The FASEB Journal express article 10.1096/fj.02-0725fje. Published online March 5, 2003.

Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin

Francis J. Descamps, Philippe E. Van den Steen, Erik Martens, Florence Ballaux,* Karel Geboes,* and Ghislain Opdenakker[1]

Rega Institute for Medical Research, Laboratory of Molecular Immunology, University of Leuven, 3000 Leuven, Belgium; and *Department of Morphology and Molecular Pathology, University of Leuven, 3000 Leuven, Belgium

[1]Correspondence: Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: ghislain.opdenakker@rega.kuleuven.ac.be

Genetic, endocrine, and environmental factors contribute to the development of diabetes. Much information has been gathered on the homeostasis mechanisms of glucose regulation by insulin-producing pancreatic β cells. Here we demonstrate high expression levels of gelatinase B (matrix metalloproteinase-9, MMP-9) by neutrophils in acute pancreatitis and by ductular epithelial cells in chronic pancreatitis. Because gelatinase B processes cytokines and chemokines, we investigated whether and how gelatinase B cleaves insulin. Pure human neutrophil gelatinase B was found to destroy insulin by cleavage at 10 sites. Pancreatic islet and ductular cells are relatively spared in comparison with the complete destruction of acinar cells of the exocrine pancreas in chronic pancreatitis. High expression levels of gelatinase B are maintained in the immediate proximity of insulin-secreting β cells. Consequently, diabetes may be worsened by enzymatic degradation of insulin by gelatinase B and by the consequent enhancement of the autoimmune process. Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin.

Key Words: diabetes • inflammation • neutrophil • islets of Langerhans

Viral infections or other insults of the pancreas may result in the life-threatening condition of acute pancreatitis. It is clinically characterized by abdominal pain, vomiting, and alterations in serum amylase and lipase levels. Metabolic complications include hypocalcemia, hyperlipidemia, and hyperglycemia. The latter is usually a complication of disintegration of the architectural structure of the pancreas, in casu the islets of Langerhans, which harbor the insulin-producing β cells. Insulitis and diabetic consequences are observed when pancreatitis evolves into the chronic stage. The pathology underlying an acute pancreatitis involves mechanisms such as the release of activated proteases. The inflammatory process is assisted by the production of soluble mediators e.g., interleukin 1β (IL-1β), tumor necrosis factor α, and platelet-activating factor. These mediators activate neutrophils and aggravate, both locally and systemically, the inflammatory process (1). Sequestration of neutrophils within the pancreas is generally believed to be an early and important event in the evolution of acute pancreatitis.

Gelatinase B (MMP-9), a member of the extracellular matrix (ECM) degrading matrix metalloproteinases, is present in considerable amounts in the granules of neutrophils and has been associated with pancreatitis (2–5). However, the literature on MMP-9 production and

activity in experimental pancreatic fibrosis is confusing. While Ng et al. documented high control levels of MMP-9 in normal pancreas tissue as detected by zymography analysis, Yokoto et al. could not visualize any zymolytic MMP-9 in control tissue.

A major substrate for gelatinase B is gelatin or denatured collagen (6). However, gelatinase B cleaves other substrates as well. In particular cases, clipping can have a regulatory function. For instance, the neutrophil chemokine IL-8 is potentiated by amino-terminal truncation of six amino acids (7). Furthermore, gelatinase B cleaves endothelin-1 yielding endothelin-1 [1–32], that activates neutrophils and promotes leukocyte-endothelial cell adhesion and consequently, neutrophil trafficking into inflamed tissues (8). Aside from these functions of gelatinase B as a regulator and effector molecule in inflammation (9), increased proteolytic activity may further mediate pathological conditions. For instance in autoimmune diseases, including multiple sclerosis and rheumatoid arthritis, gelatinase B is increased vs. control levels and may play a functional role in the generation of immunodominant peptides (6, 10).

Diabetes, which may result from pancreatitis, has been studied in detail because it is a disease with worldwide distribution with important morbidity and mortality. Moreover, type I diabetes is primarily mediated by T lymphocytes, while activated T lymphocytes have been documented as gelatinase B-producing cells (11–13). Several studies have linked the presentation of autoantigens or the production of autoantibodies with apoptosis of the insulin-producing β cells. Uptake of β cell antigens in the islets and their ferrying to the pancreatic lymph nodes permits the presentation to naive circulating β cell-reactive T lymphocytes with an autoimmune response as a consequence. Again, such cell migration needs gelatinase B for the crossing of basement membranes and connective tissues (9, 14). Here we document that gelatinase B is present in acute and chronic pancreatitis, degrades insulin into fragments and may assist in the generation of immunodominant insulin epitopes.

MATERIALS AND METHODS

Patient samples and immunohistochemistry

Autopsy or surgical specimens were obtained after informed consent by the donor or relatives in accordance with the recommendations of the local ethics committee, with the university hospital rules and the Declaration of Helsinki. Patients included 15 males and 8 females. Nine samples of chronic pancreatitis, six of acute pancreatitis, and eight specimens of surrounding normal tissue from pancreatic cancer patients were used. The samples were formalin-fixed, routinely processed and used for staining analysis and immunohistochemistry, as described previously for gelatinase B (15). A specific monoclonal antibody (REGA-2D9) against human gelatinase B was used for the detection of gelatinase B (16). The antigen was revealed with the use of an immunoperoxidase reaction and 3-amino-9-ethylcarbazole as substrate. Visualization was by a red immunoreactive staining. Insulin immunoreactivity was demonstrated with a polyclonal guinea pig anti-insulin antibody (A0564, Dako Glostrup, Denmark, dilution 1:200). The reaction was visualized with alkaline phosphatase. The black-brown reaction product was developed with the BCIP/NBT Substrate System (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium from Dako). For the double staining, the gelatinase B staining was performed first, followed by immunostaining for insulin.

