Original Article

Localization of Hedgehog Signaling Pathway Components During Exocrine Regeneration in L-Arginine-Induced Acute Pancreatitis in Rats

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Abstract: Background and Aim: Elucidating the molecular and cellular mechanisms underlying exocrine pancreatic regeneration represents a key step in developing treatment for exocrine pancreatic insufficiency. The aim of this study was to investigate the localization of Hedgehog (Hh) signaling pathway components during exocrine regeneration in L-arginine-induced acute pancreatitis in rats. Acute pancreatitis was induced by single intraperitoneal injection of L-arginine (450 mg/100g). Before sacrifice, 5-bromo-2'-deoxyuridine was intravenously injected to label proliferating cells in exocrine regeneration. Localization of the Hh receptors Smoothened (Smo) and Patched 1 (Ptch1) and of the Hh transcription factor glioblastoma (Gli) 2 and pancreatic progenitor marker pancreatic and duodenal homeobox factor-1 (Pdx1) was assessed by immunostaining. Proliferation of Pdx1-positive cells to form tubular complexes and replace necrotic tissue occurred by day 3 after L-arginine injection; acinar cell proliferation was predominant while the number of tubular complexes decreased on day 5, and exocrine regeneration was almost complete on day 14. In the control pancreata, Smo, Ptch1, and Gli2 were localized to islet and ductal cells. During exocrine regeneration, these proteins were also localized to tubular complexes. These results suggest that the Hh pathway plays an important role in regulating Pdx1-positive progenitor cell activity during exocrine regeneration.

Key Words: Hedgehog signaling pathway, Exocrine regeneration, Acute pancreatitis, Progenitor cell, Pdx1.

Introduction

The mechanisms involved in exocrine regeneration in response to acute pancreatitis or other acute

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pancreatic injury in humans are not fully understood. However, previous studies based on animal models have provided increasing evidence that exocrine regeneration in the adult pancreas can be induced in response to acute pancreatic injury, although the normal adult pancreas has a low rate of cell turnover[1-3]. The major mechanism involved in exocrine regeneration of the adult pancreas is self-duplication of acinar cells. Previous studies using various models of acute pancreatic injury have proposed that exocrine regeneration may also involve the proliferation and differentiation of stem/progenitor cells[4-8]. Understanding the mechanisms that drive the proliferation and differentiation of these progenitor cells represents a key step in generating a new therapy that involves the use of stem cells for exocrine insufficiency caused by acute necrotizing pancreatitis or chronic pancreatitis.

There are several candidate markers for progenitor cells that participate in exocrine regeneration, a commonly used marker being pancreatic and duodenal homeobox factor-1 (Pdx1). Pdx1, a member of the large family of homeodomain-containing proteins, is a transcription factor expressed in embryonic pancreatic progenitor cells that subsequently give rise to differentiated acinar, islet, and ductal cells. Pdx1 is involved in the differentiation of embryonic pancreatic progenitor cells and is essential for pancreatic development[9]. In the adult pancreas, Pdx1 expression is restricted to β cells and is required for maintaining mature β-cell function. Previous studies have shown striking proliferation of Pdx1-positive cells and newly formed duct-like structures called tubular complexes during exocrine regeneration and have suggested that Pdx1-positive cells within the tubular complexes are the progenitors of acinar cells. However, the origin of pancreatic acinar cells remains controversial, with putative pancreatic stem cells, ductal progenitors, acinar transdifferentiation, or circulating progenitors all having been suggested as sources[10-13].

The Hedgehog (Hh) signaling pathway plays an important role by regulating critical cell fate decisions, including proliferation, apoptosis, migration, and differentiation during embryonic development, adult tissue homeostasis, and regeneration of many organs; activation of this pathway is widely observed in many cancers[14-20]. In mammals, there are 3 types of Hh ligands: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Hh signaling is induced by ligand binding to the Patched 1 (Ptch1) Hh receptor on target cells, with the subsequent release of Smoothened (Smo) leading to activation of the glioblastoma (Gli) 1 and Gli2 transcription factors and upregulation of Gli target genes[21)22)]. Thus, cells that express Smo, Ptch1, and Gli2 are Hh-responsive.

