Myofibroblast proliferation, fibrosis, and defective pancreatic repair induced by cyclosporin in rats

E Vaquero, X Molero, X Tian, A Salas, J-R Malagelada

Abstract

Background—Full recovery is always achieved after caerulein induced pancreatitis. Cyclosporin stimulates transforming growth factor β (TGF-β) and may interfere with pancreatic regeneration.

Aim—to investigate the effects of cyclosporin after caerulein induced pancreatitis or after caerulein injury.

Methods—Protocol A: rats received cyclosporin daily (20 mg/kg) and caerulein pancreatitis was induced on days 2 and 8. Protocol B: six courses of caerulein pancreatitis were induced at weekly intervals. Cyclosporin was administered on induction and the day before. Rats recovered for two weeks before being killed. Control groups received saline, cyclosporin, or caerulein alone.

Results—Protocol A: plasma TGF-β1 and tissue collagenase rose after pancreatitis but decreased towards baseline values on day 15, matching a low collagen content. Morphology disclosed minimal inflammatory infiltration and some interstitial cells immunoreactive for smooth muscle α-actin (SMA). TGF-β1 increased, and remained high in cyclosporin treated groups (cyclosporin alone and cyclosporin plus caerulein). Rats treated with cyclosporin and caerulein showed severe pancreatic weight reduction, abundant inflammatory infiltrates, increased SMA immunoreactive interstitial cells, high collagen content, and delayed collagenase response. No SMA immunoreactive cells were detected in normal rats. Cyclosporin alone also increased SMA immunoreactive cells, despite the absence of inflammatory infiltration and fairly conserved pancreatic structure. Protocol B: the combined pulse treatment induced appreciable collagen deposition and resulted in a smaller pancreas than controls. Morphological examination showed atrophy, fibrosis, fibroblast proliferation, and mononuclear infiltrates.

Conclusion—Cyclosporin greatly distorts pancreatic repair, transforming caerulein induced pancreatitis into a fibrotic chronic-like disease. The mechanism involves TGF-β1, myofibroblasts, and defective collagenase activation.

Keywords: chronic pancreatitis; transforming growth factor β; caerulein; cyclosporin; fibrosis; myofibroblast

Caerulein hyperstimulation induces acute oedematous pancreatitis in rats. Despite morphological and biochemical evidence of pancreatic injury, complete recovery is the rule. Restoration of normal pancreatic architecture and function after caerulein induced pancreatitis involves controlled and transient increases in transforming growth factor β (TGF-β), fibroblast proliferation, collagen formation, and activation of tissue proteinases. The reparative process is so highly coordinated that scarring, signs of atrophy, inflammatory cells, or distortion of pancreatic morphology cannot be recognised a few days after the caerulein challenge.1–6

Moreover, even repeated bouts of caerulein induced pancreatitis fail to cause permanent damage to the pancreas,7 indicating that either the injury is not severe enough to overcome a number of non-specific tissue defences or that highly efficient regenerative mechanisms are activated. Nonetheless, experimental manipulations, such as infusion of TGF-β1 or stress8, may disrupt the reparative sequence and result in defective regeneration.

Cyclosporin is widely used in clinical practice, particularly to prevent rejection of organ transplantation. It binds to cyclophilin and inhibits calcineurin, a serine-threonine phosphatase.9 This property may account for its wide biological actions such as immunosuppression, vasoconstriction,10 and stimulation of TGF-β expression.11–14 Although cyclosporin accumulates in high concentrations in the pancreas,11–15 it only produces minor morphological or functional derangements.14–17 Even after several days of a large dose regimen, cyclosporin does not induce an acute inflammatory reaction over the pancreas.17

In the kidneys, heart, and gums, cyclosporin is known to promote collagen deposition.15–18 In the present study we tested the hypothesis that, in the pancreas, cyclosporin exerts its fibrogenic potential by interfering with the highly efficient reparative processes that are activated after caerulein hyperstimulation, resulting in poor gland regeneration and enhanced collagen deposition.

Materials and methods

MATERIALS

Caerulein, hydroxyproline, azocoll, calf thymus DNA standards, Hoechst compound 33258 (bisBenzimide) and anti-smooth muscle α-actin (SMA) monoclonal antibody were from Sigma. BCA reagent and bovine serum

Abbreviations used in this paper: SMA, smooth muscle α-actin; TGF, transforming growth factor.
Protocol A

Caerulein 20 µg/kg i.p. daily

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Days

CsA 20 mg/kg i.p. daily

Cyclosporin; i.p., intraperitoneal.

