Characterization of an Important Enzymatic Component in Collagenase That Is Essential for the Effective Digestion of the Human and Porcine Pancreas

Ruo L. Chen and Roger F. L. James

Department of Surgery, University of Leicester, RKB, Royal Infirmary, Leicester LE2 7LX, UK

Recent clinical results from Edmonton have demonstrated the feasibility of achieving normoglycemia in type I diabetic patients by islet transplantation. One of the key issues in obtaining this success was transplanting sufficient numbers of islets by sequential transplants. Although the development of semipurified endotoxin-free Clostridium histolyticum-derived collagenase (Liberase) has improved islet yields from the human pancreas, batch-to-batch variation and loss of activity with time still hampers progress in obtaining consistent islet preparations. In order to define key components of crude collagenase, a panel of monoclonal antibodies (McAbs) was raised against crude collagenase. Monoclonal antibodies were generated by fusions between splenocytes of BALB/c mice immunized with Boheringer P collagenase and the myeloma cell line NS-0. These monoclonal antibodies were used as probes to study molecular differences between effective and ineffective collagenase batches using Western blotting. Two monoclonal antibodies (LDS71 and LDS81) were raised and characterized as recognizing separate epitopes on a 125-kDa component. Western blotting indicated that the 125-kDa band was rapidly broken down by storage or by dialysis in the presence of diithiothreitol. However, this breakdown could be prevented by the addition of leupeptin (a protease inhibitor) to the dialysis buffer. On testing fractions at 5-min intervals from the "Ricordi" digestion circuit during porcine and human pancreas digestion, the 125-kDa component was rapidly broken down in relatively ineffective collagenase batches but in effective batches was present throughout the digestion process. The correlation between the presence of the 125-kDa band and effectiveness of pancreas digestion suggests that this may be a key component in the formulation of C. histolyticum collagenase.

Key words: Collagenase; Pancreas digestion; Monoclonal antibody; Western blotting

INTRODUCTION

Commercial collagenases from Clostridium histolyticum are used in the first, and most essential, step in the isolation of human pancreatic islets for transplantation. The second step in the process of isolation (i.e., isopinic density gradient purification) has been carefully studied and optimized by us and other groups (1,17), but unless islets can be "cleaved" from the acinar tissue without damage they cannot be recovered in the centrifugation process. The enzyme preparations used for pancreas digestion are very complex, containing several (at least 7) collagenase-isoenzymes, neutral protease, trypsin or trypsin-like enzymes, and several other hydrolytic enzymes (4,5). It has been known for some time that pure collagenase alone is ineffective in pancreas dissociation (14) and it is therefore necessary to utilize an enzyme formulation that will be effective in the dissociation of the many components of the pancreatic extracellular matrix, which is composed of collagen, glycoproteins, and proteoglycans (18). Even between species there are significant differences in the matrix components of the pancreas, and the enzyme combinations required to dissociate the human and porcine pancreas are different. Therefore, Roche Diagnostics now market an enzyme combination specifically designed to digest the porcine pancreas (Pig Liberase) in addition to Human Liberase. However, one striking practical problem in pancreatic dissociation is the unpredictable efficacy of the available commercial collagenases.

Several groups have tried to elucidate the roles of the different components present in collagenase preparations, and the role of the class I and II collagenases, as well neutral protease and trypsin-like activities, have been studied in some detail. However, no consensus has been agreed as to the ideal combination, as some favor a major role for certain ratios of class I versus class II collagenases while others favor the importance of neutral protease or trypsin-like activity (12,25,27,28). Even a complex series of experiments using free flow electrophoresis to purify collagenase fractions did not yield...
conclusive result (9). Although Roche Diagnostics (the main producer of collagenase) has attempted cloning of the class I and II collagenase genes to aid consistency of preparation (9,29), their favored approach now is in purifying the known important constituents from the crude lyophilized broth preparation. However, these "highly purified enzyme blends" still suffer from some inconsistency and loss of activity as was observed with the more standard preparations (7).

In order to define the active components of crude collagenase we have produced a panel of monoclonal antibodies (McAbs) against either "effective" or "ineffective" crude collagenase, where effectiveness was assessed by the outcome of porcine pancreas digestion.

MATERIALS AND METHODS

Collagenases

Collagenases used in this study are derived from Clostridium histolyticum and obtained from either Boehringer (now Roche Diagnostics), Serva, or Sigma as described in the text. The effectiveness of collagenases was assessed by the "cleavage" index (based on the percentage of islets cleaved from the exocrine tissue), fragmentation (based on the percentage of intact islets versus islet fragments), and total islet yield from porcine pancreas digestion.

