The development of cigarette smoke induced chronic pancreatitis in mice is associated with increased expression of K-Ras and NF-κB

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Abstract
Introductions and Objective. Epidemiological studies have demonstrated a strong association between cigarette smoking (CS) and chronic pancreatitis (CP); however, the exact mechanisms of this phenomenon remains unknown. The authors have previously shown that increased Ras expression activates the NF-κB mediated pathway and promotes development of CP. However, it is unclear whether a similar phenomenon occurs in CS-induced CP. Therefore, the aim of the study was to determine whether CS increases the expression of K-Ras, and promotes the development of CP in mice exposed to repeated episodes of acute pancreatitis (AP).

Materials and method. C57BL6/cmdb mice were exposed to CS or a sham treatment for 12 weeks. After one week of exposure, half of the animals from both groups were additionally subjected to repeated cerulein treatment (once a week, for 10 consecutive weeks) to mimic recurrent episodes of AP. Extension of pancreatic damage was determined histologically by H&E and Trichrome staining. The expression of K-Ras protein and downstream components (NF-κB, Cox-2, TGF-β) was evaluated by immunohistochemistry.

Results. C57BL6/cmdb mice exposed to CS or cerulein alone did not develop any chronic pancreatic damage. However, concomitant treatment with both of these agents caused focal acinar atrophy, with slight intralobular and perivascular areas of fibrosis, and inflammatory cells infiltration resembling mild CP. Moreover, immunohistochemistry examinations revealed increased pancreatic expression of K-Ras and NF-κB only in mice treated both with CS and cerulein.

Conclusions. CS promotes development of CP in mice exposed to repeated episodes of AP. This process may be, at least partially, related to increased expression of K-Ras and NF-κB protein.

Key words
mice, mutation, pancreatitis chronic, pancreatic neoplasm, tobacco

INTRODUCTION

Chronic pancreatitis (CP) is an inflammatory condition, characterized by irreversible loss of the pancreatic parenchyma, fibrosis and eventually development of exocrine and endocrine pancreatic insufficiency. It is responsible for significant deterioration in the quality of life and excess mortality [1]. Moreover, CP is also associated with pancreatic cancer with more than an 8-fold increased probability of tumour development [2]. Epidemiological data have shown an increased incidence of CP in recent years [3, 4]. Considering the high disease morbidity and mortality, clinicians should be aware of the risk factors responsible for the onset of the disease. It is well established that alcohol consumption is the most common cause of CP, responsible for about 50% cases of the disease [5]. However, it is much less appreciated that cigarette smoking is also associated with CP development and progression [6, 7, 8]. Smokers have a 2- to 3-fold higher risk of CP, compared to non-smokers [9]. Moreover, smoking cessation significantly reduces the progression of CP [9].

Cigarette smoke (CS) consists of mainstream and sidestream smoke and contains about 4,000 chemicals and at least 60 different carcinogenic factors. The components of CS interact with each other or synergize in their action. Concentration of nicotine, a main component of CS, in pancreatic juice was found to be 7 times higher in smokers than in non-smokers [10]. Nicotine has a broad spectrum of action on pancreatic cells. Long-term nicotine exposure decreases secretion while synthesis of digestive enzymes remain unchanged. As a result, an increased concentration...
of pancreatic enzymes was observed in acinar cells [11]. Nicotine has been shown to increase pancreatic damage, collagen deposition and pancreatic stellate cell activation [12]. Moreover, nicotine exposure of acinar cells isolated from mice with K-Ras mutation has been shown to hyperactivate Ras and its downstream component extracellular – the signal-regulated kinase (ERK) [13]. The most potent nicotine metabolite, 4-(methylamino)-1-(3-pyridyl)-1-butane (NNK), promotes inflammation via activation of molecules downstream of Ras, such as cyclooxygenase-2 (Cox2) and nuclear factor kappa B (NF-κB) [6].

