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Original Paper

In vivo activation of gelatinase B/MMP-9 by trypsin in acute pancreatitis is a permissive factor in streptozotocin-induced diabetes

Francis J Descamps,[1] Erik Martens,[1] Florence Ballaux,[2] Karel Geboes[2] and Ghislain Opdenakker[1]*
[1]*Rega Institute for Medical Research, Laboratory of Molecular Immunology, University of Leuven, Leuven, Belgium*
[2]*Department of Morphology and Molecular Pathology, University of Leuven, Leuven, Belgium*

**Correspondence to: Professor Ghislain Opdenakker, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: ghislain.opdenakker@rega.kuleuven.ac.be*

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Abstract

Matrix metalloproteinases, in particular gelatinase B/MMP-9, are key mediators in autoimmune diseases like multiple sclerosis and rheumatoid arthritis, but their pathogenic roles in diabetes are not well established. Gelatinase B has previously been shown to be upregulated in pancreas tissue from patients with acute and chronic pancreatitis and was suggested to exacerbate diabetes by cleaving insulin. In this study, the role of gelatinase B in diabetes was investigated using two streptozotocin-induced animal models of type I diabetes. In both a hyperacute and a subacute model, gelatinase B upregulation was found to be associated with disease activity. However, gelatinase B deficiency did not significantly protect against diabetes development, and wild-type and gelatinase B-deficient animals behaved similarly in terms of β-cell apoptosis or necrosis. The fact that gelatinase B was found almost exclusively as the inactive pro-enzyme in most of the streptozotocin-induced diabetic animals may explain the lack of a gelatinase B effect. On the contrary, gelatinase B was completely activated in a minority (15%) of wild-type animals. This coincided with exocrine pancreatic inflammation, as revealed by the presence of active trypsin. The discovery of *in vivo* activation of progelatinase B by trypsin in acute pancreatitis is extended in a model of caerulein-induced pancreatitis. In the latter model, trypsinogen activation is systematically achieved and gelatinase B is found in its active form. In conclusion, gelatinase B itself is not a causative factor but, when activated by endogenous trypsin, is a permissive factor for insulin degradation and diabetes.

Keywords: pancreatitis; diabetes; trypsin; matrix metalloproteinase-9; streptozotocin

Introduction

Type I diabetes is one of the most common autoimmune diseases and the cause of worldwide morbidity and mortality [1]. It is determined by genetic [2], endocrine, and environmental factors, and results from loss of the insulin-producing pancreatic β-cells. Environmental factors are believed to trigger the disease course by initiating processes that lead to an eventual β-cell-destructive autoimmune response [3]. In animal models of the disease, the first observable histopathological event is an initial low level of β-cell death that primes antigen-presenting cells. This leads to the proliferation of autoreactive lymphocytes and further selective elimination of the remaining β-cells.

Several reports propose that apoptosis of pancreatic β-cells may play a central role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM). Evidence for this mode of cell death comes from *in vitro* research with cultured pancreatic islets [4] and *in vivo* models using genetically predisposed NOD mice or streptozotocin (STZ)-induced diabetes [5]. During apoptosis, a group of proteolytic enzymes known as caspases are activated from their precursor forms into active catalytic species. Thus, activated initiator caspases can convert inactive procaspase-3 into active caspase-3, which is a key enzyme involved in the terminal apoptotic cascade of cell death, and therefore used as a marker for apoptosis. Necrosis is another mode of pancreatic β-cell death. This has been shown in virus-induced diabetes [6], in BB rats, which spontaneously develop diabetes [7], and in STZ-induced diabetes. For STZ-induced diabetes, it is believed that a single high dose of STZ predominantly induces necrosis of β-cells [7], while multiple low doses of STZ (MLD-STZ) induce β-cell apoptosis [8]. Necrosis of β-cells induced by STZ is well documented. After active transport to the pancreatic β-cells, STZ [2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose] predominantly methylates guanine and adenine bases. These are removed by the action of alkylpurine-DNA-N-glycosidase, leaving an apurinic/apyrimidinic (AP) site that is acted upon by an AP endonuclease.

