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Original Paper

Gli2 Mediated Activation of Hedgehog Signaling Attenuates Acute Pancreatitis via Balancing Inflammatory Cytokines in Mice

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Key Words
Acute pancreatitis • Inflammation • NF-κB • Hedgehog signaling • Gli2

Abstract
Background/Aims: Inflammatory response is a determinant in the pathological progression of acute pancreatitis (AP). Previous studies have shown that the activation of hedgehog (Hh) signaling is a remarkable change in caerulein-induced AP. However, the relationship between Hh signaling and inflammation is largely ambiguous. ***Methods:*** The AP mouse model was induced by injection of cerulein, and histological staining and serum enzymology assays were used to evaluate the establishment of AP. Western blot assay was used to determine the protein levels, cleavage of apoptotic proteins, and activation of the NF-κB signaling pathway. Cytokine array was used to screen inflammatory cytokines, and target cytokines' transcriptional expression and serum levels were examined by real-time PCR and enzyme-linked immunosorbent assay, respectively. ***Results:*** The key transcriptional factor in Hh signaling, Gli2, was upregulated in the pancreas and other tissues during the process of AP, and it seems to be a characteristic feature of local inflammation in pancreatic tissue and systemic inflammatory response in multiple organs. The inflammatory NF-κB pathway is required for the activation of Hh signaling, as blockade of the NF-κB pathway by pyrrolidine dithiocarbamate impaired the Gli2 upregulation. Manipulation of Gli2 expression altered the activation of the NF-κB pathway correspondingly, as well as the cell apoptosis in cerulein-induced AP. Moreover, Gli2 upregulation changed the cytokine expression profile in mouse pancreatic acinar cells, mainly decreasing the pro-inflammatory cytokines interleukin (IL)-6, interferon-γ, and FasL. The anti-inflammatory cytokine IL-10 was upregulated by Gli2 overexpression. Interdiction of Gli2 by the Gli-specific inhibitor GANT61 exacerbated AP in mice and altered the balance of inflammatory cytokines. **Conclusions:** This study indicates that Hh activation during AP development is a negative feedback of the inflammatory response, restricting inflammatory injury to the pancreas and other tissues. Thus, manipulation of Hh signaling should shed light on limiting inflammation and alleviating AP damage.

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Introduction

Acute pancreatitis (AP) is one of the most common gastrointestinal disorders caused by multiple factors such as pancreatic duct obstruction and alcoholism. In the clinic, approximately 10-15% of patients will develop severe AP with a higher mortality and morbidity [1, 2]. For many decades, pancreatic enzyme activation has traditionally held the spotlight as the pivotal pathogenic event of pancreatitis. However, recent experimental evidence indicates that inflammatory response is another major cellular event in pancreatitis [3, 4]. AP starts as a local inflammation of pancreatic tissue, further inducing systemic inflammation and multiple extrapancreatic organ dysfunction along with the pathogenic progression. Sustained NF-κB activation in pancreatic acinar cells is a feature of the inflammatory response independent of trypsinogen activation and is sufficient to aggravate AP [5]. Importantly, the predominant factors responsible for the morbidity and mortality of AP are systemic inflammatory response syndrome and multi-organ dysfunction. Several clinical studies have investigated the merit of circulating cytokines as predictors and markers of disease severity in AP [6]. Pancreatic acinar cells, particularly during the earlier stages of AP, secrete cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and the chemokine monocyte chemoattractant protein-1 (MCP-1). Moreover, infiltrating immune cells also release various cytokines to recruit pro-inflammatory macrophages and cause tissue injury. Thus, targeting inflammatory signaling presents a potential therapeutic strategy for altering AP progression.

The hedgehog (Hh) signaling pathway is activated by a group of secreted intercellular signaling molecules that are essential for pancreatic morphogenesis and homeostasis. The zinc finger transcription factor Gli family consists of Gli1, Gli2, and Gli3, which transmit the signals into the nucleus to evoke Hh target gene responses. Among the three Gli proteins, Gli1 and Gli2 act as agonists to mediate or expand the effects of Hh signaling, whereas Gli3 is mainly considered a negative regulator of transcription. It is well known that downstream genes of Hh signaling include *c-Myc*, *Cyclin D*, *Bcl2*, and *N-cadherin* [7, 8]. Recent evidence suggests that Hh signaling is also involved in the regulation of inflammation, damage, and repair. Our previous study revealed that sonic hedgehog (Shh) serves as an anti-inflammatory factor in cerulein-induced AP in mice [9]. Mechanistic investigation indicated that Shh upregulates the expression and secretion of IL-10 to alleviate the progression of AP. In this study, we observed that Gli2 was upregulated in cerulein-induced AP. Gli2 overexpression modified the cytokine expression profile in pancreatic acinar cells, and mainly downregulated the pro-inflammatory cytokines IL-6, interferon (IFN)-γ, and FasL. Simultaneously, Gli2 upregulation gave rise to enhanced secretion of the anti-inflammatory cytokine IL-10. Interdiction of Gli2 by the Gli-specific inhibitor GANT61 augmented pancreatic acinar cell apoptosis, and exacerbated AP in mice. Therefore, this study indicates that activation of Hh signaling or targeting its downstream cytokines sheds light on alleviating AP progress.

Materials and Methods

Animal models and enzyme assay

Animals and induction of the AP model have been described in our previous study [9]. Briefly, male C57BL mice, 6-8 weeks old, were supplied by the Experimental Animal Center of Southwest Medical University (Luzhou, China), and AP was induced by an intraperitoneal injection of cerulein at a dose of 50 μg/kg body weight at hourly intervals for a total of 7 injections. All the animal experiments were approved by The Animal Care and Welfare Committee of Southwest Medical University, and conducted according to the guidelines of the Local Animal Use and Care Committees of Luzhou as well as the National Animal Welfare Law of China.