Production of Monoclonal Antibodies

BALB/c mice were immunized with 25 μg of collagenase in adjuvant (Titremax, Sigma, UK) in the base of the tail and boosted 3 weeks later (without adjuvant) intraperitoneally. A week later mice were bled to confirm serological positivity. Mice were boosted with 10 μg of the same batch of collagenase 4 days prior to fusion of disaggregated spleen cells with the myeloma cell line NS-0 using an adaptation of the method described by Kohler and Milstein (2,13). Hybridoma supernatants were screened by enzyme linked immunosassays (ELISA) and Western blotting (see below). Cells from positive wells were cloned by limiting dilution and the supernatants retested by ELISA and Western blotting. Positive cloned anticollagenase McAb-producing cell lines were then given an LDS designation.

Enzyme Linked Immunosassays

Collagenase preparations were made up to 10 μg/ml in 0.1 M carbonate/bicarbonate buffer, pH 9.6, for coating onto specially prepared 96-well polystyrene plates. After blocking with 5% bovine serum albumin (BSA) plates were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween). Antibody-containing supernatants were incubated for 1 h at room temperature and then washed with PBS-Tween before adding alkaline phosphatase-conjugated goat anti-mouse IgG at 1:2000 (Sigma). Plates were developed with nitrophenol phosphate substrate (Sigma) before being read at 405 nm on an ELISA plate reader.

Polyacrylamide Gel Electrophoresis and Western Blotting

Collagenase components were separated by electrophoresis in the presence of sodium dodecyl sulphate (SDS) on a pair of 10% polyacrylamide gels. One gel was stained with Coomassie blue while another gel was transferred to a nitrocellulose membrane (Hybond-C, Amersham). The nitrocellulose membrane was blocked by 5% BSA at 4°C overnight, and the membrane was incubated with the primary McAb for 1 h. After washing three times, the second antibody (biotin-conjugated whole molecule of sheep anti-mouse IgG, 1:2000, Sigma) was incubated with the membrane for 1 h and, after washing, the third antibody (streptavidin-horseradish peroxide conjugate, 1:3000, Amersham) was incubated with the membrane for 1 h. The specifically bound biotin-conjugated anti-mouse antibodies were detected by incubation with freshly prepared ECL solution (Boehringer Mannheim) and developed onto film in a dark room.

Porcine and Human Pancreatic Islet Isolation

Porcine or human pancreata were digested with collagenase using the method of Ricordi et al. (17,19,20). Briefly, the pancreata were dissected by intraductal injection of collagenase solution (2 mL/g at 1.5 mg/mL) and then transferred to a stainless steel Ricordi digestion chamber. The chamber was then agitated at 37°C. When cleaved islets were seen in samples from the closed circuit the digest was collected using the open circuit with biopsies taken continuously every 5 min through the whole procedure.

Enzymatic Assays

Class II collagenase activity was measured spectrophotometrically using the FALGPA (2-furanacryloyl-1-leucylglycyl-1-propyl-1-alanine) assay (24). Other enzymatic activities were assessed by the degradation of the BAEE (N-benzoyl-L-arginine ethylester) (clostrapain) and dimethyl casein (neutral protease) (10). Protein concentrations were measured by the dye-binding Bradford system (Bio-Rad laboratories) using crystalline BSA as a standard.

RESULTS

Characterization of Monoclonal Anticollagenase Antibodies

Three fusions were made between splenocytes of BALB/c mice immunized with Boehringer type P collagenase batches 50 (B50), 82 (B82), and 92 (B92) and
cells from the mouse myeloma line NS-0. Supernatants from growing hybrids were screened by ELISA and Western blotting. Hybrids producing antibodies that showed differential reactivity against effective and ineffective batches of collagenase were selected for further study (6/286 original wells).

The effectiveness of collagenase batches was defined independently by two observers based on the outcome of porcine islet isolation. In this study we have concentrated on two batches of Boehringer collagenase P, B82, which had good digestion characteristics producing large numbers of intact porcine islets on 37 occasions from 52 digestions of porcine pancreas (71% success rate), was regarded as an effective batch of collagenase. On the other hand, B50, which gave poor results and was only used on 7 occasions yielding some islets on only 2 occasions (29% success rate), was regarded as an ineffective batch (B50 vs. B82: χ² = 4.99, p < 0.05).