The resulting DNA strand breaks activate poly(ADP-ribose)-polymerase (PARP), an abundant nuclear enzyme that initiates an energy-consuming cycle by transferring ADP ribose units from NAD^+ to nuclear proteins. This process results in rapid depletion of the intracellular NAD^+ and ATP pools, leading to cellular dysfunction and β-cell death [9].

Recently, gelatinase B/matrix metalloproteinase-9 (MMP-9) has been studied in autoimmune diseases and has been suggested to be a diabetogenic factor through proteolytic cleavage of insulin and generation of immunodominant insulin peptides [10,11]. Gelatinase B belongs to a family of matrix metalloproteinases that originally were mainly associated with connective tissue turnover, but more recently have gained much attention as regulatory proteases for the processing of signalling molecules such as cytokines, chemokines, hormones, receptors, and peptide antigens. Gelatinase B is also implicated in cell death. In experimental models of brain injury, apoptosis of retinal ganglion cells has been associated with MMP-mediated degradation of the extracellular matrix component laminin. A possible mechanism is that cell attachment to the matrix activates intracellular pathways for cell survival [12]. Recently, increased MMP activity has also been associated with endocardial endothelial apoptosis in diabetic mice [13].

Here, we studied MMP-9 expression in two models of STZ-induced diabetes and compared the evolution of glucose levels in gelatinase B wild-type versus deficient mice.

Methods

Mouse models

Wild-type C57BL/6 ($Mmp9^{+/+}$) and knockout ($Mmp9^{-/-}$) mice, back-crossed for 10 generations into a C57BL/6 genetic background, were used [14]. All experimental procedures were approved by the institutional ethics committee under licence LA 1210243 for animal welfare. Diabetes was induced with STZ (Sigma, St Louis, MO, USA), which was intraperitoneally administered in citrate buffer in a single bolus (200 mg/kg) or in five low doses (40 mg/kg) on consecutive days. Blood glucose levels were measured with a blood glucose-monitoring system (Lifescan, Beerse, Belgium). Acute pancreatitis was established by six hourly intraperitoneal injections of caerulein (Bachem, Bubendorf, Switzerland) (50 µg/kg per hour). To enhance the ongoing pancreatic inflammation, mice received a single dose of 2.5 mg/kg lipopolysaccharide (LPS) 1 h after the last caerulein injection [15].

Mice were sacrificed for pancreatectomy at specific time-points during diabetes or pancreatitis development. Pancreatic samples were placed in formalin for histological scoring or homogenates were made in lysis buffer (20 mM Tris pH 7.4, 0.5% Triton X100), containing a complete protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). DNA was destroyed by sonication and cell debris was removed by centrifugation at 30 000g. The protein concentration in the supernatants was measured according to Bradford [16].

Microscopy

Formalin-fixed samples were routinely processed and stained sections were used for microscopy. In addition, immunohistochemistry was performed using a polyclonal antibody against mouse caspase-3 (Sigma, St Louis, MO, USA). The antigen was revealed with the use of an immunoperoxidase reaction and 3-amino-9-ethylcarbazole as substrate. Visualization was by red immunoreactive staining.

Western blot analysis and gelatin zymography

300 µg protein from pancreas homogenates or 3 µl serum was applied to gelatin-sepharose affinity chromatography as described previously [17]. Briefly, the samples were diluted 1 : 2 in equilibration buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 10 mM $CaCl_2$, 0.01% Tween 20, and 5 mM o-phenanthroline), and incubated with 30 µl gelatin–Sepharose beads in mini-spin columns on a horizontal shaker. After washing, gelatinases were eluted with non-reducing SDS-PAGE loading buffer and further processed for zymography or gelatinase B western blot analysis. The latter was performed using a monoclonal mouse anti-mouse gelatinase B antibody (CDEM-ABA), which was generated in $Mmp9^{-/-}$ mice [18]. Trypsin western blot analysis was performed using a rabbit anti-trypsin polyclonal antibody (Rockland, Gilbertsville, PA, USA).

Statistical analysis

Differences in blood glucose levels between $Mmp9^{-/-}$ and $Mmp9^{+/+}$ mice or between gelatinase B levels at different time points during the disease course were evaluated using the unpaired Student's t-test. p-Values are the probability of this result assuming the null hypothesis that no difference exists between both groups.