One milliliter of blood was taken from the lateral tail vein and centrifuged at 3, 000 × *g* for 10 min at 4°C, and the supernatant was used for serum enzymology assays using kits (Nanjing Jiancheng Bioengineering

Institute, Nanjing, Jiangsu, China). All samples were in triplicate and the results represent at least three independent experiments.

Histological examination and immunohistochemistry

Briefly, 4-μm sections of paraffin-embedded tissue were prepared and deparaffinized for hematoxylin and eosin staining. For the immunohistochemical staining, sections were deparaffinized and blocked, incubated with anti-Gli2 antibody (1:100, Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight, and then incubated with horseradish peroxidase-conjugated secondary antibody at 1:200 for 1 h at room temperature. The sections were developed using a 3, 3′-diaminobenzidine tetrahydrochloride substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1-5 min and then counterstained with hematoxylin.

Cell cultures and transfection

Murine pancreatic acinar 266-6 cells were purchased from ATCC (Manassas, VA, USA) and cultured in high-glucose DMEM and FBS. Cells at 70-80% confluence were transfected with pcDNA3.1-Gli2 or Gli2-shRNA (GeneCopoeia, Rockville, MD, USA) with Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA) at a DNA:Lipofectamine 2000 ratio of 1:2 according to the manufacturer's guidelines. After 48 h of transfection, cells were harvested for further analysis.

Cytokine array and ELISA

Cytokine arrays were carried out as previously reported [10]. Briefly, the supernatants were collected from cultures of 266-6 cells transfected with Gli2-expressing plasmid for 24 h. The arrays (RayBiotech, Norcross, GA, USA) were incubated with supernatant and protein spots were detected and quantified by autoradiography according to the manufacturer's instructions. The value of the scans was adjusted based on the intensity of positive control spots on each membrane by using MultiGauge V3.0 software (Fujifilm, Tokyo, Japan). Cytokine levels in 266-6 culture media were measured by a quantitative ELISA kit (Peprotech, Rocky Hill, NJ, USA) according to the manufacturer's instructions.

Real-time PCR

Total RNA was isolated from fresh tissue (pancreas, liver, lung, small intestine, kidney) or 266-6 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 2 μg of total RNA was reverse transcribed using reverse transcriptase. One microliter of the final cDNA was used for real-time PCR amplification with SYBR Green using a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the cDNAs were amplified using the primers listed in Table 1.

Western blot

Western blot was carried out as previously described [11]. The NE-PER Nuclear and Cytoplasmic Extraction kit (ThermoFisher, Shanghai, China) was used to isolate the cytoplasmic and nuclear protein fractions. Briefly, fresh tissue or cells were lysed using 1× lysis buffer (Cell Signaling Technology, Danvers, MA, USA), and 50 μg of total protein was separated via electrophoresis in a 4-12% gel. The proteins were then transferred onto a nitrocellulose membrane, followed by incubating the membrane with primary antibodies at 4°C overnight, washing, and then incubating with secondary antibodies at room temperature for 1 h. After washing, the immunoblot was developed using a chemiluminescence substrate (Thermo Fisher Scientific). The Gli2, Gapdh, Lamin B, and actin antibodies were from Santa Cruz Biotechnology; the NF-κB p65, p-p65, cleaved caspase-3, cleaved PARP, p-IκB-α, and IκB-α antibodies were from Cell Signaling Technology. The gray-scale images of the blots were analyzed using MultiGauge V3.0 software, and the protein levels calculated relative to the mock control (shown as the numbers under the blots in the figures) using actin or Gapdh as an internal control.

Table 1. Primers used for real time PCR in this study

Gene		Sequence
Gli2	Forward	5′-GGTTGAGCGGAAGGTTGAAG -3′
	Reverse	5′-AAAGGAACAGGTTTGGGATACG-3′
Shh	Forward	5′- GGGTCTACTATGAATCCAAAG-3′
	Reverse	5′- CAGGAAGGTGAGGAAGTC-3′
Ifn-γ	Forward	5′- CACTGCATCTTGGCTTTGCA-3′
	Reverse	5′-GCTGATGGCCTGATTGTCTTTC-3′
Fasl	Forward	5′- GCAGAAGGAACTGGCAGAAC-3′
	Reverse	5′-TTAAATGGGCCACACTCCTC-3′
Il-6	Forward	5′-CGAGCCCACCAGGAACGAAAGTC-3′
	Reverse	5′-CTGGCTGGAAGTCTCTTGCGGAG-3′
G-csf	Forward	5′- AGGGAAGGAGATGGGTAAAT-3′
	Reverse	5′- TGGAGGGTGAGGGTGGAT-3′
Cxcl9	Forward	5′-AGGGTCGCTGTTCCTGCATC-3′
	Reverse	5′-TTCACATCTGCTGAATCTGGGTTTA-3′
Tnfr1a	Forward	5′-TGCCAGGAGAAACAGAACACAC-3′
	Reverse	5′-CGTTGGTAGCGATACATTA-3′
Gapdh	Forward	5′- CAGTATGACTCCACTCACGGCAA-3′
	Reverse	5′-CTCGCTCCTGGAAGATGGTGAT-3′

Statistical analysis

Experimental values are expressed as mean ± standard error of the mean if not otherwise indicated. Statistical significance was analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and determined by unpaired Student's *t* tests and one-way analysis of variance, and the survival rate was analyzed using Kaplan-Meier analysis. $P < 0.05$ was considered statistically significant. All results were reproduced in at least three independent experiments.