Hh signaling plays an important role in embryonic pancreatic development and pancreatic cancer. A few studies have described the involvement of Hh signaling in exocrine pancreatic regeneration. Fendlich et al. reported that Hh signaling was upregulated after the induction of cerulein-induced acute pancreatitis and showed that inhibition of Hh signaling by pharmacological and genetic techniques resulted in impaired exocrine regeneration, suggesting that Hh signaling is required for exocrine regeneration[23]. However, these studies did not evaluate the localization of Hh-responsive cells in detail, and the involvement of Hh signaling in exocrine regeneration has not been investigated in other experimental models of acute pancreatic injury.

Most previous studies on the mechanism of exocrine regeneration have used experimental models of acute pancreatic injury, including cerulein-induced acute pancreatitis, partial pancreatectomy, and the duct-ligation model[1)24)]. The L-arginine-induced acute pancreatitis model is an experimental acute necrotizing pancreatitis model that is induced by intraperitoneal administration of L-arginine to rats and mice. In this model, selective damage to pancreatic acinar cell leads to their almost complete depletion,

followed by acinar cell regeneration. This is therefore a useful model for evaluating the molecular mechanism that underlies exocrine regeneration[25-29].

The aims of the present study were (1) to characterize the involvement of Pdx1-positive progenitor cells in exocrine regeneration by monitoring the cell proliferation of tubular complexes and Pdx1 immunolocalization and (2) to characterize Hh-responsive cells by immunolocalization of the Hh signaling components during exocrine regeneration following L-arginine-induced acute pancreatitis in rats. These approaches were used to investigate a possible link between Hh signaling and Pdx1-positive progenitor cells in exocrine regeneration.

Materials and Methods

Animals

Male Wistar rats (Oriental Yeast, Chiba, Japan) weighing 200-230 g were provided with water and laboratory chow ad *libitum*. Experimental protocols were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine.

Induction of Experimental Acute Pancreatitis

To induce acute pancreatitis, rats were given a single intraperitoneal injection of L-arginine monohydrochloride (450 mg/100 g body weight; Nacalai Tesque, Kyoto, Japan) as a 22.5% solution in 0.1 M NaCl. Control rats were injected with an equal volume of 0.15 M NaCl. The rats were sacrificed under anesthesia by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight; Sigma-Aldrich, St. Louis, MO). L-arginine-treated rats were sacrificed on 3, 5, or 14 days after injection. The control rats were sacrificed immediately after injection (day 0). One hour before sacrifice, 5-bromo-2'-deoxyuridine (BrdU) (20 mg/kg body weight; Sigma-Aldrich) was injected intravenously into control and arginine-treated rats. Their pancreata were excised shortly after death and were immediately fixed in 4% neutral-buffered paraformaldehyde for immunohistochemical analysis.

Immunohistochemistry

Paraffin-embedded sections (4-μm thick) were prepared using standard methodology. After deparaffinization, antigen retrieval was performed by heating the sections to 95°C for 10 min in REAL target retrieval solution (Dako, Glostrup, Denmark). For BrdU staining, the sections were then incubated in 2 N HCL at room temperature for 90 min. For immunostaining, endogenous peroxidase activity was blocked by incubation for 30 min in 0.3% hydrogen peroxide in methanol. The sections were then incubated with the primary antibodies in phosphate-buffered saline overnight at 4°C. The primary antibodies were anti-BrdU (347580; Becton Dickinson, Lincoln Park, NJ; 1 : 100), anti-Pdx1 (NKR059; Transgenic, Kobe, Japan; 1 : 250), anti-Gli2 (GTX27195, Genetex, Irvine, CA; 1 : 250), anti-Smo (sc-6366; Santa Cruz Biotechnology, Santa Cruz, CA; 1 : 250), and anti-Ptch1 (sc-6149; Santa Cruz Biotechnology; 1 : 250). The sections were then incubated with the secondary antibodies for 30 min at room temperature and visualized using diaminobenzidine tetrahydrochloride. The secondary antibodies were Histofine Simple Stain Rat MAX Peroxidase (mouse) for BrdU, Rat MAX Peroxidase (rat) for Pdx1 and Gli2 staining, and Rat MAX Peroxidase (goat) for Smo and Ptch1 (Nichirei, Tokyo, Japan). The sections were counterstained with hematoxylin using standard methodology. The primary antibodies were omitted in the negative controls. The negative controls showed no immunoreactivity.

