Effects of rapamycin treatment on pancreatic fibrosis, cellular apoptosis and oxidative stress in experimental chronic pancreatitis model

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Abstract

Purpose : **Rapamycin reduces hepatic fibrosis by inhibiting hepatic stellate cell activation. The present study investigated whether rapamycin treatment could modify the degree of fibrosis, cellular apoptosis and oxidative stress (OS) in an experimental model of CP.**

Method : **Fifty-five male, Sprague-Dawley rats weighing 200-400g were randomized into four groups. CP was induced by intraductal trinitrobenzene sulfonic acid (TNBS) infusion in group A (n = 15) and group B (n = 15). Group C (n = 15) received intraductal TNBS and was killed for histologic confirmation at four weeks. Group D (n = 10) received intraductal saline instead of TNBS. Group A and group D received oral rapamycin (2 mg/kg/d) for two weeks after CP was induced while group B received oral tap water instead of rapamycin. Blood and pancreatic tissue specimens were collected and oxidative stress parameters, fibrosis and cellular apoptosis were determined.**

Results : **Tissue and blood malondialdehyde (MDA) levels were significantly lower in rapamycin treated group compared to controls ($p < 0.001$). Superoxide dismutase (SOD) and glutathion peroxidase (GSH-Px) activities were also significantly higher in the active treatment group ($p < 0.001$ for both). Tissue and blood MDA, SOD, GSH-Px measurements was similar in rapamycin group and pancreatic cannulation group ($p>0.05$). Histopathologic fibrosis scores were similar in rapamycin and control groups. Apoptotic cell counts tended to be lower in rapamycin treated animals.**

Conclusion : **Administration of rapamycin alleviated OS and, in part, prevented apoptotic cell death in experimental CP, but did not reduce fibrosis.** (Acta gastroenterol. belg., **2015**, 78, **3-7**).

Key words : rapamycin, chronic pancreaitis, apoptosis, fibrosis.

Introduction

Chronic pancreatitis (CP) is a progressive and irreversible inflammatory disease of exocrine and endocrine pancreas characterized by glandular atrophy, calcification and fibrosis (1). As in the case of hepatic stellate cells (HSCs) in the pathogenesis of liver fibrosis, pancreatic fibrosis is induced by the activation of pancreatic stellate cells (PSCs), another cell type in the diffuse stellate cell system (2,3,4). PSCs are activated due to repeated and prolonged exposure to proinflammatory cytokines, such as tumor necrosis factor-α or interleukin 1. The process of PSC activation involves transformation into a myofibroblast-like phenotype characterized by positive staining for the cytoskeletal marker protein alpha smooth muscle actin (α-SMA) and increased synthesis and secretion of extracellular matrix components including collagens, fibronectin, laminin, proteoglycans, and hyaluronan (3,4).

Inhibition or suppression of activation and/or proliferation of quiescent PSCs may decrease pancreatic damage and fibrosis in CP. Usefulness of such an approach has been shown in liver fibrosis through induction of apoptosis of stellate cells (5,6). Moreover, in previous studies, treatment with rapamycin successfully inhibited proliferation of HSCs and reduced collagen accumulation in experimental liver fibrosis (7,8).

Rapamycin is a well-characterized member of mammalian target of rapamycin (mTOR) inhibitor family, which binds to intracellular FK506-binding proteins, causing inhibition of mTOR and blockage of transition of cell cycle from S phase to G1 phase (9). Clinically, rapamycin is actively used to prevent rejection after renal transplantation (10). Although how HSC proliferation is suppressed by rapamycin has been identified partly (7,8,11), the precise mechanisms of its in-vivo antifibrotic actions have not been fully understood.

The purpose of the present study was to investigate whether rapamycin treatment could modify the degree of oxidative stress (OS), fibrosis, and cellular apoptosis in an experimental model of CP.

Materials and methods

Institutional Committee of Animal Use and Care of Gulhane School of Medicine, Ankara, Turkey, approved the study protocol. All experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Handling of Animals.

Animals and study design

Male Sprague-Dawley rats weighing 200 to 400 g were provided by the Gulhane School of Medicine

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Submission date : 09/09/2014
Acceptance date : 09/11/2014

Research Center, Ankara, Turkey. Animals were kept under a 12 h-light/dark cycle with free access to food and water. Rapamycin (Rapamune ; Wyeth-AHP, Ankara, Turkey) was purchased from the local provider.

