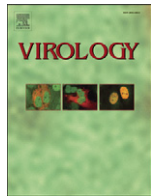




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## Increased gelatinase B/matrix metalloproteinase 9 (MMP-9) activity in a murine model of acute coxsackievirus B4-induced pancreatitis

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### ABSTRACT

Infection of mice with coxsackievirus B4 results within days in a severe acute necrotizing pancreatitis, which resolves completely within weeks. Gelatinase B or matrix metalloproteinase 9 (MMP-9) has previously been shown to be involved in several models of pancreatitis, but its role in virus-induced pancreatitis has never been investigated. We here report that MMP-9 levels are markedly increased in the pancreas of mice that developed acute pancreatitis following infection with coxsackievirus B4. Moreover, using *in situ* zymography, we demonstrated that MMP-9 is active *in vivo*. Double immunohistochemical analysis revealed that macrophages and neutrophils were the cellular source of MMP-9. Extensive tissue rearrangements involving collagen turnover were observed, and these were associated with extensive pathology and resolution of the disease. In summary, this report demonstrates that acute coxsackievirus B4-induced pancreatitis involves the action of MMP-9, which is mainly originating from macrophages and neutrophils.

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### Introduction

Coxsackievirus B4 (CVB4) belongs to the genus enterovirus within the family of the Picornaviridae (Minor et al. 2000). Usually, infections with coxsackieviruses remain asymptomatic or mild. Occasionally however, these viruses can spread to secondary target organs and lead to more serious or even life-threatening diseases (Sawyer 2002). In some instances, CVB4 infects the pancreas, which may result in a severe acute pancreatitis. The processes that underlie this pathology are not well understood, but several mechanisms have been proposed to be involved, including tissue injury as a direct result of viral infection (Huber and Ramsingh 2004; Ramsingh 1997). However, also the contribution of immune-mediated mechanisms appears to be substantial (Huber and Ramsingh 2004; Ramsingh et al. 1999). Matrix metalloproteinases (MMPs) are zinc-containing enzymes that are implicated in degradation and remodeling of components of the extracellular matrix. Increased levels of MMPs, and in particular of MMP-9 (gelatinase B), have been associated with several pathological

inflammatory conditions, including multiple sclerosis, rheumatoid arthritis, diabetes and myocarditis (Opdenakker et al. 2001b; Opdenakker et al. 2003; Van den Steen et al. 2002). It was also shown that MMP-9 is involved in the course of acute and chronic pancreatitis in several experimental rat and mouse models, and that the levels of MMP-9 correlate with the degree of disease severity (Descamps et al. 2004; Keck et al. 2002, 2006; Muhs et al. 2001). The involvement of MMP-9 was, however, never studied during the course of virus-induced pancreatitis. We employed a murine model to investigate whether MMP-9 is present and involved in CVB4-induced pancreatitis.

