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Der Einfluss von Diabetes auf Erkrankungen der Bauchspeicheldrüse

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Synopsis

Die Langerhans-Inseln als wichtiges endokrines System unseres Körpers sind als einzelne separate Entitäten in Mitte exokriner Azinuszellen der Bauchspeicheldrüse angeordnet. Diese besondere anatomische Lage inspirierte die Wissenschaft bereits im neunzehnten Jahrhundert, eine Beeinflussung des exokrinen Gewebes durch die Langerhans-Inseln zu postulieren (Jarotzky, 1899). Zudem weisen seit etwa 90 Jahren ein Vielzahl von klinischen Untersuchungen auf eine exokrine Insuffizienz bei Diabetes mellitus Typ I und in geringeren Maße bei Diabetes mellitus Typ II Patienten hin (Jones et al., 1925; Frier et al., 1976; Rathmann et al., 2001; Hardt et al., 2003; Terzin et al., 2014). Die Prävalenz exokriner Insuffizienz liegt bei den größten Einzelstudien mit Diabetes mellitus Typ I Patienten bei 23% bzw. Diabetes mellitus Typ II Patienten bei 12 % (Hardt et al., 2003; Rathmann et al., 2001). Um mögliche klinische Symptome dieser exokrinen Insuffizienz wie Steatorrhoe zu lindern, können Patienten mit Enzymen der Bauchspeicheldrüse wie z.B. Pancreatin behandelt werden. Einen Einfluss dieser Therapie auf klinisch relevante Symptome bei Diabetes assoziierter Pankreasinsuffizienz konnte jedoch in klinischen Studien nicht nachgewiesen werden (Ewald et al., 2007).

Inspiriert durch die auffällige anatomische Lage der Langerhans-Inseln und den klinischen Befunden in Diabetes Patienten, wurden in den letzten Jahrzehnten Mechanismen definiert wie Langerhans-Inseln physiologische Parameter des exokrinen Gewebes regulieren. Eine wichtige Grundlage für das Verständnis dieser Regulation beruht auf den bereits frühzeitig erkannten Besonderheiten in der Blutversorgung des Pankreasgewebes (Wharton, 1932; Thiel, 1954, Ferner, 1957). Durch ein insulo-azinäres Portalsystem fließt arterielles Blut zuerst zu den Langerhans-Inseln und danach in das exokrine Gewebe der Bauchspeicheldrüse und bildet somit eine ideale Voraussetzung für eine Kommunikation zwischen endokrinem und exokrinem Gewebe (Ohtani et al., 1983; Wayland 1997, Ballian and Brunicardi, 2007; Barreto et al., 2010). Neben diesem Portalsystem wurde auch ein insulo-venöser Blutfluss beobachtet (Ohtani et al., 1986). Möglicherweise bewirkt dieses vaskuläre System, dass Hormone der Langerhans-Inseln auch rasch an den restlichen Körper abgegeben werden (Ohtani et al., 1986). Trotz unzähliger Studien, die den insulo-azinären Blutfluss belegen, wird vor allem inselfernes exokrines Pankreasgewebe auch von intralobulären Arteriolen, die nicht durch Langerhans-Inseln führen, mit Blut versorgt (Murakami et al., 1997; Barreto et al., 2010). Dies lässt die Schlussfolgerung zu, dass inselnahe Areale des exokrinen Gewebes durch den insulo-azinären Blutfluss auf direkten Wege mit hohen Konzentrationen von Hormonen der Langerhans-Inseln versorgt werden, während inselferne Areale vorwiegend über den systemischen Blutkreislauf und intralobuläre Arteriolen mit Hormonen versorgt werden (Ohtani et al., 1986; Murakami et al., 1992). Eine

Regulation der Physiologie des exokrinen Pankreasgewebes durch Hormone der Langerhans-Inseln wird zudem durch die Fenestrierung der Kapillaren erleichtert. Diese Poren werden in den Kapillaren der Langerhans-Inseln und des exokrinen Gewebes beobachtet und erlauben einen raschen Austausch zwischen dem Kapillarbett und dem interstitiellen Gewebe (Kvietys et al. 1983).

Vor allem durch tierexperimentelle Studien wurden relevante Hormone identifiziert und deren Einfluss auf exokrine Zellen charakterisiert. So bewirkt die Ablation der Insulin produzierenden Beta-Zellen durch Administration von z.B. Streptozotocin eine verminderte Expression und Sekretion von Amylase in den Azinuszellen, wobei Insulin sowohl die basale als auch die stimulierte Amylase Sekretion erhöht (Benabdeljlil et al., 1965; Couture et al., 1972; Hegyi et al., 1999; Patel et al., 2004; Patel et al., 2006; Han and Liu 2010). Dieses Konzept einer insulinär-azinären Achse dient als wichtiges Modell für eine Erklärung der exokrinen Insuffizienz bei Diabetes mellitus Patienten (Williams and Goldfine, 1985; Barreto et al., 2010). Da zusätzlich zu Insulin auch weitere Hormone der Langerhans-Inseln wie z.B. Glucagon, Somatostatin und Pancreatic Polypeptide die Physiologie von Azinuszellen beeinflussen, wurde das ursprüngliche Modell von William und Goldfine erweitert, sodass man nun von einer Inselzell-Azinus-Achse spricht (Barreto et al., 2010). Diese Achse reguliert neben dem Nervus vagus und Hormonen des Magendarmtraktes wie z.B. Sekretin und Cholecystokinin die exokrine Funktion der Bauchspeicheldrüse.