SDS polyacrylamide gel separation followed by Coomassie blue staining did not differentiate between different collagenase preparations as all appeared to contain in excess of 30 separable protein bands (Fig. 1). Biochemical analysis of these batches also failed to show any clear differences in protein content or from the results of substrate-based enzymatic analysis (FALGPA, casein, and BAEE). However, the two MoAbs described here (LDS71 and LDS81) did show distinct patterns of reactivity against the effective (B82) and ineffective (B50) batches of collagenase. In ELISA (Fig. 2), LDS71 was strongly reactive against B50 but reacted only weakly with Liberase. On the other hand, LDS81 showed a stronger reactivity to Liberase and had weaker reactivity to B50 and B82. In Western blotting (Fig. 3), both antibodies detected a 125-kDa band against B50, but LDS71 detected a 42-kDa band against B82, B85, and B92, while LDS81 detected an 87-kDa band against the other three batches of collagenase, clearly showing their different epitope specificities. Further experiments demonstrated these bands to be breakdown products of collagenase resulting from storage of solubilized collagenase at 4°C or -20°C (Fig. 4).

**Breakdown of the 125-kDa Component**

The 125-kDa band was detected by the two MoAbs in Western blotting in all batches of freshly made crude collagenase tested. Storage at room temperature for 24 h gave complete breakdown of the 125-kDa band for most batches of Boehringer P collagenases tested, including B82, while batch B50 was more resistant to breakdown (Fig. 4). The stored samples showed a concomitant loss of FALGPA activity. It was possible to mimic the 125-kDa breakdown effect, created with storage, by dialysis against a buffer containing dithiothreitol (Fig. 5A). Using this system it was shown that in the presence of leupeptin (10 μg/ml) no breakdown of the 125-kDa component occurred (Fig. 5B).

**Analysis of 125-kDa Component Breakdown During Pancreas Digestion**

Both LDS71 (Fig. 6) and LDS81 (Fig. 7) were used to test for the presence of the 125-kDa component by Western blotting during the process of 7 human (Figs. 6A and 7A) and 7 porcine (Figs. 6B and 7B) pancreas digestions using different enzyme batches. Enzyme batches with good digestion characteristics (e.g., Liberase and B82) showed the presence of the 125-kDa band.
Figure 2. Titration of LDS71 and LDS81 against different batches of collagenase in enzyme linked immunoassay. Microtiter plates were coated with batches of collagenase at 10 μg/ml. LDS71 and LDS81 were titrated from neat supernatant (1.5–2.0 μg/ml mouse Ig) to a dilution of 1:10,000 as shown. Collagenases tested were as follows: (■) Boehringer P batch B50, (▲) Boehringer P batch B82, (●) Liberase, (●) Boehringer P batch B85, (•) Serva, (x) Sigma Type XI.

Figure 3. Western blotting of crude collagenases with LDS71 and LDS81. Lanes 1–4: Boehringer P batches B50, B82, B85, B92. Both LDS71 and LDS81 detect a 125-kDa band against B50, but LDS71 detects a 42-kDa band and LDS81 detects an 87-kDa band with the other three collagenases. The position of the 125-kDa band is indicated by the arrow.

Figure 4. Western blotting of crude collagenase preparations either freshly prepared (lanes 1, 4, 5, 7) or stored at room temperature for 24 h (lanes 2, 3, 6) with LDS71. Lanes 1 and 2: Boehringer P B50, lanes 3 and 4: Boehringer P B82, lanes 5 and 6: Boehringer Liberase, lane 7: Boehringer P B85. The position of the 125-kDa band is indicated by the arrow.

DISCUSSION

The results of human islet allotransplantation have been somewhat disappointing. In the last Islet Transplant Registry (ITR) the overall success rate for patients becoming insulin independent following transplantation was approximately 10%, although some grafts have functioned for up to 4 years (6). The reasons for this have been ascribed to inadequate numbers of islets engrafting and to the additional immunological problems associated with transplanting allografts into patients with an immune system primed to islet cells (3). It is
clear from the ITR results that insulin independence in both allo- and autoislet transplantation is rarely achieved when less than 6000 islet equivalents (IEQ) per kilogram of recipient body weight are transplanted and that most patients with long-term insulin independence receive 8000 IEQ per kilogram. This has been borne out by the recent results described by Ryan et al. (21) from Edmonton. In a series of 12 diabetic patients receiving sequential islet allografts (allowing an average of >11,000 IEQ per kg to be transplanted) 11 out of 12 patients were insulin independent with a median period of follow-up of >10 months.

As most human islet transplant centers would aim to use 1:1 donor/recipient ratios for transplantation if possible (because of both practical and immunological reasons), it has become essential to be able to produce high quality and high yields of islets on a regular basis. While the numerous variables associated with human pancreas retrieval makes this difficult, the use of intraductal collagenase delivery (15) and the Ricordi system for pancreas digestion (19) have, to some extent, helped to bring this about. However, there is little doubt that the batch of collagenase used has the most influence on the final outcome of islet yield (10). While the introduction of Liberase has brought about some improvement (16), this enzyme combination still suffers some of the problems of the more conventionally prepared collagenases in that there are batch-to-batch variations and loss of activity with time (7).