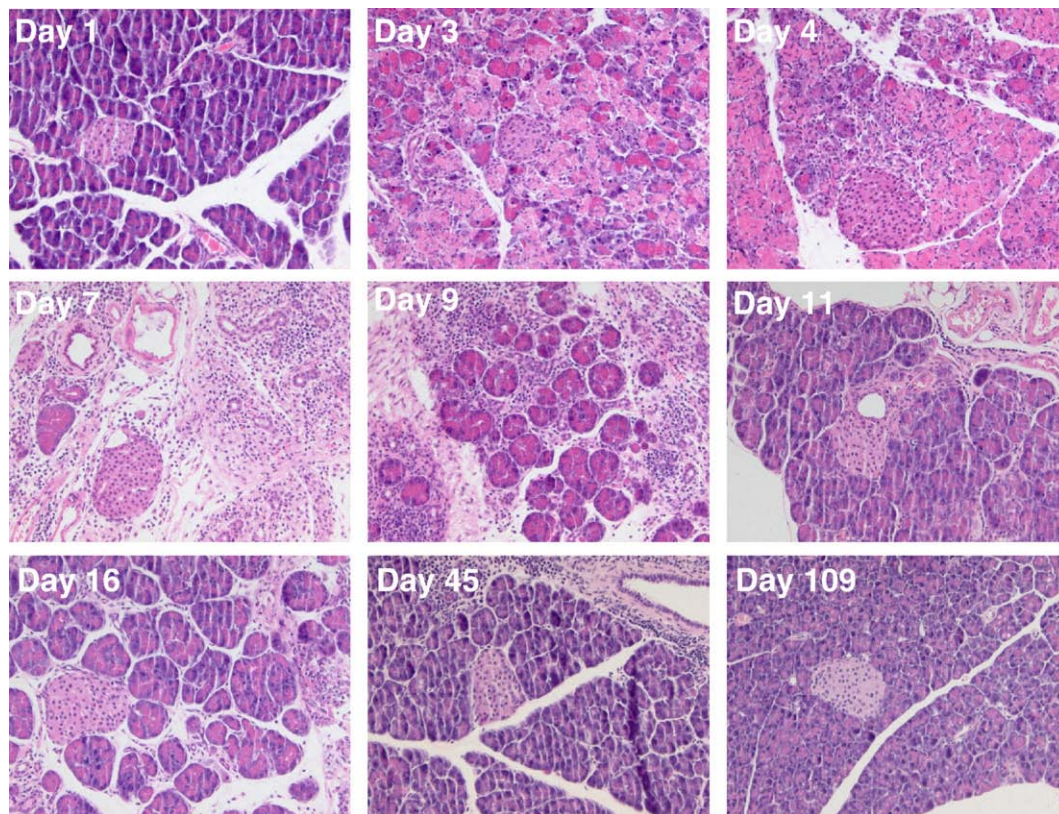
### Results

#### Histopathological analysis of CVB4-induced pancreatitis

Male SJL mice were inoculated intraperitoneally with  $1.2 \times 10^5$  PFU of coxsackievirus B4 strain E2. As early as 2 days post infection, an infiltrate consisting mainly of neutrophils and macrophages was detected in the exocrine pancreas. Around 5 days p.i. infiltration of mononuclear cells was predominated by lymphocytes, whereas neutrophils gradually

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**Fig. 1.** Histopathology of CVB4-induced pancreatitis. Mice were infected i.p. with  $2.5 \times 10^4$  PFU of CVB4 and sacrificed at the indicated times post infection. Tissue was fixed in formalin, embedded in paraffin and tissue sections were stained with H&E. (200 $\times$  magnification).

disappeared. As of 7–8 days p.i. resolution of the infiltrate started to be observed. At 2–3 days p.i. histological damage became detectable in the acinar tissue. The damage was characterized by necrosis, inflammation, edema and steatonecrosis (Fig. 1). Only the exocrine pancreas was affected, whereas the islets of Langerhans remained, as was earlier reported, largely spared (Bopegamage et al. 2005; Yap et al. 2003). Tissue injury was most pronounced at  $\sim 7$  days p.i. after which a fast and virtual complete regeneration of the exocrine pancreas was noted. By 2 weeks p. i. most of the infected pancreata were histologically indistinguishable from those of uninfected control animals. Control samples at 45 and 109 days p.i. revealed normal pancreas tissue (Fig. 1).

#### *Kinetic analysis of biochemical parameters and virus titers*

Serum lipase and amylase are standard markers for the severity of acute pancreatitis (Frossard et al. 2008). Following infection of mice with CVB4, serum amylase levels increased suddenly, peaking at 2 days p.i., and were more than 3-fold higher than levels in control animals. By 4 days p.i. serum amylase levels had returned to baseline (Fig. 2A). A similar pattern was observed for serum lipase with a 10-fold increase at 2 days p.i. (Fig. 2B). Next, virus titers were quantified in infected pancreata by means of real-time RT-qPCR. Viral RNA titers reached a maximum at 2 days p.i. after which they declined gradually. By 5 days p.i. less than 1 copy of viral RNA was present per copy of beta-actin RNA (Fig. 2C). As enteroviruses may result in persistent infections (without production of infectious virus), we next wanted to examine whether the detected viral RNA originated from infectious virus in the pancreas. The pattern of appearance and disappearance of infectious virus titers in the pancreas proved comparable to that of viral RNA, with titers peaking at 2 days p.i. By 7 days p.i. all infectious virus had been cleared from the pancreas (Fig. 2D). Finally, viral kinetics in the serum of infected mice was monitored. Again, a similar

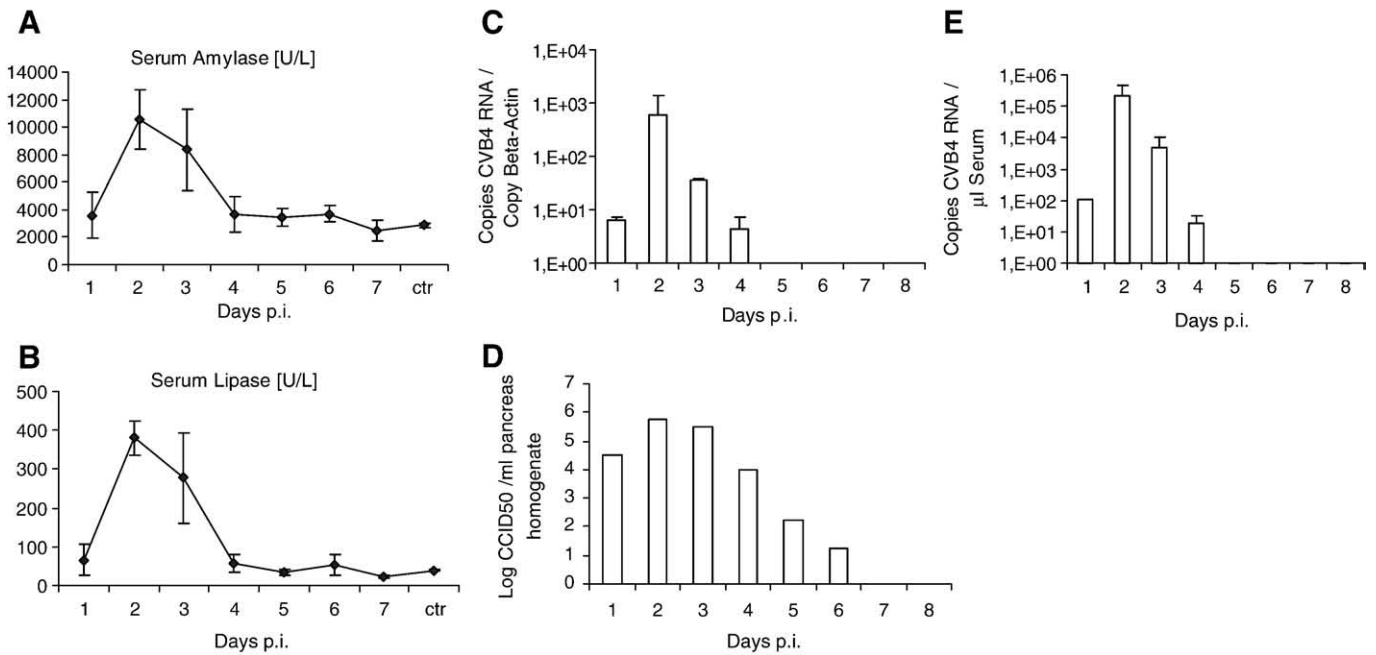
pattern was observed with peak viral titers at 2 days p.i. and very low levels (1 copy of viral RNA per  $\mu$ l of serum) beyond 5 days p.i. (Fig. 2E).