BrdU Labeling Index

The percentage of BrdU-positive nuclei was quantified in tubular complexes and acinar cells. For

each time point, sections taken randomly from 5 L-arginine-injected and 5 control animals were examined at 200× magnification. At least 150 nuclei were counted for each experimental condition.

Statistical Analysis

Data were expressed as the means ± SEM. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's post-hoc test using JMP11 software (SAS Institute, Cary, NC). *P*-values of < 0.05 were considered statistically significant.

Results

Histological Changes in the Pancreas in L-Arginine-Induced Acute Pancreatitis

On day 3, necrotic tissues were replaced by the characteristic features of exocrine regeneration, including the formation of duct-like tubular complexes, which appeared as cylindrical tubes containing flattened cuboidal cells surrounding a large lumen (Fig. 1B and 1C). On day 14, acinar cell regeneration was nearly complete and only a few tubular complexes were observed (Fig. 1D).

Proliferating Cells in L-Arginine-Induced Acute Pancreatitis

BrdU labeling was performed to evaluate the cell proliferation of tubular complexes and acinar cells after the induction of pancreatitis. In the control pancreata, very few BrdU-labeled acinar cells were detected (Fig. 2A). In all, 0.18% ± 0.01% of acinar cells were BrdU-positive in the control group (Fig. 3B). In contrast, on day 3 after L-arginine injection, many cells within the tubular complexes were BrdU-positive (17.81% ± 3.18%; Fig. 2B and 3A) and the percentage of BrdU-labeled acinar cells had slightly increased to 6.16% ± 1.05% (Fig. 2B and 3B). On day 5, BrdU-labeled acinar cells were significantly increased compared with the control group (23.00% ± 3.79% vs. 0.18% ± 0.01%, $P < 0.05$; Fig. 2C and 3B), whereas fewer tubular complexes contained BrdU-labeled cells (3.81% ± 0.53%; Fig. 2C and 3A). On day 14, far fewer acinar cells were labeled with BrdU (2.00% ± 0.80%; Fig. 2D and 3B).

Immunohistochemical Analysis of Pdx1 in L-Arginine-Induced Acute Pancreatitis

In the control pancreata, Pdx1 was detected in the nucleus of islet and ductal cells but not in acinar cells (Fig. 4A). On day 3 after L-arginine injection, Pdx1 was detected in the nucleus of tubular complexes and in the nucleus of islet and ductal cells (Fig. 4B); on day 14, it was detected in the nucleus of the remaining tubular complexes and in the nucleus of islet and ductal cells but not in acinar cells (Fig. 4C).

Immunohistochemical Localization of Hh Signaling Components in L-Arginine-Induced Acute Pancreatitis

In the control pancreata, Gli2 was detected mainly in the nucleus of islet and ductal cells, with weak staining in some acinar cells (Fig. 5A). On day 3 after L-arginine injection, Gli2 was detected in the nucleus of tubular complexes and in the nucleus of islet and ductal cells, whereas on day 14, it was detected in the nucleus of the remaining tubular complexes, islet and ductal cells, and some acinar cells (Fig. 5B and 5C). In the control pancreata, Smo was detected in the cytoplasm of islet cells but not acinar cells and duct cells (Fig. 6A). On day 3 after L-arginine injection, Smo was detected in the cytoplasm of tubular complexes and islet cells, with weak cytoplamic staining in ductal cells (Fig. 6B). On day 14 after L-arginine injection, Smo was detected in the cytoplasm of islet cells, with weak cytoplasmic staining in tubular complexes and ductal cells (Fig. 6C). In the control pancreata, Ptch1 was detected in the cytoplasm of islet and ductal cells but not in acinar cells (Fig. 7A). On day 3 and day