Preparation and activation of gelatinase B

Progelatinase B was purified from human neutrophils as described previously (6, 7). It was activated with stromelysin-1 (MMP-3) at a molar ratio of 1:100 (stromelysin-1:gelatinase B). The specific activity was controlled by cleavage of the coumarin–labeled peptide (7-methoxycoumarin-4yl)Acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 (Bachem, Switzerland). The substrate (2 μM) was incubated and stirred with gelatinase B (1–5 pmol) in a fluorimeter at 37°C as described previously (17).

Incubation of human insulin with gelatinase B

Human insulin (Roche Diagnostics; Mannheim, Germany) was incubated with activated gelatinase B in assay buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.01% Tween 20) at 37°C for 24 h (insulin concentration was 17 μM; substrate:enzyme ratio was in excess of 6.5:1). As a negative control experiment, insulin was incubated with an equivalent amount of stromelysin-1 (used for the activation of gelatinase B) under similar conditions. Alternatively, insulin was incubated with gelatinase B in the presence of 100 mM EDTA or 5 mM *o*-phenantroline. Degradation of insulin was visualized after SDS-PAGE (Tris-Tricine; 16.5% acrylamide) by silver staining analysis.

Determination of the gelatinase B cleavage sites by mass spectrometry

Reaction mixtures of insulin cleavages to completion, in assay buffer without Tween 20, were applied to a reverse phase high performance liquid chromatography (RP-HPLC) column. Each fraction was analyzed in both the oxidized and reduced state by electrospray ion-trap mass spectrometry (MS) (Esquire-LC, Bruker Daltonic, Bremen, Germany) (6). Reductions were performed in 75 mM Tris-HCl (pH 7,4) with 2% β-mercapto-ethanol. Reduced samples were desalted on C18 ZIPTIPs (Millipore Corporation, Bedford, MA) before application to the mass spectrometer. Fragment identification was done by comparison of the measured masses with the theoretical masses of all possible fragments of insulin. The peptide sequence was then confirmed by sequencing in tandem MS/MS mode on a quadrupole time-of-flight (QTOF-2, Micromass, Manchester, UK) mass spectrometer. The obtained cleavage sites were manually positioned on a three-dimensional model of insulin, based on the crystal structure (18, 19).

Enzyme kinetic studies were performed by MS analysis. A preparation of granulocyte chemotactic protein-2 (GCP-2) was used to standardize protein amount (20). Samples of the insulin-gelatinase B incubation mixture were collected at different time intervals and analyzed by MS. The relative amount of intact insulin (molecular mass is 5807 Da) was deduced from intensities of charged deconvoluted insulin peaks relative to the internal standard GCP-2 (molecular mass is 8517 Da), which was coinjected with each sample.

RESULTS

Gelatinase B is absent in normal and expressed in inflamed pancreas tissues

First, we studied the expression of gelatinase B by means of immunohistochemistry in a cohort of 23 Caucasians, 15 males and 8 females (controls included). Acute pancreatitis was diagnosed in 6 individuals, whereas 9 had chronic pancreatitis. As control pancreas tissues, we evaluated 8 specimens from normal appearing pancreas tissues obtained at the occasion of cancer surgery. Two patients with acute pancreatitis were diagnosed with diabetes at the time of biopsy. Figure 1

shows the immunohistochemical analysis on formalin-fixed and paraffin-embedded sections. Panel a illustrates the absence of gelatinase B expression in the exocrine pancreas as well as in the islets of Langerhans of a control patient. Panel b represents a biopsy from a patient in an early stage of acute pancreatitis. The microscopic slide shows marginating and extravascular leukocytes, which stained strongly with the monoclonal antibody against gelatinase B. In one case of acute pancreatitis, insulitis with polymorphonuclear cells was evident (data not shown). As indicated in panels c and d, the cases of acute pancreatitis contained many polymorphonuclear and occasional mononuclear cells expressing gelatinase B. Panels e and f show a completely different picture observed in all 9 cases of chronic pancreatitis. The exocrine pancreas is replaced by fibrotic tissue in which islets and ductules are spared. The ductular epithelial cells express considerable amounts of gelatinase B and within the islets some cells stain positively for gelatinase B.

Insulin and gelatinase B are expressed by different cell types in close proximity

Double immunostainings for gelatinase B and for insulin were performed on histological sections of patients with chronic pancreatitis in order to detect either temporal or spatial colocalisation (Fig. 2). Only occasionally, insulin-producing cells showed a weakly positive staining for gelatinase B. However, the two different cell types expressing insulin or gelatinase B were often found in close (Fig. 2*a*) or direct proximity (Fig. 2*b*). In other words, after secretion of insulin by β cells and gelatinase B by neighboring (ductular) cells, it is obvious that both molecules may interact, in particular after extensive tissue remodeling as observed in chronic pancreatitis.

Human insulin is degraded by gelatinase B

To define whether human insulin is targeted by active gelatinase B, both molecules were mixed at a substrate:enzyme ratio in excess of 6.5 and were incubated. Degradation of insulin was visualized by SDS-PAGE and silver staining analysis (Fig. 3*a*). Addition of the metalloproteinase inhibitors EDTA or *o*-phenantroline to the reaction mixture inhibited the cleavage completely (Fig. 3*a*). The fragmentation was analyzed in detail by mass spectrometry (MS). Enzyme kinetic studies were performed after standardization of the MS analysis by coinjection of known amounts of GCP-2 (20). The β chain fragment of residues 24 to 30 was the first appearing degradation product in function of time. Thus, we determined the cleavage in front of residue 24 as the most efficient. Complete digestion of insulin was obtained after a time interval of 10 h (Fig. 3*c*). Incubation of insulin with an equivalent amount of stromelysin-1, which is present in the reaction mixture for the activation of progelatinase B, showed complete absence of degradation.

