European Journal of Pharmacology 746 (2015) 245–251

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

Inhibition of Ras signalling reduces neutrophil infiltration and tissue damage in severe acute pancreatitis

Changhui Yu [a,b], Mohammed Merza [a], Lingtao Luo [a], Henrik Thorlacius [a,*]

[a] Department of Clinical Sciences, Section of Surgery, Malmö, Lund University, 20502 Malmö, Sweden
[b] Department of Gastroenterology, Zhujiang Hospital of Southern Medical University, 510282 Guangzhou, China

ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form
13 November 2014
Accepted 17 November 2014
Available online 25 November 2014

Keywords:
Amylase
Chemokines
Inflammation
Neutrophils
Pancreatitis

ABSTRACT

Neutrophil recruitment is known to be a rate-limiting step in mediating tissue injury in severe acute pancreatitis (AP). However, the signalling mechanisms controlling inflammation and organ damage in AP remain elusive. Herein, we examined the role of Ras signalling in AP. Male C57BL/6 mice were treated with a Ras inhibitor (farnesylthiosalicylic acid, FTS) before infusion of taurocholate into the pancreatic duct. Pancreatic and lung tissues as well as blood were collected 24 h after pancreatitis induction. Pretreatment with FTS decreased serum amylase levels by 82% and significantly attenuated acinar cell necrosis, tissue haemorrhage and oedema formation in taurocholate-induced pancreatitis. Inhibition of Ras signalling reduced myeloperoxidase (MPO) levels in the inflamed pancreas by 42%. In addition, administration of FTS decreased pancreatic levels of CXC chemokines as well as circulating levels of interleukin-6 and high-mobility group box 1 in animals exposed to taurocholate. Moreover, treatment with FTS reduced taurocholate-induced MPO levels in the lung. Inhibition of Ras signalling had no effect on neutrophil expression of Mac-1 in mice with pancreatitis. Moreover, FTS had no direct impact on trypsin activation in isolated pancreatic acinar cells. These results indicate that Ras signalling controls CXC chemokine formation, neutrophil recruitment and tissue injury in severe AP. Thus, our findings highlight a new signalling mechanism regulating neutrophil recruitment in the pancreas and suggest that inhibition of Ras signalling might be a useful strategy to attenuate local and systemic inflammation in severe AP.

1. Introduction

One tenth of all patients with acute pancreatitis (AP) develop a severe form associated with a high mortality rate (Mann et al., 1994). The most feared complication in AP is lung failure. Although the exact mechanisms remain elusive, it has been reported that interleukin-6 (IL-6) and high-mobility group box 1 (HMGB1) play a role in promoting lung damage in severe AP (Luan et al., 2013; Zhang et al., 2013). However, treatment of patients with severe AP is mainly limited to supporting vital functions. The lack of more specific therapies is partly related to an incomplete knowledge of the underlying pathophysiology. Trypsin activation, leucocyte recruitment and microvascular perfusion failure are well-accepted components in the induction of severe AP (Regner et al., 2008; van Acker et al., 2006; Zhang et al., 2009). Numerous studies have shown that neutrophil accumulation is a rate-limiting step in AP. For example, blocking specific adhesion molecules necessary for neutrophil trafficking, such as P-selectin, intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1) reduces tissue damage in the inflamed pancreas (Awla et al., 2011a; Hartman et al., 2012; Werner et al., 1998). Moreover, tissue accumulation of neutrophils is also coordinated by secreted chemokines, including CXCL1 and CXCL2 (Bacon and Oppenheim, 1998; Li et al., 2004). CXCR2 is the high affinity receptor on neutrophils for CXCL1 and CXCL2 (Huber et al., 1991) and targeting CXCR2 has been found to protect against tissue damage in AP (Bhatia and Hegde, 2007). Considered together, the role of adhesion molecules and chemoattractants in regulating neutrophil infiltration in the inflamed pancreas is relatively well described, however, the knowledge about signalling mechanisms orchestrating pathological inflammation in pancreatitis is limited.

We have recently shown that Rho-kinase regulates trypsin activation and neutrophil recruitment in severe AP (Awla et al., 2011b). Rho-kinase is one of many effector molecules acting downstream of the Ras supefamily, including more than 50 different small GTPases (Gutkind and Vitale-Cross, 1996). These small GTPases act as molecular switches regulating important

* Corresponding author. Tel.: +46 40 331000; fax: +46 40 336207.
E-mail address: henrik.thorlacius@med.lu.se (H. Thorlacius).

http://dx.doi.org/10.1016/j.ejphar.2014.11.020
0014-2999/

cellular functions, including vesicle transport, cytoskeleton organization, adhesion, migration and survival (Alblas et al., 2001; Itoh et al., 1999; Slotta et al., 2006). GTPases are attached to the inner side of plasma membranes where they convert inactive Ras-guanosine-diphosphate (GDP) into active Ras-guanosine-triphosphate (GTP) (Ehrhardt et al., 2002; Hancock, 2003). Farnesylthiosalisylic acid (FTS) is a synthetic derivative of carboxylic acid, resembling the farnesyl cysteine group common to all small GTPases (Nevo et al., 2011). FTS interferes with the binding of activated Ras proteins to their escort proteins and therefore inhibits Ras–plasma membrane interactions. Although Ras signalling has mainly been associated with cell growth and oncogenic transformation, recent reports suggest that Ras is also involved in the pathogenesis of arthritis, nephritis, autoimmune diseases and infections (Clarke et al., 2003; Kafri et al., 2005; Katzav et al., 2001). However, the potential effect of FTS on inflammation and tissue injury in AP has not been investigated.