Results

Gelatinase B is detected in serum of mice developing STZ-induced diabetes

STZ was administered in multiple low doses (40 mg/kg) and blood glucose levels gradually increased from 200 mg/dl to values of more than 300 mg/dl over a time interval of 3 weeks (Figure 1). When serum gelatinase B levels were determined by zymography analysis, a number of observations were made. During the induction phase of the disease, ie on the 5 consecutive days of STZ injection, no changes in serum

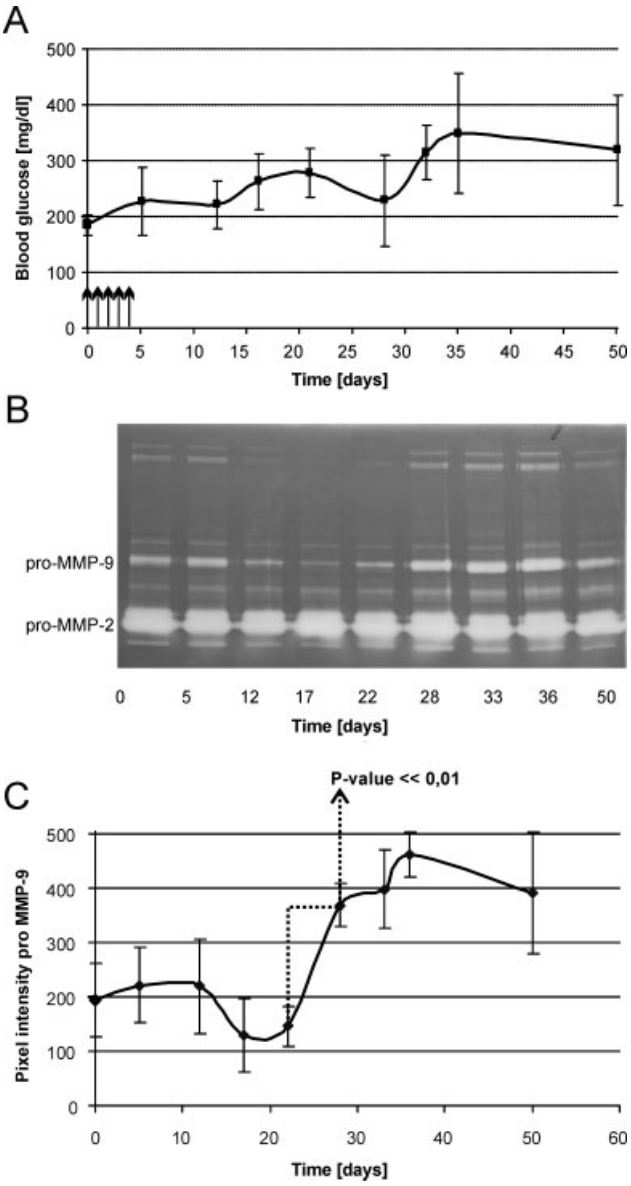

Figure 1. Levels of gelatinase B in serum of mice ($n = 10$) that had been injected with multiple low doses of streptozotocin (MLD-STZ). (A) Blood glucose levels plotted as means ± SD as a function of time. Arrows indicate consecutive STZ challenges. (B) Gelatin zymography analysis of serum samples at different time intervals during the development of diabetes. The zymogram is representative for five independent analyses of different treated animals. (C) Densitometry analysis of progelatinase B, plotted as mean pixel intensities ± SD as a function of time, shows that MLD-STZ stimulated a significant increase of gelatinase B ($p << 0.01$), when hyperglycaemia took off around day 28

gelatinase levels versus controls were detected. During the next 20 days (days 5–25) the glycaemia did not alter significantly and the serum gelatinase B had the tendency to level off. In the next phase, when hyperglycaemia took off, gelatinase B levels in the serum increased significantly until 30 days after induction and then gradually decreased to reach normal levels by 50 days post-induction, a time point after which the glycaemia levels also plateaued. Even with this association between disease activity and changes in serum gelatinase B levels, it was not clear what was cause or effect. If gelatinase B changes caused the increase of glycaemia levels, then gelatinase B-deficient mice should present with a phenotype of protection against diabetes development. Therefore, we induced subacute diabetes as described above with five consecutive challenges of 40 mg/kg STZ and compared adult $Mmp9^{+/+}$ with $Mmp9^{-/-}$ mice. As shown in Figure 2, no significant differences in blood glucose levels and diabetes development were observed between $Mmp9^{-/-}$ and $Mmp9^{+/+}$ mice.