In this study we set out to define individual components in collagenase that were associated with the effectiveness of crude collagenase by producing McAbs that differentially reacted with effective and ineffective Boehringer P batches. Out of three separate fusions (286 original wells) we found six antibody-producing lines that showed a differential effect on either ELISA or Western blotting on effective versus ineffective collagenase batches and here we describe two of these in detail (LDS71 and LDS81).

While no discernable differences between batches of collagenase can be found using standard protein separation methods, such as SDS-PAGE, as shown here (Fig. 1) and by others (27,28), the two McAbs do appear to detect components that are differentially represented in effective and ineffective batches of collagenase (Fig. 2). In the Western blotting experiments, it can be clearly seen that both McAbs detect the 125-kDa component in B50 but detect different size bands in more effective batches of collagenase, such as B82. We concluded from these experiments that both antibodies react with a different epitope on the 125-kDa component. Although the 125-kDa component has not been fully defined in this study, there are reasons to suggest that it belongs to the collagenase family as its presence is strongly associated with the level of FALGPA activity (data not shown). It was apparent that all batches of freshly made collagenase showed the presence of the 125-kDa band and that storage at room temperature rapidly led to breakdown with most batches of collagenase (Fig. 4). Most interestingly, the ineffective batch, B50, was relatively
resistant to breakdown, yielding a number of intermediate components not seen in the other enzymes. It is quite possible that the 125-kDa band represents an important collagenase precursor enzyme that must be appropriately activated by protease cleavage. On the basis that this breakdown was being caused by proteolytic components within the collagenase enzyme mixture, we attempted to mimic the effect with dialysis in the presence or absence of leupeptin (Fig. 5A, B). Clearly leupeptin completely abolishes the breakdown of the 125-kDa component, which suggests that the proteolytic component present in collagenase that leads to the breakdown of the 125-kDa component belongs to the cysteine proteinase family (22).

Finally, we used the McAbs to assess fractions coming off the Ricordi digestion system during routine preparations of human and porcine islets using different batches of collagenase (Figs. 6 and 7). Following assessment of 7 human islet preparations and 7 porcine islet preparations it was clear that there was a strong association between the continued presence of the 125-kDa band, throughout the digestion process, and successful pancreas digestion based on the numbers of cleaved islets obtained. Clearly, even for effective batches, there was partial breakdown of the 125-kDa component (and this may be related to partial cleavage by proteolytic enzymes), but for ineffective batches the breakdown of the 125-kDa component was rapid and complete within minutes of the start of pancreatic digestion. This suggests that the interaction of collagenase and other proteases is important for successful human or porcine pancreas digestion, as has been noted by others (12).

Proteolytic enzymes are considered to have dual roles in pancreatic digestion (12). On one hand, without collagenase, these proteases cannot break down native collagen but they can degrade protease-sensitive areas on proteoglycans and glycoproteins, which then allows collagenase access to collagen. On the other hand, proteases can damage islet cells irreversibly by fragmentation of the liberated islets to single cells. In the conventional one-step collagenase digestion method, proteolytic activity was seen to gradually increase as proteases were activated and released from pancreatic acinar cells.
(23,26) while collagenase activities were continually decreasing (8). Wolters et al. showed that the addition of 10% BSA during rat pancreas digestion produced better islet yields (27). BSA has no effect on collagenase activity per se but can inhibit proteolytic activity by acting as a substrate (27,28). This suggests that it would be beneficial for pancreatic islet isolation to reduce the proteolytic activity during pancreas digestion. Recently, Kenmochi et al. (11) modified the conventional one-step collagenase digestion method into a two-step digestion process. In the first step, human pancreata were digested with warm collagenase solution until cleaved islets appeared (approximately 15 min). In the second step, pancreata were digested with cold collagenase solution to avoid islet damage. This method significantly improved the islet isolation success rate over the conventional system (11). Based on the premise that the 125-kDa component plays an important role in yielding intact islets, we could predict that the presence of leupeptin at inhibitory concentrations after the first stage of pancreatic digestion (approximately 15 min) may significantly improve the yield of intact islets from the porcine and human pancreas by preventing continued breakdown of the 125-kDa component.

In conclusion, this study has identified a component of collagenase that appears to be essential for the effective digestion of both human and porcine pancreas. Its inappropriate degradation leads to loss of effectiveness of collagenase in porcine and human pancreas digestion. The presence and activity of this component could be a useful tool in monitoring the production of effective enzyme blends.

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