A selective COX-2 inhibitor suppresses chronic pancreatitis in an animal model (WBN/Kob rats): significant reduction of macrophage infiltration and fibrosis

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Abbreviations
CP   chronic pancreatitis
COX-2  cyclooxygenase-2
TGF-β transforming growth factor beta
PGE2   prostaglandin E2
fMLP N-formyl-L-methionyl-L-leucyl-L-phenylalanine.
ACE   angiotensin converting enzyme
H&E   hematoxylin and eosin
MCP-1 monocyte chemoattractant protein-1
MIP-1α macrophage inflammatory protein-1α
MPO     myeloperoxidase
TNF-α tumor necrosis factor-α
IL-6     interleukin-6
ABSTRACT

Introduction: Therapeutic strategies to treat chronic pancreatitis (CP) are very limited. Other chronic inflammatory diseases can be successfully suppressed by selective cyclooxygenase-2 (COX-2) inhibitors. Since COX-2 is elevated in CP we tried to inhibit COX-2 activity in an animal model of CP (WBN/Kob rat). We then analyzed the effect of COX-2 inhibition on macrophages, important mediators of chronic inflammation.

Methods: Male WBN/Kob rats were continuously fed the COX-2 inhibitor Rofecoxib, starting at the age of 7 weeks. Animals were sacrificed 2, 5, 9, 17, 29 weeks later. In some animals, treatment was discontinued after 17 weeks, and the animals were observed for another 24 weeks.

Results: Compared to the spontaneous development of inflammatory injury and fibrosis in WBN/Ko control rats, animals treated with Rofecoxib exhibited a significant reduction and delay (p<0.0001) of inflammation. Collagen and TGF-β synthesis was significantly reduced. Similarly, PGE₂ levels were markedly lower, indicating a strong inhibition of COX-2 activity (p<0.003). If treatment was discontinued at 24 weeks of age, all parameters of inflammation strongly increased comparable to that in untreated rats. The correlation of initial infiltration with subsequent fibrosis led us to determine the effect of Rofecoxib on macrophage migration. In chemotaxis experiments, macrophages turned insensitive to the chemoattractant fMLP in the presence of Rofecoxib.

Conclusion: In the WBN/Kob rat, chronic inflammatory changes and subsequent fibrosis can be inhibited by Rofecoxib. Initial events include infiltration of macrophages. Cell culture experiments indicate that migration of macrophages is COX-2 dependent.
INTRODUCTION
Chronic pancreatitis is a disease with a succession of pathophysiological events: inflammatory infiltration and necrosis are followed by fibrosis, sometimes pancreatic stone formation and diabetes mellitus and an increased long-term risk for pancreatic cancer. Therapeutic strategies to treat CP are mostly symptomatic and very limited. Other chronic inflammatory diseases have been successfully treated by specifically targeting COX-2.
Elevated COX-2 levels have been identified in pancreatic tissue from patients with chronic pancreatitis[1][2]. The secretory products of the COX system are prostaglandins (PG), primarily PGE2, acting in an autocrine or paracrine fashion. It is unclear whether PGE2 produced by pancreatic cells promotes inflammation. Furthermore, it is unclear whether the infiltrating inflammatory cell population of the pancreas, e.g. neutrophiles, lymphocytes, and macrophages, expresses COX-2. These inflammatory cells are attracted to the pancreas and promote the destruction of the parenchyma, and by their phagocytic activity remove dying cells and cell debris.

The infiltrating population of leukocytes in chronic pancreatitis consists of a high number of mononuclear cells, suggesting that macrophages make an important contribution to the inflammatory process[3]. Macrophages are recruited from circulating monocytes and are activated by a number of cytokines as well as by bacterial substances such as endotoxin. Activation induces phagocytic activity [4][5] as well as up-regulation of cyclooxygenase-2 (COX-2). COX-2 inhibitors have been used in a number of chronic inflammatory diseases[6][7].

In animal models of acute pancreatitis, COX-2 activity increased after induction of pancreatitis by cerulein[8]. Mice without a functional COX-2 gene on the other hand exhibited an attenuated severity of the disease[9][10], supporting the concept that the pancreas might be a target for COX-2 specific therapy.