Results

Gli2 is upregulated in the mouse AP model

Intraperitoneal injection of cerulein was used to induce the mouse AP model and control mice (mock group) received PBS injection, as we reported previously [9]. The specimens were collected for histologic examination after the animals were euthanized. As shown in Fig. 1A, hematoxylin and eosin staining revealed that cerulein injection triggered the characteristic pathological changes of AP. In contrast to the nearly normal pancreas in the control group, cerulein administration led to interstitial edema, acinar vacuolization, inflammatory cell infiltration, and pancreatic necrosis. Consistent with these observations, the activities of myeloperoxidase (MPO) increased by 7.4-fold in mice injected with cerulein compared with the control group (Fig. 1B). MPO is released by active neutrophils in the inflamed tissue, making pancreatic MPO activity a good indicator of inflammatory cell infiltration. In addition, serum amylase and lipase activities in the AP group were significantly elevated by 12.5-fold and 4.3-fold, respectively, compared with those in the control groups (Fig. 1B).

In parallel with the pathological progress of AP, immunohistochemical assay showed that Gli2, a critical ingredient of Hh signaling, was markedly upregulated in pancreatic acinar cells of the AP group (Fig. 1C). By using western blotting assay to detect the Gli2 protein level, we also found that its expression increased substantially in the AP pancreatic tissues compared with the mock control (Fig. 1D). Furthermore, real-time PCR revealed that the downstream targets of the Hh pathway, *Gli1*, *Ptch1*, and *Ccnd1* genes, were upregulated by 5.3-, 4.3-, and 6.2-fold, respectively, compared with the control (Fig. 1E). Taken together, these results indicate that Hh signaling is activated during the development of cerulein-induced AP in mice.

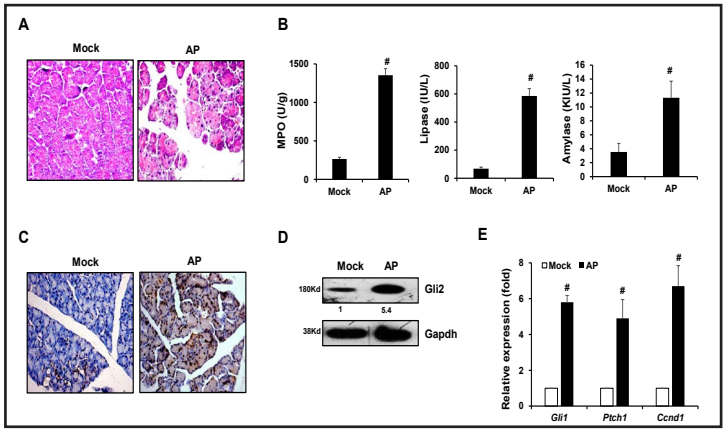

Fig. 1. Activation of hedgehog signaling pathway via Gli2 mediator in cerulein -induce acute pancreatitis in mice. (A) HE staining shows the typical histological change of the pancreas tissues in mock and acute pancreatitis mice. Magnification, 400×. (B) Serum levels of MPO, lipase and Amylase in the mock and acute pancreatitis groups. (C) Immunohistochemistry staining for the Gli2 protein level in the pancreatic tissues from mock and acute pancreatitis mice. (D) Gli2 protein level in the mock and acute pancreatic tissues detected by western blot, the Gapdh was used as the endogenous control. (E) Real-time PCR examination for the hedgehog signaling pathway downstream target gene expression in the mock and acute pancreatitis mice. Mock, mice from the mock group; AP, mice from the acute pancreatitis group. # p<0.001, compared with the mock groups, respectively.

Gli2 is upregulated in other tissues of AP mice

Severe AP leads to systemic inflammatory response syndrome and multi-organ disorders, contributing to high morbidity and mortality. Hence, we further investigated whether Hh signaling activation is a feature of systemic inflammation and organ dysfunction. Immunohistochemical examination was performed to detect the Gli2 expression *in vivo* in lung, liver, small intestine, and kidney. After the induction of AP by cerulein injection, the expression of Gli2 protein markedly increased in multiple organs and was found mainly in the nucleus, suggesting an activation of Hh signaling (Fig. 2A). Real-time PCR assay further indicated that *Gli2* mRNA was upregulated by 6.2-fold in lung, 4.7-fold in liver, 8.9-fold in small intestine, and 9.7-fold in kidney of AP mice compared with the control (Fig. 2B). Western blot also validated the upregulation of Gli2 protein in these organs (Fig. 2C). These results suggest that Gli2 upregulation is not only a feature of local inflammation in pancreatic tissue, but also a marker of systemic inflammatory response and tissue injury.

NF-κB pathway contributes to Gli2 upregulation

We next examined Gli2 expression in the murine acinar pancreatic cell line 266-6 treated with cerulein to mimic AP *in vitro*. After exposure to up to 3 μM cerulein for 24 h, the *Gli2* mRNA increased up to 20-fold over that of control in a dose-dependent manner (Fig. 3A).

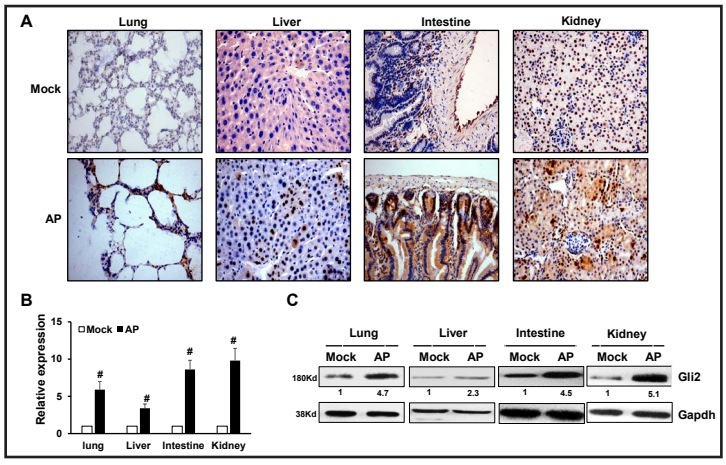

Fig. 2. Gli2 levels in organs from acute pancreatitis mice. (A) Immunohistochemistry staining for Gli2 protein levels in the lung, liver, intestine and kidney from mock and acute pancreatitis groups, respectively. (B) Detection of Gli2 mRNA level in the above organs using real time PCR. (C) Gli2 protein levels in the above organs, the Gapdh was used as the endogenous control. Mock, mice from the mock group; AP, mice from the acute pancreatitis group. # p<0.001, compared with the mock groups, respectively.