Gelatinase B plays a role in diabetes development in hyperacute STZ-induced pancreatitis in mice

In a second approach, we investigated diabetes development by a single high-dose bolus injection of STZ in mice. In this model, a more acute form of insulitis is induced than in the subacute model with repetitive lower doses of STZ. Figure 3 shows the systemic effects on blood glucose and serum gelatinase B levels, as well as the local effects in pancreas tissue. As expected, blood glucose levels increased after 48 h to values exceeding 400 mg/dl. Importantly, during the latent induction phase, ie during the first 24 h, the production of gelatinase B peaked within the pancreas at 8 h after induction with STZ and thus preceded the serum hyperglycaemia. Second, increased pancreas gelatinase B levels were maintained during the exponential and plateau phases of the hyperglycaemia. As a control, we also evaluated gelatinase A-levels, which remained constitutive (data not shown). Serum gelatinase B levels followed the same production pattern as local levels in pancreas tissue. The source of gelatinase B could be endogenous production by pancreas cells or release from infiltrating leucocytes. To distinguish between these two possibilities, we established hyperacute diabetes in mice made leucopenic with cyclophosphamide and found that gelatinase B was no longer upregulated. This observation is in line with the thesis that leucocytes are the source of gelatinase B in pancreatic tissue and serum of diabetic animals. We also compared the development of diabetes in

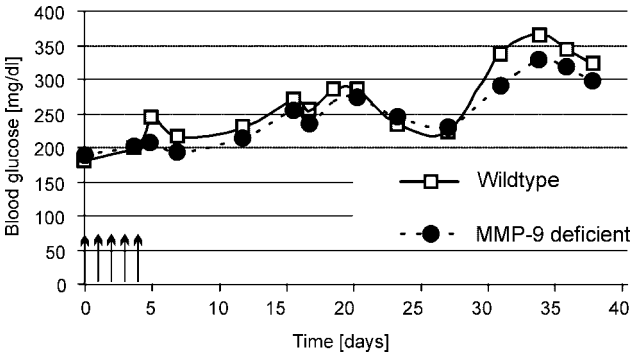

Figure 2. Blood glucose levels of $Mmp9^{+/+}$ ($n = 6$) and $Mmp9^{-/-}$ ($n = 6$) mice injected with MLD-STZ. Arrows indicate STZ challenges. No significant differences in blood glucose levels and diabetes development were observed

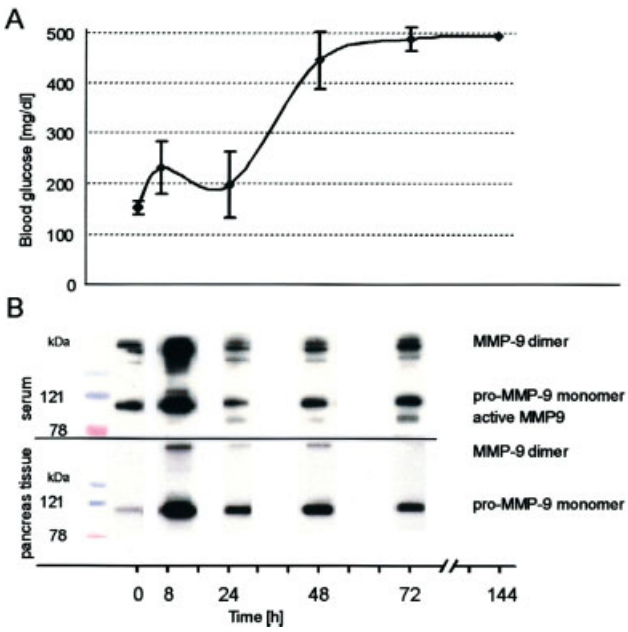

Figure 3. Levels of gelatinase B in serum and pancreas tissue of mice ($n = 10$) that had been injected with a single bolus of STZ. (A) Blood glucose levels plotted as means ± SD as a function of time. (B) Gelatinase B in serum samples and pancreas tissue extracts at different time intervals after STZ treatment, as detected by western blot analysis with a monoclonal antibody against mouse gelatinase B. Data are representative of at least five independent analyses of different treated animals. The production of gelatinase B peaked within the pancreas at 8 h post induction with STZ. Molecular marker proteins are indicated in kilodaltons and various forms of MMP-9 are defined

