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Detrimental effect of excessive collagenase class II on human islet isolation outcome

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Summary

Commercially available purified collagenases, derived from *Clostridium histolyticum*, contain two different classes of collagenase: class I collagenase (CI) and class II collagenase (CII) at a predetermined ratio. In this study, using purified CI and CII in separate vials, we had a unique opportunity to investigate the effect of the proportion between two collagenase classes on clinical human islet isolation. Pancreas organs derived from deceased donors were prospectively assigned to one of three different enzyme protocols: group A – CII:CI = 1:1 vial; group B – 1:2; group C – 1.5:1. As a result, their total collagenase activities were 2116, 2230, and 3117 Wunsch units/pancreas in groups A, B, and C, respectively but thermolysin dosage was adjusted to 624–988 Units/g pancreas. The pancreas was not efficiently digested in group C in spite of a relatively longer digestion time and significantly higher Wunsch activity, resulting in the poorest islet isolation outcome among the three groups. Additional retrospective analysis revealed that this suboptimal outcome in group C was not because of the absolute excessive amount of collagenase activity but as a result of the relatively high proportion of CII (i.e., unbalanced CII/CI ratio). Our study suggests that an excessive CII is ineffective in releasing islets from human pancreas, and rather a balanced CII/CI ratio is of paramount importance.

Introduction

The enzymatic dissociation of the pancreas represents a critical step in islet isolation for clinical transplantation. Current isolation methods include the delivery of a collagenase enzyme blend to the pancreatic duct as a means of delivering the enzyme to the islet-exocrine interface. Collagen is the major structural protein constituting islet-exocrine interface [1,2]. Because of its tight structure, collagen is not generally degraded by ordinary proteases but can only efficiently be degraded with high specificity by collagenase [3]. Therefore, collagenase is a key enzyme in the islet isolation process. Despite significant advancements in techniques of human islet isolation procedure, it remains difficult to isolate a sufficient number of high-quality islets from a single pancreas with any regularity. Indeed, the variability in efficiency among different enzyme lots has been a major obstacle for the successful recovery of islets [4,5]. We reported a rate of successful isolation that fluctuated between 0% and 75% that was highly dependent upon the enzyme lot [6].

Commercially available purified collagenases, derived from *Clostridium histolyticum*, contain two different classes of collagenase: class I collagenase (CI) and class II collagenase (CII). Genomic analysis of *Clostridium histolyticum* revealed that in this organism there are two homologous but distinct genes, ColG and ColH, which encode CI and CII, respectively [7,8]. There are fundamental similarities and differences between CI and CII. CI and CII are quite different in their primary and secondary structures, but the catalytic machinery of the two enzymes is essentially identical [9]. Both enzymes have a similar segmental structure consisting of three different segments; catalytic domain, spacing domain, and binding domain [5,10]. An important difference is that CI has tandem collagen-binding domains (CBD) but CII

possesses a single CBD [10]. Tandem CBDs of CI may have advantages for binding to collagens in the pancreas. Indeed, tandem-repeated binding domains are generally considered to be useful for the stabilization of bindings [11]. Kinetic studies evaluating the hydrolysis of collagens by CI or CII indicate a higher catalytic efficiency of CI on collagen [12]. On the other hand, CII is characterized by the ability to attack synthetic peptide substrates at a much greater rate than CI [13].

CI and CII are complementary in degrading collagen [14], and neither CI nor CII can be dispensable in islet isolation [15]. It has also been suggested that ratio between CI and CII is a good predictor of collagenase performance [6] and therefore is of significant importance in islet isolation [15]. Current commercially available products contain both CI and CII at a predetermined ratio, thus not allowing the user to adjust the ratio. In this study, using purified CI and CII in separate vials, we had a unique opportunity to investigate the effect of the proportion between two collagenase classes on clinical human islet isolation.