Während eine Beeinflussung des exokrinen Gewebes durch die Langerhans-Inseln und die Auswirkungen von Diabetes mellitus auf die Physiologie des exokrinen Pankreas seit 1899 gut charakterisiert wurden und somit zumindest teilweise verstanden werden, ist das Wissen, in wie weit Diabetes mellitus weitere Pathologien des Organs wie z.B. Pankreatitiden oder das pankreatische duktale Adenokarzinom beeinflusst, unvollständig. Durch klinische Studien ist belegt, dass Patienten mit akuter oder chronischer Pankreatitis oft temporären oder persistierenden Diabetes mellitus entwickeln. So wird bei akuter Pankreatitis eine temporäre Hyperglykämie bei bis zu 50% und ein persistierender Diabetes bei bis zu 15% der Patienten beobachtet (Czako et al., 2009; Das et al., 2014). Die Prävalenz von Diabetes bei chronischer Pankreatitis liegt abhängig von der jeweiligen Studie zwischen 30 und 83% (Angelopoulos et al., 2005; Czako et al., 2009; Chen et al., 2011). Diese Korrelationen werden zum Teil durch die wohlbekannten funktionellen Einschränkungen des endokrinen Pankreasgewebes bei Pankreatitis, den sogenannten pankreopriven Diabetes oder Diabetes mellitus Typ IIIc erklärt (Czako et al., 2009). In wie weit diese funktionelle Einschränkung des endokrinen Gewebes wiederum einen Einfluss auf den Verlauf von Pankreatitiden hat, ist noch wenig erforscht (Czako et al., 2009). Es gibt jedoch korrelative Anhaltspunkte, dass eine negative Beeinflussung möglich wäre. So ist z.B. Diabetes ein Risikofaktor bei akuter als auch chronischer Pankreatitis und Hyperglykämie ist ein Kriterium beim Ranson Score, um die

Schwere einer akuten Pankreatitis zu prognostizieren (Ranson et al., 1974; Renner et al., 1985; Seicean et al., 2006, Urushihara et al., 2012). Zudem zeigte eine klinische Studie, dass Insulinadministration bei akuter Pankreatitis die Mortalität signifikant zu senken vermag (Hallberg, 1977). Um zu überprüfen, welchen Einfluss Diabetes und Insulin auf den Verlauf einer akuten Pankreatitis hat, verglichen wir den Verlauf Cerulein induzierter akuter Pankreatitis in hyperglykämen, hypoinsulinämen Mäusen mit dem Verlauf einer akuten Pankreatitis in normoglykämen Tieren (Zechner et al., 2012; s. Anhang Teil 1). Überraschender Weise kam es in den diabetischen Mäusen zu einer starken Aggravierung der akuten Pankreatitis. Diabetes verstärkte die Inflammation, die Ödembildung und erhöhte den Zelltod im Gewebe. Diabetes hemmte auch die Regeneration von Azinuszellen, führte zu einer verstärkten azinären zu duktalen Metaplasie und zu einer verstärkten Atrophie der Bauchspeicheldrüse. Diese nachteiligen Effekte konnten durch die subkutane Administration von Insulin behoben werden. Somit kann man schlussfolgern, dass Diabetes akute Pankreatitis negativ und Insulin akute Pankreatitis positiv beeinflusst. Einige wenige weitere Publikationen unterstützen diese Hypothese. So konnte eine britische Arbeitsgruppe belegen, dass Insulin den Zelltod von Azinuszellen nach Applikation von Palmitoleinsäure in vitro hemmt (Samad et al., 2014). Eine ungarische Arbeitsgruppe berichtete, dass Diabetes in Ratten die Cholecystokinin stimulierte Pankreasregeneration nach Arginin induzierter Pankreatitis verringert und dass Insulin die durch Cholecystokinin stimulierte Proliferation von Azinuszellen erhöht (Takács et al., 2001, Hegyi et al., 2004). Insgesamt unterstützen diese Studien die Hypothese, dass Diabetes akute Pankreatitis verschlimmert, während Insulin das Gegenteil bewirkt. Laut einer Meta-Analyse beobachtet man bei 37% der Patienten mit akuter Pankreatitis eine Hyperglykämie, wobei jedoch nur 15% der Patienten mit Insulin behandelt werden (Das et al., 2014). In wie weit eine Insulinadministration einen Vorteil für weitere Patientengruppen darstellen könnte und ob man Hyperglykämie bei akuter Pankreatitis generell mit Insulin behandeln sollte, müsste nun in weiteren klinischen Studien evaluiert werden.

Die molekularen Mechanismen, wie Diabetes Pankreatitis beeinflusst, werden bisher nur marginal verstanden. Tierexperimentelle Ansätze definierten jedoch ein sogenanntes Notfallprogramm während einer akuten Pankreatitis (Fiedler et al., 1998). Hierbei wird die Expression von Genen induziert, die den Zelltod und die Inflammation während einer Pankreatitis hemmen und somit einen protektiven Effekt auf die Bauchspeicheldrüse haben (Fiedler et al., 1998). Gene, die während dieses Notfallprogrammes induziert werden, sind z.B. der Ko-Transkriptionsfaktor p8, der wiederum die Expression weiterer Gene wie z.B. Reg3β induziert (Vasseur et al., 2004; Closa et al., 2004). Die Expression von Reg3β reduziert Inflammation und den Zelltod im exokrinen Gewebe und hat somit eine hemmende Wirkung auf den Verlauf der Pankreatitis (Zhang et al., 2004; Gironella et al., 2007). Die Expression

dieses protektiven Gens wird jedoch durch Diabetes gehemmt (Zechner et al., 2012; s. Anhang Teil 1). Ein weiteres Protein, dem eine Funktion bei inflammatorischen Erkrankungen aber auch bei von Diabetes mellitus verursachten Pathologien zugeschrieben wird, ist der Rezeptor für Advanced Glycation Endproducts (RAGE), der durch glykierte Proteine oder auch Proteine wie High-Mobility-Group-Protein-Box-1 (HMGB1), welches bei Nekrose freigesetzt wird, aktiviert wird (Yamagishi et al., 2005; Chuah et al., 2013). Während dem zytomembranständigen RAGE meist eine pro-inflammatorische Funktion zugesprochen wird, haben N-terminale proteolytische Abbauprodukte bzw. Splicevarianten, die zur Expression einer löslichen Isoform von RAGE (sRAGE, esRAGE) führen, eine anti-inflammatorische Wirkung (Ramasamy et al., 2009). Diese löslichen Isoformen von RAGE binden RAGE-Liganden und hemmen somit deren pro-inflammatorische Wirkung (Ramasamy et al., 2009). Interessanter Weise reduziert Diabetes die Expression kurzer N-terminaler RAGE Isoformen in der entzündeten Bauchspeicheldrüse (Zechner et al., 2013; s. Anhang Teil 2). Somit könnte die Aggravierung akuter Pankreatitis durch Diabetes zumindest teilweise durch die Reduktion in der Konzentration von Reg3β und anti-inflammatorischen RAGE Isoformen im Pankreasgewebe erklärt werden.