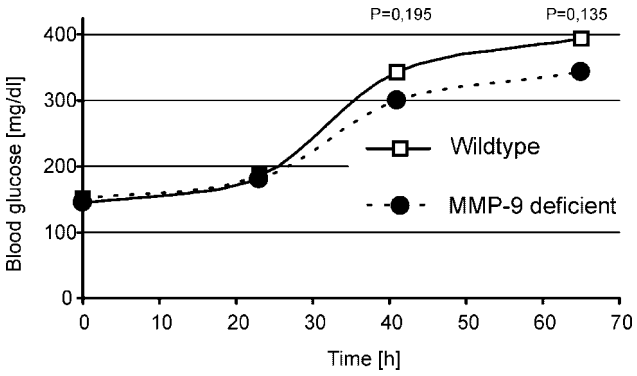

Figure 4. Blood glucose levels of $Mmp9^{+/+}$ ($n = 34$) and $Mmp9^{-/-}$ ($n = 35$) mice injected with a single bolus of STZ. Hyperglycaemia in MMP-$9^{-/-}$ mice develops more slowly and reaches a plateau level that is lower than that of MMP-$9^{+/+}$ mice. *p*-Values, calculated using the unpaired Student's *t*-test, do not indicate significant differences between $Mmp9^{+/+}$- and $Mmp9^{-/-}$ mice

this hyperacute model with single-bolus STZ induction in $Mmp9^{+/+}$ versus $Mmp9^{-/-}$ mice. Figure 4 documents the trend that in $Mmp9^{-/-}$ mice the hyperglycaemia develops slower and reaches a plateau level that is lower than that of $Mmp9^{+/+}$ mice but differences were not significant at any time point during the disease course, though a considerable number of animals were tested.

Destruction of β-cells in hyperacute STZ-treated mice is mediated by apoptosis and necrosis

Gelatinase B has recently been pinpointed to induce apoptosis of endothelial cells in diabetes [13]. To investigate whether gelatinase B is an apoptotic factor for pancreatic β-cells, we investigated apoptosis and necrosis of β-cells in the islets of Langerhans in $Mmp9^{-/-}$ and $Mmp9^{+/+}$ mice that were treated with STZ. On the basis that gelatinase B expression in the pancreas is more pronounced in mice treated with a single high dose of STZ and that gelatinase B ablation has more impact on glucose levels for this model in comparison to the MLD-STZ model, we focused on the acute model. Pancreas samples were collected 8 h after STZ treatment, ie when peak levels of gelatinase B were reached. Under light microscopy, islets almost entirely depleted of endocrine cells and/or islets with apoptotic islet cells identifiable by the presence of pyknotic nuclei were recognized (Figure 5). For the $Mmp9^{-/-}$ and $Mmp9^{+/+}$ mice, no difference in islet morphology was observed. The involvement of the apoptotic pathway in the damaged cells was supported by immunohistochemical staining analysis with an antibody directed against mouse caspase-3. Pancreas sections from STZ-treated mice stained positive for caspase-3 and cellular immunoreactivity predominantly coincided with pyknosis of the islet β-cell nuclei. No appreciable difference in caspase-3 immunoreactivity was observed between $Mmp9^{-/-}$ and $Mmp9^{+/+}$ mice.