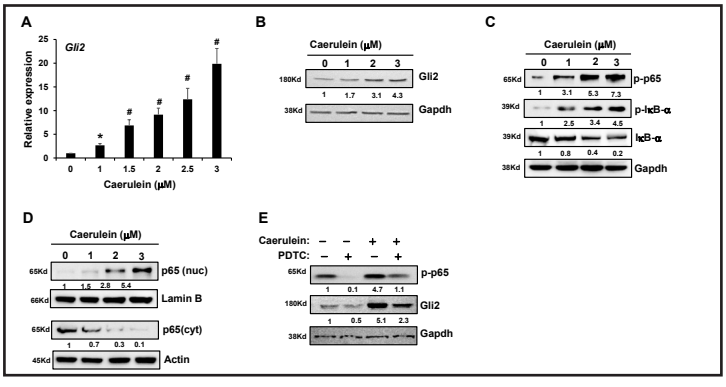

Fig. 3. NF-κB pathway activation in caerulein treated 266-6 cells in vitro. (A) Real time PCR assay showed the Gli2 mRNA level in 266-6 cells treated with increasing dosage of caerulein for 24 hours. (B) Western blotting showed the Gli2 protein level in 266-6 cells treated with increasing dosage of caerulein for 24 hours. (C) Protein levels of the phosphor-p65, phosphor-IκB and total IκB was analyzed to indicate the activation of NF-κB signaling in 266-6 cells treated with different dosage of caerulein. (D) NF-κB p65 protein level in the cytoplasm and nucleus was detected in 266-6 cells treated with different dosage of caerulein, β-actin and Lamin B were used as the cytoplasmic and nuclear loading control, respectively. (E) Western blotting assay showed the effect of co-treatment of NF-κB pathway inhibitor PDTC (100μM) and caerulein (3 μM) for 24 hours in 266-6 cells. *, p<0.05; # p<0.001, compared with the control, respectively.

Furthermore, the Gli2 protein level increased in response to increasing concentrations of cerulein as well (Fig. 3B). The inflammatory NF-κB signaling pathway was also activated by cerulein treatment, as the phosphorylation of the NF-κB subunit p65 (p-p65) increased with increasing cerulein concentration, as well as that of the antagonist of NF-κB signaling, IκB-α (p-IκB-α) (Fig. 3C). Phosphorylation of IκB-α leads to its ubiquitination and proteasomal degradation, freeing NF-κB/REL complexes and activating the NF-κB signaling pathway. In addition, NF-κB (p65) in the nucleus increased gradually, but the cytoplasmic NF-κB decreased accordantly, which indicated the obvious cyto-nuclear translocation (Fig. 3D). These findings demonstrated that Gli2 upregulation is a cellular response to cerulein, and there might be a functional association between Gli2 and NF-κB.

We further explored the mechanisms underlying Gli2 upregulation. Pyrrolidine dithiocarbamate (PDTC) was utilized to abrogate the inflammatory NF-κB pathway. We found that PDTC impaired NF-κB activation to a certain degree, but dramatically reversed the increase in Gli2 and p-p65 in the presence of 3 μM cerulein (Fig. 3E). Thus, our results suggest that Gli2 is a marker of cerulein-induced AP, and the upregulation of Gli2 in AP is dependent on NF-κB activation.

Manipulation of Gli2 altered the activation of the NF-κB pathway in cerulein-induced AP

To further ascertain the role of Gli2 activation in cerulein-induced AP, we established acinar pancreatic 266-6 cells transiently expressing Gli2. Western blotting assay showed that the Gli2 protein level was successfully elevated after transfecting the Gli2-expressing plasmid (Gli2-OE) (Fig. 4A, upper panel). Gli2 overexpression had virtually no effect on the basal level of NF-κB protein; however, the inducible level of p-p65 in cerulein treatment was extensively blocked by the forced expression of Gli2 (Fig. 4A). Moreover, Gli2-overexpressing cells were more resistant to cerulein-induced apoptosis, as the apoptotic rate in these cells decreased from 33% to 20% ($P < 0.01$) as estimated by flow cytometric analysis using annexin V-FITC and propidium iodide staining (Fig. 4B). Western blot showed that cerulein treatment effectively induced PARP and caspase-3 cleavage in the control cells, while Gli2 overexpression in 266-6 cells substantially decreased the extent of cleavage (Fig. 4C). In contrast, when Gli2 was knocked down using Gli2-shRNA (Gli2-KD), the level of p-p65 induced by cerulein treatment was enhanced (Fig. 4D), the apoptotic rate in the Gli2-KD cells was significantly augmented from 37% to 74% ($P < 0.01$) (Fig. 4E), and the level of cleaved PARP and caspase-3 increased (Fig. 4F). Therefore, Gli2 seems to be a protective factor in the cerulein-induced inflammation.