Data from animal experiments have shown that CS exposure causes inflammation and development of pathological lesions within the pancreas [14, 15]. This action may be mediated, at least partially, by the Ras signaling pathway. Ras protein regulates many intracellular processes, including cell proliferation, differentiation, apoptosis, and migration. The authors have shown that Ras protein is strongly involved in the process of pancreatic inflammation [16]. External stimuli, e.g. infection, high fat diet or cholecystokinin analogue – ceruline, can easily upregulate the Ras signaling pathway, leading to the development of CP and eventually pancreatic cancer [16, 17, 18]. Moreover, our data suggest that transcription factor NF-κB, which is activated downstream of Ras, is also involved in this process. It is tempting to speculate that similar mechanisms are engaged in the development of smoking-induced pancreatic injury.

Despite many attempts, the exact molecular mechanisms responsible for smoking-induced CP remain to be discovered. In order to achieve this goal, a clinically-relevant animal model of smoke-related pancreatic injury has to be developed. Currently available experimental models, based on the exposition of animals to a CS or its components, do not fully reproduce the pathological lesions found in humans. The reason for that, in our opinion, is lack of concomitant risk factors acting together with CS. Therefore, the aim of this study was to determine whether CS promotes the development of CP in mice exposed to repeated episodes of acute pancreatitis (AP). The secondary aim was to evaluate whether K-Ras protein and its downstream effectors are involved in this process.

MATERIALS AND METHOD

Treatments in animals. Animal experiments were carried out at the Centre for Experimental Medicine (CEM) animal facility, Medical University of Białystok, Poland, according to EU Directive 2010/63/EU and approved by the Local Ethic Committee for Experiments with the Use of Laboratory Animal (20/15, 25.02.2015). 60-day-old male C57BL6/cmdb mice (a strain developed in CEM) were subjected to a smoking protocol was used (2 sec., 3 puff, 1 per min., total of 9 puffs). Mice were placed in the rodent exposure chamber and exposed to CS for 90 min (20 cigarettes per session) daily for 3 months. Sham treated mice were exposed to the room air at the same time.

Exposure to cigarette smoke (CS). Mice were randomly assigned to a CS exposure group (CS, n=10) or sham treatment group (Ctr, n=10) for 12 weeks (Fig. 1). The 3 months exposure duration was based on previous publications determining CS-induced CP [14, 20]. To mimic real-life tobacco exposure, a microprocessor-controlled cigarette smoking apparatus (Teague TE-10C Davis, CA, USA) was used. The system consisted of the smoking machine, a smoke control hood with mixing and dilution chamber, exposure chambers with air moving and flow measuring equipment. To simulate ‘active smoking’ animals were exposed to the mixture of the side-stream (85%) and mainstream (15%) smoke from Marlboro Red Box cigarettes (Philip Morris International, 1 mg Nicotine, 13 mg Tar), one of the most popular brands of cigarettes in Poland. Marlboro cigarettes were preferred instead of reference cigarettes because the latter are lacking about 600 potentially harmful compounds included in commercial cigarettes, nor are they smoked in real life. Cigarettes were kept at 4 °C and 48 h before use, they were transferred to a chamber with glycerin and water to achieve humidity of 60% at room temperature. Federal Trade Commission cigarette smoking protocol was used (2 sec., 35cm² puff, 1 per min., total of 9 puffs). Mice were placed in the rodent exposure chamber and exposed to CS for 90 min (20 cigarettes per session) daily for 3 months. Sham treated mice were exposed to the room air at the same time.

Ceruline treatment. To mimic recurrent episodes of AP, one week after smoke exposure, CS (n=5) and Ctr (n=5) mice were randomly divided and treated with ceruline or vehicle only injections. Ceruline (Sigma-Aldrich) was injected i.p., hourly over 7 hours (50 μg/kg of cerulean in 100μl of saline with 0.1% BSA), once a week, for 10 consecutive weeks (Fig. 1). The last course of ceruline treatment was performed 2 weeks before the end of the study to allow the mice to recover from AP.

Tissue collection. After 12 weeks of CS or sham exposure, mice were sacrificed by cardiac puncture. Pancreata were completely removed and washed in sterile saline. Weight of the pancreas was measured and determined as a percentage of the total body weight (pancreas weight / body weight × 100%).