Materials and methods

Islet isolation

Human pancreases were procured from deceased donors with informed consent and were processed according to our facilities standard isolation protocol with the exception of the collagenase dosages. The pancreas was perfused with 350 ml of cold solution (Perfusion solution; Mediatech, Herndon, VA, USA) containing CI, CII and thermolysin through the cannulated pancreatic duct. The distended pancreas was transferred to a Ricordi chamber and digested by re-circulating the enzyme solution through the chamber at 37 °C [16]. The time required from the start of enzyme circulation in the Ricordi chamber to the beginning of digest collection was defined as digestion time. Islets were purified on a continuous gradient consisting of Biocoll (Biochrome AG, Berlin, Germany) and Viaspan (Barr Pharmaceuticals Pomona, NY, USA) using a refrigerated COBE 2991 cell separator [17]. We conducted all islet isolations in an attempt to use for clinical transplantation regardless of whether the donor was marginal or not.

Islet evaluation

Islet preparations were evaluated by two independent investigators for islet number, size, purity, and morphology [scored from 0 (fragmented) to 10 (intact)] by dithizone staining. Islet masses recovered after digestion phase and after purification were expressed as islet equivalent (IE). Islet recovery rate was calculated as ratio of postpurification IE to prepurification IE. The percentage of dead and live tissues was estimated by fluorescent staining with SYTO® 13 (Molecular Probes, Eugene, OR, USA)/ethidium bromide [18].

Enzyme blending

We obtained three different enzyme products from Roche Applied Science (Indianapolis, IN, USA): Liberase™ Collagenase Type I (containing only CI), Liberase™ Collagenase Type II (containing only CII), and Liberase™ Thermolysin (containing thermolysin-derived from *Bacillus thermoproteolytics*). Roche Applied Science employs Wunsch assay and caseinase assay for the measurement of collagenase (WU) and thermolysin (NP) activities, respectively. The Wunsch assay which involves the hydrolysis of a short synthetic peptide is the preferred assay for CII. The caseinase assay is based on fluorescence-labeled casein, and widely used for the measurement of nonspecific protease activities [19]. Details of each product are listed in Table 1 and values were based on Roche's certificate of analysis. Pancreases were prospectively assigned to one of three different enzyme protocols: group A – Type II:Type I = 1:1 vial; group B – Type II:Type I = 1:2 vial; group C – Type II:Type I = 1.5:1 vial. As a result, their total collagenase activities were 2116, 2230, and 3117 WU/pancreas for groups A, B, and C, respectively. Randomization was done by assignment of the pancreases in consecutive order to the different protocols without knowing any donor-related information. Thermolysin dosage was adjusted to 624–988 NP/g pancreas based on our previous findings [6] because a narrow dosing window was recommended for this enzyme. Collagenase dosage was not adjusted according to pancreas weight.

High performance liquid chromatography

Anion exchange high performance liquid chromatography (HPLC) system comprised a System Gold HPLC (Gold 126 Solvent Module and System Gold 168 Detector) and a System Gold Model 508 Autosampler (Beckman Coulter, Fullerton, CA, USA). Separation was performed

Table 1. Characteristics of enzyme products.

Product name	Lot #	Wunsch assay WU/vial	Caseinase assay NP/vial	Endotoxin EU/mg
Liberase™ Collagenase Type I	93502420	114	–	23.96
Liberase™ Collagenase Type II	93502520	2002	–	20.61
Liberase™ Thermolysin	93477220	–	61143	–

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on a Mono-Q 5/50GL column (GE Healthcare, Uppsala, Sweden) that was maintained at 18 °C. The mobile phase was composed of a Tris–$CaCl_2$ with a NaCl linear gradient at pH 7.5 with a flow rate of 1.5 ml/min. Approximately 1.0 ml of collagenase solution containing both CI and CII was sampled before use in an islet isolation. 200 μl of collagenase solution was diluted with 600 μl of Tris–$CaCl_2$ buffer. A volume of 499 μl was injected by the autosampler and UV detection of the eluted protein was performed at 280 nm and integrated using the 32 Karat software (Beckman Coulter, Fullerton, CA, USA). Two main peaks were identified on chromatogram. Area under the curve (AUC) of CI and CII fraction was calculated using the 32 Karat software.