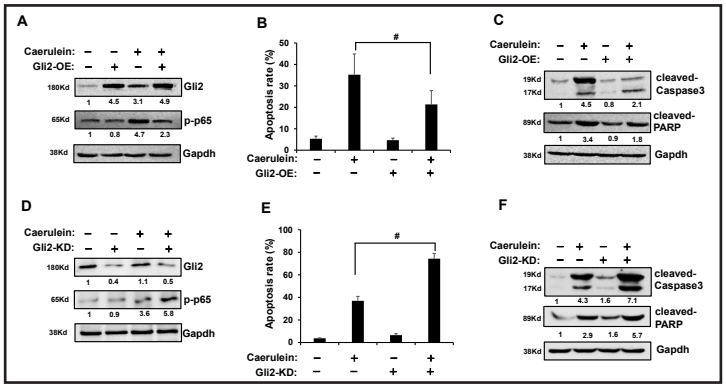

Fig. 4. Gli2 protects 266-6 cells from caerulein induced apoptosis. (A). Western blot assay detected the Gli2 and NF-κB level in 266-6 cells transfected with Gli2-expressing plasmid (Gli2-OE) with or without caerulein (3 μM) treatment for 24 hours. (B) Quantitative analysis of apoptotic 266-6 cells transfected with GLi2-expressing plasmid with or without caerulein treatment for 24 hours. (C) Western blot assay showed the activation of Caspase 3 and PARP in response to the caerulein treatment for 24 hours with or without Gli2 overexpression. In 266-6 cells transfected with Gli2-shRNA (Gli2-KD) with or without caerulein (3 μM) cells, (D) the Gli2 and NF-κB level, (E) flow cytometry assay for apoptosis rates, and (F) western blot for cleavage of PARP and Caspase-3 were shown.*, $p<0.05$; #, $p<0.001$, compared with the controls as indicated, respectively.

Gli2 overexpression reduces the cytokine expression profile

To further examine the effect of Gli2 on pancreatic inflammation, we performed cytokine arrays to screen for alterations in cytokine expression. Representative images of cytokine arrays in Fig. 5A show that Gli2 overexpression dramatically changed the cytokine expression profile in murine pancreatic acinar cells. Gli2 overexpression effectively decreased the pro-inflammatory cytokines IL-6, IFN-γ, and FasL (Fig. 5B). At the same time, Gli2 also triggered an increase in anti-inflammatory cytokines such as IL-10. However, Gli2 also enhanced the expression of some pro-inflammatory cytokines such as G-CSF and MIG (Cxcl9). Obviously, the outcome of Gli2 activation can be ascribed to a comprehensive effect of multiple cytokines affected by Gli2. Real-time PCR further confirmed the array results and showed that the mRNA levels of three cytokines, IL-10, IFN-γ, and FasL, were greatly altered by Gli2 overexpression (Fig. 5C). IL-10 is an anti-inflammatory cytokine, FasL belongs to the TNF family and its binding with the receptor induces cell apoptosis, and IFN-γ is an important inflammation inducer. Thus, we hypothesized that Gli2 overexpression inhibits inflammation.

Gli2 antagonism exacerbates pancreatic injury

The overall effect of Gli2 overexpression is cell protective and anti-inflammatory in cultured 266-6 cells, as mentioned above. Gli2 also alters the expression of anti-inflammatory

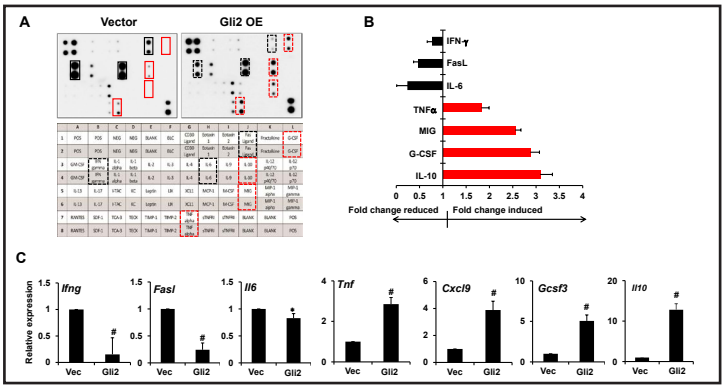

Fig. 5. Gli2 mediated cytokine expression profile change in vitro. (A) Cytokine array for 266-6 cells transfected with vector control (Vector) or Gli2 expressing plasmids (Gli2-OE). The table underneath shows the cytokine profile map. (B) Quantification of three downregulated cytokines and four upregulated cytokines by densitometry analysis. (C) Confirmation of the cytokines changed upon Gli2 overexpression using real time PCR for 266-6 cell transfected with vector (Vec) or Gli2 expressing plasmid (Gli2). *, p<0.05; #, p<0.001, compared with the vector control, respectively.

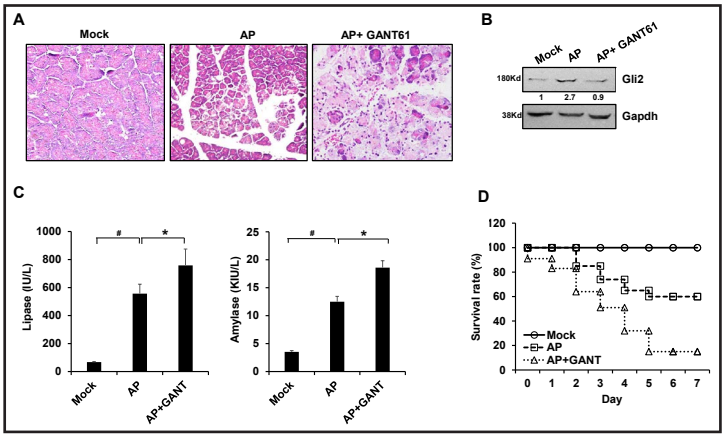

Fig. 6. Interdiction of Gli2 by GANT61 exacerbate acute pancreatitis in mice. (A) HE staining shows the typical histological change of the pancreas tissues in mock, acute pancreatitis, and acute pancreatitis plus GANT61 mice. Magnification, 400×. (B) Gli2 protein level in tissues from the mice in the control group (mock), acute pancreatitis, and acute pancreatitis plus GANT61 mice detected by western blot, the Gapdh was used as the endogenous control. (C) Serum levels of lipase and Amylase in the mock, acute pancreatitis, and acute pancreatitis plus GANT61 mice. (D) Survival rate of the mice in the mock, acute pancreatitis, and acute pancreatitis plus GANT61 groups. *, p<0.05; #, p<0.001, compared with the control groups as indicated.