The study included 4 groups of animals. CP was induced in groups A (n = 15), B (n = 15) and C (n = 15). Animals in group D also underwent pancreatic duct cannulation but they were injected with 0.4 ml of saline instead of active chemical. CP was induced by a single dose trinitrobenzene sulfonic acid (TNBS) according to a previously described method (12,13). Briefly, after laparotomy performed 1 cm under xiphoid, pancreatic duct was cannulated and 2% TNBS dissolved in 0.4 ml of 10% alcohol (pH : 8) was infused. Because pancreatic fibrosis is known to develop 3 weeks after TNBS administration, rapamycin treatment was started by the end of the 4th week after surgery. Rapamycin was given via an orogastric tube at a dose of 2 mg/kg/day for 2 weeks in groups A and D, while group B animals received water (2 ml/kg/day) using the same approach. Animals in group C were killed 4 weeks after CP was induced followed by appropriate sampling. Group A, B and D animals were killed 3 weeks after the end of treatment. Tissue and blood samples were collected for biochemical parameters and histologic analyses. Blood samples were taken directly from the heart and collected in EDTA tubes. Tissue samples were kept in 10% formaldehyde solution until processed.

All animals received Crystallized penicillin (20.000U, im) to prevent postoperative infections, and weight changes were monitored weekly. One rat in groups A and C, and 2 rats in group B died after pancreatic duct cannulation.

Blood and tissue oxidative stress parameters

Malondialdehyde (MDA) level, superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) activity, indirect markers of OS were measured by previously described methods (14). Tissue SOD and GPx enzyme activities were reported in units per gram wet weight. Tissue MDA level was expressed in nanomoles per gram wet weight. Blood SOD and GPx enzyme activities were reported in units per milliliter. Blood MDA level was expressed in nanomoles per milliliter.

Histopathologic analyses

Tissue samples were fixed with 10% phosphate-buffered formalin at room temperature overnight and were embedded in paraffin. 4-6 micron thick sections taken from paraffin-embedded tissues were used for histopathologic examination (hematoxylin-eosin and Masson-Trichrome stain). All sections were examined under a light microscope (Leica DM400B) and were portrayed (Leica DFC280). Histomorphological evaluation was performed by using a previously described scoring system with small modifications (15). Cell infiltration, atrophy, fibrosis, degenerative changes and pseudotubule formation were evaluated semiquantitatively by two blinded pathologists.

Apoptotic cell counts were determined under light microscope and apoptotic indexes were calculated using TUNEL system. TUNEL staining was performed based on the instructions of manufacturer (In Situ Cell Death Detection Kit, ROCHE). TUNEL stained cell counts were scored as 0, 1+, 2+, 3+ when none, < 25%, 25-50% and > 50% of cells were noted positive, respectively. Intensity grading of apoptotic cells in pancreatic endocrine glands, exocrine glands, intralobar ductal and interlobar ductal epithelial cells were reported as none, mild, moderate and marked when scored as 0, 1+, 2+, and 3+, respectively. Scores for each region were calculated and sum of the scores were recorded.

Statistical analysis

The data were analyzed by using SPSS PC version 15.0 (SPSS Inc. USA). Categorical values (histologic examination) were expressed as median and percentiles (25%-75%). Mann-Whitney U test was used to evaluate the differences of the groups. OS parameters were expressed as mean ± SEM. One-way analysis of variance test and Tukey's test significant difference as post hoc were used to evaluate the differences among the groups. $P < 0.05$ was considered statistically significant.

Results

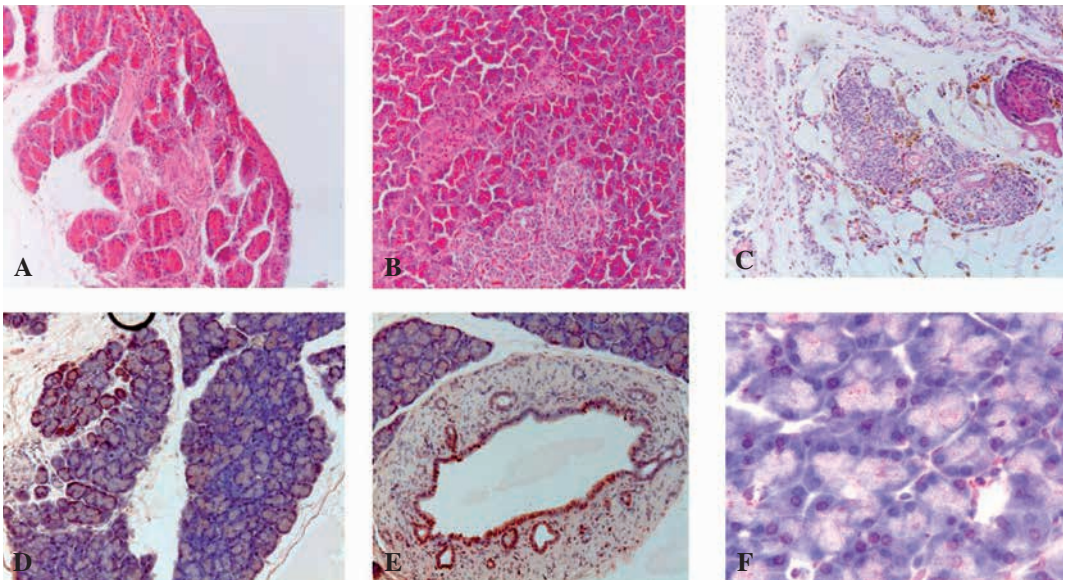
Animals in groups A, B and D showed significant weight gain by the study end (p = 0.003, p = 0.003 and p = 0.036, respectively).There was no difference among the groups in terms of weight gain. Group C animals (untreated CP) which were killed 4 weeks after TNBS infusion to confirm establishment of CP showed morphologic findings of pancreatitis including lobular atrophy and marked collagen deposition.

