI
<i>n</i>	25	51
Age (years)	45.7 ± 2.3	44.8 ± 1.7
BMI	26.9 ± 0.8	26.2 ± 0.6
Perfusion volume (l)	5.5 ± 0.5	5.6 ± 0.2
Warm ischemia (min)	37.2 ± 2.3	45.7 ± 2.7
Cold ischemia (h)	8.5 ± 0.5	7.7 ± 0.3
Pancreas weight (g)	76.5 ± 3.3	84.7 ± 3.2

Data are *n* or means ± SE. Statistical analysis revealed no significance (Mann-Whitney test).

nant collagenase than with Liberase HI (32.0 vs. 17.6%; $P < 0.05$).

Digestion variables. Time for recirculation during digestion was 35 ± 2 and 37 ± 2 min (NS by Mann-Whitney test) using recombinant collagenase and Liberase HI, respectively. Pancreas dissociation was completed after 59 ± 2 min if performed by recombinant collagenase and 66 ± 3 min using Liberase HI (NS). The packed tissue volume obtained after digestion with recombinant collagenase was 29 ± 2 ml, which was not different from the tissue volume after dissociation with Liberase HI (33 ± 2 ml; NS). Before purification, the percent of exocrine-attached or -mantled islets isolated with recombinant collagenase was slightly lower compared with islet entrapment obtained by Liberase HI (39 ± 4 vs. 48 ± 4%; NS by Mann-Whitney test). Nevertheless, after purification, the proportion of exocrine-contaminated islets was nearly identical, regardless of whether recombinant collagenase or Liberase HI (27 ± 5 vs. 28 ± 4%; NS) was used.

Isolation outcome. As detailed in Table 2, before or after purification, no significant differences were observed between the experimental groups regarding total islet yield and islet yield per gram of pancreatic tissue. However, a small but significant difference was found before ($P < 0.05$) and after purification ($P < 0.01$) in the ratio of IEQ to islet numbers, calculated as the isolation index (Table 2); this indicated that isolation with recombinant collagenase resulted in a larger diameter of islets compared with those isolated with Liberase HI. The estimated purity of final

TABLE 2

Islet yield counted before and after purification of pancreata digested with recombinant collagenase or Liberase HI

	Recombinant collagenase	Liberase HI
<i>n</i>	25	51
Total IEQ		
Before	474,850 ± 48,940	458,490 ± 28,120
After	237,910 ± 26,100	249,800 ± 16,560
IEQ/g		
Before	6500 ± 757	5780 ± 400
After	3290 ± 410	3160 ± 270
IEQ recovery (%)	53.1 ± 7.9	57.0 ± 3.6
Isolation index		
Before	2.8 ± 0.2*	2.4 ± 0.9
After	3.9 ± 0.2†	3.2 ± 0.1

Data are *n* or means ± SE. * $P < 0.05$; † $P < 0.01$ (Mann-Whitney test).

TABLE 3
Intracellular insulin content and glucose-stimulated insulin release of human islets during static incubation

Insulin	Recombinant collagenase	Liberase HI
<i>n</i>	20	34
Content ($\mu\text{U}/\text{IEQ}$)	159.3 ± 47.0	136.6 ± 23.2
Basal release ($\mu\text{U} \cdot \text{IEQ}^{-1} \cdot 90 \text{ min}^{-1}$)	2.5 ± 0.9	2.7 ± 0.7
Stimulated release ($\mu\text{U} \cdot \text{IEQ}^{-1} \cdot 90 \text{ min}^{-1}$)	15.0 ± 5.6	13.7 ± 2.0
Stimulation index	13.1 ± 2.7	11.9 ± 1.8

Data are *n* or means \pm SE. Statistical analysis revealed no significance (Mann-Whitney test).

islet suspension did not differ between the recombinant and Liberase HI group (63 ± 3 vs. $62 \pm 2\%$; NS).