Histology. After fixation with 10% formalin, the pancreata were embedded in paraffin, cut into 5-μm sections, and stained with haematoxylin and eosin (H&E). All stained specimens of pancreatic tissue were randomly examined at ×100 magnification to determine potential pathological lesions. To detect collagen deposits in the pancreas, trichrome staining (Sigma-Aldrich) was performed as described previously [16].

Figure 1. Treatment schedule. Animals were exposed to CS or a sham treatment (no smoke exposure) for 90 min. once a day for 12 weeks. To develop recurrent AP, half of the mice from both groups were additionally treated with cerulein for 10 consecutive weeks. Animals were sacrificed at week 12, 14 days after the last ceruline or vehicle only treatment.
Immunohistochemistry. In order to determine the involvement of K-Ras protein and its downstream signaling pathway components, immunohistochemical staining for K-Ras (K-Ras-2B Antibody (C-19); sc-521), NF-kB (NFkB p65 Antibody (C-20); sc-372), TGF-beta (TGFβ1 Antibody (3C11); sc-130348), Cox-2 (Cox-2 Antibody (29); sc-19999) (Santa Cruz Biotechnology), was performed in pancreatic paraffin sections. Presence of inflammatory cells was measured with the use of antibodies against CD45 antigen (CD45 Antibody (30-F11); sc-53665) (Santa Cruz Biotechnology). To visualize activation of pancreatic stellate cells, an alpha-Smooth Muscle Actin antibody (anti–α-SMA Antibody (B4); sc-53142) was used. To performed immunohistochemical staining, slides were deparaffinized first, antigens were retrieved in the EnVision Flex Target Retrieval Solution (DAKO) at low pH, and unspecific binding was blocked. Primary antibodies were applied for 60 min at room temperature, followed by administration of visualization reagent EnVision (DAKO) for 30 min., and DAB solution for 10 min. Counterstaining of the slides was performed with the use of haematoxylin. All slides were assessed in the random 10 fields (×20). Positive and negative controls were stained for each antibody. The histopathological score included 2 variables: 1/ % of positive cells, 2/ intensity of staining. Percentage of K-Ras, NFkB, TGF-beta and Cox-2 expression was evaluated as follows: 0 – when the expression was seen in less than 5% of the cells; 1 – when the expression was seen in more than 5% and less than 30%; 2 – when the expression was seen in more than 30% and less than 60%; 3 – when the expression was seen in more than 60% of the examined cells. α-SMA expression was evaluated as focal (when less than 30% of the examined fields showed expression) or diffuse (when more than 30% of the examined fields showed expression). The intensity was evaluated as negative – 0, weak – 1 or strong – 2. The final result was calculated as a score of percentage and intensity.

Blood glucose. Blood samples were collected by tail-tip amputation without anesthesia to avoid the effects of drugs on the glucose levels. Blood glucose level was determined with standard glucometer (ACCU-CHECK, Roche) commonly used in clinical practice.

Serum amylase activity. The serum level of pancreatic hydrolytic enzyme, amylase, was determined to exclude ongoing AP after cerulein injections with the use of the Phadebas test (Pharmacia Diagnostics), as described previously [21].

Statistics. For statistics analysis, a two-sided unpaired Student’s t test was used, with p < 0.05 considered as statistically significant. All values were presented as mean ± SD.

RESULTS

Combined exposure to different environmental factors has caused pathological changes in the pancreas. After 12 weeks of the experiment, the average pancreatic weight was significantly higher in animals exposed to both CS and cerulein, compared to control animals (1.32% ± 0.11 vs. 0.91% ± 0.24; p < 0.01) (Fig. 2a). Moreover, pancreata of these animals tended to be bigger than the pancreata of cerulein only (1.15 ± 0.19) or CS only treated mice (1.15 ± 0.14); however, the differences were not statistically significant. Macroscopically, the pancreata of mice with the combined exposure to CS and cerulein appeared more swollen than the other animals. To rule out the possibility of organ enlargement due to ongoing AP, the level of serum amylase was determined. No differences in serum amylase values were found between all study groups (Fig. 2c). Blood glucose level, a parameter of endocrine insufficiency, was also analyzed but no significant differences were observed between the treatment groups (Fig. 2b).