#### *Quantification of gelatinase activity in CVB4-infected pancreata*

MMPs have been reported to be involved in inflammatory diseases of connective tissues and levels of the enzyme have been shown to be increased in non-infectious pancreatitis (Descamps et al. 2004; Muhs et al. 2001). To investigate whether also virus (in this case CVB4)-induced pancreatitis is associated with increased gelatinase activity, gelatin zymography was performed on pancreas tissue homogenates. As is evident from Fig. 3A, gelatinase levels were markedly increased at 3 days p.i. as compared to uninfected controls. The 70 kDa band represents the presence of MMP-2 (constitutively expressed gelatinase A). The band at higher molecular weight reflects gelatinase B (MMP-9). As with other examples of inflammatory diseases, gelatinase A (MMP-2) remained unaffected, whereas gelatinase B (MMP-9) levels were increased considerably during the inflammation phase (Opdenakker et al. 2001a,b). Quantification of MMP-9/MMP-2 levels indicated that gelatinase B was markedly increased at 2 and 3 days p.i. after which levels returned to baseline by 6 days p.i.

#### *Detection of in vivo activated MMP-9 in the pancreas*

Next, the presence and the activity of gelatinase B in pancreas tissue sections of infected animals were monitored. Immunohistochemical staining (Fig. 4A) confirmed the presence of MMP-9 in the exocrine pancreas, revealing a “spotty” localization of the enzyme. MMP-9 levels appeared to be maximal at 2 and 3 days p.i. and decreased gradually thereafter. To demonstrate not only the presence but also the in vivo activity of gelatinase, in situ zymography was performed on infected tissue sections and this corroborated the previous data (Fig. 4B).



**Fig. 2.** Kinetic parameters of CVB4-induced pancreatitis. Mice ( $n=3$ ) were infected with  $1.2 \times 10^5$  PFU of CVB4. Serum was collected from all animals and amylase (A) and lipase (B) were quantified using an enzymatic colorimetric assay at the indicated times post infection. The presence of virus in the pancreas was measured using quantification of viral RNA copies by means of real-time RT-qPCR (C) or infectious virus by endpoint titration of tissue homogenates on confluent cell cultures (D). Serum titers of virus are presented in (E). Data represent averages  $\pm$  SD.

*Identification of the cellular source of MMP-9*

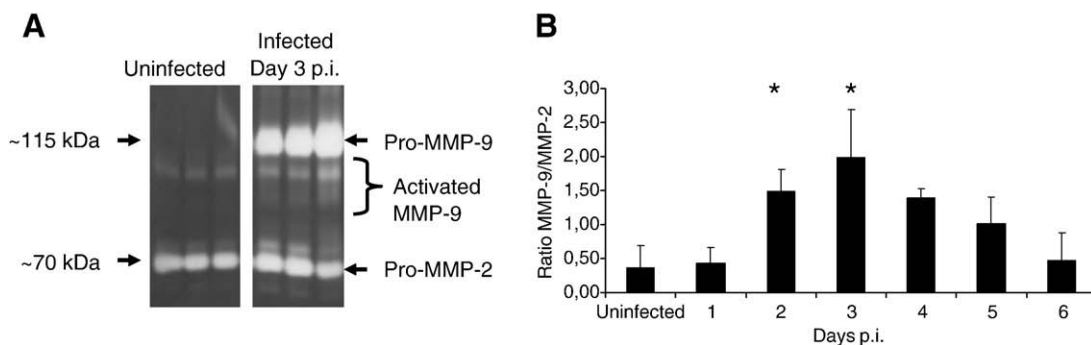
In order to determine the cellular source of MMP-9, a dual immunohistochemical analysis was performed combining MMP-9 with a marker for either T cells, B cells, macrophages or neutrophils. As illustrated in Figs. 5A and D, single stainings for macrophage and neutrophil markers were mainly membranous and linear, whereas the presence of MMP-9 was detected intracellularly by a diffuse and cytoplasmic staining of macrophages and an intense overall staining of neutrophils (Figs. 5B and E). Double immunohistochemistry revealed the presence of MMP-9 in macrophages and in neutrophils (Figs. 5C and F). No MMP-9 was detected originating from T cells or B cells (data not shown).