Protocol B

Caerulein 10 µg/kg x 2

1 2 3 4 5 6 7

Wks

CsA 20 mg/kg x 2 i.p. weekly

To examine the effects of cyclosporin on pancreatic regeneration after acute oedematous pancreatitis, two main protocols were followed (fig 1). In protocol A we evaluated the early cyclosporin induced events after acute oedematous pancreatitis (fig 1). Intra-peritoneal daily doses of cyclosporin (20 mg/kg diluted 1:4 in saline) were administered for 15 days. On days 1 and 8, acute pancreatitis was induced by four intra-peritoneal injections of caerulein (20 µg/kg) one hour apart (CR+CsA group). Three other groups followed the same experimental protocol except that they received vehicle, cyclosporin, or caerulein alone (n = 12 per group).

**Morphology**

Pancreatic specimens were fixed in 10% formalin and embedded in paraffin wax. Several sections were cut and stained with haematoxylin-eosin or Masson trichrome stain. When indicated, immunohistochemistry was performed with the monoclonal anti-SMA antibody (α-SMA) to identify myofibroblasts (interstitial α-SMA positive cells with fibroblast-like shape) using the avidin-biotin-peroxidase complex system (LSAB kit, Dako Corporation). Quantitative analysis of interstitial α-SMA positive cells was performed at 400× final magnification. Ten non-overlapping fields were evaluated for each animal in each treatment group.

**Biochemical determinations**

For biochemical assays, total pancreata were excised, trimmed of fat and lymph nodes, weighed, frozen, and lyophilised. A powder was made in a mortar from the dried residue and resuspended in ice cold distilled water (100 mg/ml). Pancreatic homogenates were prepared by serial use of a motor driven shearer (Tissue Tearor 985-370; Biospec Products Inc, Drewel, Wisconsin, USA) and Dounce tissue homogenisers on ice.

Total protein and hydroxyproline content were quantified from crude homogenates. Protein concentration was determined by BCA reagent. Hydroxyproline content was determined as described with minor modifications. Briefly, an aliquot of 200 µl of crude homogenate was placed in a 10 ml amoule containing 2 ml 6 M HCl and sealed. Hydrolysis was performed at 110°C for 16 hours. The hydrolysate was dried, and the sediment redisolved in 2 ml distilled water and dried again. The latter step was repeated three times before the residue was resuspended in 3 ml 0.2 M citrate buffer (pH 2.2). A 1 ml portion of the sample or 1 ml of hydroxyproline standards (to establish the calibration curve) was placed in a Pyrex test tube and mixed with 1 ml chloramine T solution prepared as described by Rojkind and Gonzalez. After a 20 minute incubation period, the reaction was stopped by the sequential addition of 0.5 ml sodium thiosul-
Cyclosporin interferes with pancreatic repair

Cyclosporin interferes with pancreatic repair in 50 ml 50 mM Tris/HCl/1 mM CaCl2 (pH 7.8), but temporarily increases the oedematous acute pancreatitis induced by caerulein. It was measured in pancreatic homogenates using a previously described method, that measures myeloperoxidase activity, and both remained slightly elevated in the CR group but had returned to baseline values by the end of the observation period. *p<0.05 v caerulein alone.

Assessment of pancreatitis severity

To elucidate whether or not cyclosporin worsened the edematous acute pancreatitis induced by caerulein, we measured amylase activity, tissue oedema, and myeloperoxidase activity six hours after inducing acute pancreatitis. A specimen from the head of the pancreas was also processed for histological examination. Amylase activity was determined by the ß-amylase EPS test (Bohringer) for BM/Hitachi system 717. Aliquots (200 µl) of 1:800 dilutions of the sample were used in the assay.

To measure pancreatic oedema, excised pancreata were wet weighed, desiccated at 160°C for 24 hours, and reweighed. Pancreatic water content was calculated as percentage of total wet weight.

Tissue myeloperoxidase activity was used as a biochemical marker of polymorphonuclear infiltration. It was measured in pancreatic homogenates using a previously described method26-27 that measures myeloperoxidase activity as the result of H2O2 dependent oxidation of 3′,5′,5′-tetramethylbenzidine and expressed as units per mg of protein.

Statistical analysis

Values are expressed as mean (SEM). Student’s t test was used when two variables were compared. When more than two variables were present, group means were compared by analysis of variance followed by Fisher’s protected least significant differences test. Differences were regarded as significant at p<0.05.