Insulin degradation by gelatinase B in function of enzyme:substrate ratios was studied. We titrated a twofold dilution series of the enzyme on insulin conversion. Reaction mixtures were incubated for 24 h and analyzed by MS. To detect insulin degradation, we focused on the formation of the β chain 24–30 fragment (MW = 903.1 Da), noticed to be the first generated degradation product in our kinetic study and which is not further degraded (*vide infra*). The enzyme:substrate activity on insulin as a substrate was readily detectable for enzyme:substrate ratios between 1:6.5 and 1:208.

Site-specificities of insulin degradation by gelatinase B

The reaction mixture of a complete digestion of 50 μg human insulin by gelatinase B was separated by RP-HPLC on a C18 column (Fig. 4*a*) with on-line mass spectrometry analysis. First, exact molecular masses were derived from the spectra and were fitted to virtual insulin fragments. Predicted amino-acid sequences were confirmed by MS/MS analysis of the corresponding peptides. Next, a different set of fractions was analyzed after reduction with β-mercapto-ethanol. Reduction resulted in the separation of fragments, linked by one of the three disulfide bridges present in the insulin molecule. As expected, molecular masses of the nonreduced fragments were exactly in accordance with one or the summation of two fragments in the reduced state. In Fig. 4*b*, the generated insulin fragments are shown and indicated with their RP-HPLC fraction number. By indicating the amino acids following the cleavage sites (P1' residues) in green (Fig. 4*b–d*), the accessibility for gelatinase B can be demonstrated on the three-dimensional crystal structure of human insulin (18, 19) (Fig. 4*c*). With these data, we were able to elucidate previously undefined peaks from the mass spectra obtained after short incubation times or at low enzyme concentrations in the kinetic studies. Association of the appearance of specific peaks in function of incubation time is indicative of the relative efficiency of the respective cleavage sites. In Fig. 4*d*, the 10 cleavage sites were aligned from the most to the least efficient cleavage. The P1' residue of the cleavage was six times a leucine. This clear preference for hydrophobic residues at P1' is in agreement with digestion of synthetic peptides (21) and the previously published cleavage sites in denatured bovine collagen II (6). From our data, it is obvious that insulin is progressively degraded into fragments by gelatinase B, rather than processed by a unique clipping, as observed for IL-1β (22), IL-8 (7), and endothelin-1 (8).

DISCUSSION

Gelatinase B expression has been functionally associated with various autoimmune diseases including multiple sclerosis (10) and rheumatoid arthritis (6). For diabetes as an autoimmune disease, only circumstantial evidence and phenomenological data are available (23), but so far no functional link has been demonstrated between gelatinase B and insulin or any other islet antigen (24).

To evaluate the expression in human pancreas, we first studied by immunohistochemistry which cell types produce gelatinase B. The enzyme is not expressed in normal exocrine and endocrine pancreas. As expected (9), it is abundantly produced by inflammatory cells in acute pancreatitis; mainly neutrophils and less extensively mononuclear leukocytes expressed gelatinase B. Surprisingly, in chronic pancreatitis ductular epithelial cells produce abundantly gelatinase B, evidenced by their strong immunoreactivity. Even in a fibrotic exocrine pancreas, the ductuli express considerable amounts of gelatinase B, often in close association with the islets of Langerhans. The effects of the expression of gelatinase B, in particular its diabetogenic role, is exemplified in Fig. 5.

Whereas it is understandable that, by its virtue to cleave ECM components, gelatinase B may contribute to the aforementioned extensive tissue remodeling and fibrosis in pancreatitis, it may exert other functions. For instance, gelatinase B has been shown to potentiate IL-1β and the neutrophil chemokine IL-8 and it degrades other CXC chemokines. Here we demonstrate the processing of the classical polypeptide hormone insulin by gelatinase B. To evaluate the possibility of a coexistence of insulin and gelatinase B in time and space, we performed double immunohistochemical stainings. In chronic pancreatitis tissues, insulin-producing β cells were

frequently found adjacent to gelatinase B-positive cells. Previously, a model how gelatinase B may play a role in autoimmunity was developed (10). Cytokine-regulated extracellular proteases (including gelatinase B) play a central role in this model by degrading secreted proteins into fragments that activate T lymphocytes. For instance, degradation of myelin basic protein (25) and denatured type II collagen (6) by gelatinase B effectively generates fragments corresponding to immunodominant peptides for multiple sclerosis and rheumatoid arthritis, respectively. For type I diabetes, the neuroendocrine enzyme glutamic acid decarboxylase (26), tyrosine phosphatase (27) and insulin (28) are important autoantigens (24). For instance, the fragment of the insulin β-chain, containing residues 9 to 23, constitutes an immunodominant epitope (29). From our data, it is clear that the splitting by gelatinase B in front of residue 24 is the most efficient of all 10 cleavages. This implies that such a remnant fragment, containing residues 1 to 23 of the β chain, may be presented in MHC-II either after processing into the immunodominant epitope [9 to 23] or after direct uptake by antigen-presenting cells (30). However, if the fragment 1 to 23 persists in the extracellular milieu for prolonged time intervals, then it will be further degraded by the action of gelatinase B. In fact, human insulin is completely destroyed by gelatinase B. We used for our studies pure natural human gelatinase B, excluding the possibility that the observed cleavages were by some other contaminating protease (7). This was further corroborated by control experiments with the MMP-inhibitors EDTA and *o*-phenantroline, which completely inhibited the degradation of insulin by gelatinase B.