Macroscopic changes in the pancreas after combined CS + cerulein exposure were accompanied by histological changes resembling mild chronic pancreatitis. 12-weeks exposure to CS only or 10 repeated episodes of cerulein-induced AP in mice exposed to room air, did not cause development of macroscopic or microscopic lesions within the pancreas (Fig. 3a–c). The pancreata of these animals were completely normal, similar to the control mice. However, concomitant exposure to CS and cerulein resulted in the development of local lesions resembling early stage CP. We found local acinar atrophy and slight intralobular areas of oedema (Fig. 3d). The lesions were very limited (<5% of pancreatic tissue) and had patchy localization, independently of pancreatic region. Moreover, Trichrome Masson staining revealed enhanced collagen depositions in perivascular areas and infiltrating the surrounding pancreatic tissue (interstitial fibrosis), only in animals exposed simultaneously to both environmental factors (Fig. 3b). As with acinar atrophy, positive collagen
TGF-β was evaluated. Interestingly, immunohistochemistry staining showed increased pancreatic expression of K-Ras protein only in animals after combined exposure to CS and cerulein, which means in mice with histological features resembling mild CP (Fig. 4a-d). In this group of animals, we observed diffuse K-Ras expression with strong intensity of staining in more than 30% of the acinar cells (score 2+2). Moreover, differences were found in pancreatic NF-kB expression between the groups. Positive staining of the p65 subunit was lower and patchy in pancreatic acini exposed to CS alone, and strong, and diffuse (score 2+2) in CS + cerulein treated mice (Fig. 4e-h). No significant changes were observed in COX-2 and TGF-beta expression in the examined groups (data not shown).

**DISCUSSION**

CP is characterized by loss of acinar cells and replacement of the normal pancreatic parenchyma with fibrous connective tissue. As a result, pancreatic exocrine and endocrine insufficiency is observed. Despite excessive studies analyzing pathogenesis of CP, the mechanisms responsible for the onset and progression of the disease remain unknown. For example, everyone knows that alcohol is a major risk factor for CP; however, we do not know why only 3% of alcoholics develop CP. This phenomenon suggests that additional factors can be involved in this process. Clinical data have established that cigarette smoking is an independent risk factor for CP [6, 7, 8]. The effect of smoking on CP development is stronger than immoderate alcohol consumption [8]. Moreover, the likelihood of developing CP depends on the number of cigarettes smoked per day. The relative risk of CP for people smoking < 1 pack / day is 1.69 and for subjects smoking > 1 pack/day increases to 3.35 [7]. Interestingly, many patients as well as physicians do not acknowledge smoking as a potential reason for CP development [22].

Despite strong clinical evidences showing association between smoking and CP, there are limited experimental data regarding exact disease pathogenesis. To explore the mechanisms responsible for smoking-induced CP, a reliable and clinically-relevant experimental model has to be established, which will help recognize the key steps involved in development of pancreatitis, and facilitate finding potential therapeutic targets. So far, few animal models have
been developed. In one of the studies, 60% of rodents treated with a high but not low-dose of CS for 12 weeks, developed microscopic lesions resembling mild CP, very similar to the lesions observed in our experiment [20]. However, in our study we induced CP with low-dose of CS exposure. The reason for that may be concomitant treatment with another external stimuli – cerulein. Moreover, Marlboro Red Box cigarettes, the one we used in our experiment, contain more nicotine (1.0mg vs. 0.8mg) and tar (13mg vs. 10mg) than 1R4F cigarettes used in the other study. Unfortunately, although typical features of CP were found (acinar atrophy, fibrosis, inflammatory cells infiltration), the lesions were very limited and patchy. In our experiment, only a small proportion of the pancreas (~5%) was affected by the disease. Our results are very similar to other studies where only very focal and limited lesions were observed [14, 20]. Generally, pancreatic inflammation caused by CS in the current and others studies was milder, compared to CP observed in humans. In our opinion, the reason for this is the relatively short treatment period, compared to real-life smoke exposure.

