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ORIGINAL RESEARCH

# Inhibitors of Arg-Gly-Asp-Binding Integrins Reduce Development of Pancreatic Fibrosis in Mice

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## SUMMARY

Arg-Gly-Asp–binding integrins are critically involved in cerulein-induced pancreatic fibrogenesis in mice, a model of chronic pancreatitis. Small-molecule Arg-Gly-Asp integrin antagonists have the potential to be developed for effective treatment of pancreatic fibrotic diseases.

**BACKGROUND & AIMS:** Pancreatic stellate cells (PSCs) regulate the development of chronic pancreatitis (CP) and are activated by the cytokine transforming growth factor $\beta$ (TGFB). Integrins of the $\alpha$v family promote TGFB signaling in mice, probably by interacting with the Arg-Gly-Asp (RGD) sequence of the TGFB latency-associated peptide, which frees TGFB to bind its cellular receptors. However, little is known about the role of integrins in the development of CP. We investigated the effects of small-molecule integrin inhibitors in a mouse model of CP.

**METHODS:** We induced CP in C57BL/6 female mice by repeated cerulein administration. An active RGD peptidomimetic compound (Center for World Health and Medicine [CWHM]-12) was delivered by continuous infusion, starting 3 days before or 5 days after cerulein administration began. Pancreata were collected and parenchymal atrophy, fibrosis, and activation of PSCs were assessed by histologic, gene, and protein expression analyses. We measured CWHM-12 effects on activation of TGFB in co-culture assays in which rat PSC cells (large T immortalized cells [LTC-14]) activate expression of a TGFB-sensitive promoter in reporter cells.

**RESULTS:** Pancreatic tissues of mice expressed messenger RNAs encoding subunits of RGD-binding integrins. Cerulein administration increased expression of these integrins, altered pancreatic cell morphology, and induced fibrosis. The integrin inhibitor CWHM-12 decreased acinar cell atrophy and loss, and substantially reduced fibrosis, activation of PSCs, and expression of genes regulated by TGFB. CWHM-12 also reduced established fibrosis in mice and blocked activation of TGFB in cultured cells.

**CONCLUSIONS:** Based on studies of a mouse model of CP and cultured PSCs, integrins that bind RGD sequences activate PSCs and promote the development of pancreatic fibrogenesis in mice. Small-molecule antagonists of this interaction might be developed for treatment of pancreatic fibrotic diseases. *(Cell Mol Gastroenterol Hepatol 2016;2:499–518; http://dx.doi.org/10.1016/j.jcmgh.2016.03.004)*

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Chronic pancreatitis (CP) is a slowly progressive disease that causes substantial loss of quality of life from chronic pain, malnutrition, and diarrhea stemming from exocrine insufficiency and finally endocrine insufficiency over decades. The prevalence of CP is approximately 1/2000 persons, and patients with CP have a shortened survival compared with the general population. Extensive pancreatic fibrosis is the primary pathologic feature of CP.[1] No disease-specific treatments are available, but a major advance in the field was the discovery of specialized cells in the pancreas, named *pancreatic stellate cells* (PSCs), which are responsible for the development of fibrosis upon activation.[2–4]

The published literature strongly supports the central importance of cytokine transforming growth factor $\beta$ (TGFB) in the activation of PSC and in driving pancreatic fibrogenesis.[5] Triggering of the TGFB pathway is the primary regulatory mechanism found in several fibroproliferative diseases including pulmonary fibrosis and cirrhosis.[6] TGFB initially is produced by cells in an inactive state through association with latency-associated peptide (LAP) and requires activation to produce its effects.[7] The latent complex is abundantly present in most tissues, including the pancreas,[8] and thus activation control may be a more important mechanism of regulating its biological effects than control of expression. Integrins of the $\alpha$v family bind

***Abbreviations used in this paper:*** **Col1a1, collagen type I $\alpha$1; CP, chronic pancreatitis; CTGF, connective tissue growth factor; CWHM, Center for World Health and Medicine; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FBS, fetal bovine serum; $IC_{50}$, median inhibitory concentration; LAP, latency-associated peptide; LTC-14, large T immortalized cells; MLEC, mink lung epithelial cell; MMP, matrix metallopeptidase; mPSC, mouse pancreatic stellate cell; mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PSC, pancreatic stellate cell; p-SMAD, phosphorylated SMAD; RGD, arginine-glycine-aspartic acid; $\alpha$-SMA, $\alpha$-smooth muscle actin; TGFB, transforming growth factor $\beta$.**

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LAP via its Arg-Gly-Asp (RGD) sequence, and have been implicated in TGFB activation in several organs.[9–11] However, the mechanism of TGFB activation in pancreatic fibrogenesis has not been studied.

Integrins are a large family of transmembrane cell adhesion and signaling receptors consisting of $\alpha$ and $\beta$ subunits connecting the inner cytoskeleton with the outer extracellular matrix.[12] In mammals, a total of 18 $\alpha$ and 8 $\beta$ integrin subunits noncovalently associate to form 24 different integrin heterodimers. This diversity and complexity allows highly tissue-specific expression of different heterodimers with different ligand affinities. Of the 24 integrin heterodimers, 6 have been shown to bind and activate latent TGFB in vitro by binding to the amino acid sequence RGD of LAP.[13–18] These include all $\alpha$v integrins ($\alpha$v$\beta$1, $\alpha$v$\beta$3, $\alpha$v$\beta$5, $\alpha$v$\beta$6, and $\alpha$v$\beta$8) and $\alpha$8$\beta$1.