To study CP, the WBN/Kob rat is a widely used model [11]. It mimics pathophysiological processes of chronic inflammation and fibrosis, although the initiation differs from human CP. This model has been used to test potential therapeutic agents, e.g. prednisolon[12] and troglitazone[13], which had a limited anti-inflammatory effect. So far, the most successful drugs suppressing fibrosis are Lisinopril, an angiotensin converting enzyme (ACE) inhibitor, and Candesartan, an angiotensin II receptor antagonist [14][15].

In this report, we address the question whether the COX-2 inhibitor Rofecoxib suppresses inflammation and subsequent fibrosis in the WBN/Kob rat model of chronic pancreatitis.
We show that due to Rofecoxib the progression of the disease is significantly suppressed and delayed and suggest a direct effect of the inhibitor on macrophage migration.

MATERIALS AND METHODS

Animals
Male rats were purchased from BRL Füllinsdorf, Switzerland (Wistar) and WBN/Kob rats from Japan LSC Inc., Shizuoka and TGC INC, Tokyo Japan [16]. Rats were housed as reported previously[16].
Prior to sacrifice, the rats were deprived of food overnight (16–18 hrs) with free access to water. All manipulations conformed with the Swiss federal guidelines on animal experiments and were approved by the local ethics committee.
Treatment with Rofecoxib or Lisinopril

After an adaptation period of three weeks, animals were fed the pure cyclooxygenase-2 inhibitor Rofecoxib, a gift from Merck, USA, mixed with powdered rat chow. According to the manufacturers recommendations, 10 mg/kg body weight per day were applied. A defined amount of food (50 g/rat per day) was given with a Rofecoxib content of 50 mg/kg. Control rats received the same amount of food without Rofecoxib. Food consumption was monitored in 2-day intervals. The assigned amount was completely consumed by both, the control and the treatment group animals. Their body weight development displayed no significant differences.

The treatment regime was as follows: starting at age 7 weeks, animals were given the inhibitor continuously until they were sacrifized at week 9, 12, 16, 24 and 36 of age. In treatment regime II, the same dose was applied up to week 24, then control food was given for an additional 12 (age 36) or 24 weeks (age 48) when the rats were sacrificed.

Lisinopril, a generous gift from AstraZeneca (Switzerland) was supplied continuously (10 mg/rat/day)[14], starting at age 7 weeks, until 16 weeks, when the animals were sacrifized.

Specimen
Organs were harvested as reported previously [16].

Histopathology and immunohistochemistry
A score describing early and late inflammatory changes of the exocrine pancreatic tissue was established based on criteria shown in Figure 3. Tissue damage was scored by assessing various parameters: infiltration by inflammatory cells (macrophages, neutrophilic granulocytes and lymphocytes), acinar cell apoptosis, edema, hemorrhage, formation of granulation tissue, proliferation of myofibroblastic cells, fibrosis and scarring. Changes were graded according to their extent: Score 1: changes affecting a single lobule. Score 2: changes affecting two or more distinct lobules. Score 3: confluent changes leading to an effacement of the lobular structure. Score 4: changes affecting the majority of the section, defined by at least 3 areas of confluent change. The scoring of the different parameters was summarized to form a histology score. This histology scoring was performed in a blinded fashion by a pathologist (A.P.) experienced in pancreatic pathology.

To determine the extent of fibrosis, sections were stained by Sirius-Red, which preferentially labels collagen fibrils with red color. Non-fibrotic areas retain a blue color. The morphometry software ‘Analysis’ was employed to analyze 10 low-power fields per section. The percentage of the collagen positive area is given. For detection of macrophages, antibodies against CD68 (ED1) and CD163 (ED2) were used. Positive cells were counted in 10 independent fields of vision per section and expressed as cells per 0.6 mm². For COX-2 immunohistochemistry, an antibody against rat COX-2 (Cayman) was used at a dilution of 1:200. The slides were pretreated by boiling in TRIS buffer pH8.5 for 30 minutes on an automated slide processing system (Ventana Benchmark, Tucson, AZ).
RNA extraction and real-time PCR
RNA was extracted and used for real-time PCR as described previously[16]. The primers were ordered from Applied Biosystems based on the rat genes coding for COX-1, COX-2, TNF-α and IL-6.