An intriguing question is what mechanisms are involved in smoking-induced CP? This knowledge can be of great practical importance and may help to prevent development of the disease. So far, the mechanisms by which smoking contributes to pancreatic inflammation are still poorly understood. Wittel et al. showed increased vulnerability to pancreatic self-digestion caused by inappropriate ratio between trypsinogen and pancreas-specific trypsin inhibitor (PSTI) in rodents exposed to CS [14]. In the smoke exposed animals, the trypsinogen level was elevated, while PSTI was unchanged, making the animals more susceptible to pancreatitis. Nicotine affects acinar cells function in multiple mechanisms. Long-term treatment with nicotine reduced pancreatic secretion, leading to increased concentration of enzymes within the cells (increased amylase concentration and trypsinogen 2 to trypsinogen 1 ratio) [23]. Moreover, nicotine exposure can up-regulate epidermal growth factor receptor and influence Ras activation. It has been shown that pancreatic inflammation and resulting cascade of inflammatory mediators can be mediated by the Ras signaling pathway.

Our previous results strongly suggested that the Ras protein is a key player in the process of CP development [16, 17, 18]. In mice with pancreatic K-Ras mutation, physiological levels of oncogenic K-Ras were without pathologic effect. However, exposure of these mice to environmental factors, such as high fat diet or inflammatory agents (cerulein, lipopolysaccharide), caused strong increase of Ras activity and further stimulation of downstream molecules, e.g. NF-κB and Cox-2 that promoted inflammation [16]. In the current study, we found that the development of CP after CS exposure was accompanied by increase expression of both K-Ras and NF-κB in the pancreas. Although we did not examine Ras activity, it is highly probable that it would also be increased. To-date, very limited experimental data exploring the effect of K-Ras protein on smoking-induced pancreatic injury are available. Long-term nicotine exposure in mice with mutant K-Ras expression in the pancreas, caused lesions resembling pancreatic intraepithelial neoplasia (PanIN) [13]. This process was accompanied by increased infiltration of macrophages into the stroma compartment, similar to that observed in our study. Moreover, chronic nicotine treatment has caused hyperactivation of oncogenic K-Ras. Exposure of mice with pancreatic K-Ras mutation to CS caused the development of pre-malignant lesions with infiltration of macrophages and pro-inflammatory cytokines [24]. In our experiment, no development of any PanINs was observed; however, we used mice without artificial K-Ras mutation. In our opinion, this model is clinically more reliable as most smokers do not have genetic abnormalities in the pancreas. In a meta-analysis of pancreatic cancer incidence, cigarette smoking history was not significantly correlated with a higher incidence of K-Ras mutations (OR=1.26) [25]. These results suggest that tobacco smoke affects the risk of pancreatitis and cancer independently of K-Ras mutations. As shown in the current study, chronic activation of the Ras protein by components of cigarette smoke may result in increased expression of NF-κB, leading to the progression of inflammation.

This study has some limitations. Primarily, we developed the mice model of CP, so we cannot directly extrapolate the findings of our research to people with CP. Patients with CP are subjected to many additional, potentially harmful agents that can affect the severity of the disease. Being aware of these limitations, in the current study an attempt was made to mimic real-life conditions. Therefore, recurrent episodes of AP were induced in animals exposed to cigarette smoke with the use of processor-controlled apparatus to simulate active smoking. Moreover, we used commercially available cigarettes in the experiment, which is more clinically relevant. Secondly, though we obtained development of CP in mice, the extent of the lesion was relatively small, probably due to the short cigarette smoke exposition period. It would be interesting to determine if prolonged exposition to cigarette smoke would exaggerate the severity of CP. Finally, we did not evaluate the effect of cigarette smoke condensate on Ras expression and activity in isolated pancreatic acinar cells to confirm its role in the development of CP.

CONCLUSIONS

Cigarette smoke promotes the development of chronic pancreatitis in mice exposed to repeated episodes of acute pancreatitis. This phenomenon may be connected to increased expression of K-Ras protein and its downstream signaling component – transcriptional actor NF-κB. Further research is needed to clarify the mechanisms of action of K-Ras and its effectors in chronic pancreatitis caused by cigarette smoke.

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