The obtained cleavage pattern of insulin by gelatinase B was in accordance with other studies on substrate cleavage. In particular, a hydrophobic amino acid was detected most often as the P1' residue, and the most efficient cleavage occurred after a glycine, as was observed in collagen II (6). Our study implies that MMP inhibitors may have a stabilizing effect on insulin levels and that in some cases of diabetes, insulin therapy may be assisted by MMP inhibition. Anyhow, insulin is an important and novel substrate of gelatinase B. Because inflammatory (in acute pancreatitis) and immune cells (in chronic pancreatitis and diabetes) are often powerful sources of gelatinase B, our findings constitute another link between the innate immune and endocrine systems.

REFERENCES

1. Banks, R. E., Evans, S. W., Alexander, D., McMahon, M. J., and Whicher, J. T. (1991) Is fatal pancreatitis a consequence of excessive leukocyte stimulation? The role of tumor necrosis factor alpha. *Cytokine* **3,** 12–16

2. Muhs, B. E., Patel, S., Yee, H., Marcus, S., and Shamamian, P. (2001) Increased matrix metalloproteinase expression and activation following experimental acute pancreatitis. *J. Surg. Res.* **101,** 21–28

3. Ishihara, T., Hayasaka, A., Yamaguchi, T., Kondo, F., and Saisho, H. (1998) Immunohistochemical study of transforming growth factor-beta 1, matrix metalloproteinase-2,9, tissue inhibitors of metalloproteinase-1,2, and basement membrane components at pancreatic ducts in chronic pancreatitis. *Pancreas* **17,** 412–418

4. Ng, E. K., Barent, B. L., Smith, G. S., Joehl, R. J., and Murayama, K. M. (2001) Decreased type IV collagenase activity in experimental pancreatic fibrosis. *J. Surg. Res.* **96,** 6–9

5. Yokota, T., Denham, W., Murayama, K., Pelham, C., Joehl, R., and Bell, R. H., Jr. (2002) Pancreatic stellate cell activation and MMP production in experimental pancreatic fibrosis. *J. Surg. Res.* **104,** 106–111

6. Van den Steen, P. E., Proost, P., Grillet, B., Brand, D. D., Kang, A. H., Van Damme, J., and Opdenakker, G. (2002) Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity, post-translational modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis. *FASEB J.* **16,** 379–389

7. Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., and Opdenakker, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by amino-terminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* **96,** 2673–2681

8. Fernandez-Patron, C., Zouki, C., Whittal, R., Chan, J. S., Davidge, S. T., and Filep, J. G. (2001) Matrix metalloproteinases regulate neutrophil-endothelial cell adhesion through generation of endothelin-1. *FASEB J.* **15,** 2230–2240 (1-32)

9. Opdenakker, G., Van den Steen, P. E., and Van Damme, J. (2001) Gelatinase B: a tuner and amplifier of immune functions. *Trends Immunol.* **22,** 571–579

10. Opdenakker, G., and Van Damme, J. (1994) Cytokine-regulated proteases in autoimmune diseases. *Immunol. Today* **15,** 103–107

11. Zhou, H., Bernhard, E. J., Fox, F. E., and Billings, P. C. (1993) Induction of metalloproteinase activity in human T-lymphocytes. *Biochim. Biophys. Acta* **1177,** 174–178

12. Montgomery, A. M., Sabzevari, H., and Reisfeld, R. A. (1993) Production and regulation of gelatinase B by human T-cells. *Biochim. Biophys. Acta* **1176,** 265–268

13. Weeks, B. S., Schnaper, H. W., Handy, M., Holloway, E., and Kleinman, H. K. (1993) Human T lymphocytes synthesize the 92 kDa type IV collagenase (gelatinase B). *J. Cell. Physiol.* **157,** 644–649

14. Kobayashi, Y., Matsumoto, M., Kotani, M., and Makino, T. (1999) Possible involvement of matrix metalloproteinase-9 in Langerhans cell migration and maturation. *J. Immunol.* **163,** 5989–5993

15. Grillet, B., Dequeker, J., Paemen, L., Van Damme, B., and Opdenakker, G. (1997) Gelatinase B in chronic synovitis: immunolocalization with a monoclonal antibody. *Br. J. Rheumatol.* **36,** 744–747

16. Paemen, L., Martens, E., Masure, S., and Opdenakker, G. (1995) Monoclonal antibodies specific for natural human neutrophil gelatinase B used for affinity purification, quantitation by two-site ELISA and inhibition of enzymatic activity. *Eur. J. Biochem.* **234,** 759–765

17. Murphy, G., and Willenbrock, F. (1995) Tissue inhibitors of matrix metalloendopeptidases. *Methods Enzymol.* **248,** 496–510

18. Adams, M. J., Baker, E. N., Blundell, T. L., Harding, M. M., Dodson, E. J., Hodgkin, D. C., Dodson, G. G., Rimmer, B., Vijayan, M., and Sheats, S. (1969) Structure of rhombohedral 2 zinc insulin crystals. *Nature (London)* **224,** 491–495

19. Smith, G. D., Pangborn, W. A., and Blessing, R. H. (2001) Phase changes in T(3)R(3)(f) human insulin: temperature or pressure induced? *Acta Crystallogr. D Biol. Crystallogr.* **57,** 1091–1100

20. Proost, P., Wolf-Peeters, C., Conings, R., Opdenakker, G., Billiau, A., and Van Damme, J. (1993) Identification of a novel granulocyte chemotactic protein (GCP-2) from human tumor cells. In vitro and in vivo comparison with natural forms of GRO, IP-10, and IL-8. *J. Immunol.* **150,** 1000–1010