Eine akute Pankreatitis kann bei bis zu 20% von Patienten zu schweren Symptomen wie lokalen Nekrosen in der Bauchspeicheldrüse oder durch eine systemische Ausschüttung von Entzündungsmediatoren zu einem systemischen inflammatorischen Response Syndrom (SIRS) führen (Forsmark and Baillie, 2007). Dies führt oft zu akuten Lungenversagen und sogar zu multiplen Organversagen und ist teilweise verantwortlich für die beobachtete Mortalität bei akuter Pankreatitis. Um zu überprüfen, welchen Einfluss Diabetes auf systemische Inflammation und die Lunge während einer akuten Pankreatitis hat, verglichen wir sowohl Interleukin-6 Konzentration im Blut als auch Inflammation und Zelltod in der Lunge in hyperglykämen, hypoinsulinämen Mäusen mit normoglykämen Tieren (Zechner et al., 2014; s. Anhang Teil 3). Obwohl diabetische Tiere im Vergleich zu normoglykämen Tieren stark erhöhte IL-6 Konzentrationen bei akuter Pankreatitis im Blut aufwiesen, war die Anzahl von Granulozyten in den Lungen dieser Tiere nur moderat erhöht, und eine signifikante Induktion von Zelltod konnte nicht im Lungengewebe nachgewiesen werden. Somit führte Diabetes zu einer wesentlichen Verschlechterung in der lokalen Symptomatik einer akuten Pankreatitis und in der Folge zu erhöhten Entzündungsparameter wie z.B. einer erhöhten IL-6 Konzentration im Blut. Diabetes führte jedoch nicht zu einer stark erhöhten Lungeninflammation bzw. einer Schädigung des Lungenparenchyms. Da sich diese Daten bisher nur auf ein Mausmodell beziehen, kann jedoch nur eingeschränkt die Schlussfolgerung gezogen werden, dass Diabetes generell lokale Komplikationen bei akuter Pankreatitis aggraviert, jedoch dies nicht die Gefahr für ein Lungenversagen erhöht. Eine derartige Schlussfolgerung wäre jedoch konsistent mit klinischen Studien und mit tierexperimentellen Ansätzen, die belegen, dass

Diabetes eine Inflammation und Lungenschädigung in unterschiedlichen Situationen meist nicht verstärkt, sondern hemmt (Honiden and Gong., 2009; Esper et al., 2009; Koh et al., 2012; Filgueiras et al., 2012; Gu et al., 2014; Filgueiras et al., 2014).

Während akute Pankreatitis vorwiegend zu temporärer Hyperglykämie führt (Czako et al., 2009; Das et al., 2014), entwickeln Patienten mit chronischer Pankreatitis oft einen persistierenden Diabetes (Angelopoulos et al., 2005; Czako et al., 2009; Chen et al., 2011). Um zu überprüfen, welchen Einfluss Diabetes auf den Verlauf einer chronischen Pankreatitis hat, verglichen wir Cerulein induzierte chronische Pankreatitis in hyperglykämen, hypoinsulinämen Mäusen mit chronischer Pankreatitis in normoglykämen Tieren (Zechner et al., 2014; s. Anhang Teil 4). Diabetes verstärkte die Infiltration inflammatorischer Zellen in das Pankreasgewebe, die Proliferation pankreatischer Sternzellen und die Bildung von Kollagen I. Diabetes erhöhte den Zelltod von Azinuszellen und führte somit zu einer verstärkten Pankreasatrophie. Diese Daten unterstützen die Hypothese, dass Diabetes den Verlauf chronischer Pankreatitis verschlimmert. In wie weit eine intensiviertere Insulintherapie vom Vorteil für diabetische Patienten bei chronischer Pankreatitis sein könnte, müsste jedoch sorgfältig in klinischen Studien evaluiert werden. Aufgrund der bei chronischer Pankreatitis meist vorliegenden eingeschränkten Glukagonsekretion und der damit verbundenen Gefahr von hypoglykämen Phasen wird meist eine konservative Insulintherapie empfohlen (Forsmark et al., 2007; Braganza et al., 2011; Rickels et al., 2013). Jedoch belegt eine klinische Studie, dass eine intensiviertere Insulintherapie bei chronischer Pankreatitis relativ gefahrlos durchgeführt werden kann (Terzin et al., 2012). Ob dies jedoch auch einen Vorteil für den Patienten darstellt, müsste noch evaluiert werden.

Neben Pankreatitiden stellt das duktale pankreatische Adenokarzinom (PDA) eine weitere wichtige Erkrankung der Bauchspeicheldrüse dar. Wegen einer meist späten Diagnose, einer schnellen Metastasierung und einem schlechtes Ansprechen auf Therapien liegt die 5-Jahres-Überlebensrate dieser Erkrankung bei etwa 6-7% (Jemal et al., 2010, Lemke et al., 2014). Gut charakterisierte Risikofaktoren für die Entwicklung von PDA sind z.B. chronische Pankreatitis, aber auch Diabetes mellitus Typ 2 (Berrington et al., 2003; Huxley et al., 2005; Duell et al., 2012). Es ist unumstritten, dass diese Faktoren, das Risiko an PDA zu erkranken, erhöhen. Jedoch scheitert der Versuch, dies mechanistisch zu verstehen, am mangelnden Verständnis, wie sich präkanzerogene Läsionen entwickeln. Ursprünglich vermutete man, dass PDA sich über mehrere Zwischenstufen, den sogenannten pankreatischen intraepithelialen Neoplasien (PanINs), aus Gangepithel entwickeln (Kopp und Sander 2014). Untersuchungen an gentechnisch veränderten Mäusen zeigten jedoch, dass duktales Epithel kaum durch Onkogene transformiert werden kann, während die Expression von onkogenen K-Ras in Azinuszellen insbesondere bei gleichzeitiger chronischer Pankreatitis mittels azinär-duktaler Metaplasie zur Bildung von PanINs und PDA führt (Brembeck et al.