COX-1_Forward  CCAGCCCAACTCCCTC
COX-1_FAM  CATCTCTATCATGCTCTCCCCAAA
COX-1_Reverse  GGGCTGATGCTGGAGAAGTG
COX-2_Forward  CCATGTCAGGGATCTTTCTTTTCTCA
COX-2_FAM  CTTCCTACGCCAGCAATCTGA
COX-2_Reverse  CAAGGAAGGTCTGATTGTC
IL-6_Forward  GCCCTTCAGGAACAGCTATGA
IL-6_FAM  CATCAGTCCCAAGAAGGCAACT
IL-6_Reverse  TCCGCAAGAGACTTC
TNF-α_Forward  GCTCCCTCTCATCAGTTCCAT
TNF-α_FAM  GGCTTGTCACTCGAGTTTTGAGAA
TNF-α_Reverse  CCTCACACTCAGATCAT

Primers coding for collagen α1(III), TGF-β, MCP-1 and MiP-1α were designed as published[17][18]. Real-time PCR was run on a Taqman 7000 (AppliedBiosystems, Switzerland) under standard conditions. Transcript levels were quantified using 18S RNA (Applied Biosystems) as a reference and normalized to 6 week old Wistar rat[16], or 9 week old WBN/Kob control rats, respectively.

PGE2-ELISA
PGE2 was purified from pancreatic tissue using Amprep™ Octadecyl C18 Minicolumns (Amersham) according to the manufacturer’s instructions. The collected Ethyl Acetate fractions were evaporated to dryness under nitrogen. The purified PGE2 were quantified with an EIA PGE2 kit (Amersham).

Cell culture experiments
The cell line RAW264.7 was purchased from ATCC, Manassas, Va, USA. Cell culture medium and supplements were purchased from Invitrogen Life Technologies (Carsbad, USA). TPP plastic wares and cell culture inserts for chemotaxis assays (8µM pore size) were obtained from Milian (Geneva, Switzerland). For chemotaxis assays, 0.5x10^8 viable cells (above 95%) were seeded on top of 24-well filter inserts. Medium containing various amounts of fMLP (Sigma Buchs, Switzerland) was placed at the bottom for one hour before harvesting the cells and quantitative analysis by a cell counter (Becton-Dickinson FACSCalibur).

Protein quantification
Protein contents in tissue homogenates were determined by a commercially available reagent (Pierce, Socochim, Lausanne, Switzerland) and quantified against bovine serum albumin as a standard.
Statistics
The effect of the treatment was statistically analyzed by two-way ANOVA. When single time points were analyzed, a one-way ANOVA with Dunnetts Multiple comparison post-test was used.

RESULTS
Cyclooxygenase-1 & -2 mRNA increase in WBN/Kob rats during inflammation
To test whether cyclooxygenase levels are increased during the inflammatory process in the WBN/Kob rat, we determined transcript levels of COX-1 and COX-2 in both, WBN/Kob and Wistar rats, from an experiment previously described[16]. At week 16 and 24, COX-1 levels increased more than 25-fold in the WBN/Kob rat compared to the control Wistar rats (Fig. 1A). COX-2 levels increased about 15-fold at the same time (Fig. 1B).

Chronic application of a COX-2 inhibitor reduces COX transcript levels
Based on the increased COX transcript levels, we administered a COX-2 inhibitor (Rofecoxib) continuously to WBN/Kob rats, starting at seven weeks of age, to test whether selective inhibition of COX-2 would have a beneficial effect on pancreatic inflammation and fibrosis. Control WBN/Kob rats received the same amount of chow without Rofecoxib. The rats were treated up to week 36 to test the longterm effects of the drug. COX transcript levels of treated rats were clearly reduced compared to untreated controls. There was no selective regulation of one of the genes coding for COX-1 (Fig. 1C) and COX-2 (Fig. 1D). We conclude that the chronic application of COX-2 inhibitor targets inflammatory cells that migrate into the pancreas, and that due to the relative paucity of inflammatory cells in treated rats both COX-1 and COX-2 are present in lower amounts.