Based on the above, the aim of the present study was to define the functional significance of Ras signalling in regulating trypsin activation, CXC chemokine production, neutrophil accumulation and tissue damage in severe AP.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (20–25 g) were housed on a 12–12 h light dark cycle and fed a laboratory diet and water ad libitum. All experiments were approved by the local ethical committee at Lund University. Mice were anesthetized intraperitoneally (i.p.) with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight.

2.2. Acute pancreatitis

Through a midline laparotomy in anaesthetized animals were the second part of duodenum and papilla of vater identified. Traction sutures were placed 1 cm from the papilla and a small puncture was made through the duodenal wall (23 G needle) in parallel to the papilla of vater. A polyethylene catheter connected to a microinfusion pump (CMA/100, Carnegie Medical, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the common bile duct. The common hepatic duct was temporarily clamped at the liver hilum. 10 μl of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) was infused into the pancreatic duct for 10 min. Then, the catheter and the common hepatic duct clamp were removed. The duodenal puncture was closed with a purse-string suture. Traction sutures were removed and the abdomen was closed. Using this protocol we observed a total mortality rate of 5% within 24 h of pancreatitis induction. Sham mice underwent laparotomy and phosphate buffered saline (PBS) infusion into the pancreatic duct and were pretreated i.p. with vehicle (PBS, $n=5$). Vehicle (PBS) or the Ras inhibitor, FTS (10 mg/kg, Cayman Chemical, Boston, USA) was given i.p. prior to bile duct cannulation and induction of AP. One group of mice received 10 mg/kg of FTS alone without bile duct cannulation. This dose and scheme of administration of FTS was based on a previous investigation (Zhang et al., 2014). All animals were killed 24 h after induction of pancreatitis.

2.3. Amylase measurements

Blood amylase levels were determined in blood collected from the tail vein by use of a commercially available assay (Reflotron®, Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Myeloperoxidase (MPO) activity

A piece of the pancreatic head and lung tissue were harvested for MPO analyses. All frozen tissues were pre-weighed and homogenized in 1 ml mixture (4:1) of PBS and aprotinin 10,000 KIE per ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenates were centrifuged (15,339g, 10 min) and the supernatant was stored at −20 °C. The pellet was used for MPO assay as previously described (Laschke et al., 2007). All pellets were mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide and the samples were frozen for 24 h. Thawed samples were sonicated for 90 s, incubated in a water bath at 60 °C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25 °C). Values are expressed as MPO units per g tissue.

2.5. Histology

Tissue pieces from the head of the pancreas were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. 6 μm sections were stained (haematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system, including oedema, acinar cell necrosis, haemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scales as previously described (Schmidt et al., 1992).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Pancreatic levels of CXCL1 and CXCL2 as well as plasma levels of IL-6 and HMGB1 were determined by use of a double-antibody Quantikine enzyme linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine CXCL1, CXCL2, IL-6 and HMGB1 as standards. The minimal detectable protein concentration is less than 0.5 pg/ml.

2.7. Mac-1 expression on neutrophils

Blood was collected in acid citrate dextrose and incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labelling. Neutrophils were stained with APC-conjugated anti-Mouse Ly-6C (Clone: AL-21, BD Biosciences Pharmingen, San Jose, CA, USA), phycoerythrin-conjugated anti-mouse Ly-6G (clone 1A8, BD Biosciences) and fluorescein isothiocyanatee-conjugated anti-mouse CD11b (clone M1/70, BD Biosciences) antibodies. Cells were fixed and erythrocytes were lysed. Neutrophils were recovered following centrifugation and defined as $Ly\text{-}6G^+/Ly\text{-}6C^+$ cells. Flow-cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with Cell-Quest Pro software (BD Bioscience).

2.8. Acinar cell isolation

Acinar cells from the pancreas were prepared by collagenase digestion and gentle shearing as described previously (Saluja et al., 1999). Cells were suspended in HEPES–Ringer buffer (pH 7.4) and passed through a 150 μm cell strainer (Partec, Görlitz, Germany). Isolated acinar cells (1×10^7 cells per well) were immediately preincubated with PBS or FTS (10 or 100 μM, 30 min) and stimulated with cerulein (100 nM, 30 min) in duplicates. The buffer was then discarded and the cells were washed twice with buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3-(morpholino) propanesulphonic acid and 1 mM MgSO4. The cells were next