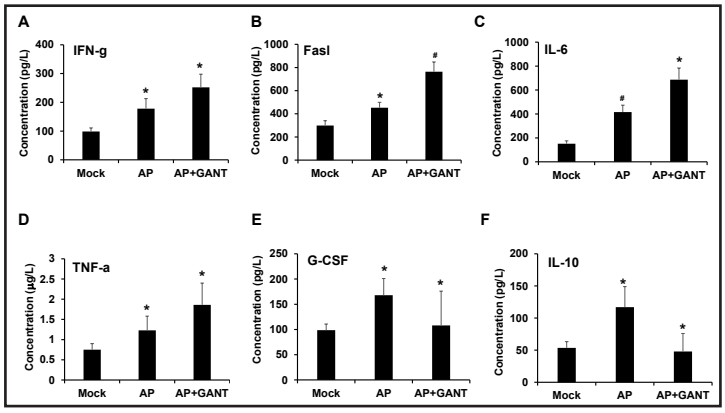

Fig. 7. Cytokine profile change in the acute pancreatitis mice treated with GANT61. ELISA assay showed the serum cytokine levels for (A) IFN-γ, (B) Fasl, (C) IL-6, (D) TNFα, (E) IL-10 in the acute pancreatitis group and acute pancreatitis plus GANT61 groups. Data shown represents for three independent experiments. *p<0.05 VS AP group, #p<0.05 VS AP+GANT group.

and pro-inflammatory cytokines. We speculated that activation of Hh signaling and inducible expression of Gli2 in AP is beneficial to limit local inflammation. To test this assumption, we utilized GANT61, a specific inhibitor for Gli transcriptional factors, in the cerulein-induced AP model. As shown in Fig. 6A, the extent of cell edema and inflammatory cell infiltration in the cerulein plus GANT61 group was more pronounced compared with the AP and control groups. Thus, the AP development in mice co-treated with cerulein plus GANT61 was more extensive than that in the group treated with cerulein alone. Furthermore, GANT61 successfully antagonized the increase in Gli2 in the presence of cerulein (Fig. 6B). As predicted, lipase and amylase levels were obviously higher in the cerulein plus GANT61 group when compared with the untreated or cerulein-treated group (Fig. 6C). Additionally, Gli suppression decreased the survival rate in mice with AP (Fig. 6D). Collectively, these results show that Gli inhibition by GANT61 aggravated cerulein-induced pancreatic injury.

Further experiments indicated that GANT61 treatment enhanced the expression of inflammatory cytokines IFN-γ, FasL, IL-6, and TNF-α (Fig. 7A–D, respectively) as determined by enzyme-linked immunosorbent assay (ELISA). The secretion of G-CSF was slightly lower in the GANT61-treated group compared with the AP group (Fig. 7E). In contrast, IL-10 expression was significantly inhibited by GANT61 (Fig. 7F). Collectively, these results show that Gli inhibition altered the inflammation-associated cytokine profile, possibly accounting for the greater pancreatic injury.

Discussion

Most cases of AP start as a local and self-limiting inflammation of pancreatic tissue with only a brief need of clinical support. However, approximately 10-15% of patients develop severe AP and of those, 10-30% may die due to increases in systemic inflammation and dysfunction in multiple extrapancreatic organs. The variability in inflammatory responses could be a crucial determinant of the severity of AP. However, it remains unclear how the acinar cell injury from the initial inciting event results in local inflammation and its escalation into systemic inflammation [1, 2].

An increasing number of experimental studies have implicated NF-κB activation in AP as an early and important event in the progression of inflammation [5]. NF-κB might be a central molecule that links the initial acinar injury to systemic inflammation and perpetuate the inflammation. It is well established that the expression and activity of NF-κB subunits such as p65 are upregulated in experimental and clinical AP. NF-κB activation during AP is capable of producing a considerable variety of cytokines such as TNF-α, IL-1, IL-2, IL-6, and IL-18, various chemokines such as IL-8 and MCP-1, and adhesion molecules. Several experimental AP models have clearly shown that pancreatic acinar cells produce cytokines via activation of NF-κB. NF-κB inhibition in the AP mouse model reduced the cytokine expression and local injury. The cytokines released from acinar cells after NF-κB

activation in turn initiate the infiltration of neutrophils and lymphocytes into the pancreas and intestinal tracts, thereby amplifying the inflammatory response. Thus, the activation of the NF-κB pathway is intensified and expanded. As a positive feedback, NF-κB activation in acinar cells produces pro-inflammatory cytokines, specifically TNF-α and IL-1β, which have an emerging role in driving the pancreatic inflammation to systemic inflammation [3, 4]. Therefore, direct NF-κB inhibition strategies have already been attempted to alleviate pancreatic and systemic injury in animal AP models. Some agents such as aspirin, ω-3 fatty acids, fentanyl, and castanospermine have been shown to protect mice from severe pancreatitis and complications by inhibiting NF-κB activity [12-15].

In this study, we revealed a novel crosstalk between NF-κB signaling and Hh signaling. It is well established that Hh signaling participates in fate determination of the gut tube, as well as in early pancreatic development and islet cell function. Deregulation of Hh signaling also contributes to the pathogenesis and progression of pancreatic diseases, including diabetes mellitus, pancreatitis, and pancreatic cancer [7, 8]. Previous studies have demonstrated that the expression of the Hh signaling molecules, such as Shh, Ptch1, and Smo, was nearly absent in normal pancreatic tissues, and in the context of experimental AP, the Hh pathway members were greatly augmented, indicating a potential link between Hh signaling and the severity of AP [9, 16-18]. In this study, we found that Gli2, a crucial activator of Hh signaling, was greatly upregulated in damaged pancreas and other tissues such as lung, liver, small intestine, and kidney. Seemingly, Gli2 upregulation in the process of AP can reflect local inflammation in pancreatic tissue, and systemic inflammatory response in other organs. We further revealed that the upregulation of Gli2 is a result of inflammation-associated NF-κB activation. Antagonism of NF-κB by PDTC inhibited this Gli2 upregulation.