Inhibition of COX-2 affects prostaglandin E2 levels in the pancreas
The presence of COXs is demonstrated indirectly by assessing prostaglandin E2 (PGE2), the active secretory product of the enzymatic cascade initiated by cyclooxygenases. Figure 2 demonstrates a significant reduction of PGE2 levels up to week 24 in animals treated with Rofecoxib. We conclude that systemic inhibition of COX-2 lowers PGE2 secretion in the pancreas. In comparison, animals treated with Lisinopril for nine weeks (an angiotensin converting enzyme inhibitor), exhibited a similar reduction in PGE2 levels (data not shown).

Continuous application of a COX-2 inhibitor improves histopathologic parameters
Application of the COX-2 inhibitor Rofecoxib reduced the presence of COX-1 &-2 mRNA significantly and caused a reduction of PGE2 levels in pancreatic tissue. Therefore, we investigated whether tissue injury and subsequent fibrosis were also alleviated. This was carried out by analysis of H&E stained sections: Chronic pancreatitis in the WBN/Kob rat is characterized by focal infiltration of macrophages and neutrophils together with edema, the early phase of the disease (Fig. 3AB). Late changes include the appearance of fibrosis, collagen deposition, granulation tissue and the formation of tubular complexes (Figure 3C and inset in 3C). The histopathological assessment demonstrated a significant deposition of collagen fibrils as shown by Sirius red staining (Figure 3D). An experienced pathologist evaluated the sections in a blinded manner. There was an obvious reduction of macrophages. To quantitate these cells, we performed immunohistochemistry of ED2 (CD163), an indicator of macrophages (Figure 3E). The foam-like cells were found predominantly
in the vicinity of parenchymal destruction. Furthermore, we stained similar sections with an antibody against COX-2. Figure 3F clearly demonstrates that COX-2 is localized to infiltrating cells again with a foam cell like appearance, suggesting that these cells are macrophages containing COX-2. To further substantiate that COX-2 is expressed in macrophages, double staining of ED2/COX-2 was performed on frozen sections. Figure 3G-I demonstrates that a significant population of macrophages is COX-2 positive.

The histological assessment in figure 4A clearly demonstrates a significant inhibition of inflammatory infiltration. As a next step, we investigated the effect of treatment on markers of acute and chronic infiltration. Neutrophilic granulocytes, markers of acute inflammation stained by myeloperoxidase, were significantly reduced (Figure 4B). Macrophages are promoters of chronic inflammation; we counted them by immunohistochemistry using ED2 (CD163). While they were present in rather high numbers in untreated WBN/Kob rats, these numbers decreased in the COX-2 inhibitor treated animals (Figure 4C).

**Inhibition of COX-2 affects fibrosis**

In support of the observation that the initial inflammation determines chronic changes, we quantified the extent of collagen fibril deposition in 24 week old rats. Sections were stained with Sirius-red, differentially highlighting collagen fibrils (Fig. 3D). The sections were then analyzed morphometrically. In untreated rats, collagen deposition was found in the parenchyma covering 20% of the tissue area. Rofecoxib-treated rats exhibited a low collagen deposition, indicating that the suppression of inflammatory infiltration had a decisive effect on fibrosis (Fig. 4C).

**Termination of treatment is followed by an increase in inflammation and fibrosis**

The data presented in figure 4 strongly suggest that pancreatic inflammation is associated with fibrosis. To further test whether in the WBN/Kob rat fibrosis is inflammation-dependent, some rats received Rofecoxib treatment until week 24 and were kept for another 12 or 24 weeks (ages 36 and 48 weeks) without Rofecoxib. Animals treated continuously until week 36 demonstrated (Fig. 4A) a reduction in the histopathological score after a peak at week 24. When treatment was discontinued (dashed line, Fig. 4A), the histopathological scores increased to values seen in untreated rats.

We conclude that acute inflammation is a prerequisite for extensive fibrosis and that COX-2 inhibitors can significantly reduce both.

The observation that inflammation increased again after discontinuation of Rofecoxib-treatment was also supported by the detection of mRNAs coding for COX-1 and COX-2, which significantly increased after the treatment with Rofecoxib was discontinued (Fig. 5AB).