21. Netzel-Arnett, S., Sang, Q. X., Moore, W. G., Navre, M., Birkedal-Hansen, H., and Van Wart, H. E. (1993) Comparative sequence specificities of human 72- and 92-kDa gelatinases (type IV collagenases) and PUMP (matrilysin). *Biochemistry* **32,** 6427–6432

22. Schönbeck, U., Mach, F., and Libby, P. (1998) Generation of biologically active IL-1□ by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1□ processing. *J. Immunol.* **161,** 3340–3346

23. Ebihara, I., Nakamura, T., Shimada, N., and Koide, H. (1998) Increased plasma metalloproteinase-9 concentrations precede development of microalbuminuria in non-insulin-dependent diabetes mellitus. *Am. J. Kidney Dis.* **32,** 544–550

24. Immunology of Diabetes *Autoimmune mechanisms and the prevention and cure of type 1 diabetes*. (2002) (Sanjeevi, C. B., ed) Vol. 958. *Ann. New York Acad. Sci*.

25. Proost, P., Van Damme, J., and Opdenakker, G. (1993) Leukocyte gelatinase B cleavage releases encephalitogens from human myelin basic protein. *Biochem. Biophys. Res. Commun.* **192,** 53–56

26. Baekkeskov, S., Aanstoot, H. J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., DeCamilli, P., and Camilli, P. D. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature (London)* **347,** 151–156

27. Christie, M. R., Vohra, G., Champagne, P., Daneman, D., and Delovitch, T. L. (1990) Distinct antibody specificities to a 64-kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. *J. Exp. Med.* **172,** 789–794

28. Wegmann, D. R., and Eisenbarth, G. S. (2000) It's insulin. *J. Autoimmun.* **15,** 286–291

29. Wegmann, D. R., Norbury-Glaser, M., and Daniel, D. (1994) Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol.* **24,** 1853–1857

30. Pu, Z., Carrero, J. A., and Unanue, E. R. (2002) Distinct recognition by two subsets of T cells of an MHC class II-peptide complex. *Proc. Natl. Acad. Sci. USA* **99,** 8844–8849

Received August 28, 2002; accepted December 19, 2002.

