Blockade of JAK2 signaling produces immunomodulatory effect to preserve pancreatic homeostasis in severe acute pancreatitis

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ARTICLE INFO

Keywords:
JAK-STAT
Pancreatitis
Immunomodulation
sTREM-1

ABSTRACT

JAK/STAT plays an important role in cytokine signal transduction and it is potentially involved in the proinflammatory response during the early phase of severe acute pancreatitis (SAP). However, whether JAK2 activity is upregulated and whether JAK2 inhibition plays a role in the maintenance of pancreatic homeostasis during SAP is incompletely understood. Here we show that JAK2/STAT3 activity is highly elevated in SAP and blockade of JAK2 by AG-490 protects against SAP-induced pancreatic inflammation and injury. Gene expression and ELISA studies showed that JAK2 inhibition altered the cytokine profiles in both the circulation and pancreases. Further analysis revealed that JAK2 inhibition restored the level of cytokines critical for macrophage polarization towards M2 macrophage. Our findings suggest that pharmacological targeting at JAK2/STAT signalling may be an effective choice of therapeutic interventions against SAP.

1. Introduction

Severe acute pancreatitis (SAP) is a life-threatening disorder that induces a signalling cascade leading to the rapid amplification of the proinflammatory response at early phase [1–3]. If inflammation remains severe and uncontrolled, SAP eventually results in a state of uncontrolled tissue inflammation to cause failure of multiple organs such as the lung [2,3]. Therefore, it is imperative to identify key signaling targets to render the inflammation under control during the early occurrence and development of SAP.

Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is a critical cascade transducing biological signaling downstream of Type I and Type II cytokine receptors [4,5]. JAK/STAT pathway therefore plays an important role in the regulation of the expression of pro-inflammatory genes in a variety of cell types [4]. Inhibitors of JAKs had been developed to block the proinflammatory action of multiple cytokines to treat inflammatory and immune-related diseases including rheumatoid arthritis and inflammatory bowel disease [6,7]. Previous studies on pancreatic STAT3 reported that STAT3 has different roles in chronic and acute pancreatitis (CP and AP) in mice, with protective effect against CP but detrimental role in local and pulmonary inflammation during AP [8,9]. However, currently it is poorly understood whether JAK inhibition can benefit pancreatic homeostasis during SAP. In addition, the direct in vivo evidences showing the activation of JAK/STAT signaling in pancreas during SAP are still lacking.

In the present study, we focused to investigate the effect of pharmacological inhibition of JAK2 signalling on pancreatic homeostasis in a rat model of SAP. We aimed to uncover whether JAK2 inhibition modulates immune response in SAP by determination of key immunoregulatory factors in both peripheral blood mononuclear cells and pancreases. Our results showed that inhibition of activated JAK2 signaling in SAP protected against SAP-induced inflammation and pancreatic tissue damage.

2. Materials and methods

2.1. Animals, chemicals and antibodies

All rats were raised in the laboratory fed with standard diet and have free access to water. All animal experiments complied with the ARRIVE guidelines [10], and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. The experiment was approved by the ethical committee of animal research of Bengbu Medical College (NO. 2020-206). The rats were fasted for 12 h
before surgery and 30 male Sprague Dawley rats (4-week old, provided by Cavens Animal Laboratory Center, Changzhou, Jiangsu) were randomly divided into three groups: Control (n = 8), Severe Acute Pancreatitis (SAP, n = 11) and SAP plus AG-490 treatment (n = 11). The rats were anesthetized with intraperitoneal injection of 7% chloral hydrate (0.5 mL/kg). AG-490 (8 mg/kg, JAK2 inhibitor, MCE), was dissolved in DMSO and 20% SBE-β-CD (for increasing solubility), then mixed well in physiological saline solution. Phospho-JAK2 (Tyr1007/1008) antibody (1:1000, Cell Signalling Technology, #3776), JAK2 Polyclonal antibody (1:500, Proteintech, 17670-1-AP), Phospho-STAT3 (Ser 727) antibody (1:1000, HUA BIO, ET1607-39), STAT3 antibody (1:1000, Proteintech, 10253-2-AP), GAPDH antibody (1:20000, Proteintech, 60004-1-lg).