**mRNA levels of chemoattractants and cytokines correlate with infiltration and tissue injury**

To determine whether chemoattractant proteins are generated in the pancreas of untreated WBN/Kob rats, mRNA levels of monocyte chemoattractant protein-1 (MCP-1, Fig. 5C) and of macrophage inflammatory protein –1α (MIP-1α, Fig. 5D) were determined. MCP-1 increased parallel to the initial inflammation. In treated animals, there was a significant reduction indicating that the inhibitor reduced expression
levels of this chemoattractant. Similarly, MIP-1α levels were affected, although absolute expression levels appear much lower (data not shown).

Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been detected in WBN/Kob rats in the early phase of inflammation[19]. Figure 5E clearly demonstrates increasing TNF-α mRNA (almost 50-fold) in rats 16 weeks of age compared to 9 week old WBN/Kob control rats. Thereafter, there is a slow decline of TNF-α transcripts over the remaining period of inflammation and fibrosis. In animals which received Rofecoxib, the TNF-α mRNA levels were significantly lower, while levels increased as soon as treatment was stopped, reaching levels similar to those in untreated controls at 16 weeks.

The levels for IL-6 mRNA also significantly increased and reached levels, approximately 600-fold higher than in 9 week old rats (Fig. 5F). Again, treatment with Rofecoxib caused a significant reduction of IL-6 mRNA levels in the pancreas. However, these levels were still 150-fold higher than in 9 week old rats and in contrast to COX-1, COX-2 and TNF-α, the IL-6 peak was reached only at 24 weeks. After discontinuation of Rofecoxib feeding, the levels strongly increased (450-fold higher than 9 week old rats).

We conclude, that both cytokines are present over the whole period of inflammation, with a correlation to the disease activity.

**Molecular markers of fibrosis are dependent on inflammation**

Finally, we asked whether molecular markers of fibrosis, i.e. TGF-β and collagen, were affected by the treatment. Collagen α1(III) mRNA levels strongly increased in the untreated rats reflecting the activation of collagen synthesis presumably by myofibroblasts (Fig. 5G). In Rofecoxib-treated rats, mRNA levels were drastically reduced. In those animals in which treatment was discontinued, the mRNA levels rose to those contents found in untreated rats. Similar to collagen mRNA, TGF-β mRNAs were significantly increased in untreated controls while Rofecoxib treatment reduced the levels considerably (Fig. 5H). Parallel to the other parameters of fibrosis, TGF-β levels strongly rose in animals where treatment was discontinued.

**COX-2 dependent macrophage migration**

Based on the observation that macrophage infiltration was retarded in COX-2 inhibitor treated rats, we asked whether COX-2 activity might be involved in the regulation of macrophage migration. We performed cell culture assays in which a mouse macrophage cell line (RAW 264.7) was exposed to a known chemoattractant, fMLP, in a migration chamber. Various concentrations of fMLP were tested to demonstrate that the typical bell shaped dose response curve was achieved. Migration was accelerated at 10^{-10} M fMLP and reduced to control levels at 10^{-11} M. After addition of a COX-2 inhibitor, the fMLP mediated migration was abolished, suggesting that COX-2 activity was required for the directional response (Figure 6).

**DISCUSSION**

Recent therapeutic advances in chronic inflammatory diseases using COX-2 specific inhibitors led us to explore whether such an inhibitor was effective in an animal model of chronic pancreatitis. We found a significant reduction in severity of inflammation and in fibrotic changes in the male WBN/Kob rats.

Although Rofecoxib has recently been withdrawn from the market due to previously unknown cardiovascular side effects, COX-2 inhibitors nevertheless seem excellent tools to understand the mechanism of inflammation in chronic pancreatitis.
The WBN/Kob model spontaneously develops chronic inflammatory changes of the pancreas (for an overview, see[16]). The main characteristics of human chronic pancreatitis, infiltration of inflammatory cells and fibrosis replacing the progressively destroyed exocrine parenchyma, are present in the WBN/Kob model. Furthermore, the spontaneous course of the disease can be observed in its early phases without experimental interference. The initiation of inflammation may, however, differ from human CP.