Fig. 1

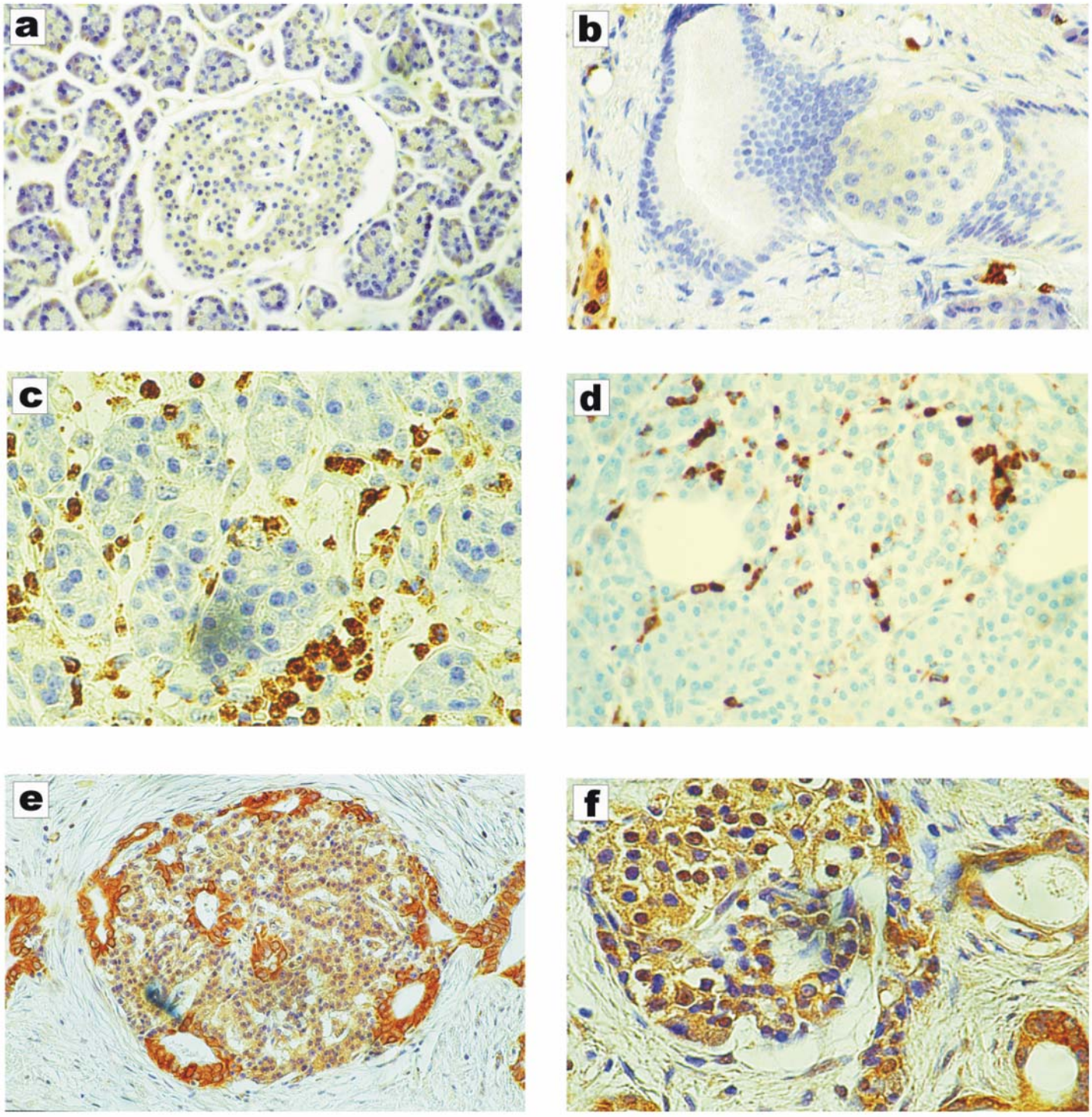

Figure 1. Gelatinase B expression in acute and chronic pancreatitis. In panel **a** (125×), normal exocrine pancreas and islets of Langerhans show no immunoreactivity for gelatinase B. At the onset of acute pancreatitis, the infiltration of inflammatory cells starts by margination and extravasation of polymorphonuclear and mononuclear cells expressing high levels of gelatinase B, as shown in panel **b** (250×). Patients with acute pancreatitis show massive infiltration of gelatinase B positive cells (**c**, 500×; **d**, 250×). After evolution into the chronic stage of pancreatitis, mainly ductular and insular mononuclear cells express gelatinase B (**e**, 125×; **f**, 500×).

Fig. 2

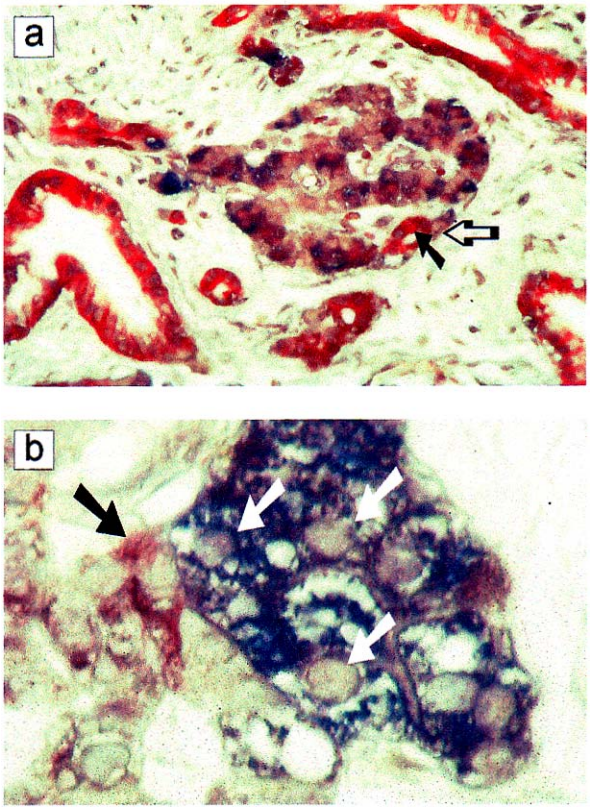

Figure 2. Spatial relationship between insulin-positive and gelatinase B-positive cells. Pancreas tissue from patients with chronic pancreatitis was immunostained with antibodies against insulin to reveal the β cells (black-purple) and with the monoclonal antibody against gelatinase B (red). Panel **a** (250×) shows an islet and the general distribution of gelatinase B in ductular cells (black arrows) and insulin in the β cells (white arrows). At higher magnification in panel **b** (1250×), the hormone and the enzyme were clearly detected in adjacent cells. All arrows point to the respective cell nuclei.