Acute pancreatitis with involvement of the exocrine pancreas leads to severe diabetes

Since we could not observe a significant general effect of gelatinase B ablation on glycaemia levels, despite obvious correlations between disease activity and gelatinase B levels, we presumed that upregulated gelatinase B was insufficiently activated in the endocrine pancreas to be effective. Indeed, the majority of hyperacute diabetic mice (17/20) had a gelatinase B pattern as represented above, with high upregulation of progelatinase B 8 h post-induction, but relatively low levels of active gelatinase B. Interestingly, we did observe active gelatinase B in a subgroup of hyperacute diabetic mice (3/20) by zymography and western blot analysis. In addition, this observation paralleled with signs of pancreatitis as reflected by local trypsinogen activation and degradation (Figure 6). In addition, we also observed, to a lesser extent, that gelatinase A/MMP-2 had been activated. The simultaneous occurrence of trypsin is in line with the finding of trypsin to be the activator of progelatinase B. Indeed, trypsin has previously been described as a potent activator of progelatinase B *in vitro* [19]. In fact, the present data show, for the first time, activation of pro-MMP-9 and pro-MMP-2 by endogenous trypsin *in vivo*. In order to reinforce these data, we induced systematic activation of trypsinogen in the pancreas

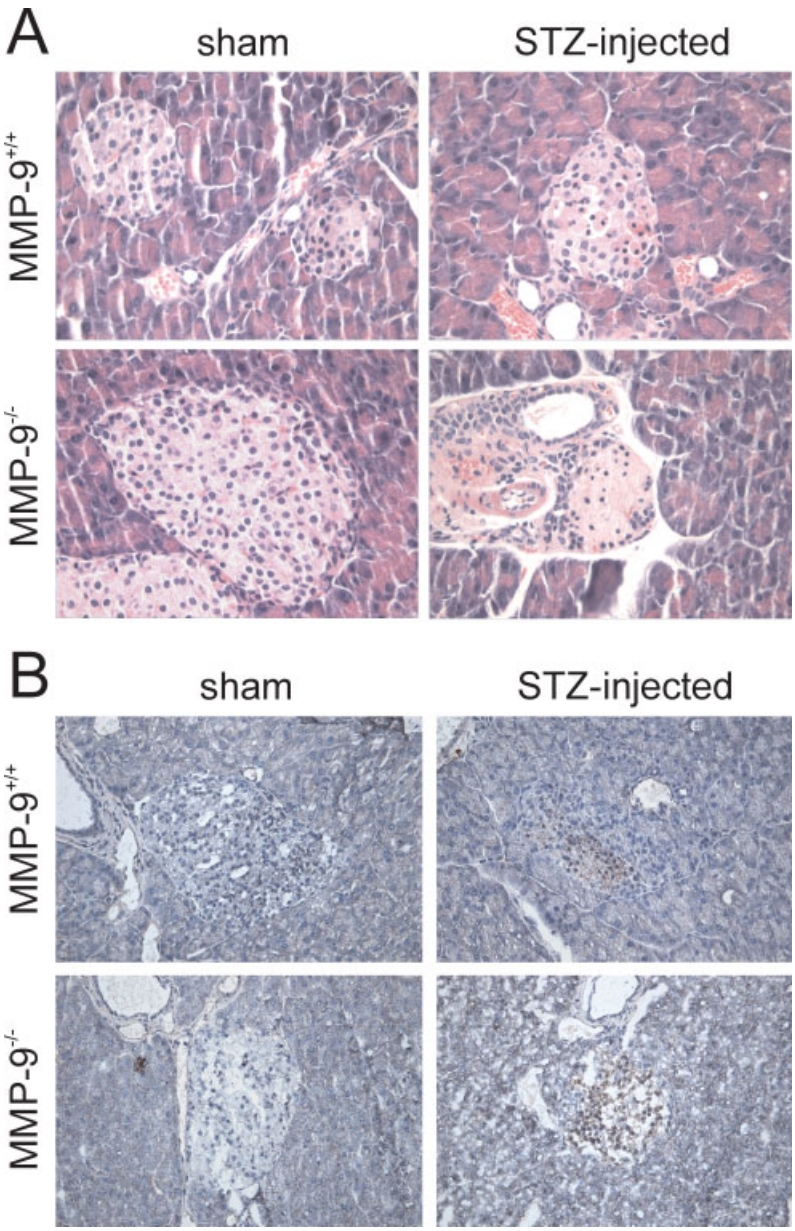

Figure 5. Apoptosis and necrosis in $Mmp9^{+/+}$ and $Mmp9^{-/-}$ mice treated with a single high bolus of STZ. (A) Pancreas sections of $Mmp9^{+/+}$ and $Mmp9^{-/-}$ mice that were collected 8 h after STZ treatment in addition to sections from corresponding sham-injected animals. Pyknotic cell nuclei of STZ-treated animals indicate necrotic cell death. (B) Anti-caspase-3 immunohistochemistry of parallel pancreas sections. Positive staining in the islets of STZ-treated animals indicates involvement of apoptosis as a mode of pancreatic β-cell death