Changes in prostaglandin synthesis are an early key event in inflammatory processes. Cyclooxygenases play an important role in prostaglandin synthesis. While COX-1 is constitutively expressed in many tissues and involved in modulation of normal physiological processes (cf[20]), COX-2 is regulated and inducible by injury through mediators of inflammation[20][21]. Both COX isoforms are integrated in the enzymatic cascade resulting in the generation of PGG2, PGH2 and finally PGE2. During inflammation in the WBN/Kob rat, COX-1&-2 transcript levels increased up to 20-fold (Figure 1). Treatment with Rofecoxib completely suppressed this increase. In (untreated) WBN/Kob rats with pancreatitis, PGE2 levels were 2.5-fold. This increase of PGE2-levels demonstrates the relevance of elevated COX-2 expression. Furthermore, in support of the COX-2 transcript levels, we were able to demonstrate a significant reduction of PGE2-levels in Rofecoxib treated rats.

In pancreatic tissue homogenates, the inducible COX-2 transcripts would be expected to be produced by inflammatory cells, particularly by macrophages. The reason for the concurrently increased COX-1 levels seems unclear, but it is probable that they represent constitutive expression in the infiltrating cells.

In an acute model of pancreatitis, it was shown that a selective COX-2 inhibitor (NS-398) reduced prostaglandin E2 serum levels by a factor of two and improved systemic effects of pancreatitis [22]. In other acute pancreatitis models, celecoxib [23] and parecoxib [24] partially ameliorated pancreatic and systemic injury.

In our hands, COX-2 immunoreactivity localized to macrophages, some other types of immune cells and possibly stellate cells. The latter play an important role with regard to the development of fibrosis in chronic pancreatitis [25][26][27][28]. In our experimental work presented here, we have concentrated on inflammatory cell infiltration, especially that of macrophages. In human pancreas from patients with chronic pancreatitis COX-2 immunoreactivity has been detected in ductal cells and to some degree in degenerating acinar cells [1][2]; conflicting results have been published regarding the question whether COX-2 is up-regulated in mononuclear inflammatory cell infiltrates[2] or not [1].

In addition to pancreatic mRNA-levels of COX-1 and COX-2, those of the chemoattractants MCP-1, MIP-1α and the cytokines TNF-α, IL-6 and TGF-β were significantly diminished in Rofecoxib-treated rats as compared to untreated WBN/Kob rats, reflecting diminished inflammation and fibrosis.

A blinded assessment of edema, extent of infiltration of inflammatory cells, destruction of acinar tissue and fibrosis, demonstrated that treatment caused a significant reduction and delay of pancreatitis. Termination of treatment resulted in a full re-establishment of pancreatitis. This indicates that COX-2 promotes inflammation during all phases of the disease including fibrosis. To evaluate the extent of protection we included a drug recently reported to suppress fibrosis in the WBN/Kob rat[14]. For this purpose, animals received lisinopril for 9 weeks prior to analysis at
week 16. The histopathological score was similar to that of Rofecoxib-treated rats, indicating that the inflammatory cascade as well as fibrosis were significantly affected.

A similar effect was described after administration of an angiotensin II receptor antagonist [15]. We do at present not know whether this is a mechanism independent of COX-2 driven processes, or whether there is an interaction of the two mechanisms, a question, however, worthwhile to be followed. We used immunohistochemistry for MPO and ED2 (CD163) to dissect the composition of the inflammatory cells. The number of neutrophilic granulocytes as well as macrophages was dramatically reduced by COX-2 inhibition. In a model of acute pancreatitis neutrophil infiltration was reduced to 40-50% [9][10] in COX-2-/- mice, a finding that is in agreement with our own data on neutrophils. In a further study of the role of macrophages, important mediators of chronic inflammation, we found a reduction after treatment and were able to localize COX-2 protein to macrophages. To test the hypothesis that pancreatic infiltration by macrophages is COX-2 dependent, we used in vitro migration assays. fMLP, a known chemoattractant, induced migration of RAW264.7 cells. Rofecoxib reduced the response to the chemoattractant signal significantly (Fig. 6), without affecting the viability of the cells (data not shown). This suggests that systemically applied inhibitor may disrupt the signaling cascade for monocyte attraction and hence reduce the inflammatory response. Expression of COX-2 in macrophages as well as a reduced migration after COX-2 inhibition argues in favor of a direct action of Rofecoxib on macrophages.