*Analysis of extracellular matrix components*

During the course of the viral pancreatitis, extensive rearrangements in the extracellular matrix (ECM) were observed. Therefore, two major ECM proteins, collagen and laminin, were examined more closely as a function of time. As is evident from Fig. 6 at 1 day p.i.,

collagen levels were almost undetectable in the exocrine tissue but were visualized perivascularly (Sirius red staining). At later time points however, a gradual increase in collagen synthesis was observed, reaching maximum intensity at 7 days p.i. (Fig. 6). Thereafter, as regeneration of the exocrine tissue took place, the collagen was gradually degraded and returned to baseline levels (at 21 days p.i. in Fig. 6).

In normal pancreatic tissue, laminin is present as a major component of the basement membrane, surrounding one or several acini in the exocrine tissue (day 1 in Fig. 6). As the inflammation proceeded and necrosis of the exocrine tissue became histologically apparent, it was observed that the laminin structures remained present, albeit in a more loosely organized fashion (day 7 Fig. 6). As depopulation of the acini proceeded due to cell necrosis, the disappearing cells led to a “collapse” of the basement membrane, although the contours remained preserved (day 11 Fig. 6). By day 21 p.i. when regeneration of the pancreas was essentially complete, the “collapsed” structures had been repopulated with new acinar cells and were reshaped to their original constitution, albeit in a less well organized form.

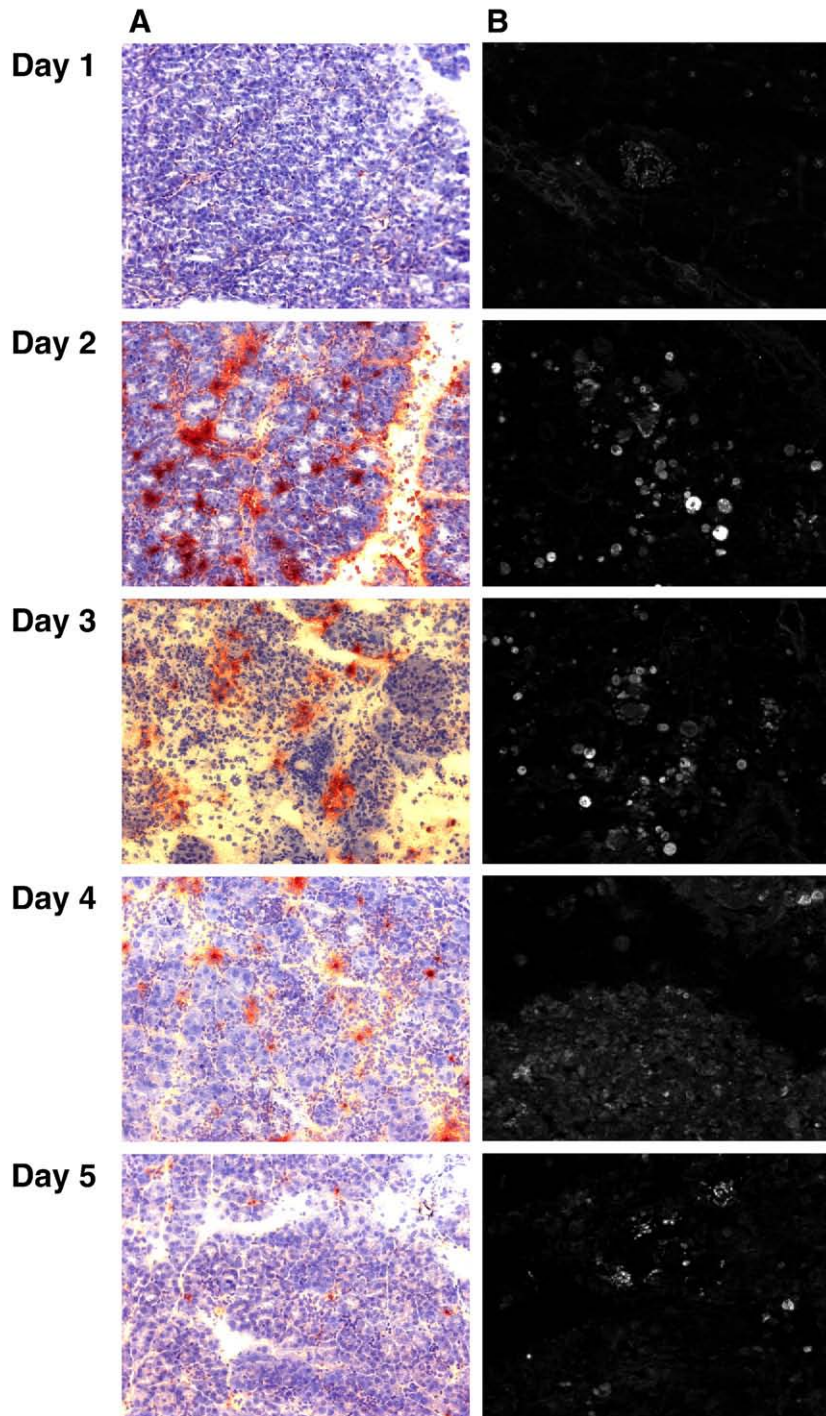


**Fig. 3.** Levels of MMP-9 and MMP-2 in pancreas of CVB4-infected mice. The presence of gelatinases in pancreas homogenates (5% w/v) from 3 mice was detected using gelatin zymography (A). The levels of gelatinase B (MMP-9) were quantified relatively to the constitutively present gelatinase A (MMP-2) and expressed as a ratio (B). Bars represent averages  $\pm$  SD (\*)  $P < 0.05$ .