Statistical analysis

Results are expressed as mean ± SE. Statistical comparisons were performed with one-way analysis of variance followed by within-group comparison with Fisher's protected least significant difference test. In the part of retrospective study, differences between two groups were analyzed using unpaired Student's *t*-test. Levels of statistical significance were set at $P < 0.05$.

Results

When preparing enzyme solutions prior to islet isolation, we noted that CII dissolved faster in the solution than CI (~15 min vs. ~30 min). This observation and the fact that CII elutes earlier than CI in reverse-phase HPLC as shown in the previous studies [15,20], indicate CII as being less hydrophobic than CI.

Representative chromatograms of enzyme solution for each group are shown in Fig. 1. The ratio between the AUC of CII and the AUC of CI was 0.647 ± 0.014 for group A. This value is very close to the value obtained from 120 islet isolations using three lots of Liberase™ HI and four lots of Liberase™ Collagenase Blend (0.632 ± 0.011). Expectedly, the values (CII/CI) for group B (0.349 ± 0.009, $P < 0.001$) and C (1.061 ± 0.018, $P < 0.001$) were significantly lower and higher, respectively, than those for group A.

Variables of pancreas donor and islet isolation outcomes in the three study groups are listed in Table 2. Donors were distributed evenly in terms of body surface area, body mass index, glycosylated hemoglobin A_{1c} levels and cold ischemia time. The donor age was younger in group A than in group B. The pancreas weight tended to be lighter in group B than in group C. The mean digestion time in group C was the longest among the groups, although not statistically significant. Nevertheless, the pancreas was not efficiently digested in group C as indicated by a relatively higher percent undigested pancreas, calculated by dividing the undigested pancreas weight by the pancreas weight. Following recombination of the unpurified pancreatic digest, only 2270 ± 195 IE/g pancreas was recovered in group C, which was significantly lower than the 4316 ± 681 IE/g pancreas obtained in group A and tended to be lower than in group B. Postpurification islet yield in group C was 1956 ± 230 IE/g pancreas, which also tended to be lower than in groups A and B. In group C, no islet preparations contained more than 300×10^3 IE, thus failing to reach the mass requirement of greater than 5000 IE/kg of recipient body weight. Islet morphology in group C was inferior to those in groups A and B. Percentage of trapped islet, islet recovery rate, islet purity, percentage of islet volume, packed tissue volume, and tissue viability all showed that they were not significantly affected by the proportion of CII/CI.

Suboptimal outcome of group C may be because of the absolute higher amount of collagenase activity. To address this issue, we conducted retrospective review of the records of islet isolations where >3000 WU of collagenase was used but thermolysin dosage was ranged between 624 and 988 NP/g pancreas. There were six cases over a six month period just prior to the introduction of Liberase™ Collagenase Type I/Type II. These six cases are referred to as group D. Mean enzyme dosage used was 3318 ± 154 WU/pancreas ranging from 3034 to 3920 WU for collagenase, and 773 ± 23 NP/g pancreas for thermolysin. All values above relating to enzymes did not significantly differ from those of group C with one exception – the ratio between the AUC of CII and the AUC of CI was significantly lower in group D than in group C (0.636 ± 0.016 vs. 1.061 ± 0.018, $P < 0.001$). Regardless of similar donor characteristics and enzyme activities, isolation outcome of group D was remarkably superior to group C (Table 2). Pancreas was efficiently and rapidly digested as indicated by a lower percent undigested pancreas (22.1 ± 3.6%, $P = 0.027$) and by a relatively shorter digestion time (10.3 ± 0.8, $P = 0.085$). Prepurification islet yield was 4931 ± 779 IE/g pancreas ($P = 0.014$), which was significantly higher when compared with group C. Postpurification islet yield was significantly higher as well ($417 \pm 31 \times 10^3$ IE or 3494 ± 352 IE/g pancreas). Five of six cases (83%) met the minimum requirement of 5000 IE/kg of recipient body weight and were therefore utilized for clinical transplantation.