Results

Protocol A: Effects of Daily Cyclosporin Treatment on the Early Phase of Pancreatic Regeneration

Cyclosporin treatment did not aggravate caerulein induced pancreatitis, as assessed by serum amylase (45.7 (6.9) v 70.7 (13.7) U/ml; p = 0.12), tissue oedema (83.2 (2.3) v 87.1 (0.7)%; p = 0.4), and myeloperoxidase activity (311 (54) v 344 (70) mU/mg protein; p = 0.8). Furthermore, contrary to previous reports,28 no haemorrhages could be identified in pancreata from rats receiving the combined treatment of cyclosporin plus caerulein, by either macroscopic or microscopic inspection.

Plasma TGF-β concentration

In close agreement with previous reports in humans and mice,2, 9 plasma levels of TGF-β in rats treated with cyclosporin were found to be well above control values of 19 (3) ng/ml, in both the CR+CsA and CsA groups (fig 2). TGF-β also increased, but to a lesser extent, in the CR group but had returned to baseline values by the end of the observation period.

Hydroxyproline concentration and collagenase activity in pancreatic homogenates

Tissue hydroxyproline increased soon after pancreatitis in the CR group. This enhancement in collagen formation was matched by a reciprocal increase in pancreatic collagenase activity, and both remained slightly elevated in response to a second induction of pancreatitis (fig 3). In contrast, tissue collagenase in the CR+CsA group was low after pancreatitis, despite an elevated hydroxyproline content, but increased with time and became very high by the end of the observation period in the face of increased tissue hydroxyproline concentration (fig 3), which suggests defective extracellular matrix remodelling.
Pancreatic weight and total protein
Both variables are widely used to quantify pancreatic gland atrophy. Pancreatic weight and protein content decreased after each bout of pancreatitis in the caerulein alone group, but recovered thereafter (fig 4). In contrast, when rats also received cyclosporin, pancreatic weight and protein decreased further and did not recover. Cyclosporin alone had no measurable effects after 15 days of treatment on pancreatic weight or total protein content (3.2 (0.1) g/kg body weight and 102 (5.2) mg) as compared with saline (3.2 (0.2) and 97 (10); p>0.05 for both variables).

Morphological findings
At the end of the observation period, neither cyclosporin nor caerulein alone had produced significant alterations in pancreatic structure. Loose connections between adjacent acini, a certain degree of fat atrophy, and some cells with cytoplasmic vacuolisation could be observed after cyclosporin, and, two weeks after the initial caerulein challenge, pancreatic morphology in the caerulein alone group was grossly restored, except for occasional inflammatory infiltrates (fig 5). In contrast, pancreatic specimens from the CR+CsA group showed diffuse infiltration with inflammatory mononuclear and polynuclear cells, surrounding areas of gland atrophy. There was also increased connective tissue and fine bands of collagen that were best visualised by Masson trichromic staining (data not shown).

Fibroblast-like cells immunoreactive for α-SMA have contractile capabilities and are considered to be myofibroblasts. Immunostaining with anti-α-SMA allowed quantification of myofibroblasts in pancreatic parenchyma (fig 6). Normal pancreatic tissue contained no interstitial cells exhibiting immunoreactivity for α-SMA. At the end of the observation period in the caerulein alone group, myofibroblasts (interstitial α-SMA immunoreactive cells) displayed an elongated
shape and could be observed scattered among well structured acini throughout the parenchyma of the pancreas (fig 6B). In the CR+CsA group, myofibroblasts were significantly increased in number compared with the CR group (figs 6D and 7). They were mainly distributed in association with areas of excess connective tissue accumulation and inflammatory cell infiltrates. Interestingly, in the pancreas of rats receiving cyclosporin alone, myofibroblasts were also a prominent feature on morphological inspection (figs 6C and 7). They were increased in number as compared with controls and were found evenly distributed in parenchyma with minimal structural disturbances and no inflammatory infiltrates.

**PROTOCOL B: EFFECTS OF WEEKLY PULSES OF CYCLOSPORIN ON LONG TERM PANCREATIC RECOVERY AFTER REPEATED BOUTS OF CAERULEIN INDUCED PANCREATITIS**

The main aim of this protocol was to assess the persistence of pancreatic collagen deposition on morphological inspection (figs 6C and 7). They were increased in number as compared with controls and were found evenly distributed in parenchyma with minimal structural disturbances and no inflammatory infiltrates.