with an established model of caerulein-induced pancreatitis in mice.

Six-week-old mice ($n = 10$) received six hourly doses of caerulein (50 µg/kg per hour) or saline ($n = 4$). Five caerulein-injected and one saline-injected mouse were sacrificed 1 h after the last injection (Figure 7, left panel). Serum amylase levels were 5201 IU/L ± 174 in caerulein-treated mice and 385 IU/L in the saline-injected mouse. Gelatinase B levels in pancreas homogenates were highly upregulated in comparison with saline-injected mice, but no activation was detected at this time point. To enhance the ongoing pancreatic inflammation, three of the remaining caerulein-treated mice and two control mice received a single dose of LPS, 1 h after the last caerulein injection (2.5 mg/kg) [15]. All mice (Figure 7, right panel) were sacrificed 30 h after the first caerulein injection. Serum amylase levels in mice that received six caerulein injections and one LPS injection were 3016 IU/L ± 630 at this time point. Zymography analysis revealed that LPS alone induced, as expected, gelatinase B and that gelatinase B was most upregulated in the group treated with caerulein and LPS. Importantly, in the animals with severe pancreatitis after caerulein plus LPS, gelatinase B was clearly present in its active form. The activation of progelatinase B in the caerulein-induced pancreatitis model demonstrates independently that the mechanism of progelatinase B activation by endogenous trypsin is relevant *in vivo*.

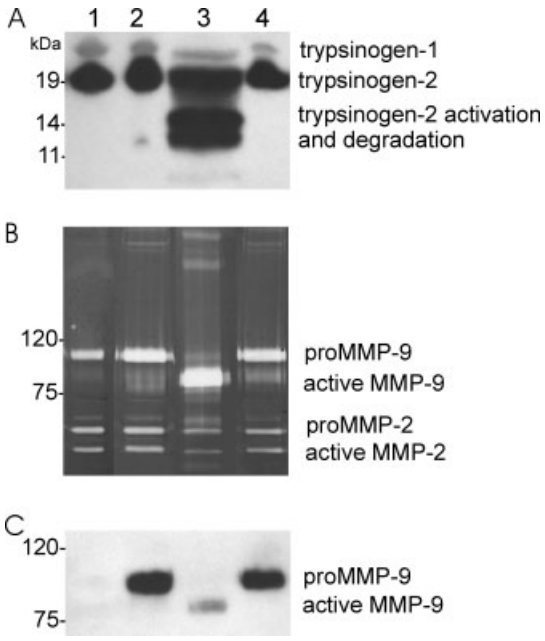

Figure 6. *In vivo* activation of progelatinase B by active trypsin. (A) Western blot analysis with a polyclonal antibody against mouse trypsin shows trypsinogen-2 activation and degradation in pancreas extract from animals that developed severe pancreatitis 8 h after STZ induction (lane 3, representative of 3 cases). Trypsinogen-1 and trypsinogen-2 remain intact in animals that were not treated with STZ (lane 1) and in animals that did not develop pancreatitis 8 h after STZ treatment (lane 2 and 4, representative of 17 animals). (B) Gelatin zymography analysis of corresponding pancreas extracts. Lane 3 shows almost complete activation of progelatinase B and partial activation of progelatinase A. (C) Western blot analysis of pancreas extracts with a monoclonal antibody against mouse gelatinase B demonstrates the complete conversion of progelatinase B into its active form. Samples correspond to those of panels A and B