2.2. Animal model of experimental SAP

Briefly, the rat abdomen was disinfected with iodophor and shaved before the surgery. For the sham group, 1.5 cm midline incision was created in the abdomen, and the duodenum and pancreas were turned for several times. For the SAP group and treatment group, expose the pancreaticobiliary duct to the duodenum, and pierce the tip of a 5-gauge needle into the duodenal intestinal cavity and enter the pancreaticobiliary duct by opening about 0.5 cm. Meanwhile, close the pancreaticobiliary duct with arteriole forceps near the hepatic portal to prevent the pancreatic duct injection from returning to the liver. After completion, inject freshly prepared 5% sodium taurocholate (1.5 ml/kg). Clamp the pancreatic duct near the nipple with arteriole clamp for about 30 min. The white layer where PBMC is located is pipetted to wash again in 1× PBS for 3 times and subsequently incubated with secondary antibodies (1:5000) conjugated with horseradish peroxidase (Bio-Rad, USA) for 1 h at 37 °C. The PVDF membranes were washed again in 1× TBST for 3 times before development with enhanced chemiluminescence detected using the ChemiSence S300 Pro system.

2.6. Real-time PCR

Samples from PBMCs and pancreatic tissues were homogenized and efficiently lysed in the Trizol reagent (Invitrogen) before the isolation of total RNA according to the manufacturer’s instruction. 1 μg total RNA was reversely transcribed using RevertAid First Strand cDNA Reverse Transcription Kit (Thermo Scientific #K1622). After mixing the cDNA template and primer with SYBR Green reagent (Thermo Scientific #F415XL), the quantitative PCR reaction was performed on the ABI-7500 real-time PCR system (Applied Biosystems) with Gapdh as the house-keeping control. Relative gene expression level was calculated using the comparative C_T method. Please refer to Table 1 for the DNA sequences of primers.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The blood samples from rats were centrifuged at 3000 rpm for 10 min before serum collection. Rat IL-10, TGF-β and sTREM-1 ELISA Kits were used to determine the circulating level of IL-10, TGF-β and sTREM-1 using a microplate reader (Thermo MK3). To determine the level of IL-10, TGF-β and sTREM-1 in pancreas, normal saline was added to the tissue to mash it. Supernatant was collected for ELISA assay using Kits after centrifuge at 3000 rpm for 10 min.

2.8. Statistical analysis

All the results represent mean ± SD. GraphPad Prism (Version 6.0) was used for statistical analysis. The relative expression of genes and proteins was normalized to GAPDH. Prior to statistical difference test, normality and equal variance tests were performed. The data in this study exhibit normal distribution and differences were determined by unpaired two-tailed student’s t-test. The p value < 0.05 was considered as statistically different.

### Table 1 Real-time PCR Primer sequences.

| Primer | Sequence (5’-3’) | length |
|--------|------------------|--------|
| Foxp3-F | TGTGGCCCTGAGTGAGCACAG | 118 bp |
| Foxp3-R | TCTCCGACACAGAACAAACAG | |
| Il10-F | AAGAGGCGGCTACACAGAGAA | 213 bp |
| Il10-R | TTGTGTGGATCTGTGGGCTT | 114 bp |
| Il6-F | CACCCTCAAGTGGCAGGGCT | 167 bp |
| Il6-R | TCTGACATGTCATGAGCCT | |
| Tgfb1-F | AGGGCATCCATGCACTCACC | 168 bp |
| Tgfb1-R | CACCTGGATAAGAGAGGATCC | |
| Trem1-F | AGGAGGCTGTTGGCAGAGGC | 215 bp |
| Trem1-R | AAGGAGGGTGTGGCTGGAGAT | |
| Tnfa-F | TAAAGGTTCTATTGGTTCGG | 104 bp |
| Tnfa-R | AATGTCTCTGAAGTTCCCTTG | |
| Gapdh-F | AGAGGCTCTTCCTTGACCCCAT | 148 bp |
| Gapdh-R | GACACGTTGCTGTCATACCG | |
| HATF000101 | TGGAGAGAGGTGAGTGACCAA | 185 bp |
3. Results