**Figure 5** Histological features of the pancreas 15 days after initiation of protocol A schedule (two pancreatitis inductions one week apart). (A) Caerulein alone group (haematoxylin and eosin stain; original magnification ×200). (B) Caerulein + cyclosporin group (haematoxylin and eosin stain; original magnification ×250). Pancreas from the caerulein alone group had a normal appearing parenchyma with occasional discrete mononuclear infiltrates. Specimens from the caerulein + cyclosporin group showed evident signs of persistent tissue damage such as increased connective tissue deposition associated with diffuse lymphomononuclear infiltrates and areas of gland atrophy.

**Figure 6** Representative examples of pancreatic specimens from controls (A), caerulein alone (B), cyclosporin alone (C) or caerulein + cyclosporin(D) groups as seen after immunostaining for smooth muscle α-actin (SMA). The antibody binds to cells in the vessel walls (A). No immunoreactive interstitial cells were identified in controls, while some SMA positive cells were observed in the caerulein alone and cyclosporin alone groups, scattered throughout a well preserved pancreatic parenchyma. Vessel walls display strong staining. Abundant SMA positive cells were present in pancreatic specimens from caerulein + cyclosporin rats, often in the proximity of inflammatory infiltrates and increased deposition of connective tissue (original magnification 400×).
generated by cyclosporin, administered by a weekly pulse regimen in association with repeated bouts of oedematous acute pancreatitis.

Once again, cyclosporin was the inducer of an inappropriate regenerative pancreatic response. Biochemical markers of gland atrophy such as pancreatic weight, total protein, and DNA content were all significantly reduced in the group of rats receiving the combination regimen, even after two weeks of the last bout of pancreatitis (fig 8). Furthermore, collagen deposition was greatly enhanced in this group as measured by tissue hydroxyproline concentration (fig 8) and examination of the histological appearance (fig 9). Indeed, two weeks after the last pancreatitis induction there was appreciable atrophy of peripancreatic fat, areas of gland atrophy, and large bundles of fibrosis with diffuse lymphomonocytic infiltrates and proliferation of fibroblasts. Fine bands of fibrotic tissue extended inside the better preserved lobuli, indicating a generalised fibrotic process.

These findings contrasted with the absence of substantial morphological or biochemical abnormalities in groups of rats treated with either cyclosporin or caerulein alone.

Discussion

In this paper we describe the transformation of a highly efficient and well coordinated process of tissue repair—that is, regeneration after caerulein induced pancreatitis—into chronic-like pancreatitis featuring gland atrophy, persistent mononuclear infiltrates, and enhanced collagen deposition, all under the influence of a single pharmacological manipulation.

Cyclosporin interfered with pancreatic regeneration even when it was administered at weekly pulse doses (protocol B), noteworthy, without concurrence of any surgical interventions or anaesthetics. The latter may be a critical point as pentobarbital and other anaesthetics inactivate or inhibit the action of nitric oxide and therefore may reduce splanchnic blood flow. Cyclosporin also reduces pancreatic blood flow, and consequently the combination of cyclosporin and anaesthetics may well potentiate damage to the gland.

Cyclosporin did not aggravate the severity of caerulein induced acute pancreatitis based on factors such as amylasaemia, tissue oedema,
Cyclosporin interferes with pancreatic repair

Infiltrates or fibrosis are observed (haematoxylin and eosin stain; original magnification at weekly intervals). (A) Representative example of pancreatic tissue from caerulein alone group. Pancreatic architecture is normal. No inflammatory infiltrates or fibrosis are observed (haematoxylin and eosin stain; original magnification ×250). (B) A remarkably different picture is observed in specimens from the caerulein + cyclosporin group. Prominent areas of perilobular and intralobular fibrosis are present. Lymphomonocytic infiltrates are abundant, especially in areas of increased connective tissue and acinar atrophy (Masson trichromic stain; original magnification ×250).

Figure 9 Histological findings two weeks after the last pancreatitis in pancreatic specimens from protocol B (induction of six courses of acute pancreatitis with caerulein and cyclosporin). (A) Representative example of pancreatic tissue from caerulein alone group. Pancreatic architecture is normal. No inflammatory infiltrates or fibrosis are observed (haematoxylin and eosin stain; original magnification at weekly intervals). (B) A remarkably different picture is observed in specimens from the caerulein + cyclosporin group. Prominent areas of perilobular and intralobular fibrosis are present. Lymphomonocytic infiltrates are abundant, especially in areas of increased connective tissue and acinar atrophy (Masson trichromic stain; original magnification ×250).