The most clinically relevant criterion of islet isolation outcome is the functional graft performance following transplantation. Although the sample size was too small for any meaningful comparison (2, 1, 0, and 5 for groups A, B, C, and D, respectively), the quality of our eight

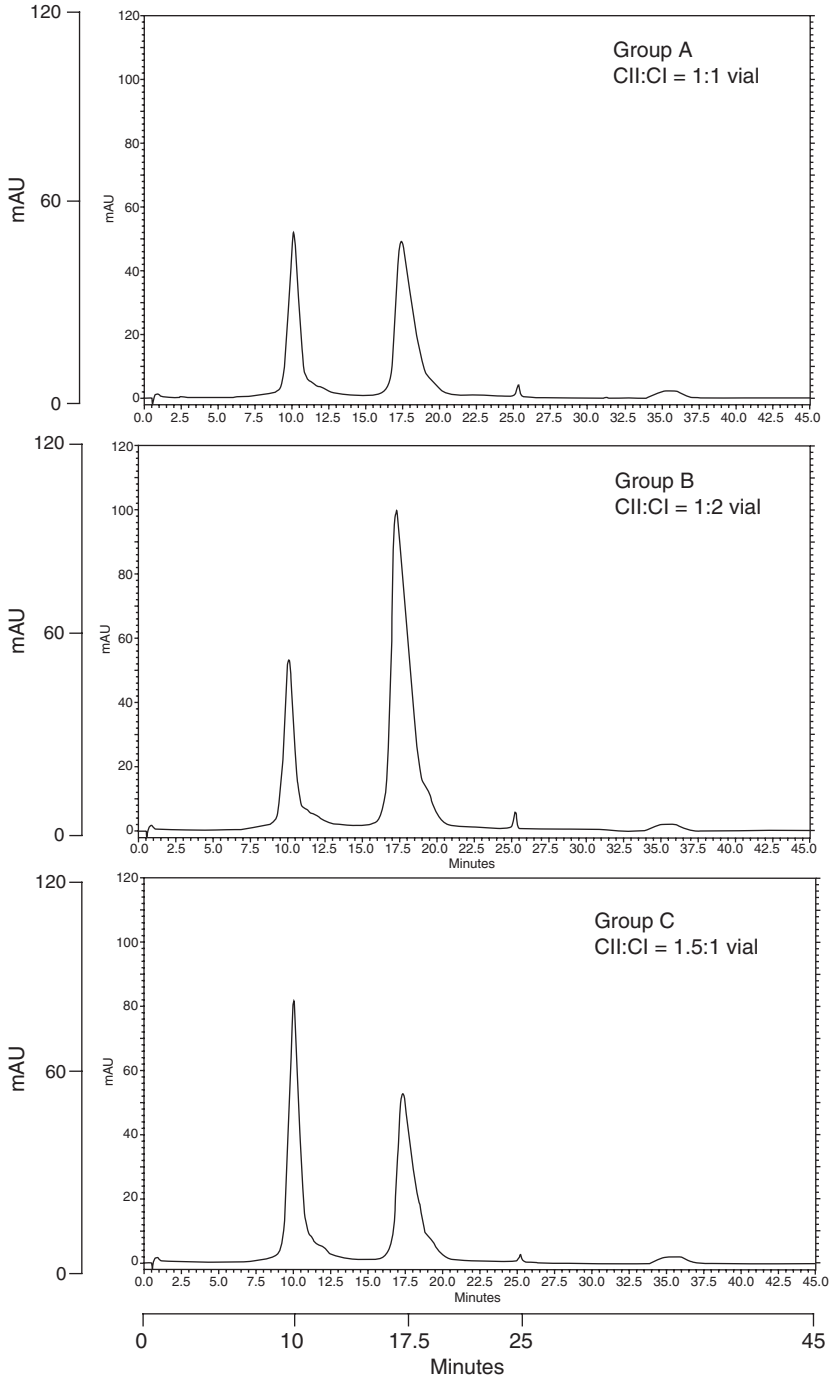

Figure 1 Anion-exchange high performance liquid chromatograms of enzyme sample from group A (upper panel), group B (middle panel), and group C (lower panel). The first and second peaks represent CII and CI, respectively.