3.1. Activation of JAK-STAT pathway in the experimental rat model of SAP

To show the direct evidence supporting upregulated JAK2 activity during SAP, we first determined the phosphorylation level of JAK2/STAT3 in pancreatic tissues. Western blotting results showed that, compared with the control group, while the total expression of JAK2 and STAT3 was not changed, the levels of p-JAK2 and p-STAT3 were significantly increased in the pancreas of the SAP model (Fig. 1 A and B). Inhibition of JAK2 by its inhibitor AG-490 significantly reduced the elevated ratio of p-JAK2/JAK2 and p-STAT3/STAT3 (Fig. 1 A and B). These results demonstrate that JAK2-STAT3 pathway is activated during SAP and suggest that JAK2 may mediate the pathological alteration of pancreas.

3.2. Inhibition of JAK2 protects against pancreatic damage and reduces inflammation

To examine whether inhibition of JAK2 protects against SAP-induced damage and inflammation of pancreases, H&E staining of pancreatic sections was performed to assess pancreatic pathology. The pathological analysis of the pancreas of the rats in each group showed that the rats in the sham operation group had neither edema in the pancreatic lobules nor leukocyte infiltration, bleeding, necrosis, or rupture (Fig. 2 A). In the SAP model group, the pancreatic lobular space was widened; edema, bleeding and necrosis occurred in the acinar cells. In addition, the integrity of the acinar was damaged, and the acinar fluid overflowed (Fig. 2A). The JAK2 inhibitor AG-490 suppressed the infiltration of proinflammatory cells, lobular edema, bleeding, and necrosis (Fig. 2A). The overall pathological score of pancreases was also significantly reduced by AG-490 treatment (Fig. 2B). These observations support the notion that JAK2 inhibition provides beneficial effects to pancreas in SAP.

3.3. JAK2 inhibition suppressed proinflammatory gene expression in PBMCs and pancreas

To reveal the effect of AG-490 on pancreatic homeostasis during SAP at the molecular level, the present study focused on the detection of the expression of proinflammatory genes in both PBMC and pancreases. Compared with the control group, the mRNA expression of genes (Foxp3, Il10, Tgfb1 and Il4) involved in regulatory T cell activation and macrophage polarization was significantly reduced in isolated PBMCs from the SAP model group, whereas the mRNA level of Il6 (IL-6) and Trem1 (sTREM-1) was significantly increased (Fig. 3 A). After injection of AG-490, the expression of Foxp3, Il10, Tgfb1 and Il4 was significantly increased, reaching a level comparable to those in the control group. Importantly, the expression of proinflammatory genes Il6 and Trem1 was effectively brought down by AG-490 (Fig. 3 A) (see Fig. 4).

Similar to the gene expression pattern seen in PBMCs, in contrast to the elevated expression level of proinflammatory genes (Ifng-encoding IFN-γ, Tnfa, Il6 and Trem1), the level of genes (Foxp3 and Il10) holding

![Fig. 1. The expression and activity of JAK2 and STAT3 in pancreatic tissues of SAP rats. (A) The protein level of p-JAK2, JAK2, p-STAT3, STAT3 and GAPDH in rat pancreases were determined by western blotting. (B) The quantitative analysis result of p-JAK2/JAK2 ratio. n = 8–11. (C) The quantitative analysis result of p-STAT3/STAT3 ratio. n = 8–11, all data are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs Control. *P < 0.05 vs SAP+5% DMSO, unpaired two-tailed t-test. SAP: severe acute pancreatitis.](image-url)
anti-inflammatory function was markedly reduced in pancreas (Fig. 3B). Strikingly, the administration of AG-490 reversed SAP-induced effects on the expression of aforementioned genes (Fig. 3B).

3.4. JAK2 inhibition balanced the relative levels of proinflammatory and anti-inflammatory cytokines

To further verify the immunomodulatory effect of JAK2 inhibition, we assayed the levels of key cytokines in both the circulation and pancreases using ELISA. Compared with the control group, the content of IL-10 and TGF-β was decreased whereas the content of sTREM-1 was increased. By contrast, injection of AG-490 restored the circulating levels of IL-10 and TGF-β and reduced the amount of sTREM-1 in the blood stream. In the pancreases, TNF-α and sTREM-1 were markedly elevated while the level of IL-10 was downregulated, which was also reversed by AG-490 treatment. Taken together, the profiling of these key cytokines suggests that JAK2 plays a regulatory function in immune response to regulate resolution of inflammatory signaling.