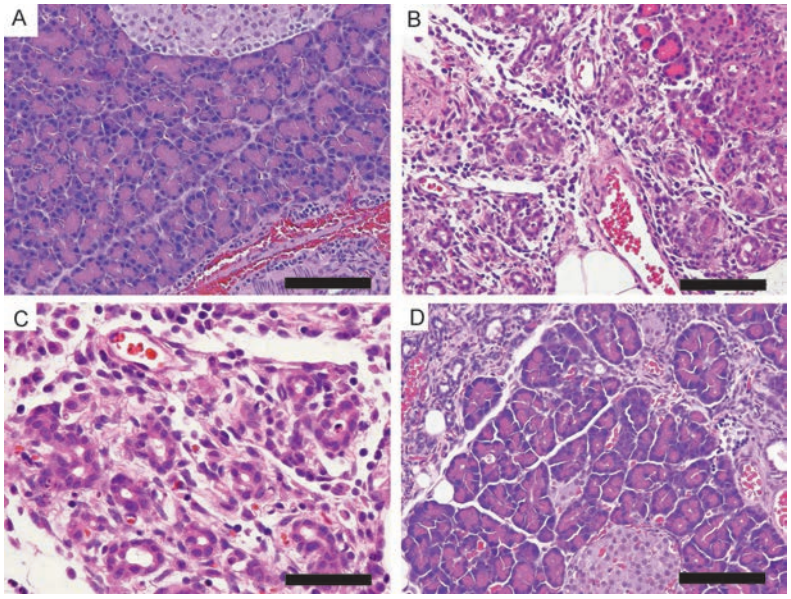

Fig. 1. Histological progression of exocrine regeneration in L-arginine-induced acute pancreatitis. (A) Pancreatic tissue injected with saline as a control. (B) On day 3, necrotic cells were replaced by tubular complexes. (C) Tubular complexes appeared as cylindrical tubes with flattened cuboidal cells surrounding a large lumen. (D) On day 14, acinar cell regeneration was nearly complete and there were fewer tubular complexes. Hematoxylin and eosin staining. Scale bar, 100 μm (A, B, and D); 200 μm (C).

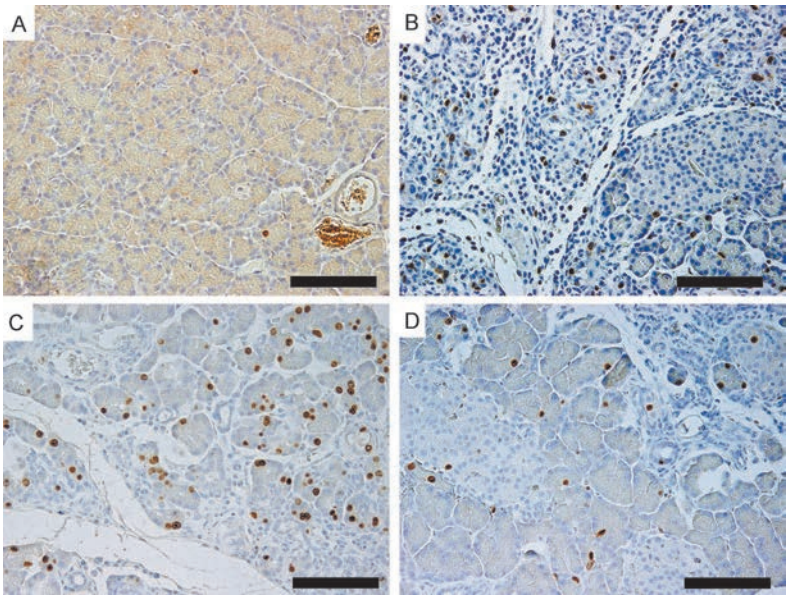

Fig. 2. Changes in pancreatic BrdU immunostaining in L-arginine-induced acute pancreatitis (scale bar = 100 μm). (A) In control pancreata, very few BrdU-labeled acinar cells were detected. (B) On day 3 after L-arginine injection, many tubular complex cells were positive for BrdU staining. (C) On day 5, many acinar cells were positive for BrdU, whereas fewer tubular complex cells were positive for BrdU. (D) On day 14, a few BrdU-labeled acinar cells were detected.

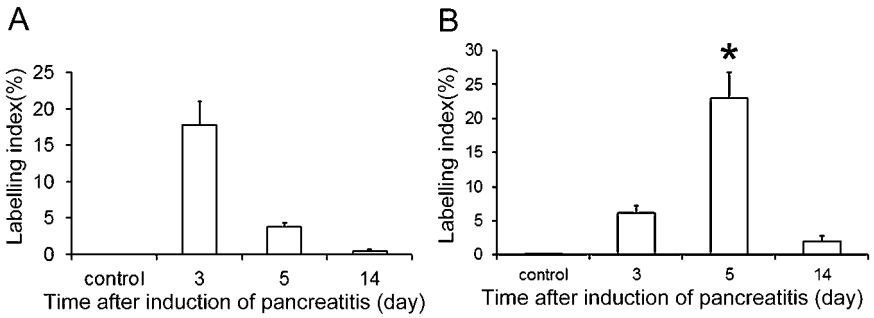

Fig. 3. BrdU labeling indices for tubular complexes (A) and acinar cells (B) in L-arginine-induced acute pancreatitis. Data represent the mean ± SEM ($n = 5$ in each group). *$P < 0.05$ vs. controls (day 0).