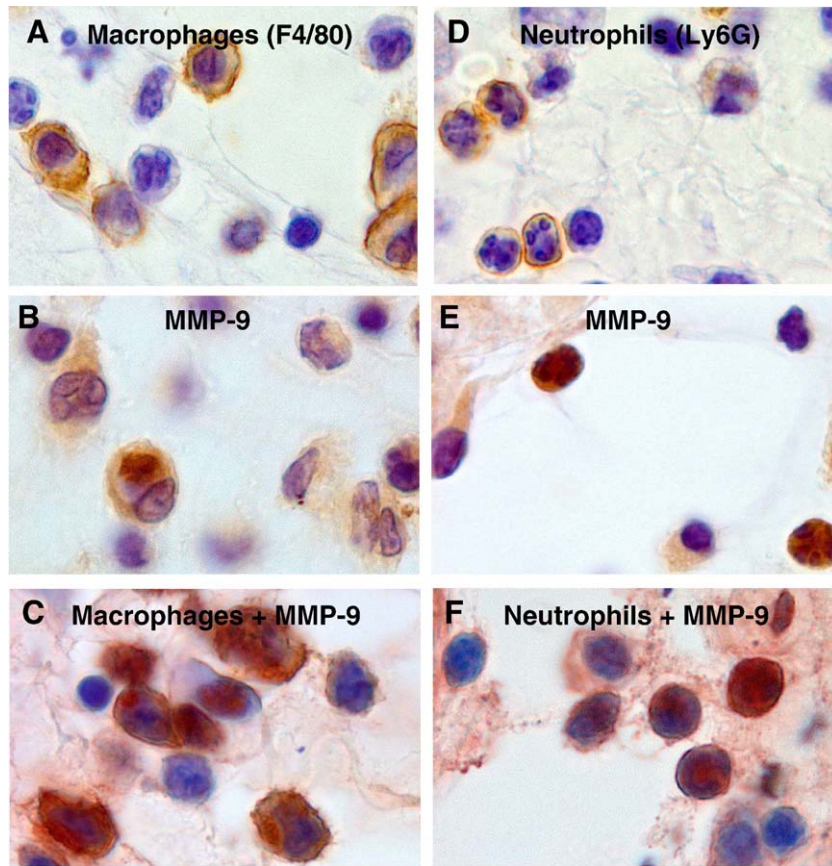
## Discussion

Acute pancreatitis is an inflammatory disease of the abdomen, affecting about 0.01% of the population in Western countries (Steinberg and Tenner 1994). The most common causes of acute pancreatitis in humans are alcohol abuse and gallstone migration into the common bile duct. Another 15–25% of all cases however, remain of unknown origin (Frossard et al. 2008). Viruses have been considered as etiological agents for acute pancreatitis of unknown origin and in

particular coxsackievirus B4 (CVB4) has often been used in models of experimental viral pancreatitis (Ramsingh 1997). Infection of mice with CVB4 results in a severe acute necrotizing pancreatitis, but the exact mechanisms that contribute to disease severity remain elusive. The role of T cells and direct virus-mediated effects in the pathogenesis of viral pancreatitis have been studied extensively (Huber and Ramsingh 2004; Ramsingh et al. 1999), whereas a role for perforin-mediated cytotoxicity by natural killer cells was excluded (Mena et al. 2000).



**Fig. 4.** Detection of MMP-9 by immunohistochemical staining and in situ zymography. The presence of gelatinase B was detected by immunohistochemical analysis (red staining) on H&E-counterstained tissue sections from mice infected for the indicated time intervals (A) (20× magnification). Parallel cryosections were processed for in situ gelatinolysis with a quenched fluorogenic substrate (B) (200× magnification). Gelatinolytic activity is indicated by fluorescence on a dark background. The overall gelatinase levels in panel B are paralleled by the red staining in panel A.



**Fig. 5.** Identification of the cellular source of MMP-9. The source of MMP-9 was determined by double immunohistochemical staining for MMP-9 and either macrophages or neutrophils. Single membranous staining of macrophages for F4/80 and neutrophils for Ly6G (brown in panels A and D). Single cytoplasmic staining of MMP-9 in either macrophages or neutrophils (brown in panels B and E). Double immunohistochemical staining of MMP-9 (red cytoplasm) and either macrophages or neutrophils (brown membranes) (panels C and F).