2003; Guerra et al., 2007; Kopp et al., 2012). Erst kürzlich wurden spezifische Ausstülpungen der Pankreasgänge, sogenannte Pancreatic Duct Glands (PDGs), in Mäusen und Menschen entdeckt (Strobel et al., 2010). Diese PDGs exprimieren Gene charakteristisch für Vorläuferzellen wie z.B. pdx1 und können nach Stimulation sich zu PanIN ähnlichen Strukturen entwickeln (Gier et al., 2012; Wang et al., 2013). Um zu überprüfen, welchen Einfluss Risikofaktoren wie Diabetes mellitus Typ II und chronische Pankreatitis, auf PDGs haben, verglichen wir PDGs in diabetischen B6.V-Lep$^{ob/ob}$ mit PDGs in nichtdiabetischen B6.V-Lep$^{+/?}$ Mäusen nach bzw. ohne Induktion einer chronischen Pankreatitis (Bobrowski et al., 2013; s. Anhang Teil 5). Wir beobachteten, dass eine Diabetes Typ II ähnliche Symptomatik und chronische Pankreatitis die Proliferation der epithelialen Zellen spezifisch in PDGs stimulieren und beide Risikofaktoren Charakteristika von PDA wie z.B. nukleares S100P und Produktion von Mucin 5a, in diesen PDGs induzieren. PDGs stellen somit interessante Strukturen dar, aus denen sich PDA entwickeln könnten. Um diese Hypothese zu unterstützen, müsste man spezifisch in PDGs onkogenes Ras und/oder weitere Onkogene exprimieren und das Entstehen von PDA beobachten. Um dies technisch umzusetzen, ist jedoch zuerst eine weitere Charakterisierung spezifischer Genexpression in PDGs notwendig.

Während einige Publikationen den Einfluss von Diabetes mellitus Typ II und chronische Pankreatitis auf potentielle präkanzerogene Läsionen im Pankreas belegen, gibt es kaum Informationen, wie diese Faktoren die Pathophysiologie etablierter Karzinome beeinflussen (Guerra et al., 2007; Strobel et al., 2010; Guerra et al., 2011; Daniluk et al., 2012; Bobrowski et al., 2013). Um zu überprüfen, welchen Einfluss diese Faktoren auf etablierte Pankreaskarzinome haben, injizierten wir 6606PDA Zellen in den Pankreaskopf von diabetischen B6.V-Lep$^{ob/ob}$ und nichtdiabetischen B6.V-Lep$^{+/?}$ Mäusen und induzierten in jeweils einer Hälfte der Mäuse eine chronische Pankreatitis (Zechner et al., 2014; s. Anhang Teil 6). Wir beobachteten, dass Diabetes die Proliferation dieser Karzinomzellen verstärkte und zu erhöhtem Tumorgewicht führte, während die chronische Pankreatitis nur einen minimalen Einfluss auf die Pathophysiologie der Karzinome in diesem syngenen orthotopen Pankreaskarzinommodell hatte. Diese Daten unterstützen die Hypothese, dass Diabetes mellitus sowohl präkanzerogene Läsionen stimuliert und somit das Risiko erhöht, an PDA zu erkranken, als auch die Proliferationsrate eines etablierten PDAs verstärkt. Diese Daten sind auch konsistent mit der Hypothese, dass chronische Inflammation primär die frühe Phase der Tumorgenese beeinflusst und somit das Risiko erhöht, an PDA zu erkranken, jedoch bei einem etablierten Karzinom zu keiner weiteren Stimulation pathophysiologischer Vorgänge führt. Klinische und tierexperimentelle Studien unterstützen diese These. So reduzieren anti-inflammatorische Medikamente die Progression präkanzerogener Läsionen, haben jedoch nicht automatisch therapeutisches Potenzial bei voll etablierten PDA (Furukawa et al., 2003; El-Rayes et al., 2005; Funahashi et al., 2007; Mukherjee et. al., 2009). Des Weiteren konnten

wir beobachten, dass Tumorzellproliferation und Tumorgröße durch Behandlung mit Metformin, einem traditionellen Diabetes Medikament, gehemmt wurde (Zechner et al., 2014; s. Anhang Teil 6). Dieses Ergebnis mit murinen Zelllinien ist konsistent zu ähnlichen Ergebnissen mit humanen PDA Zellen (Lonardo et al., 2013; Kisfalvi et al., 2013). In wie weit eine anti-tumorigene Therapie von PDA mit Metformin an Patienten sinnvoll ist, wird momentan mit mehreren klinischen Studien evaluiert, wobei eine endgültige Schlussfolgerung noch aussteht (http://clinicaltrials.gov/; Pollak, 2014; Burney et al., 2014).

Aus den hier dargestellten Studien geht hervor, dass eine diabetische Stoffwechsellage einen starken Einfluss auf Erkrankungen der Bauchspeicheldrüse hat. Hyperglykämie bei gleichzeitiger Hypoinsulinämie wie sie bei Diabetes mellitus Typ I oder Typ 3c auftreten, aggraviert den Verlauf akuter und chronischer Pankreatitis. Hyperglykämie bei gleichzeitiger Hyperinsulinämie wie sie im Frühstadium von Diabetes mellitus Typ 2 auftritt, stimuliert PDGs, eine neuentdeckte anatomische Struktur im Pankreas, aus denen sich möglicher Weise PanINs und PDA entwickeln können. Zudem verstärkt ein dem Diabetes Typ 2 ähnliche metabolische Stoffwechsellage die Proliferation von Karzinomzellen in etablierten PDA. Diese Daten unterstützen die Hypothese, dass das klassische Konzept der Inselzell-Azinus-Achse, das die Kommunikation zwischen endokrinen und exokrinen Kompartiment beschreibt, eine besonders wichtige Bedeutung und evtl. auch klinische Relevanz bei unterschiedlichen Erkrankungen der Bauchspeicheldrüse hat.

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5. Bobrowski A, Spitzner M, Bethge S, Mueller-Graf F, Vollmar B, Zechner D. Risk factors for pancreatic ductal adenocarcinoma specifically stimulate pancreatic duct glands in mice. Am J Pathol. 2013;182:965-74.