Islet quality control. Basal and stimulated insulin secretory capacity during static glucose incubation and the calculated stimulation index did not vary significantly between islets isolated with Liberase HI or recombinant collagenase (Table 3). Likewise, no difference was found regarding the intracellular insulin content. A small, but significant, difference existed between the viability of control islets and islets isolated with recombinant collagenase, with the latter expressing a slightly lower viability (92.4 ± 0.8 vs. $85.6 \pm 2.9\%$; $P < 0.05$ by Mann-Whitney test).

As demonstrated in Fig. 1, transplantation of 22°C -cultured islets into diabetic nude mice resulted in immediate reversal of hyperglycemia. In each experimental group, normoglycemia was maintained for 34 days until nephrectomy of graft-bearing kidneys was performed, leading to the prompt return of hyperglycemia.

DISCUSSION

Although the problem of variability between the activity of different collagenase batches persists from the very begin-

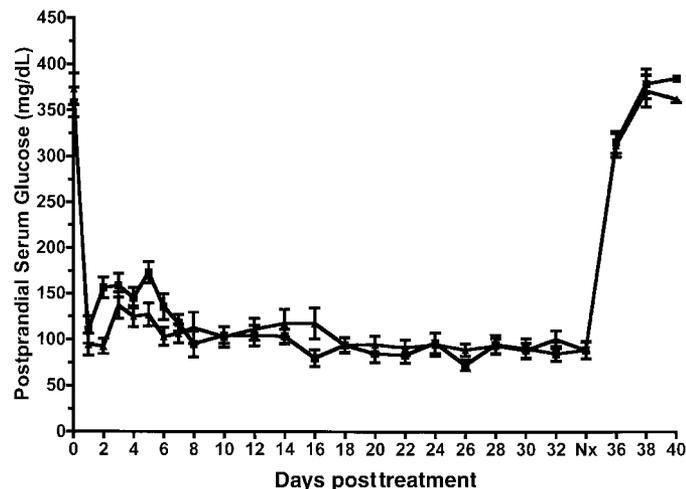


FIG. 1. In vivo function of human islets isolated with recombinant collagenase (\blacktriangle ; $n = 24$) or Liberase HI (\blacksquare ; $n = 31$) after transplantation beneath the kidney capsule of streptozotocin-treated NMRI nude mice. Graft removal through nephrectomy (Nx) was performed as indicated at day 34 after transplant. Data are means \pm SE from 10 (Liberase HI) and 7 (recombinant collagenase) individual islet preparations.

ning of enzymatic islet isolation (19), recent progress in the quality of collagenase blends has not completely eliminated the variability among different enzyme lots (9). The production of highly purified recombinant collagenase blends as an essential tool for human islet isolation (10) represents a prerequisite for long-term reproducibility of successful islet isolations. In the present study, we evaluated the efficacy of recombinant collagenase blends for successful islet release from pancreata of multiorgan donors.

No significant differences were found regarding yield, purity, cleavage, or in vitro function of islets isolated by recombinant collagenase in comparison with Liberase HI, which was previously tested for its suitability for successful human islet isolation (12). Likewise, the in vivo function of islets transplanted into diabetic nude mice did not differ between experimental groups. Digestion time and packed tissue volume suggested that both blends expressed comparable enzyme activities. Nevertheless, to a small extent, the viability of islets isolated with recombinant collagenase was significantly lower compared with Liberase-isolated islets. It seems unlikely that this observation can be related to detrimental protease activities included in the recombinant blend, as the ratio between IEQ and islet numbers indicated a lesser fragmentation in islets isolated with recombinant collagenase than with Liberase HI. Because the percentage of donor pancreata suffering from fibrosis and fatty degeneration was significantly higher in the group digested with recombinant collagenase, it has to be considered that these donor-specific variables might have a significant adverse impact on yield and viability of human islets (20,21).

In summary, the present study successfully demonstrated the suitability of recombinant collagenase blends for the mass isolation of viable human islets, making long-term reproducibility of successful islet isolations feasible in the near future. Nevertheless, the balancing of enzyme activities within recombinant mixtures has to be optimized, with the aim of a gentle and maximal release of islets from acinar tissue.

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