We employed a mouse model of CVB4-induced pancreatitis to assess several parameters that are associated with disease development. Histological damage appeared as soon as 3 days p.i. and peaked around 7 days p.i. In line with earlier reports, tissue injury remained restricted to the exocrine parenchyma, while sparing the islets of Langerhans (Bopegamage et al. 2005; Vella et al. 1992; Yap et al. 2003). Histopathologically, necrotizing acinar cells, edema, inflammation, steatonecrosis and an inflammatory infiltrate rich in neutrophils, macrophages and lymphocytes were observed in the exocrine tissue. Immediately after the acute phase, a spectacular regeneration of the pancreas was noticed and complete repair of the pancreas was observed within 2 weeks p.i. which is also in line with previous findings (Huber and Ramsingh 2004). The mechanisms underlying this pancreatic regeneration are yet not completely understood, but two major views are currently supported. One possible explanation is that precursor cells in the pancreatic duct system can differentiate into mature pancreatic cells, whereas other studies support the idea that each differentiated cell type in the pancreas is able to rapidly restore its own population after cell loss (Huber and Ramsingh 2004).

Serum lipase and amylase were evaluated as typical markers for pancreatitis. Their levels were found to be increased, respectively, more than 10-fold (lipase) and more than 3-fold (amylase) at day 2 p.i. and levels returned to baseline by day 4 p.i. Virus titers were quantified in pancreas tissue and in serum and were also found to peak 2 days p.i. One week after infection, all infectious virus in the pancreas had dropped to undetectable levels.

Recently, matrix metalloproteinases (MMPs), and in particular MMP-9 (gelatinase B), have gained much attention and have been

shown to be involved in a multitude of processes, both pathological and non-pathological (Opdenakker et al. 2001a,b). The role of MMP-9 in a model of viral pancreatitis was, however, never addressed before.

To study whether MMP-9 was upregulated in the pancreas following infection with CVB4, purified pancreas homogenates were analyzed by means of gel zymography. This revealed that MMP-9 levels were markedly increased and peaked at 2 days p.i. after which the levels returned to baseline within days. By means of immunohistochemical staining, the presence of MMP-9 was confirmed and appeared in “spot-like” shapes throughout the exocrine tissue. In situ zymography confirmed the biological activity of MMP-9 in the infected pancreas. Double immunohistochemical staining analysis revealed that macrophages and neutrophils were the major cell types responsible for the increased levels of MMP-9. By contrast, T and B lymphocytes did not appear to be a cellular source of MMP-9.

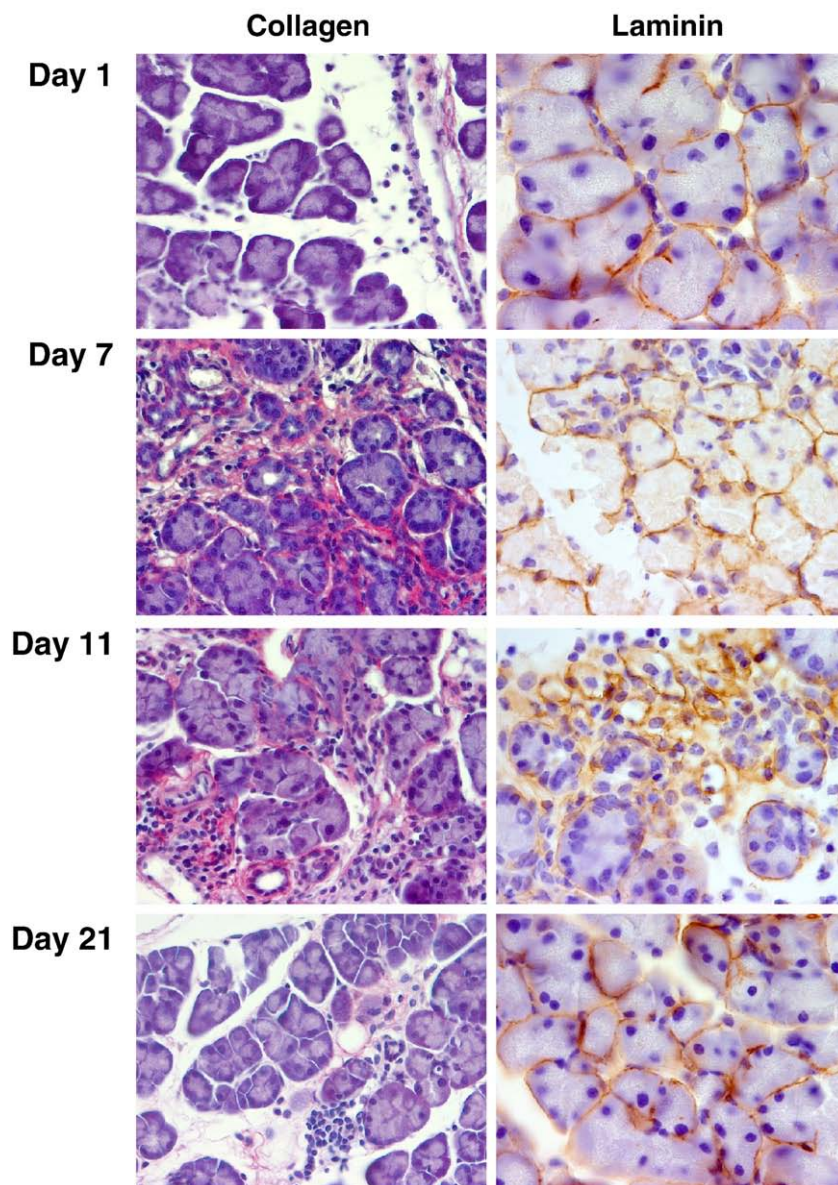
Despite the fact that this study clearly provides evidence that MMP-9 is upregulated in correlation with histopathological damage by the virus, the exact function of this enzyme in CVB4-induced pancreatitis remains to be studied. Other studies on the role of MMP-9 in non-viral pancreatitis have convincingly demonstrated that elevated levels of MMP-9 correlate with the severity of disease, or that reducing MMP-9 expression attenuates disease severity (Aynaci et al. 2006; Chen et al. 2006; Keck et al. 2002). Similarly, in a model of CVB3-induced myocarditis, MMP-9 expression has been shown to be associated with cardiac injury (Heymans et al. 2006), although these findings were recently challenged by a study using MMP-9 knock-out mice (Cheung et al. 2008).