We may not yet have reached the point where chronic pancreatitis in humans can be therapeutically influenced. The recent detection of cardiovascular side effects of COX-2 inhibitors restricts the use of these drugs [29]. However, by studying and forthwith understanding processes that seem to be controlled by COX-2 or angiotensin II, new therapeutic approaches for a hitherto incurable disease might evolve with time.

In conclusion, we have shown in an animal model of chronic pancreatitis that pancreatic inflammation, particularly macrophage invasion, is strongly reduced after application of a COX-2 inhibitor. Concurrently, histological and molecular markers of fibrosis indicated a significant inhibition. We could also demonstrate that COX-2 inhibitors might directly affect the migratory behaviour of macrophages which contribute significantly to the acute and chronic phases of this disease.

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The authors wish to express that they do not have any competing interest.
REFERENCES


FIGURE LEGENDS

Figure 1
A, B: Presence of COX-1 and COX-2 transcripts in the pancreas of WBN/Kob and Wistar rats. RNA was extracted from pancreata of both strains. After reverse transcription, the cDNA was amplified using specific primers for COX-1 and COX-2 DNA. Upon real-time PCR, the number of cycles were corrected by an internal standard (18S). Finally, the number of cycles of the experimental animals was calculated by using the Wistar rats at six weeks as a reference point \(2^{\Delta \Delta C_t}\). A: COX-1 levels of Wistar (●) and WBN/Kob (■). B: COX-2 levels. Mean ± SEM is given. Both genes are significantly different between strains (ANOVA, p<0.01).

C, D: Presence of COX-1 and COX-2 transcripts in the pancreas of control and Rofecoxib treated WBN/Kob rats as determined by real-time PCR. C: COX-1, D: COX-2. Mean ± SEM is given for WBN/Kob controls (■) and Rofecoxib (▲) treated WBN/Kob rats. Curves were significantly different (p<0.001, two-way ANOVA).

Note: Experiments presented in A/B were performed with WBN/Kob rats bred several years ago, when the strain demonstrated peak inflammatory changes around week 24. Over the years, the disease development in this strain seems to have accelerated, and in more recent times, peak changes are observed around week 16 (cf C/D).

Figure 2
Demonstration of prostaglandin E\(_2\) production in WBN/Kob rats with and without Rofecoxib treatment. Prostaglandins were extracted from pancreatic tissue, purified and quantified by an ELISA. The amount of prostaglandin E\(_2\) was normalized to the protein in the extract. Mean ± SEM is given for controls (■) and Rofecoxib (▲) treated rats. The difference between controls and Rofecoxib treatment was significant (p<0.0003, two-way ANOVA). Inset: PGE\(_2\)-levels at week 16 after treatment with Rofecoxib and Lisinopril compared to controls.

Figure 3
Analysis of histological changes in the pancreas of the WBN/Kob rat. A represents early changes with edema, apoptosis of acinar cells as well as interacinar inflammatory infiltrates consisting of histiocytes, lymphocytes and neutrophilic granulocytes (week 12). B illustrates the confluent early changes affecting a group of adjacent lobules (week 16). C illustrates confluent late changes with destruction of the lobular architecture, inset demonstrates formation of tubular complexes (week 24). D Sirius red staining of collagen fibrils. E Immunohistochemistry for ED2 (CD163), staining macrophages (red stain). F Immunohistochemistry for COX-2 (brown stain). G, H, I colocalization of ED1 (CD68) (G) and COX-2 (H) and superposition of the two pictures (I) on frozen sections.

Figure 4
Histopathology of the pancreas with and without treatment with a specific COX-2 inhibitor (Rofecoxib). The animals were sacrificed at the indicated time-points and processed for sectioning and H&E staining, immunohistochemistry or Sirius-red staining. The score included early and late inflammatory changes and fibrosis as described in figure 3 and in methods: a score of 1 was given when one lobule was affected, 2 when two or more were affected, 3 when inflammation or fibrosis was confluent over several lobules, 4 for extended changes. The tissue specimen were scored in a blinded fashion and are graphically represented by means ± SEM. A:
histopathology score; controls (■) and Rofecoxib (▲) treated rats were significantly different (ANOVA, p<0.001). Rats treated until week 24 (dashed line, ♦); rats treated with Lisinopril (●)

B: number of MPO positive cells (neutrophilic granulocytes) per 0.3 mm². Sections from animals age 16 weeks were analyzed i.e. non-treated controls and Rofecoxib treated rats. Means ± SEM are given. C: number of ED2 positive cells (macrophages) per 0.6 mm² in sections from animals 16 weeks of age. D: morphometric quantification of collagen-fibrils by Sirius-red staining in 24 week old animals. Treatment groups in BCD are significantly different from untreated groups (ANOVA, p<0.01).