transplanted preparations was confirmed by the decrease of insulin requirement at 1 month after transplantation (62.6 ± 7.6% reduction).

Discussion

Enzymatic digestion at a CII/CI ratio of 1.061 ± 0.018 in group C was characterized as being less effective in pancreas dissociation in spite of having longer digestion times, leading to the least desirable islet isolation outcome. One may argue that this inferior result is because of the absolute excessive amount of collagenase activity (3117 WU), not because of the relatively high proportion of CII. However, pancreas dissociation using a similar amount of collagenase (3277 WU) with a balanced CII/CI ratio (0.636 ± 0.016) in group D resulted in a signifi-

Table 2. Donor variables and isolation outcomes.

	Group A $n = 5$	Group B $n = 5$	Group C $n = 5$	*P*-value Fisher's PLSD test	Group D $n = 6$	*P*-value* *t*-test
Donor variables						
Age (years)	43.4 ± 2.5	52.6 ± 2.7	46.6 ± 3.2	0.038 (A vs. B)	42.1 ± 4.8	NS
Body surface area (m^2)†	2.00 ± 0.15	1.85 ± 0.17	1.99 ± 0.07	NS	2.14 ± 0.05	NS
Body mass index (kg/m^2)	27.4 ± 1.84	26.2 ± 4.3	25.6 ± 1.5	NS	29.0 ± 2.1	NS
HbA_{1c} (%)	5.6 ± 0.3	5.3 ± 0.2	5.3 ± 0.2	NS	5.5 ± 0.2	NS
Cold ischemia time (h)	9.1 ± 2.2	8.3 ± 1.3	9.2 ± 1.1	NS	9.5 ± 1.2	NS
Pancreas weight (g)	99.1 ± 9.4	82.6 ± 11.0	110.5 ± 9.4	0.071 (B vs. C)	121.4 ± 6.1	NS
Digestion variables						
Liberase Collagenase Type I	1 vial	2 vials	1 vial		–	
Liberase Collagenase Type II	1 vial	1 vial	1.5 vials		–	
Liberase Collagenase Blend‡	–	–	–		1.25 ∼ 1.5 vials	
Digestion time (min)	12.5 ± 1.6	11.6 ± 1.1	15.3 ± 2.3	NS	10.8 ± 0.8	0.085
Percentage of undigested tissue (%)	37.0 ± 6.4	26.9 ± 5.6	44.7 ± 8.4	0.093 (B vs. C)	22.1 ± 3.6	0.027
Percentage of trapped islet (%)	22.0 ± 5.7	30.9 ± 10.3	27.9 ± 13.0	NS	28.2 ± 6.8	NS
Prepurification islet mass ($\times 10^3$ IE)	412 ± 60	298 ± 50	249 ± 27	0.034 (A vs. C)	582 ± 78	0.005
Prepurification islet mass/g pancreas (IE/g)	4316 ± 681	3865 ± 769	2270 ± 195	0.034 (A vs. C), 0.086 (B vs. C)	4931 ± 779	0.014
Purification outcome						
Postpurification islet mass ($\times 10^3$ IE)	308 ± 47	255 ± 36	213 ± 25	0.097 (A vs. C)	417 ± 31	0.001
Postpurification islet mass/g pancreas (IE/g)	3188 ± 460	3208 ± 493	1956 ± 230	0.056 (A vs. C), 0.053 (B vs. C)	3494 ± 352	0.007
Islet recovery rate (%)	75.7 ± 6.1	87.6 ± 7.8	85.9 ± 5.6	NS	77.0 ± 9.9	NS
Islet purity based on dithizone stain (%)	57.7 ± 8.2	46.6 ± 4.9	62.0 ± 6.0	NS	70.8 ± 2.3	NS
Percentage of islet volume (%)§	12.6 ± 2.9	12.8 ± 1.7	14.3 ± 2.7	NS	17.7 ± 1.2	NS
Packed tissue volume (ml)	4.9 ± 0.7	3.9 ± 1.0	3.1 ± 0.6	NS	4.3 ± 0.4	NS
Morphology score	8.0 ± 0.5	7.3 ± 0.1	6.0 ± 0.7	0.011 (A vs. C), 0.073 (B vs. C)	7.3 ± 0.4	NS
Tissue viability (%)	83.9 ± 5.3	79.6 ± 3.9	89.7 ± 4.1	NS	79.0 ± 4.6	NS