The roles of Hh signaling in inflammation remain controversial. Local inhibition of the Hh pathway facilitated endoneurial and perineurial vascular permeability following trigeminal nerve injury, thereby facilitating immunocyte infiltration and neuroinflammation [19]. Shh expression was gradually increased in the MPTP (1-methyl-4-phenyl-1, 2,3, 6-tetrahydropyridine) model of Parkinson's disease, and was involved in the recovery from neurotoxin-induced injury [20]. It was found that Shh protected dopaminergic neurons and attenuated the inflammatory response by stimulating the PI3K/Akt pathway and increasing the expression of transforming growth factor-β (TGF-β) [21]. The induction of immune-modulatory IL-10 and $CD4^+Foxp3^+$ regulatory T cells is required for the protective effect of the Hh pathway in colitis [22]. Disruption of Hh signaling in inflammatory bowel disease fostered chronic intestinal inflammation. Associated mechanisms included downregulating inflammatory cytokines, and repressing monocyte chemo-attraction and fibroblast proliferation. Hh blockade increased the abundance of TNF-α, IL-17, and TGF-β [23]. However, Hh signaling was found to promote the induction and progression of inflammation and neoplastic transformation in infection with the pathogen *Helicobacter pylori* [24]. Shh has also been shown to positively regulate the expression of cytokines during *H. pylori* infection, such as IL-12, IL-1β, IL-10, IFN-γ, and MIP-2 [25]. Moreover, exogenous Shh could amplify the production of IL-2, IFN-γ, and IL-10 in activated $CD4^+$ T cells [26]. As for AP, solid evidence has shown that the Hh pathway plays a protective role. Our previous studies found that neutralizing Shh caused more severe AP injury in mice. Another report indicated that Gli1 activity is required for pancreas recovery [27]. Deletion of a single allele of *Gli1* caused improper stromal remodeling and persistence of the inflammatory infiltration, and hampered fibroblast migration. Mechanistic insight showed that partial loss of *Gli1* downregulated a subset of cytokines including IL-6, IL-8, MCP-1, and M-CSF. In the case of chronic pancreatitis, the Hh pathway was found to interact with pancreatic stellate cells, leading to destructive parenchymal fibrosis and atrophy, as well as to irregular tissue remodeling with an increase in tumorigenesis [7, 28]. In this study, we validated the positive roles of Gli2 in AP. Gli2-overexpressing acinar cells exhibited greater potency to counteract cerulein-induced apoptosis, as evaluated by flow cytometric analysis. Our studies further demonstrated that Gli2 overexpression compromised NF-κB activation in cerulein-induced inflammation. Thus, it seems that Gli2 upregulation responding to NF-κB activation produces a negative feedback

to limit NF-κB activity and associated inflammation. However, an important point to be emphasized is that Gli1 and Gli2 are both transcriptional factors of hedgehog signaling and their expression increased in the AP mice. Because we used GANT61, an inhibitor of both Gli1- and Gli2-induced transcription, we cannot exclude a partial contribution of Gli1.

Our further investigation revealed the complexity of Gli2 in modulating inflammation. The analysis of the cytokine profile in Gli2-overexpressing acinar cells revealed that several pro-inflammatory cytokines including IL-6, IFN-γ, and FasL were downregulated, which may contribute to the protective roles of Gli2. Interestingly, Gli2 also upregulated the expression of anti-inflammatory cytokine IL-10 as well as pro-inflammatory G-CSF and MIG. These results suggest that Hh signaling and Gli transactivators may exert different effects on the distinct aspects of AP such as injury, inflammation, and repair. However, the precise mechanism and phase of Gli action remain elusive. Further investigation is required to draw a more nuanced picture of the functional aspects of the Hh pathway or Gli activators in AP.

In summary, our study emphasizes the protective function of Hh signaling in AP and proposes a role of Gli2 upregulation as a marker for local and systemic inflammation. An altered cytokine profile after Gli2 upregulation paves the path for cell defense against inflammatory injury. Thus, manipulating the Hh pathway might be an effective therapeutic guideline for clinical AP treatment.

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Disclosure Statement

All authors declare to have no conflict of interests.

References

1 Singh P, Garg PK: Pathophysiological mechanisms in acute pancreatitis: Current understanding. Indian J Gastroenterol 2016;35:153-166.
2 Manohar M, Verma AK, Venkateshaiah SU, Sanders NL, Mishra A: Pathogenic mechanisms of pancreatitis. World J Gastrointest Pharmacol Ther 2017;8:10-25.
3 Minkov GA, Halacheva KS, Yovtchev YP, Gulubova MV: Pathophysiological mechanisms of acute pancreatitis define inflammatory markers of clinical prognosis. Pancreas 2015;44:713-717.
4 Habtezion A: Inflammation in acute and chronic pancreatitis. Curr Opin Gastroenterol 2015;31:395-399.
5 Jakkampudi A, Jangala R, Reddy BR, Mitnala S, Nageshwar Reddy D, Talukdar R: NF-kappaB in acute pancreatitis: Mechanisms and therapeutic potential. Pancreatology 2016;16:477-488.
6 Rao SA, Kunte AR: Interleukin-6: An Early Predictive Marker for Severity of Acute Pancreatitis. Indian J Crit Care Med 2017;21:424-428.
7 Klieser E, Swierczynski S, Mayr C, Jager T, Schmidt J, Neureiter D, Kiesslich T, Illig R: Differential role of Hedgehog signaling in human pancreatic (patho-) physiology: An up to date review. World J Gastrointest Pathophysiol 2016;7:199-210.
8 Lau J, Kawahira H, Hebrok M: Hedgehog signaling in pancreas development and disease. Cell Mol Life Sci 2006;63:642-652.
9 Zhou X, Liu Z, Jang F, Xiang C, Li Y, He Y: Autocrine Sonic hedgehog attenuates inflammation in cerulein-induced acute pancreatitis in mice via upregulation of IL-10. PloS one 2012;7:e44121.