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Anhang - Teil 1

Diabetes aggravates acute pancreatitis and inhibits pancreas regeneration in mice

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ARTICLE

Diabetes aggravates acute pancreatitis and inhibits pancreas regeneration in mice

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Abstract

Aims/hypothesis It is well established that acute pancreatitis often causes diabetes and that a high blood glucose level associated with pancreatitis is a marker of poor prognosis. The aim of this study was to evaluate if diabetes merely reflects the severity of pancreatitis or whether it can also aggravate the progression of this disease in a vicious circle.

Methods Reversible acute oedematous pancreatitis was induced in untreated and streptozotocin-treated diabetic mice by injection of cerulein. Progression of pancreatitis was studied by immunohistochemistry, ELISA and various other enzyme assays. The production of regenerating islet-derived 3β (REG3β) was determined by western blot and immunohistochemistry.

Results While cerulein treatment in non-diabetic mice resulted in acute pancreatitis followed by regeneration of the pancreas within 7 days, diabetes aggravated pancreatitis, inhibited the regeneration of the exocrine tissue and led to strong atrophy of the pancreas. The aggravation of pancreatitis by diabetes was characterised by decreased production of the anti-inflammatory protein REG3β, increased inflammation, augmented oedema formation and increased cell death during the acute phase of pancreatitis ($p<0.05$). During the regenerative phase, diabetes augmented inflammation, increased cell death, reduced acinar cell expansion and increased the expansion of duct as well as interstitial cells, resulting in the formation of tubular complexes ($p<0.05$). Administration of insulin reversed the observed phenotype in diabetic mice.

Conclusions/interpretation Diabetes aggravates acute pancreatitis and suppresses regeneration of the exocrine tissue. Thus, diabetes is not just a concomitant phenomenon of pancreatitis, but can have a fundamental influence on the progression of acute pancreatitis.

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Abbreviations

AP	Acute pancreatitis
CAE	Chloroacetate esterase
REG3β	Regenerating islet-derived 3β
STZ	Streptozotocin
s.c.	Subcutaneously
TAP	Trypsinogen-activating peptide

Introduction

Acute pancreatitis (AP) is an inflammatory disease of variable severity with an overall mortality rate of 2–3% [1]. In most cases AP is mild and heals without any further complications. However, 15–20% of patients develop a severe form of AP, which is characterised by presence of organ failure and/or local pancreatic complications such as the development of a pseudocyst, abscess or parenchymal necrosis [1]. Most clinical centres report mortality rates for severe AP of 5–15% and some reports even present mortality rates of up to 25% [1, 2].

There is a well-recognised correlation between hyperglycaemia and acute as well as chronic pancreatitis in patients [3, 4]. For example, temporary hyperglycaemia

can be observed in up to 50% of patients with AP, persistent diabetes may affect 1–15% of patients with AP, and the prevalence of diabetes in chronic pancreatitis varies between 30% and 83%, depending on the study [5, 6]. This correlation is usually explained by the accepted fact that pancreatitis can cause the development of diabetes mellitus [3]. The investigation into whether diabetes can also cause the aggravation of pancreatitis is less tangible [3]. However, several publications suggest that this cause-and-effect relationship might exist. For example, patients with diabetes have been reported to have a higher risk for pancreatitis [7–10], and hyperglycaemia may predispose patients with acute pancreatitis to systemic organ failure [11]. Blood glucose level is an accurate predictor of outcome in gallstone pancreatitis [12] and an important criterion for the Ranson score, which is used to assess the prognosis of AP [13]. All of these clinical studies support the hypothesis that hyperglycaemia may aggravate pancreatitis. However, these correlative studies cannot prove a causal relationship.

An animal model widely used to study reversible oedematous AP relies on the induction of pancreatic injury with the administration of cerulein, an analogue of the pancreatic secretagogue cholecystokinin. Administration of a supraphysiological dose of cerulein activates trypsinogen within acinar cells, possibly by causing a co-localisation of digestive zymogens with lysosomal enzymes [14, 15]. After the acute phase of pancreatitis, acinar cells respond to the tissue injury by dedifferentiation to immature progenitor-like cells, followed by massive proliferation and redifferentiation into acinar cells. This leads to a recovery of the exocrine tissue within 7 days after AP [16–18]. During the acute phase of pancreatitis the pancreas initiates an acute emergency programme for protecting the parenchyma. As part of this emergency programme, the expression of genes such as *Reg3β* (also known as *Pap, PapI, Reg-III* and *Hip*), is strongly upregulated [19]. By using blocking antibodies, antisense oligonucleotides or genetic manipulation of mice, it has been demonstrated that regenerating islet-derived 3β (REG3β) inhibits inflammation and protects the pancreas after cerulein-induced pancreatitis [20–22]. Thus, REG3β is a key factor of the acute emergency programme.

The purpose of this present study was to explore the question of whether diabetes can influence the progression of AP and to analyse which aspects of this disease are affected by diabetes.

Methods

Induction of diabetes and experimental pancreatitis Male C57BL/6 J mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and were used at 8–12 weeks of age with a body weight of 22–30 g or at 12 months of age with a body weight of 30–35 g. Animals were allowed access to water and standard laboratory chow ad libitum. Diabetes was induced by i.p. injection of 50 mg/kg streptozotocin (STZ; Sigma-Aldrich, Steinheim, Germany) on 5 consecutive days. Progression of diabetes was monitored with the blood glucose meter Contour (Bayer Vital, Leverkusen, Germany). For insulin treatment, mice received on day 15 one pellet of insulin subcutaneously (s.c.; Linshin Canada, Toronto, ON, Canada; release rate, 0.1 U insulin per day). For treatment with REG3β, mice received 100 μg/kg body weight REG3β (Dynabio, Marseille, France) i.v. 2 h before the first cerulein injection on days 22 and 23. Pancreatitis was induced by administration of eight i.p. injections of 50 μg/kg cerulein (Sigma-Aldrich) at a rate of one every hour over 2 consecutive days. All control mice were sham treated appropriately (0.9% wt/vol. NaCl solution instead of cerulein, 50 mmol/l sodium citrate pH 4.5 instead of STZ, wounding of the skin with a trocar instead of insulin pellets, PBS instead of REG3β). At 2 h before induction of pancreatitis and up to the time point of tissue preservation all mice received drinking water containing 800 mg/l metamizol (Ratiopharm, Ulm, Germany) and 1 g/l BrdU (Sigma-Aldrich). BrdU administration had no effect on pancreas atrophy as animals without administration of BrdU had a similar pancreas/body weight ratio on day 30 (STZ + cerulein with BrdU 0.0027±0.0012, n=4; STZ + cerulein without BrdU 0.0026±0.0012, n=3). Animals were anaesthetised with 75 mg/kg ketamine (bela-pharm, Vechta, Germany) and 5 mg/kg xylacine (Bayer Health Care, Leverkusen, Germany), before blood samples and organs were taken at the indicated time points. All experiments were approved by the local animal welfare committee and were performed in accordance with the German legislation and the principles of laboratory animal care (NIH publication no. 85–23, revised 1985).