Oxidative stress parameters

Blood and tissue MDA levels in rapamycin treated animals with CP (group A) were significantly lower than those in controls (group B) (for both results, p < 0.001) (Table 1). In addition, blood and tissue SOD and GPx enzyme activities in group A were induced significantly compared to group B (for all results, p < 0.001). Blood and tissue mean MDA levels in group D were lower than those in group A, but neither difference was significant. Groups A and D showed similar blood and tissue SOD and GPx enzyme activities (Table 1).

Histopathologic examination

Atrophic changes and cellular infiltration were found similar in rapamycin treated group A compared to vehicle treated group B animals with CP. Although degenerative changes were more pronounced in group A, it was graded as ''moderate'' in both group A and group B.



A. Intralobular fibrosis, group A, HE, ×100 ; B. Degenerative changes in exocrine glands, intralobular band fibrosis, group B, HE, ×100 ; C. Atrophy, pseudolob formation and fibrosis, group C, HE, ×100 ; D. Apoptotic changes in exocrine gland epithelial cells, group A, TUNEL, ×100 ; E. Apoptotic changes in interlober duct epithelial cells, group B, TUNEL, ×100 ; F. Apoptotic changes in exocrine gland epithelial cells, group C, TUNEL, ×400.

Fig. 1. — Histopathological findings and apoptotic activity microscopy.

Interlobular and intralobular fibrosis were observed in a relatively mild pattern (Fig. 1A and 1B) in these two groups. In group C, histopathologic findings were in a milder pattern compared to group A or B (Fig. 1C). Histopathologic findings were almost normal and cell infiltration was detected only in one sample from group D animals.

Rapamycin treated animals with CP had significantly higher scores for fibrosis compared to other groups ($p < 0.05$, for all comparisons). Fibrosis severity was similar in groups B and C, whereas it was lowest in group D compared to other three groups ($p < 0.05$) (Table 1).

Moderate grade of cellular apoptosis was detected in group A and B, as scattered nuclei in exocrine glands and ductular epithelial cells (Fig. 1D and 1E). Apoptotic activity in group B was higher than that observed in group A, but the difference was not significant. In group B, apoptosis was also observed in islet cell, though in a less severe pattern. CP control animals (group C) showed lesser apoptotic activity in acinar cells (Fig. 1F). Apoptosis was detected in rare areas in group D (Table 1).

Discussion

In this study, we showed significant improvements in blood and tissue markers of OS in experimental CP after treatment with rapamycin. However, rapamycin treatment failed to alleviate fibrosis and acinar cell apoptosis on histologic examination, suggesting that collagen producing cells of the pancreas (i.e., PSCs) were not responsive to inhibition of mTOR signaling. Although modification of stellate cell proliferation by rapamycin was demonstrated in rats with liver fibrosis (7), natural course of CP may be more destructive to the organ because of distinctive morphological features and insufficient regeneration capacity.

At present, pathophysiological mechanisms of CP have not been clearly understood. The main theories proposed so far includes ductal obstruction, necrosis-fibrosis, toxic metabolites and OS all of which eventually lead to collagen accumulation and disruption of the pancreas. Increased OS is a dominant figure in the pathogenesis of pancreatic injury in response to an acute event (16), resulting in activation of nuclear factor kappa B (NF-κB) (16). Repeated exposures to reactive oxygen species (ROS) trigger synthesis of proinflammatory cytokines impairs antioxidant capacity of the pancreas, eventually causing tissue damage and fibrosis (17). PSCs, key elements that produce collagen in the pancreas during chronic injury, were shown to be directly activated after xanthine oxidase-derived free radicals exposure (18). Accordingly, reduction in collagen accumulation after improvement of OS has been shown in experimental CP (13,14). In the present study, rapamycin treatment ameliorated tissue OS parameters in rats with CP ; however, to the best of our knowledge, it has never been studied whether such a treatment specifically targets a particular cell type in the pancreas. In a stem cell study, rapamycin was found to stimulate OS response genes in a favorable manner (19). On the other hand, treatment with rapamycin might also cause divergent effects in different health or disease conditions. As an example,