NS, not significant.

*Group C versus group D.

†Body surface area = sqrt[body weight (kg) × height (cm)/3600] [Ref 24].

‡This product contains both class I and class II collagenases but not thermolysin.

§Percentage of islet volume = Postpurification IE × 1.766×10^{-6}/packed tissue volume (ml) × 100.

cantly higher islet yield. Although each case of group D was retrospectively selected for comparison, both groups C and D utilized the identical procedure for islet isolation from comparable donor pancreases. Our observations clearly show that a higher proportion of CII has a detrimental effect on islet isolation, counteracting the beneficial effects of absolute higher collagenase activity.

This study confirms the previous studies [15,21] showing the importance of CII/CI ratio in enzymatic islet release from pancreas. Antonioli *et al.* retrospectively analyzed enzyme components using microelectrophoresis for 163 human islet isolations [21]. They found that islet isolation outcomes were significantly improved when an enzyme blend having a narrow range of CII/CI ratio was utilized for pancreas dissociation. Further, Brandhorst *et al.* digested rat pancreases at a variety of ratios between CII and CI, and found that the highest islet yield was obtained using a CII/CI ratio of 1.0 determined by reverse phase HPLC analysis [15].

An earlier study by Wolters *et al.* showed that CII plays a predominant role in rat pancreas dissociation whereas CI is not a key player [22]. However, this view has been challenged by a recent study demonstrating that neither CI nor CII alone is able to release islets from rat pancreas [15]. Our works are in conflict with Wolters' view, too. We showed that the stability of intact CI is of great importance to the quality of the blend [23] and that a better islet isolation outcome is ascribed to a higher proportion of CI rather than a higher proportion of CII [6].

Our original intention was to conduct this study with greater power per group, but the potential risk of prion disease transmission associated with the use of bovine brain heart infusion growth media during the manufacturing of the Liberase™ has been preventing us from car-

rying out additional islet isolations using this enzyme at the present time. The small size in each group is certainly recognized as a potential limitation of this study. Unfortunately, we did not obtain improved outcome utilizing separated vials of CI and CII, however, our study suggests that an excessive CII is ineffective in releasing islets from human pancreas, and rather a balanced CII/CI ratio is of paramount importance. Our study also confirms the previous report [6] indicating that collagenase activity based on Wunsch assay alone is not suitable to predict a successful performance. Liberase™ will likely be replaced shortly with alternative collagenase enzyme blends manufactured in the strict absence of bovine products, but the effect of balance between the different classes of collagenase will still be relevant and important as early clinical testing of these new enzyme blends move forward. Further research into developing enzyme strategies to improve not only quantity but also quality of isolated islets will be required for widespread clinical application of islet transplantation.

Authorship

Tatsuya Kin: drafted the manuscript, performed study, collected data. Xiaojun Zhai: performed HPLC analysis. Doug O'Gorman: collected data, performed study. AM James Shapiro: performed study, gave critical input for the analysis.

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