14 after L-arginine injection, Ptch1 was detected in the cytoplasm of tubular complexes and in the cytoplasm of islet and ductal cells (Fig. 7B and 7C).

Discussion

In the present study, we demonstrated that exocrine regeneration after L-arginine-induced acute pancreatitis was initiated by Pdx1-positive progenitor cell proliferation for tubular complex formation, acinar cell proliferation, and acinar cell-mediated regeneration coincident with the disappearance of tubular complexes. We also demonstrated that expression of these Hh signaling components was almost completely restricted to islet and ductal cells in the control pancreata; however, expression of these proteins was also observed in tubular complexes during exocrine regeneration, indicating that the Pdx1-positive progenitor cells within tubular complexes are Hh-responsive.

Previous studies in various rodent experimental models of acute pancreatic injury have suggested that Pdx1-positive progenitor cells forming tubular complexes have stem cell-like properties that allow them to differentiate into acinar cells based on the following: 1) the coincidence of the proliferation of acinar cells and the disappearance of tubular complexes; 2) the continuum of differentiation from tubular complex to acinar cells; and 3) expression of markers of other embryonic pancreatic progenitor cells, such as Tcf2 and Sox9, Hnf1b, and Foxa2[4)12)13)]. We first examined the proliferation of Pdx1-positive progenitor cells that occurred during exocrine regeneration. Our results indicated that exocrine regeneration after L-arginine-induced acute pancreatitis was initiated by the appearance and proliferation of Pdx1-positive progenitor cells to form tubular complexes and acinar cells and the coincidence of the progression of acinar cell proliferation and the disappearance of tubular complexes. These results indicate that exocrine regeneration in L-arginine-induced acute pancreatitis involves both transiently proliferating Pdx1-positive progenitor cell proliferation to form tubular complexes and acinar cell proliferation, supporting previous notions that Pdx1-positive progenitor cells may have the potential to differentiate into acinar cells.

Elucidating the mechanism that regulates Pdx1-positive cells in exocrine regeneration may provide an important basis for understanding how Pdx1-positive progenitor cells proliferate and differentiate into new acinar cells. Hh signaling regulates the regeneration of many organs and tissues through its

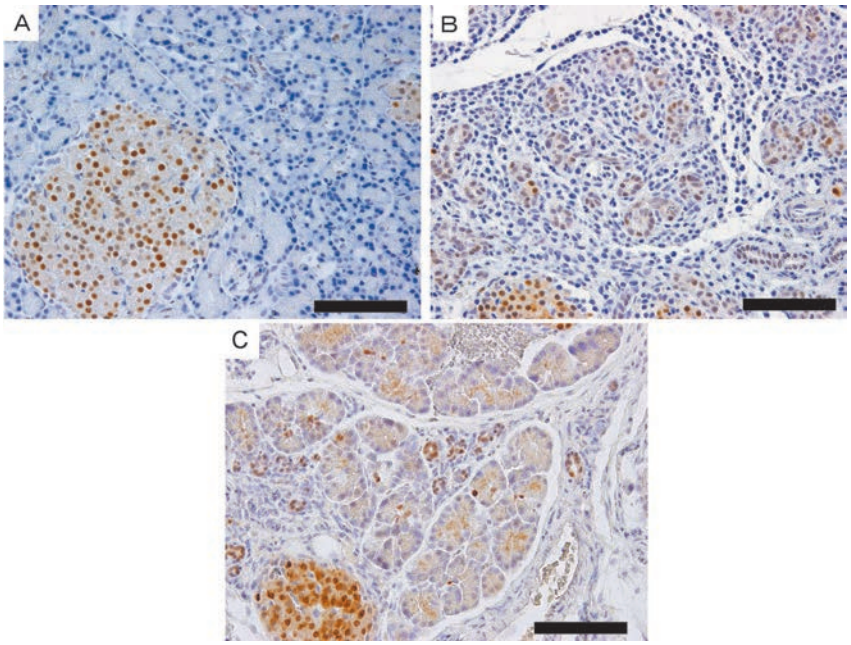

Fig. 4. Immunohistochemical analysis of Pdx1 expression in L-arginine-induced acute pancreatitis (scale bar = 100 μm). In control pancreata (A), Pdx1 was expressed in islet and ductal cells. During exocrine regeneration, Pdx1 was also expressed in tubular complexes on day 3 (B) and day 14 (C) after L-arginine injection.