Fig. 3

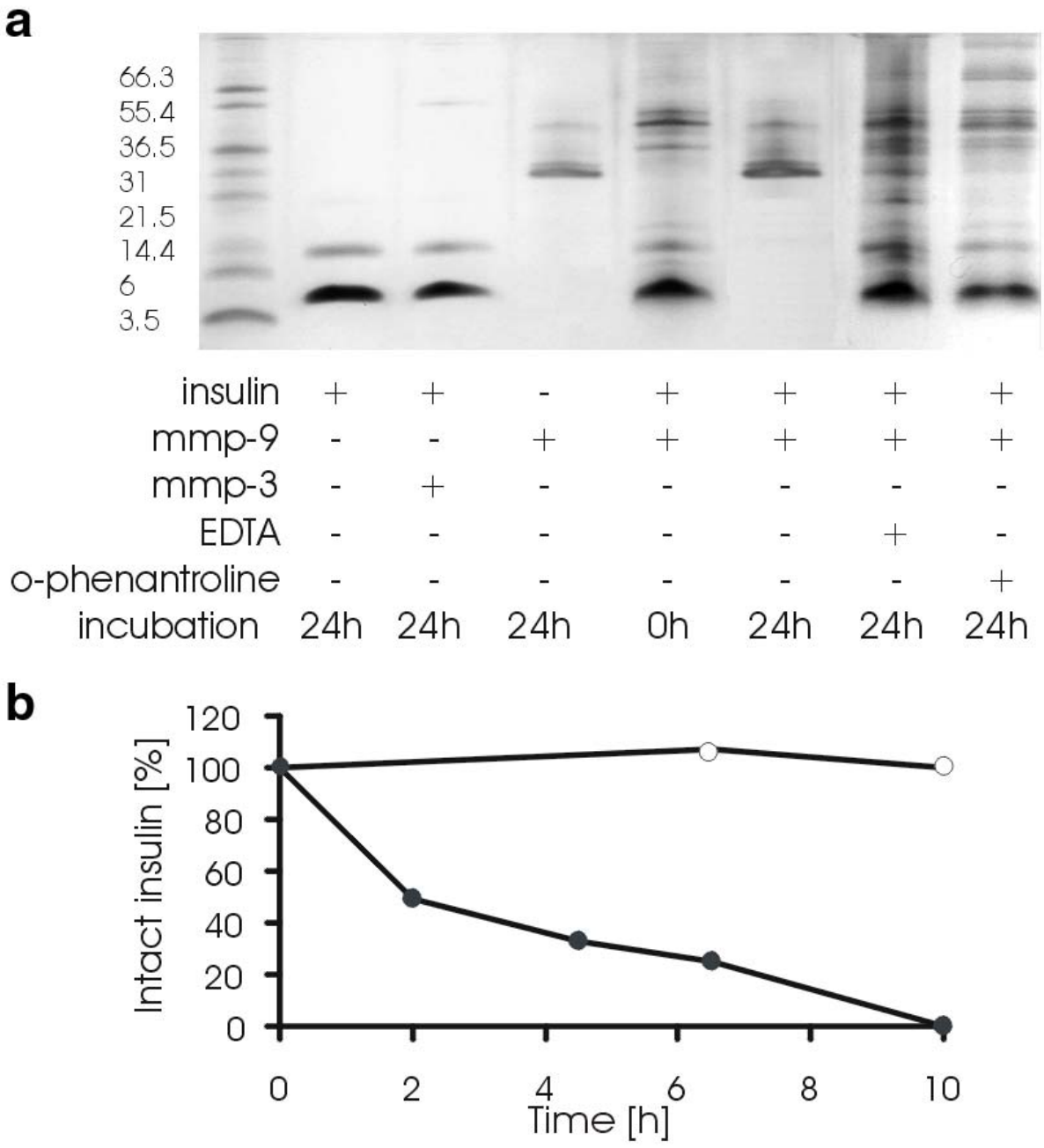

Figure 3. Degradation of insulin by activated neutrophil gelatinase B. a) SDS-PAGE under nonreducing conditions. Incubation of insulin with activated MMP-9 at a substrate:enzyme ratio of 6,5:1 leads to degradation of insulin. MMP-3 at a ratio of 650:1 is a negative control. Other controls include incubations with enzyme or substrate only or substrate:enzyme incubations in the presence of EDTA and o-phenantroline. Higher molecular weight bands are gelatinase B degradation products present in the enzyme preparation and these are further degraded during the incubation. Molecular markers, indicated in kDa, are shown in the left lane. **b)** Degradation of insulin by gelatinase B was quantified for incremental time intervals using mass spectrometry analysis (●). Granulocyte chemotactic protein-2 was used as an internal standard (O).