The Center for World Health and Medicine (CWHM) at Saint Louis University has synthesized many small-molecule RGD peptidomimetic compounds that inhibit the ligand-binding activities of integrins involved in TGFB activation. One of these compounds, a broad range antagonist of RGD-binding integrins called CWHM-12, recently was tested in mouse models of lung and liver fibrosis and showed significant efficacy in fibrosis prevention and reversal.[11]

The present studies evaluate the effects of pharmacologic inhibition of RGD-binding integrins by CWHM-12 in a cerulein-induced injury mouse model of CP. This model reproduces the histopathologic features found in human CP, including fibrosis, inflammation, acinar atrophy, and tubular complex formation.[19–21] Moreover, the TGFB pathway was shown to play a central role in fibrosis development in this model.[22–24] We show a critical role of RGD-binding integrins in CP and the promising potential to arrest or possibly even reverse pancreatic fibrosis using a pharmacologic approach to inhibiting integrin-mediated TGFB activation.

## Materials and Methods

### Animals

Experiments were performed with C57BL/6 female 7- to 8-week-old mice obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in standard facilities under controlled conditions of temperature, humidity, and a 12-/12-hour light/dark cycle, and were maintained on standard rodent chow with free access to water. Animal care and all procedures were approved by the Institutional Animal Care and Use Committee of Saint Louis University.

### Induction of Pancreatic Fibrosis and Tissue Processing

Mice were divided randomly into treatment groups of 10 animals each. Pancreatic fibrogenesis was induced by repetitive intraperitoneal injections of 50 $\mu$g/kg cerulein (Sigma, Saint Louis, MO) as described in detail previously.[25–27] Briefly, cerulein treatments (one intraperitoneal injection every hour for 6 hours) were given to mice every other day so that each animal received 3 courses of the injury agent. The control group received comparable injections of sterile 0.9% sodium chloride (saline). Mice then were euthanized by $CO_2$ asphyxiation and this was performed 3 days after the last injection to allow resolution of acute changes. Blood was collected by cardiac puncture in heparin tubes (Lithium Heparin Separator MiniCollect; Greiner Bio-One, Kremsmünster, Austria). The pancreas from each mouse was removed, weighed, and divided into sections. Sections were either immediately frozen in liquid nitrogen and stored at -80°C for subsequent protein extraction and Western blot analysis, fixed in 10% neutral buffered formalin solution (Sigma) for histologic analysis, or placed in an RNA stabilization solution (RNAlater; Ambion, Austin, TX) and stored overnight at 4°C for RNA isolation and subsequent real-time quantitative polymerase chain reaction (PCR) assays.

### Induction of Acute Pancreatic Injury and Tissue Processing

Mice were divided into groups of 4–6 animals each. To determine the effects of acute pancreatic injury, mice were subjected to a single course of cerulein treatment (ie, 6 hourly intraperitoneal injections of 50 $\mu$g/kg each). Sex- and age-matched control mice received comparable injections of sterile saline solution. Nine hours after the first injection, the mice were euthanized by $CO_2$ asphyxiation and blood was collected by cardiac puncture for amylase analysis. Each pancreas was removed, weighed, and placed in 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO) for histologic analysis.

### Administration of Integrin Antagonist Compounds

The small-molecular-weight integrin antagonist, CWHM-12, and its inactive enantiomer control compound, CWHM-96, were synthesized by the Center for the World Health and Medicine (Saint Louis University, St. Louis, MO). Syntheses and structures of these compounds have been described previously.[11] This prior report also showed that CWHM-12 has excellent potency (median inhibitory concentration [$IC_{50}$] in the low nanomolar range) against 4 $\alpha$v integrins ($\alpha$v$\beta$1, $\alpha$v$\beta$3, $\alpha$v$\beta$6, and $\alpha$v$\beta$8) and integrin $\alpha$5$\beta$1 in in vitro ligand-binding assays, and also has good but somewhat lesser potency against $\alpha$v$\beta$5 (IC50 $<$ 100 nmol/L). In contrast, the R-isomer of CWHM-12, called CWHM-96, which differs from CWHM-12 only in the orientation of its carboxyl group, did not inhibit any of these integrins in in vitro ligand-binding assays.[11] To evaluate prevention of pancreatic fibrogenesis (preventive mode), CWHM-12 was delivered by continuous infusion at 100 mg/kg/day (50% dimethyl sulfoxide [DMSO], 50% phosphate-buffered saline [PBS]) using Alzet mini-osmotic pumps (Durect, Cupertino, CA) implanted subcutaneously in mice 3 days before the first cerulein treatment. Minipumps with vehicle (50% DMSO, 50% PBS) also were implanted in cerulein-treated and control saline-treated groups of mice. To evaluate the effect of therapeutic rather than preventive compound administration, minipumps with CWHM-12, CWHM-96, or vehicle (for the cerulein- and saline-treated groups) were implanted on day 5 (relative to the first day of cerulein treatment). To evaluate effects on acute pancreatic injury,