A common feature of MMPs is their potential to extensively remodel the extracellular matrix (ECM) (Visse and Nagase 2003). We

analyzed laminin and collagen, two major components of the ECM. Pronounced collagen synthesis started several days after infection and was maximal when exocrine tissue damage was most extensive. As tissue regeneration proceeded, collagen was degraded, and eventually returned to normal levels. These observations are in sharp contrast with the events that take place when pancreatitis progresses to chronic pancreatic fibrosis. In the latter case, collagen synthesis is not followed by a turnover of the protein and hence generates fibrous tissue (Apte et al. 1999). An important role for the regulation of collagen (and, more generally, of ECM) homeostasis has recently been attributed to TGF- $\beta$ 1 (Shek et al. 2002). By inhibiting the expression of MMPs that normally degrade collagens, TGF- $\beta$ 1 can enhance the formation of fibrous tissue (Shek et al. 2002). As in our observations collagen is degraded rapidly after its synthesis, one might postulate that TGF- $\beta$ 1 is in this case not precluding the collagenolytic activity of MMP-9. The precise function of TGF- $\beta$ 1 in this model of virus-induced pancreatitis remains however to be studied.

In contrast to collagen, levels of laminin remained relatively constant throughout the course of virus infection, but the stability and the rigidity of the basement membranes surrounding the acini changed. In fact, as a result of depopulation of the acini due to cell necrosis, the basement membranes “collapsed” during the acute phase of the disease. As repopulation of the acini with new acinar cells progressed, the original structures were obtained.

In summary, infection of SJL mice with CVB4 leads to a severe acute necrotizing pancreatitis that remains restricted to the exocrine tissue and which usually resolves within weeks. Levels of gelatinase B or MMP-9, originating from neutrophils and macrophages, are upregulated during several days post infection, and return to baseline levels soon thereafter. Rearrangements of the extracellular matrix during the course of the disease are associated with changes in levels and/or structural organization of collagen and laminin. The exact role or contribution of MMP-9 in acute viral pancreatitis remains to be addressed in future studies.



**Fig. 6.** Analysis of extracellular matrix components. Collagen and laminin were detected with Sirius red and by immunohistochemical staining, respectively. Collagen levels (stained in red) increase gradually till 7 days p.i. but return to baseline thereafter. Levels of laminin, the major component of the basement membrane, remain constant throughout the course of the acute pancreatitis. (400 $\times$  magnification for collagen, 630 $\times$  magnification for laminin).

## Materials and methods

### Mouse model and virus

Five-to six-week-old male SJL mice (Harlan, The Netherlands) weighing 20–25 g ( $n=3$  per group) were inoculated intraperitoneally with  $1.2 \times 10^5$  plaque forming units (PFU) of coxsackievirus B4 (CVB4) strain Edwards (E2) (Yoon et al. 1979) in a final volume of 250  $\mu$ l saline. Virus was kindly provided by Prof JW Yoon (University of Calgary, Alberta, Canada). All experimental procedures were in accordance with the institutional ethics committee. Virus stocks were grown on Vero cells (ATCC CCL-81). Briefly, confluent cell monolayers were inoculated with 0.1 PFU of CVB4 and incubated at 37 °C in a 5% CO<sub>2</sub> incubator until extensive cytopathic effect was apparent. Next, the supernatant was centrifuged at 4 °C and 15,000 g for 15 min. Viral titers were determined by endpoint titration on confluent Vero cell cultures in 96-well plates and aliquots were stored at –80 °C until use.

### Histology and immunohistochemistry

At 3 days post infection, mice were euthanized using pentobarbital (Nembutal®). Following extensive transcardial perfusion with PBS, the pancreas was removed and divided in 3 parts. One part was fixed in 4% formaldehyde for 24 h and embedded in paraffin. Tissue sections (4  $\mu$ m) were stained with hematoxylin and eosin for routine histological examination. The other parts were used for quantification of viral RNA and infectious virus, as described below. Immunohistochemical staining for gelatinase B was performed with a commercial goat anti-mouse antibody from R&D Systems (Minnesota, MN). Laminin and collagen were stained using a rabbit anti-laminin antibody (Sigma, Bornem, Belgium) and Sirius red, respectively. Neutrophils were stained with rat anti-mouse Ly6G (Beckton Dickinson, Erembodegem, Belgium), B cells with a monoclonal anti-mouse B220 antibody (R&D Systems, Minnesota, MN), T cells with a monoclonal anti-mouse CD3 antibody (R&D Systems, Minnesota, MN) and macrophages with a rat anti-mouse F4/80 antibody (Serotec, Düsseldorf, Germany).