Fig. 4

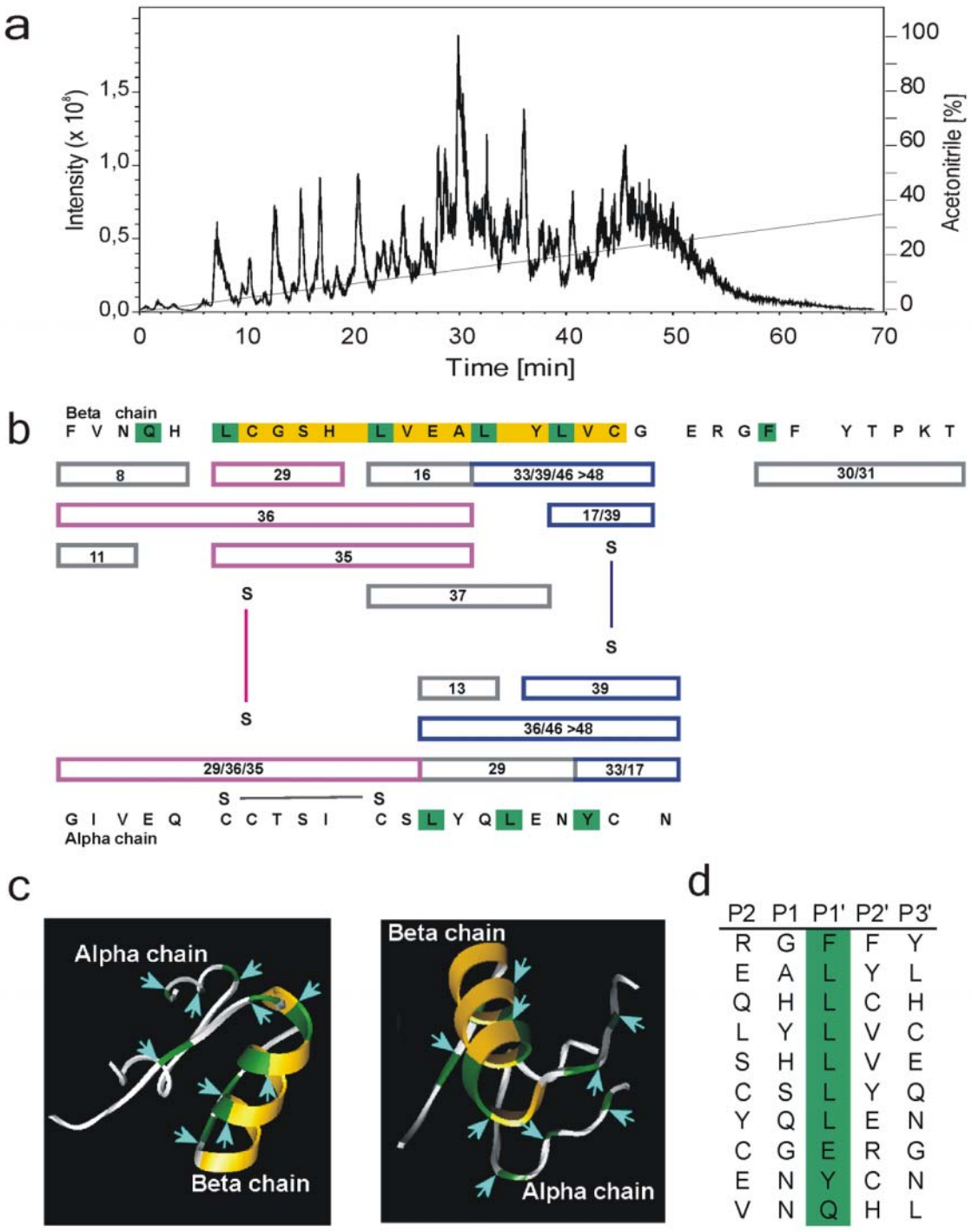

Figure 4. Identification of insulin fragments generated by gelatinase B. a) On-line total ion count chromatogram of the RP-HPLC separation of the insulin fragments; the acetonitrile gradient is indicated on the chromatogram by a thin line. **b)** Insulin α and β chain sequences and fragmentation by gelatinase B, as determined by MS analysis of the successive fractions. Insulin cleavage products are indicated as red or blue boxes, connected by disulfide bridges in the corresponding color or as gray boxes, representing fragments that are not connected by disulfide bridges. Numbers in the boxes are the RP-HPLC fraction numbers. **c)** Three-dimensional views of the crystal structure of insulin. Cleavage sites by gelatinase B are indicated by means of blue arrows to demonstrate the accessibility of the enzyme and the resulting complete degradation of insulin. **d)** Alignment of the insulin cleavage sites from the most to the least efficient proteolysis. **b–d)** Amino acids that follow a cleavage site (P_1' positions) are indicated in green. The α helix present in the insulin β chain is shown in yellow in panels **b** and **c**.

Fig. 5

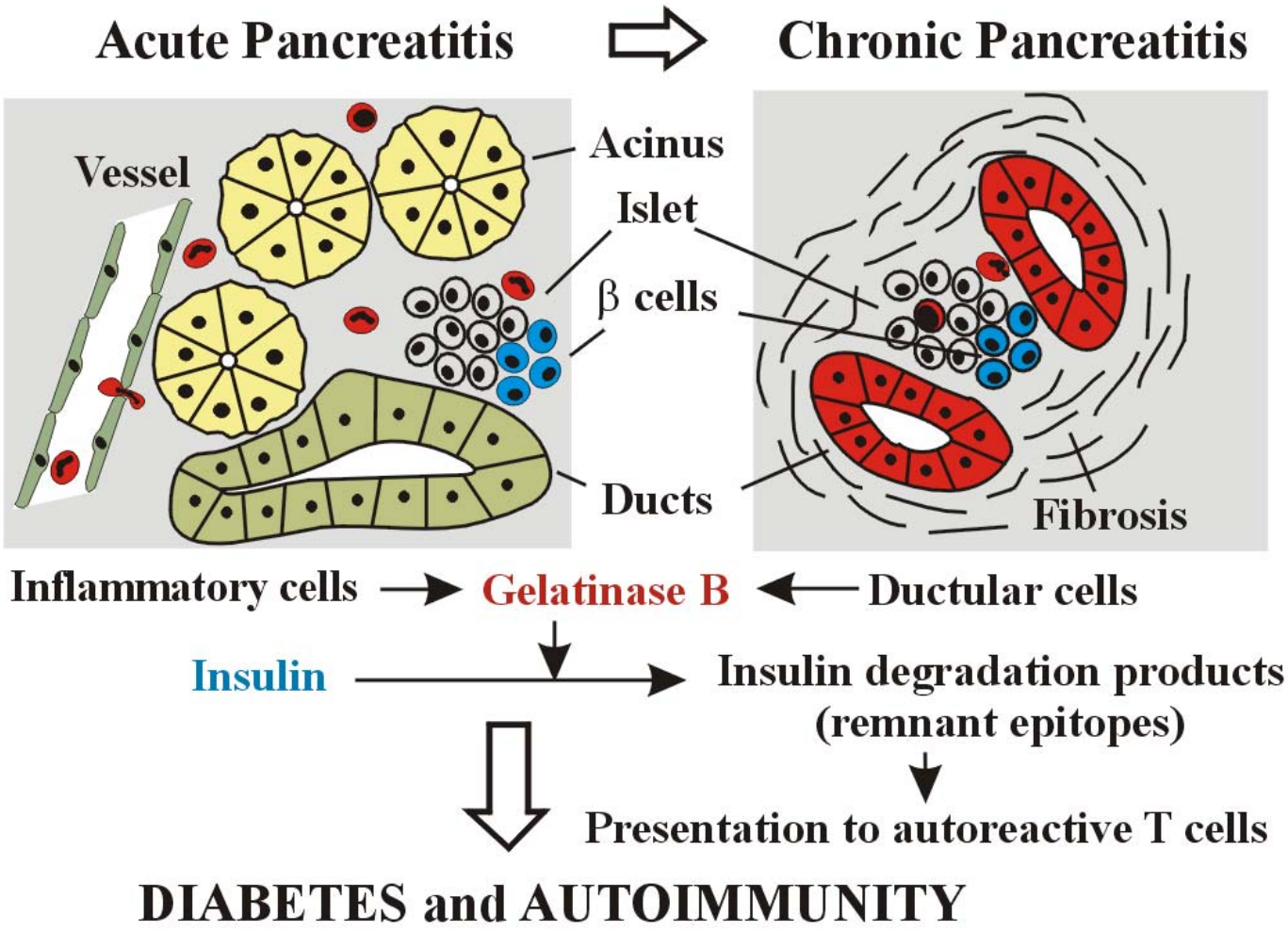

Figure 5. Cleavage of insulin by gelatinase B in the pathophysiology of diabetes. Inflammatory and ductular cells produce gelatinase B (red) in acute and chronic pancreatitis, respectively. Gelatinase B cleaves insulin (blue), secreted by β cells in close proximity. Degradation of insulin leads to diabetes. Remnant epitopes of insulin trigger autoimmunity after presentation to autoreactive T cells and aggravate the diabetes by loss of β cells.