Figure 5
Presence of mRNA coding for COX-1, COX-2, MCP-1-α, TNF-α, IL-6, TGF-β and collagen α1(III) in pancreata of non-treated control WBN/Kob rats, in Rofecoxib-treated rats and in those with treatment discontinued at week 24. mRNAs were determined by reverse transcription and real-time PCR. Mean ± SEM are given. Controls (■) and Rofecoxib (▲) treated rats and those treated until week 24 (dashed line, ♦); animals at week 16 that were Lisinopril-treated (●). A: COX-1, B: COX-2, C: MCP-1, D: MIP1-α, IL-6, E: TNF-α, G: collagen α1(III), H: TGF-β.

Figure 6
Migration of mouse macrophages towards a chemoattractant gradient. RAW264.7 cells were seeded in a chemotaxis chamber and exposed to 0, 10⁻¹⁰ and 10⁻¹¹ M fMLP. After one hour, migrated cells were collected and counted. This experiment was repeated four times, the unstimulated number of migrating cells was normalized to 1. Lightly hatched bars: no treatment, darkly hatched bars: Rofecoxib (10 µM) treatment. The treatment groups were significantly different (ANOVA, p<0.03).

Figure 7
Acinar cell damage (*) leads to the nearby accumulation of activated macrophages (#) due to chemoattraction, e.g. exerted by MCP-1 which is secreted in the vicinity of injured acinar cells. Activated macrophages produce and shed various cytokines, chemokines, prostaglandines and TGF-β. The latter stimulates the activation of stellate cells which consequently synthesize and deposit collagen fibers, eventually leading to fibrosis. COX-2 inhibitors interfere with different processes: they inhibit chemoattraction and the secretory activity of macrophages, especially the secretion of TGF-β.
In our WBN/Kob rats we have shown that infiltration of macrophages and subsequent fibrosis are significantly reduced and delayed if Rofecoxib, a COX-2 inhibitor, is administered.
Inset: In vitro experiments using a macrophage cell line (RAW 264.7) demonstrated a similar effect of Rofecoxib: the directed migration of macrophages caused by the chemoattractant fMLP was significantly reduced with Rofecoxib. We therefore hypothesize that the infiltration of activated macrophages represents an essential step in the pathogenesis of chronic pancreatitis. Full arrow: substances secreted by a cell. Broken arrows: positive regulation. Line with a dash at the end: negative regulation.
Figure 2

The graph shows the change in PGE2 (pg/mg) over weeks for two groups: Control and Rofecoxib. The x-axis represents weeks, and the y-axis represents the concentration of PGE2. The data points are marked with vertical lines indicating variability. The Control group shows a decrease in PGE2 from week 8 to week 16, followed by an increase to week 24. The Rofecoxib group shows a slight increase from week 8 to week 12, followed by a decrease to week 24.
Figure 5

A. Cox-1

B. Cox-2

C. MCP-1

D. MIP-1α

E. TNFα

F. IL-6

G. Collagen

H. TGF-β
WBN/ Kob rats

in vivo

RAW 264.7 macrophage cell line

migration

fMLP (chemo-attractant)

Cox-2 Inhibitor

MCP-1

WBN/Kob rats

in vivo

Cytokines
Chemokines
Prostaglandines
TGF-β

Cox-2 Inhibitor

quiescent stellate cell

Activated stellate cell

Collagen
A selective COX-2 inhibitor suppresses chronic pancreatitis in an animal model (WBN/Kob rats): significant reduction of macrophage infiltration and fibrosis

Theresia V Reding, Daniel R Bimmler, Aurel Perren, Li-Kang Sun, Franco Fortunato, Federico Storni and Rolf Graf

*Gut* published online December 1, 2005

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http://gut.bmj.com/content/early/2005/12/01/gut.2005.077925.citation

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