Discussion

Our study demonstrates alterations in the expression levels of gelatinase B in serum and pancreas tissue of mice with STZ-induced diabetes. When multiple low doses of STZ were applied, the increase in gelatinase B levels in the serum coincided with development of hyperglycaemia [11]. Gelatinase B was previously suggested to aggravate hyperglycaemia by cleaving insulin. However, the insignificant effect of genetic gelatinase B ablation on diabetes development argues against a crucial role for gelatinase B as a cause of STZ-induced diabetes. Obviously, the degradation of insulin by gelatinase B is not a central cause of diabetes in general, certainly not in streptozotocin-induced diabetes, for which it has been shown that streptozotocin has a deleterious effect on pancreatic β-cells by apoptosis and necrosis pathways. Importantly, the toxic effect of STZ was equally observed in gelatinase B knockout and wild-type mice. Since we could only observe a marginal protection by gelatinase B deficiency, we conclude that insulin degradation was insufficient in wild-type mice with STZ-induced diabetes to really affect glucose levels. In fact, this outcome was not really surprising, in view of the relative lack of active gelatinase B in pancreatic tissue of STZ-treated animals.

In contrast to the lack of gelatinase B activity in pancreatic tissue of STZ-treated animals, we have demonstrated for the first time that gelatinase B can be efficiently activated by endogenous trypsin, which is present in the pancreas in cases of pancreatitis. A recent publication on the role of exogenously administered trypsin on progelatinase B activation in a rat ischaemia–reperfusion model is in line with our observations [20]. Initially, trypsin was described as a potent activator of progelatinase B *in vitro* [19]. In fact, the involvement of exocrine pancreatic enzymes in diabetes is not unusual. In acute pancreatitis, temporary hyperglycaemia can be observed in about 50% of the patients and persisting diabetes might affect 1–15% [21,22]. In chronic pancreatitis about 60% of patients are reported to have diabetes, of whom 30% are insulin-dependent [23]. Another study reported abnormal circulating pancreatic enzyme activities in more than 25% of recent-onset insulin-dependent diabetic patients [24]. In line with these data, a novel subtype of type I diabetes has recently been characterized by high serum pancreatic enzyme concentrations and a remarkably abrupt onset [25]. On the basis of the latter reports and our *in vivo* observations with the

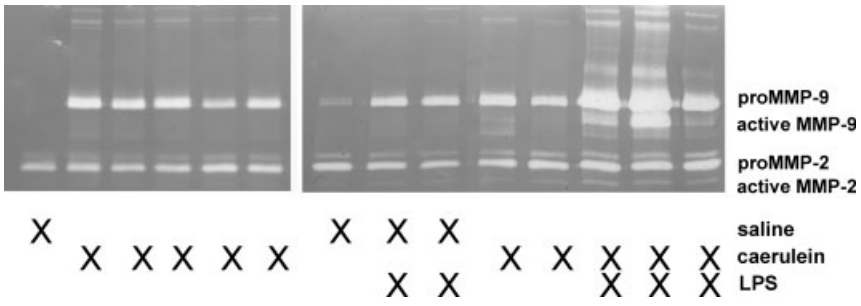

Figure 7. Activation of progelatinase B in caerulein-induced pancreatitis. The experimental design and treatment of each individual animal are tabulated below the two panels of the figure. Saline and caerulein injections were administered as six hourly doses from the start of the experiment. LPS was administered as a single dose 1 hour after the last caerulein or saline injection. Left panel: samples taken 6 h after the initial saline or caerulein injection. Right panel: samples taken 30 h after the initial saline or caerulein injection. Whereas caerulein and LPS (right panel) independently induce progelatinase B, the combination of both results in acute pancreatitis with concomitant *in vivo* activation of gelatinase B

acute STZ-induced diabetes model and the caerulein-induced pancreatitis model, we document in mice, and propose, that exocrine pancreas enzymes may play an exacerbating role in diabetes development through activation of gelatinase B.

Finally, we want to underscore that active gelatinase B may also have indirect effects on the diabetic process in acute pancreatitis, apart from insulin degradation, which results in a direct hyperglycaemic effect. Active gelatinase B is indeed a key mediator for the inflammatory process. This is exemplified by its capability to potentiate chemokines like IL-8 or to assist in the passage of leucocytes that carry along other insulin-degrading enzymes [26].

Acknowledgements

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