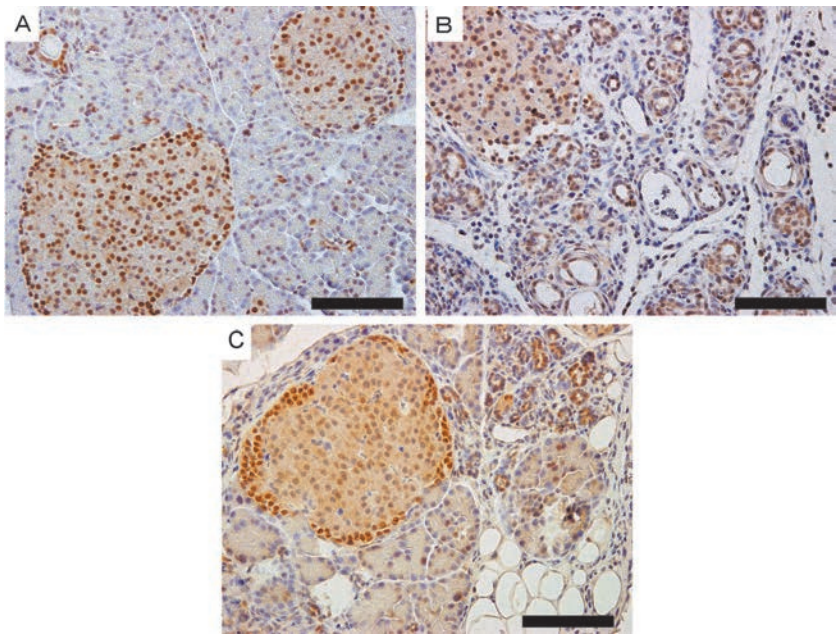

Fig. 5. Immunohistochemical analysis of Gli2 expression in L-arginine-induced acute pancreatitis (scale bar = 100 μm). In control pancreata (A), Gli2 was expressed in islet and ductal cells and weakly expressed in a few acinar cells. During exocrine regeneration, Gli2 was also expressed in tubular complexes on day 3 (B) and day 14 (C) after L-arginine injection.

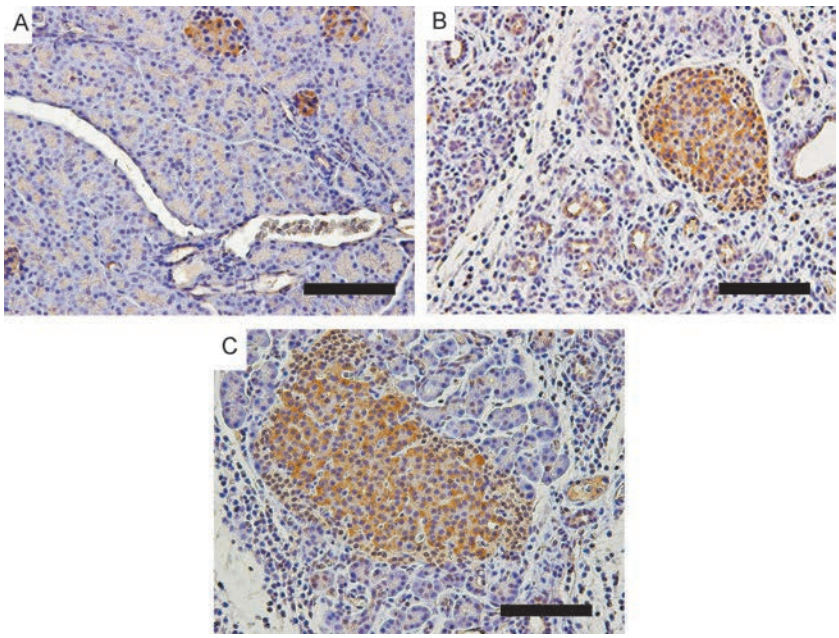

Fig. 6. Immunohistochemical analysis of Smo expression in L-arginine-induced acute pancreatitis (scale bar = 100 μm). In control pancreata (A), Smo was expressed in islet cells. During exocrine regeneration, Smo was also expressed in tubular complexes on day 3 (B) and day 14 (C) after L-arginine injection.