Analysis of plasma Blood samples were taken 2 h after the last cerulein injection. The activity of lipase and amylase in plasma was analysed using the Cobas c111 spectrophotometer (Roche Diagnostics, Mannheim, Germany). The concentration of the trypsinogen-activating peptide (TAP) in plasma was determined by an ELISA kit for TAP (Uscn Life Science, Wuhan, People's Republic of China).

Histology, quantification of oedema formation, immunohistochemistry and western blots Tissue was preserved 2 h after the last cerulein injection on days 23 or 30. The tissue was either frozen for analysis of protein concentrations or fixed in 4% (wt/vol.) phosphate-buffered formalin for 2–3 days, embedded in paraffin and 4 μm sections were cut. Histology was evaluated after staining the sections with haematoxylin and eosin. To evaluate the cellular inflammatory response, naphthol AS-D chloroacetate esterase (CAE) staining was performed on sections. Oedema formation was quantified

as pancreas wet/dry weight ratio by dividing the weight of the pancreas after drying (at 60°C for 48 h) by the weight of the native pancreas. Cell proliferation was evaluated by immunohistochemistry using mouse anti-BrdU (clone Bu20a, dilution 1:50) and the Universal LSAB$^+$ Kit/HRP kit (Dako, Hamburg, Germany). Cell death was analysed using the ApopTag Plus Peroxidase in situ detection kit (Millipore, Eschborn, Germany). Immunohistochemistry for REG3β and insulin was performed using a rat-anti-REG3β antibody (R&D Systems, Minneapolis, MN, USA; code MAB5110, dilution 1:800) or a guinea pig-anti-insulin antibody (Abcam, Cambridge, UK; ab7842, dilution 1:500). For quantification, at least ten fields (each field with 300 to 650 cells using a ×40 objective) were evaluated per mouse. Acinar cells were identified by large round nuclei, granulated cytoplasm and non-fibroblast-like appearance. Duct cells were identified by a cobblestone-like appearance and direct proximity to a duct. Interstitial cells were identified by interstitial localisation in the exocrine tissue (criterion for exclusion was identification as acinar cell, duct cell or endothelial cell). Western blots were performed by separating 25 mg lysed tissue on 14% (wt/vol.) SDS gels and transferring the proteins to a polyvinyldifluoride membrane (Immobilon-P; Millipore). After blockade with 5% (wt/vol.) milk powder, membranes were incubated overnight at 4°C with a rat-anti-REG3β antibody (R&D Systems; code MAB5110, dilution 1:1,000), followed by a secondary peroxidase-linked anti-rat antibody (Santa Cruz Biotechnology, Santa Cruz, USA; code sc3823, dilution 1:10,000). For analysis of β-actin production, membranes were stripped, blocked by 2.5% (wt/vol.) BSA and incubated with mouse anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA; code A5441, dilution 1:20,000) followed by peroxidase-conjugated anti-mouse antibody (Sigma-Aldrich [USA]; code A9044, dilution 1:60,000). Protein production was visualised by luminol-enhanced chemiluminescence (ECL plus; GE Healthcare, Munich, Germany) and digitalised with ChemiDoc XRS System (Bio-Rad Laboratories, Munich, Germany). Signals were densitometrically assessed and corrected with the signal intensity of β-actin (Quantity One; Bio-Rad Laboratories).

Statistics Data are given as means and standard deviations. The significance of data was assessed by SigmaStat3.5 software (SigmaStat, Jandel Corporation, San Rafael, CA, USA). In cases where the assumption of normality and homogeneity of variance across groups was proven, differences between the groups were calculated using the unpaired Student's t test, including correction of the α-error according to the Bonferroni probabilities for repeated analysis. In cases where the assumption of normality or the homogeneity of variance across groups failed, the Mann–Whitney rank sum test was performed, including correction of the α-error according to the Bonferroni probabilities for repeated analysis. The criterion for significance was $p<0.05$.

Results

Diabetes inhibits the remission of cerulein-induced pancreatitis To test whether diabetes influences the progression of pancreatitis, we induced diabetes in C57BL/6 J mice by i.p. injection of STZ (for experimental procedure see Fig. 1a). Diabetes was characterised by average blood glucose concentrations of 20 mmol/l on day 22 (Fig. 1b) and a reduction in the number of insulin-producing beta cells in the islets of Langerhans (Fig. 1c, d). STZ-treated mice lost 2.7±4.3% of their body weight, whereas non-diabetic mice gained 4.9±3.2% body weight by day 22. We induced AP by repeated i.p. injections of cerulein on days 22 and 23 in diabetic (group: STZ + cerulein) as well as in non-diabetic mice (group: cerulein) and compared them with diabetic (group: STZ) and non-diabetic (group: sham) mice without cerulein exposure.