### Quantification of serum lipase/amylase

At various days post infection, 500  $\mu$ l of blood was collected by orbital sinus bleeding. After 1 hour at room temperature (RT), the blood was centrifuged at 17,000 g for 10 min. Serum amylase and lipase were quantified using an enzymatic colorimetric reaction (Cobas/Roche, Basel, CH) on the Modular P Analyzer (Roche Diagnostics, Basel, CH).

### Gelatin zymography

Five mg of protein from pancreas homogenates (5% w/v) was applied to gelatin-Sepharose affinity chromatography as described previously (Descamps et al. 2002). Briefly, samples were diluted 1:2 in equilibration buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 10 mM CaCl<sub>2</sub>, 0.01% Tween20 and 5 mM *o*-phenantroline), and incubated with 50  $\mu$ l gelatin-Sepharose beads in mini-spin columns on a horizontal shaker. After washing, gelatinases were eluted with non-reducing SDS-PAGE loading buffer and 40  $\mu$ l of purified eluate were further processed for zymography.

### In situ zymography

Gelatinolytic activity of MMPs was localized on frozen tissue sections by *in situ* zymography. As a substrate, FITC-labeled DQ gelatin was used that is intramolecularly quenched (gelatinase/collagenase assay kit (EnzChek; Molecular Probes, Eugene, OR)). Proteolysis by gelatinases yields cleaved gelatin-FITC peptides that are fluorescent.

The localization of fluorescence indicates the sites of net gelatinolytic activity. Briefly, frozen tissue sections were incubated with 20  $\mu$ g/ml DQ gelatin for 30 min in a moist chamber at 37 °C. At the end of the incubation period, gelatinolytic activity of MMPs was localized and the fluorescence microscopy slides were photographed.

### Quantification of viral RNA using RT-qPCR

One third of the isolated pancreata was used for quantification of viral RNA and cellular beta actin. Tissue homogenates in lysis buffer (5% w/v) were prepared in 2 ml tubes containing ceramic beads that were shaken at a high frequency in an automated homogenizer (Precellys24, Bertin, France). Briefly, homogenization was performed at 6500 rpm for 3 cycles of 5 s, with intervals of 5 s. Next, the tubes were centrifuged for 15 min at 4 °C and 13,000 rpm and 600  $\mu$ l of the cleared supernatant (corresponding with 30 mg tissue) was used for total RNA extraction using the Qiagen RNeasy mini kit (Qiagen, Venlo, The Netherlands). Quantification of RNA using real-time RT-qPCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). For CVB4, the following primers and probe were used: a forward primer 5'-GTA GTC CTC CGG CCC CT-3', a reverse primer 5'-AAT TGT CAC CAT AAG CAG CCA-3' and a Taqman probe 5'-FAM-ATG CGG CTA ATC CTA ACT GCG GAG-TAMRA-3' as described by Brilot et al. (Brilot et al. 2004). For quantification of mouse beta actin, the following primers and probe were used: a forward primer 5'-AGA GGG AAA TCG TGC GTG AC-3', a reverse primer 5'-CAA TAG TGA TGA CCT GGC CGT-3' and a Taqman probe 5'-JOE-CAC TGC CGC ATC CTC TTC CTC CC-TAMRA-3'. Each reaction was performed in 25  $\mu$ l of a PCR reagent mixture (One-Step RT-qPCR Mix, Eurogentec, Seraing, Belgium) containing 900 nM of each primer and 200 nM of the specific Taqman probe. The PCR consisted of a reverse transcription step (30 min at 48 °C), a *Taq* activation step (10 min at 95 °C), and 50 cycles of denaturation (15 s at 94 °C) and annealing (1 min at 60 °C). The RNA copy number in each sample was determined by a standard curve generated from increasing copy numbers of a synthetic transcript corresponding to the PCR-amplified region.

For quantification of viral RNA in serum, 150  $\mu$ l serum was obtained as described above and viral RNA was isolated using the Qamp Viral RNA Min Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions.

### Quantification of infectious virus

One third of pancreas tissue was homogenized similarly as described above, the sole difference being that saline was used instead of lysis buffer. The clarified supernatants were assayed for infectious virus content by endpoint titration on confluent Vero cell cultures in 96-well plates. Data were expressed as 50% cell culture infective doses (CCID<sub>50</sub>) and were calculated according to the method of Reed and Muench (Reed and Muench, 1938).

### Statistical analysis

For several parameters, the differences between treated and untreated groups were statistically evaluated using the unpaired Student's *t*-test (two-tailed – unequal variance). *P*-values indicate the probability of the obtained result, assuming the null hypothesis that no difference exists between the two groups.

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