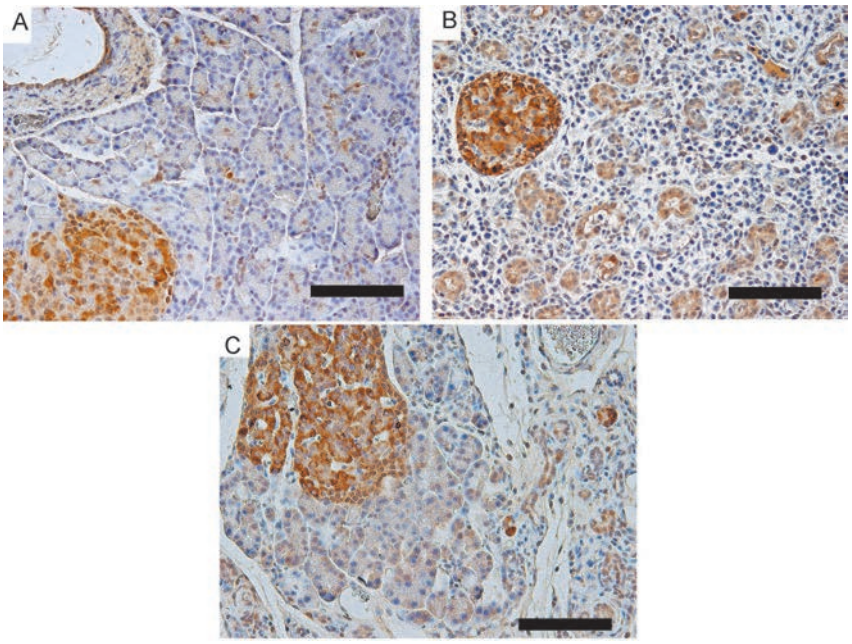

Fig. 7. Immunohistochemical analysis of Ptch1 expression in L-arginine-induced acute pancreatitis (scale bar = 100 μm). In control pancreata (A), Ptch1 was expressed in islet and ductal cells. During exocrine regeneration, Ptch1 was also expressed in tubular complexes on day 3 (B) and day 14 (C) after L-arginine injection.

effects on stem/progenitor cells[18)28)]. Previous studies using a cerulein-induced acute pancreatitis mouse model showed that the blocking of Hh signaling inhibited acinar cell regeneration but did not inhibit Pdx-1-positive progenitor cell proliferation in tubular complexes, suggesting that Hh signaling is involved in Pdx1-positive progenitor cell differentiation within tubular complexes during exocrine regeneration[23)]. To gain some insight into a possible link between Hh signaling and Pdx1-positive progenitor cells in exocrine regeneration, we aimed to identify and localize Hh-responsive cells using a different rodent model of acute pancreatic injury. Our results indicated that Hh signaling is activated in tubular complexes during exocrine regeneration, underscoring the importance of Hh signaling in controlling Pdx1-positive progenitor cells to form tubular complexes during exocrine regeneration, although we did not investigate the function of Hh signaling in the regeneration of Pdx1-positive progenitor cells in detail. Further research is needed to elucidate the roles of Hh signaling in regulating cell fate decisions in acinar cell regeneration, such as Hh target gene responses, which may play important roles in regulating the proliferation or differentiation of Pdx1-positive progenitor cells.