On day 30 normal morphology of the pancreas was observed in cerulein-, STZ- and sham-treated mice (Fig. 2a [not shown

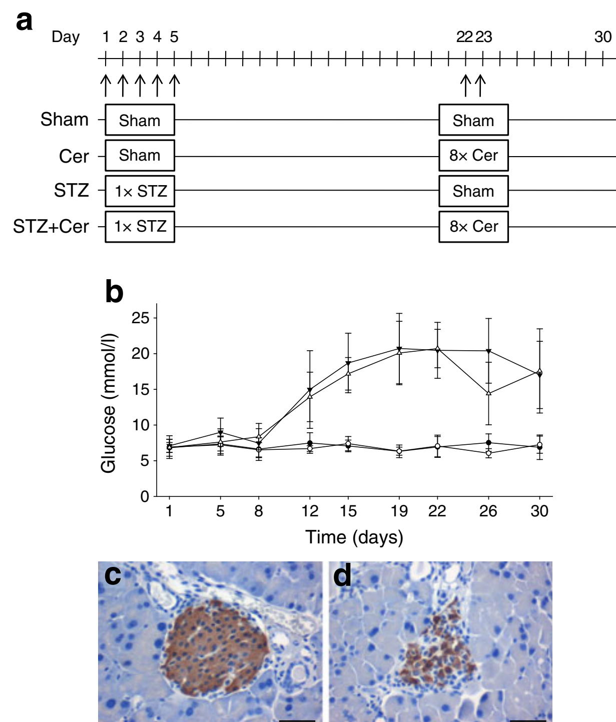

Fig. 1 Experimental protocol, blood glucose levels and insulin production. **a** Two groups of mice (STZ n=22; STZ + cerulein n=30) were injected i.p. on days 1–5 with STZ to induce diabetes. Control mice (sham n=16; cerulein n=26) received only the vehicle solution. On days 22 and 23 pancreatitis was induced in two groups of mice ('cerulein' and 'STZ + cerulein') by eight i.p. injection of cerulein at a rate of one every hour, whereas control mice received vehicle solution ('sham' and 'STZ'). **b** Average blood glucose levels in animals of the four experimental groups. Black diamond, sham; white diamond, cerulein; black triangle, STZ; white triangle, STZ + cerulein. **c**,**d** Representative images of anti-insulin immunohistochemistry on day 30. The brown colour depicts insulin-positive beta cells of sham-treated (**c**) and STZ-treated (**d**) mice. Values denote mean±SD. Scale bar, 50 µm. Cer, cerulein

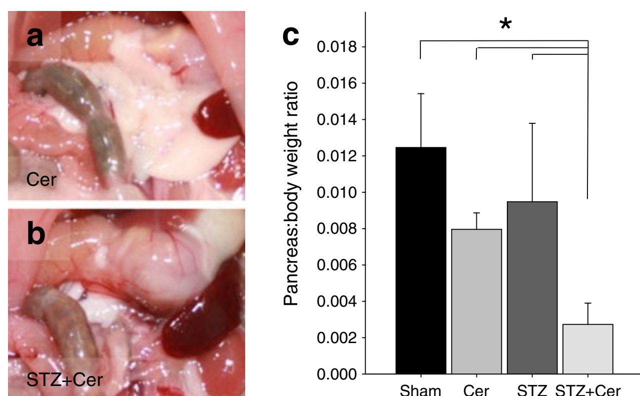

Fig. 2 Pancreas morphology and pancreas/body weight ratio on day 30. Pancreases of a cerulein-treated (**a**) and a STZ + cerulein-treated (**b**) mouse. Quantification of the pancreas/body weight ratio revealed profound atrophy of the pancreas in cerulein-treated diabetic mice (**c**). Values denote mean±SD (sham n=4; cerulein n=4; STZ n=4; STZ + cerulein n=4). Significant differences are marked (*). Cer, cerulein

for sham and STZ group]). At that time point, the pancreas had regenerated after cerulein application. However, in mice treated with STZ + cerulein only remnants of the pancreas were visible (Fig. 2b). The pancreas/body weight ratio was reduced in these mice by 78% when compared with sham-treated animals (Fig. 2c). The reduction in pancreas/body weight ratio of STZ + cerulein-treated mice was significant when compared with the respective values of sham-, cerulein- and STZ-treated animals (Fig. 2c).

Histological analysis of the pancreatic tissue on day 23 revealed the induction of AP in cerulein- as well as in STZ + cerulein-treated animals (Fig. 3a,c). The pancreatic tissue of these mice exhibited marked oedema and the typical feature of tissue-infiltrating inflammatory cells consisting mainly of neutrophils (Fig. 3a,c). The histology of the exocrine tissue was normal in STZ- and sham-treated mice (Fig. 3b). On day 30, no major histological difference was observed between cerulein-, STZ- or sham-treated mice (Fig. 3d,e). However, the pancreases of STZ + cerulein-treated animals were characterised by loss of acinar cells and the appearance of tubular complexes.

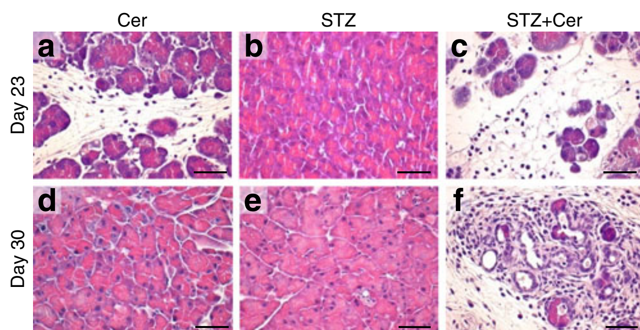

Fig. 3 Haematoxylin and eosin staining of pancreatic tissue specimens. Representative images on day 23 (**a**–**c**) and day 30 (**d**–**f**) of mice treated with cerulein (**a**,**d**; n=3), STZ (**b**,**e**; n=6) and STZ + cerulein (**c**,**f**; n=6). Scale bar, 50 μm. Cer, cerulein