4. Discussion

JAK2, a non-receptor tyrosine kinase downstream of cytokine receptors, regulates expression of a wide range of proinflammatory genes via control of STAT transcription factors [14]. The activity of JAK2 is tightly controlled at multiple levels by cytokines, growth factor and hormones [15,16]. Severe acute pancreatitis (SAP) is an inflammatory disease with elevated level of a myriad of proinflammatory cytokines [17]. Our findings of the anti-inflammatory effect of JAK2 inhibition through modulation of cytokine profile in SAP reveal a new molecular link bridging JAK2 with pancreatic inflammation.

In the present study, we observed that AG-490 at a concentration (8 mg/kg) did not cause signs of toxicity and the AG-490-administered rats appeared improved vitality when compared to the sham treated rats. Previous studies done in mice also reported that administration of...
AG490 was very well tolerated and treated showed no signs of toxicity and appeared healthier compared to control group [18, 19]. These findings suggest the use of AG-490 is generally safe and will not cause significant toxicity in vivo.

We aimed to reveal whether JAK2/STAT3 activity is regulated under SAP. The present results showed that phosphorylated level of both JAK2 and STAT3 was increased in the pancreas during SAP, and such increase was abolished by AG-490 administration. Our in vivo experiments also found that inhibition of JAK2 activity has a beneficial effect on pancreas. These results are consistent with the previous reports on the bad role of hyperactivity of JAK/STAT pathway in other forms of pancreatitis [20, 21]. Further studies on the cellular mediator(s), for example - macrophage phenotype, in pancreases for inhibiting JAK2 activity will help uncover any unknown pathophysiological roles of JAK2 in SAP.

We next sought to investigate the potential molecular basis of JAK2 inhibition-mediated anti-inflammatory property. We used qPCR and ELISA approaches to examine the expression of a panel of key factors involved in macrophage polarization. The results indicated that JAK2 may modulate macrophage polarization by repressing the expression of IL-10 and TGF-β, key cytokines for alternatively activated macrophages that participate in the resolution of tissue inflammation and repair [22, 23]. We also determined factors and cytokines that serve as the markers of classically activated macrophages [24]. TNF-α and IL-6 are potent proinflammatory cytokines signaling through NF-κB and JAK/STAT pathways [25, 26] and sTREM-1 amplifies monocyte-mediated inflammatory responses by stimulation of the release of pro-inflammatory mediators [27, 28]. AG-490 treatment abolished the upregulated level of IL-6 and sTREM-1. Interestingly, the promoter regions of IL-6 and sTREM-1 all contain putative binding sites for STATs, indicating a forward regulatory mechanism between JAK/STAT and the target genes. Moreover, the reduced level of Foxp3, the master regulator of regulatory T cells [29], was restored by JAK2 inhibition, suggesting that JAK2 also holds regulatory function for T cells maturation. Altogether, these findings indicate JAK2 is a crucial immunomodulatory kinase in SAP although future mechanistic studies are needed to reveal the detailed mechanisms by which JAK2 regulates the differentiation and activation of critical immune cells.

In conclusion, the present study provides the first line of evidences that inhibition of JAK2 modulates immune and inflammatory responses and preserves pancreatic homeostasis in SAP. The suppressive effect of JAK2 inhibitor on inflammation is highly likely attributed to immunomodulatory function of JAK2. Thus, the presented results suggest that pharmacological targeting at JAK2/STAT signalling hold therapeutic potential against acute pancreatitis.

Data availability statement

Data are available upon reasonable request.

Author statement

Z.Q. and Z.W. performed the majority of the experiments, data analysis, and contributed to manuscript preparation. P.Y. assisted in the animal experiments. Z.B., L.L. provided technical supports and made critical comments on the manuscript. F.C., H.J., F.Z contributed to the study design and the preparation of the manuscript. F.X. supervised the study and provided with materials and contributed to the experimental design and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial
interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by Key Project of Translational Medicine, Bengbu Medical College, Anhui Province (BYTM2019017) and China Primary Health Care Foundation (YLGX-JZ-20200012).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101133.

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