Table 1. — Biochemical and histologic results

	Group A CP (+), Rapamycin (+)	Group B CP (+), Placebo (+)	Group C CP (+)	Group D CP (-), Rapamycin (+)
Tissue OS parameters				
MDA (nmol/gr)	0.89 ± 0.22	1.59 ± 0.33	2.43 ± 0.3	0.8 ± 0.29
SOD (U/gr)	41.9 ± 5.39	31.08 ± 5.76	19.72 ± 2.93	44.94 ± 4.49
GSH-Px (U/gr)	28.5 ± 3.94	20.22 ± 3.5	13.18 ± 1.96	30.01 ± 3
Blood OS parameters				
MDA (nmol/ml)	2.86 ± 0.71	5.07 ± 1.06	7.78 ± 0.97	2.65 ± 0.97
SOD (U/ml)	712.21 ± 91.74	515.3 ± 90.61	335.36 ± 49.84	764.89 ± 80.83
GSH-Px (U/ml)	1.76 ± 0.23	1.27 ± 0.22	0.83 ± 0.13	1.89 ± 0.19
Histology				
Fibrosis	8 ± 3.26	4.23 ± 3.11	3.14 ± 2.54	0.20 ± 0.63
Apoptosis	4.43 ± 4.24	5.69 ± 6.82	1.36 ± 2.13	0

OS parameters *;* $P > 0.05$ for groups A vs. D, $P < 0.001$ for all other results, Fibrosis ; $P > 0.05$ for groups B vs. C, $P < 0.05$ for all other results, Apoptosis ; $P < 0.05$ for groups A vs. D and B vs. D, $P > 0.05$ for all other results.

mTOR inhibition in kidneys exposed to ischemia reperfusion injury led to decreased cytoprotective capacity in kidneys, but in chronic kidney disease models, inhibition of mTOR with rapamycin ameliorated glomerular hypertrophy, decreased renal expression of proinflammatory and profibrotic cytokines, and retarded the development of tubulointerstitial inflammation and fibrosis (20). Both rapamycin and FK-506 were shown to decrease intracellular generation of ROS, inducible nitric oxide synthase production and nitric oxide expression at mRNA level through inhibition of NF-kappa B activation in rat hepatocytes (21). Accordingly, rapamycin treatment in animals with either established cirrhosis or early portal hypertension improved fibrosis (11). In our study on an animal CP model, we found rapamycin to reduce OS parameters in blood and pancreas tissue, close to the level that was observed in the negative control group.

Despite improvements in OS parameters, rapamycin treatment showed no benefit of fibrosis regression in the pancreas in the present work. The primary reason of this finding may be that degree of scar formation in the pancreas after TNBS infusion might have exceeded the point of possible regression. This was indeed clearly evident in the specimens obtained from CP animals killed by the end of the forth week. Studies from experimental cirrhosis and fibrosis of liver consistently showed that recovery of matrix deposition and reversibility of fibrosis cannot be achieved after destruction of the core metabolic unit of the organ. Because we did not include one more group to start rapamycin treatment simultaneously with TNBS infusion, any preventive role of mTOR inhibiton in the course chronic injury could not be determined. Another concern lays over the method of CP induction on animals. In liver studies, rapamycin treatment could decrease fibrosis more successfully in bile duct ligation model compared to that in thioacetamide model in which primarily affected cells are hepatocytes but not bile duct cells (11). TNBS infusion triggers chronic injury by affecting on pancreatic acinary cells ; thus, whether an obstruction model for CP would be more responsive to recovery could not be estimated in our study.

Apoptosis is considered as a vital component of various processes including normal cell turnover, development and functioning of immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (22). Bateman *et al*. reported that apoptosis plays a significant role in acinar cell loss in chronic pancreatitis, and apoptotic activity also starts in islet cells concurrently with the increase in inflammatory processes (23). In another study, rapamycin decreased the proliferative index and enhanced apoptosis in rats with coronary artery injury (24). In our experiment, we detected significant increase in apoptotic activity 4 weeks after pancreas injury, primarily among acinar cells. As pancreas inflammation progressed, apoptotic activity spread to islet cells. Although rapamycin treatment did not significantly decrease the amount of cells undergoing apoptosis, a preventive action was evident in some samples.

A major limitation of the present investigation was the absence of evaluation of α-SMA expression in pancreas tissues to identify activated PSCs and the degree of apoptosis via TUNEL staining specifically in these cells.

In conclusion, the present study showed that rapamycin treatment ameliorated tissue and blood OS and, to some extent, prevented apoptotic cell death in rats with experimental CP. Rapamycin showed no effects on fibrosis in TNBS induced experimental CP.

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