Previous studies have proposed that the mechanisms underlying exocrine regeneration recapitulate some aspects of embryonic pancreatic development. Many studies have reported that Pdx1-positive progenitor cells within tubular complexes have characteristics similar to embryonic pancreatic progenitor cells during early pancreatic development, e.g., the loss of mature acinar cell markers such as amylase, co-expression of embryonic pancreatic progenitor cell markers, and activation of other embryonic signaling pathways such as the Notch and Wnt signaling pathways[10)30)31)]. Hh signaling is also essential for embryonic pancreatic development. During the early stages, Hh signaling must specifically be downregulated in embryonic pancreatic progenitor cells for normal development to occur[32)33)]. In contrast, at later stages of pancreatic development and in adults, Hh signaling is activated in islet and ductal cells[34)]. Previous studies on cerulein-induced acute pancreatitis have indicated that a blockade of Hh signaling results in the inhibition of exocrine regeneration, suggesting the positive role of Hh signaling in exocrine regeneration, in contrast to its negative role in pancreatic development[23)]. Our study indicated that Pdx1-positive progenitor cells within tubular complexes during exocrine regeneration are Hh-responsive, in contrast to the exclusion of Hh signaling in embryonic pancreatic progenitor cells. These results provide additional insights into the differences between the molecular mechanisms by which Hh signaling regulates exocrine regeneration and embryonic pancreatic development. There is accumulating evidence that tight regulation of Hh signaling is essential for adult tissue regeneration after acute injury. This process involves Hh signaling being transiently and reversibly activated after acute injury, with the expansion of Hh-responsive cells, as well as the loss of Hh-responsive cells when recovery is completed[18)]. In contrast, chronic or persistent injury results in a prolonged increase in Hh signaling, leading to Hh-responsive cell proliferation for as long as the injury persists and finally resulting in irreversible activation of the signaling pathway, which leads to cancer formation[28)]. In this study, we demonstrated that transient activation of Hh signaling in exocrine regeneration is characterized by a transient expansion of Hh-responsive cells to form tubular complexes and reduction of Hh-responsive cells as regeneration progresses, suggesting that transient activation and tight control of Hh signaling is important in exocrine regeneration. On the other hand, constitutive and uncontrolled Hh pathway activation has been implicated in the development of chronic pancreatitis and pancreatic cancer[35-38)]. In chronic pancreatitis, the pancreatic parenchyma is destroyed and replaced by persistent tubular complexes. These tubular complexes in chronic pancreatitis also express

embryonic pancreatic progenitor markers (including Pdx1) and exhibit activation of Hh and Notch signaling, showing morphological and molecular characteristics similar to those of tubular complexes in acute pancreatic injury. However, aberrant activation of Hh signaling in tubular complexes during chronic inflammation, which can be induced through NK-κB activation during inflammation, may have a role in the pathogenesis and progression of chronic pancreatitis, further correlating with pancreatic cancer development[39]. Further studies are required to elucidate the mechanisms underlying the tight regulation of Hh signaling in exocrine regeneration without uncontrolled activation that possibly correlates with the development of pancreatic cancer.

Conclusions

We demonstrated that exocrine regeneration after L-arginine-induced acute pancreatitis involves Pdx1-positive progenitor cell proliferation to form tubular complexes and that transient activation of Hh signaling is characterized by the expression of Hh components in tubular complexes during exocrine regeneration. These findings suggest that Hh signaling plays an important role in controlling Pdx1-positive progenitor cell function during exocrine regeneration, a finding that needs to be explored further.

Acknowledgments

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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〈和文抄録〉

膵腺房細胞再生におけるヘッジホッグシグナル伝達系の局在の評価

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急性膵障害後の膵腺房細胞の再生過程で増殖する Pdx1 陽性細胞が，腺房細胞へと分化する前駆細胞の可能性が示唆され，このメカニズムを解明することが慢性膵炎などの膵外分泌腺機能不全に対する再生医療を開発する上で有用であると考えられる．本研究ではラットアルギニン急性膵炎モデルを用いて，腺房細胞再生過程におけるヘッジホッグシグナル伝達系（以下 Hh 系）と Pdx1 陽性細胞の関連を評価した．アルギニン投与後3日目に膵炎発症に伴う腺房細胞の壊死消失，Pdx1陽性細胞からなる Tubular complex の出現増殖を認めた．投与後 5 日目に腺房細胞の増殖が優位になる一方，Tubular complexの減少を認め，投与後14日目には腺房細胞の再生はほぼ終了する．Hh系のレセプターである Smo と Ptch1，転写因子である Gli2 は，正常膵ではラ氏島と導管に発現を認めたが，アルギニン投与後 3 日目及び 14 日目には Tubular complex にも発現を認めた．本研究において，Hh 系が Pdx1 陽性細胞を制御することにより膵腺房細胞の再生に関与している可能性が示唆された．

キーワード：ヘッジホッグシグナル伝達系，膵腺房細胞の再生，急性膵炎，膵前駆細胞，Pdx1.