10 Liu Z, Xu J, Li H, Zheng Y, He J, Liu H, Zhong Y, Lu Y, Hong B, Zhang M, Lin P, Du J, Hou J, Qian J, Kwak LW, Yi Q, Yang J: Bone marrow stromal cells derived MCP-1 reverses the inhibitory effects of multiple myeloma cells on osteoclastogenesis by upregulating the RANK expression. PloS one 2013;8:e82453.
11 Liu Z, Xu J, He J, Zheng Y, Li H, Lu Y, Qian J, Lin P, Weber DM, Yang J, Yi Q: A critical role of autocrine sonic hedgehog signaling in human CD138+ myeloma cell survival and drug resistance. Blood 2014;124:2061-2071.
12 Lu G, Tong Z, Ding Y, Liu J, Pan Y, Gao L, Tu J, Wang Y, Liu G, Li W: Aspirin Protects against Acinar Cells Necrosis in Severe Acute Pancreatitis in Mice. Biomed Res Int 2016;2016:6089430.
13 Hong YP, Chen C, Guo WY, Zhao L, Mei FC, Xiang MW, Wang WX: Effects of Castanospermine on Inflammatory Response in a Rat Model of Experimental Severe Acute Pancreatitis. Arch Med Res 2016;47:436-445.
14 Wang B, Wu XW, Guo MX, Li ML, Xu XB, Jin XX, Zhang XH: Effects of omega-3 fatty acids on toll-like receptor 4 and nuclear factor-kappaB p56 in lungs of rats with severe acute pancreatitis. World J Gastroenterol 2016;22:9784-9793.
15 Wang Y, Chen M: Fentanyl Ameliorates Severe Acute Pancreatitis-Induced Myocardial Injury in Rats by Regulating NF-kappaB Signaling Pathway. Med Sci Monit 2017;23:3276-3283.
16 Wang LW, Lin H, Lu Y, Xia W, Gao J, Li ZS: Sonic hedgehog expression in a rat model of chronic pancreatitis. World J Gastroenterol 2014;20:4712-4717.
17 Wei-Guo H, Xi-Wang L, Qi-Ping L: Expression of sonic hedgehog signaling pathways in a rat model of chronic pancreatitis. Saudi Med J 2010;31:14-17.
18 Fendrich V, Esni F, Garay MVR, Feldmann G, Habbe N, Jensen JN, Dor Y, Stoffers D, Jensen J, Leach SD, Maitra A: Hedgehog signaling is required for effective regeneration of exocrine pancreas. Gastroenterology 2008;135:621-631.
19 Moreau N, Dieb W, Mauborgne A, Bourgoin S, Villanueva L, Pohl M, Boucher Y: Hedgehog Pathway-Mediated Vascular Alterations Following Trigeminal Nerve Injury. J Dent Res 2017;96:450-457.
20 Lee JH, Chung YC, Bok E, Lee H, Huh SH, Lee JE, Jin BK, Ko HW: Injury-stimulated Sonic hedgehog expression in microglia contributes to neuroinflammatory response in the MPTP model of Parkinson's disease. Biochem Biophys Res Commun 2017;482:980-986.
21 Liu H, Peng F, Liu Z, Jiang F, Li L, Gao S, Wang G, Song J, Ruan E, Shao Z, Fu R: CYR61/CCN1 stimulates proliferation and differentiation of osteoblasts *in vitro* and contributes to bone remodeling *in vivo* in myeloma bone disease. International journal of oncology 2017;50:631-639.
22 Lee JJ, Rothenberg ME, Seeley ES, Zimdahl B, Kawano S, Lu WJ, Shin K, Sakata-Kato T, Chen JK, Diehn M, Clarke MF, Beachy PA: Control of inflammation by stromal Hedgehog pathway activation restrains colitis. Proc Natl Acad Sci U S A 2016;113:E7545-E7553.
23 Buongusto F, Bernardazzi C, Yoshimoto AN, Nanini HF, Coutinho RL, Carneiro AJV, Castelo-Branco MT, de Souza HS: Disruption of the Hedgehog signaling pathway in inflammatory bowel disease fosters chronic intestinal inflammation. Clin Exp Med 2017;17:351-369.
24 Wessler S, Krisch LM, Elmer DP, Aberger F: From inflammation to gastric cancer - the importance of Hedgehog/GLI signaling in Helicobacter pylori-induced chronic inflammatory and neoplastic diseases. Cell Commun Signal 2017;15:15.
25 Marwaha S, Schumacher MA, Zavros Y, Eghbalnia HR: Crosstalks between Cytokines and Sonic Hedgehog in Helicobacter pylori Infection: A Mathematical Model. Plos One 2014;9
26 Stewart GA, Lowrey JA, Wakelin SJ, Fitch PM, Lindey S, Dallman MJ, Lamb JR, Howie SE: Sonic hedgehog signaling modulates activation of and cytokine production by human peripheral CD4+ T cells. J Immunol 2002;169:5451-5457.
27 Mathew E, Collins MA, Fernandez-Barrena MG, Holtz AM, Yan W, Hogan JO, Tata Z, Allen BL, Fernandez-Zapico ME, di Magliano MP: The transcription factor GLI1 modulates the inflammatory response during pancreatic tissue remodeling. J Biol Chem 2014;289:27727-27743.
28 Jung IH, Jung DE, Park YN, Song SY, Park SW: Aberrant Hedgehog ligands induce progressive pancreatic fibrosis by paracrine activation of myofibroblasts and ductular cells in transgenic zebrafish. PLoS One 2011;6:e27941.