Collagenase activity is expressed by extracellular matrix metalloproteases that can be regulated by a number of cytokines such as interleukin 1β or TGF-β.35–39 The main sources of collagenases are fibroblasts and neutrophils.39 Both tissue collagenase activity and fibroblast dependent collagen synthesis can be modulated by TGF-β.40

Indeed, the multifunctional TGF-β family of cytokines play a central role in the regulation of the coordinated events that characterise optimal wound healing.36–39 However, tissue fibrosis is prone to develop in response to sustained production of TGF-β.37–39 This cytokine activates stellate cells and stimulates their differentiation into myofibroblasts in the liver,36–42 inhibits matrix degrading proteases (collagenase I, II, and III),35–41 and increases the production of collagenase inhibitors, facilitating fibrogenesis.41 TGF-β is also known to inhibit the growth of epithelial cells and to stimulate fibroblast proliferation, and has been implicated in the pathogenesis of fibrosis in a number of other organs such as the kidney, lung, skin, and arteries.42

There are data suggesting that TGF-β also modulates extracellular matrix formation in the pancreas. Thus, TGF-β stimulates collagen synthesis from fibroblasts derived from human pancreas,43 and transgenic mice overexpressing TGF-β show accumulation of extracellular matrix in the pancreas and fibroblast proliferation.44 Administration of a neutralising antibody against TGF-β reduces fibronectin expression in rats with caerulein induced pancreatitis,45 whereas intravenous infusion of excess TGF-β leads to increased collagen deposition and gland atrophy in mice.46 Moreover, TGF-β expression seems to be enhanced in human pancreas with chronic pancreatitis.45–46

We speculate that steady increased plasma levels of TGF-β, induced by cyclosporin, may account for a number of our experimental findings such as parenchymal atrophy, defective collagenase activity, and myofibroblast proliferation. After induction of caerulein pancreatitis, TGF-β expression in the pancreas has been reported to be transiently elevated in the form of either a sharp peak of 2–3 days duration37 or as a double wave peak occurring during the first week.38 Our data on plasma TGF-β concentration after caerulein pancreatitis are in agreement with the above reports.

Cyclosporin is widely used in clinical practice, on account of its immunomodulatory properties.47 In addition, cyclosporin increases plasma TGF-β in humans and mice39–42 and has been shown to promote fibrogenesis in kidney, heart, and gums.39–40 In vitro, cyclosporin induces collagen synthesis in fibroblasts.48 Cyclosporin toxicity to the pancreas is regarded to be of lesser relevance. Given for periods of weeks in large dose regimens, cyclosporin causes a reduction in stimulated pancreatic secretion and some acinar cell...
vacuolisation. However, no inflammatory reaction has been shown, and our results corroborate this point.

We have found, however, that cyclosporin induces the expression of pancreatic α-SMA in interstitial fibroblast-like cells (myofibroblasts) and stimulates their proliferation even in the absence of an inflammatory reaction. In the liver, myofibroblasts (fibroblast-like cells expressing α-SMA) represent the activated form of stellate cells, the main cell type involved in the synthesis of extracellular matrix components. In the pancreas, fibroblasts are involved in the reparative processes activated after caerulein induced acute pancreatitis and are known to proliferate after the initial injury. The normal pancreas contains interstitial cells that express desmin and vimentin, intermediate filaments that are also present in hepatic stellate cells. In fibroblast-like cells from human pancreas in culture, TGF-β enhances expression of α-SMA and collagen synthesis. The basic nature of these observations has now been confirmed and expanded by other investigators who have renamed pancreatic myofibroblasts as activated stellate cells of the pancreas.

However, despite increased plasma TGF-β and myofibroblast transformation, we could not show any enhancement in pancreatic collagen deposition in response to cyclosporin alone. In our model, stimulation of extracellular matrix accumulation (fibrosis) required the participation of an additional aggressive factor, such as oedematous pancreatitis, a mild inflammatory disease. Under such combined conditions of injury and background cyclosporin, the normal pancreatic repair pattern characterised by a transient rise in plasma TGF-β, myofibroblast proliferation, collagen generation, and collagenase activity was substituted by a pathological post-injury pattern of steady high plasma levels of TGF-β, myofibroblast proliferation, increased collagen deposition, and inappropriate collagenase activation. The end result of this pathological process was that, instead of the customary restoration of normal pancreatic architecture expected after an acute caerulein challenge, there was a catastrophic disturbance of the regenerative sequence. The accentuated gland atrophy, persistent inflammatory reaction, and enhanced fibrosis formation brought about a morphological transformation that closely resembles human chronic pancreatic disease.

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