Regulation of cell expansion and cell death by diabetes To assess the expansion of specific cell types in the diseased pancreas, BrdU incorporation into replicating nuclei was evaluated at the end of the acute phase on day 23 and at the end of the regenerative phase on day 30. On day 23 no differences in the number of BrdU-positive acinar or duct cells between the distinct experimental groups were observed (data not shown), but an increase in the number of BrdU-positive interstitial cells was noticed in cerulein- and STZ + cerulein-treated mice (17.4±4.2 and 21.3±5.7 cells per field, respectively) when compared with control animals (sham 1.4±0.7 cells per field and STZ 2.7±0.2 cells per field). On day 30 the number of BrdU-positive interstitial cells was strongly increased in the STZ + cerulein-treated mice compared with sham-, cerulein- and STZ-treated animals (Fig. 4a–d). The number of BrdU-positive acinar cells was significantly elevated in cerulein-treated mice (12-fold) when compared with sham-treated animals (Fig. 4e). However, in STZ + cerulein-treated animals only a modest increase (2.4-fold) in the number of BrdU-positive acinar cells was noticed when compared with STZ-treated animals (Fig. 4e). Interestingly, significantly fewer BrdU-positive acinar cells were also observed in the pancreas of STZ-treated mice when compared with sham-treated animals (Fig. 4e). The number of BrdU-positive duct cells in the pancreas was highly increased in the STZ + cerulein-treated mice when compared with sham-, STZ- and cerulein-treated animals (Fig. 4a,b,f). In order to evaluate the extent of cell death during pancreatitis, we detected double-stranded DNA breaks on histological sections. On days 23 and 30, cells with DNA breaks were primarily observed in cerulein- and STZ + cerulein-treated mice (Fig. 5a–d). At both time points STZ + cerulein-treated animals had a significant increase in the number of cells with DNA breaks when compared with sham-, cerulein- or STZ-treated animals (Fig. 5e,f).

Diabetes affects pancreatic inflammation As cerulein-induced pancreatitis is characterised by infiltration of granulocytes, which strongly express CAE, we evaluated the number of tissue-infiltrating CAE-positive cells in the pancreas. On day 23, almost no CAE-positive cells were observed in STZ- and sham-treated mice, while after treatment with cerulein an increase in the number of CAE-positive infiltrated cells was observed (electronic supplementary material [ESM] Fig. 1a,b; Fig. 6a). An even stronger increase in the number of CAE-positive cells was observed after treatment with STZ + cerulein (ESM Fig. 1c; Fig. 6a). To compare the infiltration of immune cells to the magnitude of tissue oedema, we analysed the pancreas wet/dry weight ratio. In sham-treated mice the wet/dry weight ratio was 3.51±0.04. After treatment with cerulein a significant increase in the pancreas wet/dry

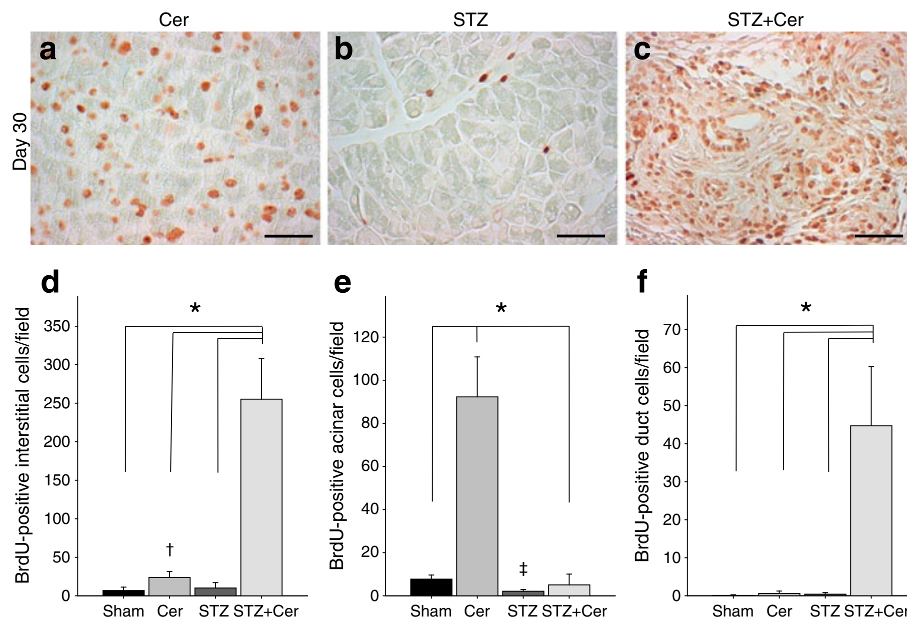

Fig. 4 Detection of proliferative cells by anti-BrdU immunohistochemistry on day 30. The brown colour depicts BrdU-positive cells from mice treated with cerulein (**a**), STZ (**b**) and STZ + cerulein (**c**). Quantification of BrdU-positive interstitial cells per field (**d**), of BrdU-positive acinar cells per field on day 30 (**e**) and of BrdU-positive duct cells per field (**f**) reveals a cell-type-specific increase in the number of BrdU-positive cells in diabetic mice after induction of pancreatitis. Values denote mean±SD (sham n=7; cerulein n=7; STZ n=9; STZ + Cer n=8). Symbols denote significant differences between the indicated groups (*), between cerulein- and sham-treated mice (†) or between STZ- and sham-treated mice (‡). Scale bar, 50 μm. Cer, cerulein

weight ratio to 4.58±0.03 was observed (Fig. 6b). Treatment with STZ + cerulein resulted in a significant rise in the wet/dry weight ratio to 10.31±0.19 (Fig. 6b).

On day 30 almost no CAE-positive infiltrating cells were observed in STZ-, cerulein- or sham-treated animals while in STZ + cerulein-treated animals a considerable number of CAE-positive cells were observed (ESM Fig. 1d–f; Fig. 6c).

Activation of trypsinogen and suppression of REG3β production As inappropriate intracellular proteolytic activation of trypsinogen to trypsin has been considered to be the pathological event that initiates pancreatitis [23], we evaluated whether diabetes modulates trypsinogen activation. For this purpose we measured the concentration of TAP in the plasma 2 h after the last cerulein injection. After treatment with cerulein or STZ + cerulein a twofold increase in the concentration of TAP was observed (Fig. 7a). Importantly, no difference in the concentration of TAP was observed between cerulein- and STZ + cerulein-treated mice (Fig. 7a). We also analysed lipase activity and amylase activity in the plasma. Lipase activity in the plasma was induced eightfold in cerulein-treated mice compared with sham-treated animals and 29-fold in STZ + cerulein-treated mice compared with STZ-treated animals (Fig. 7b). Similarly, the activity of amylase was induced sixfold in cerulein-treated mice compared with sham-treated animals and 13-fold in STZ + cerulein-treated mice compared with STZ-treated animals (Fig. 7c).

In order to evaluate, whether the production of REG3β, an important protein induced by the emergency programme during pancreatitis, is altered in diabetic mice, we analysed the production of this protein by immunohistochemistry. REG3β production was not detected in the acinar cells of sham- or STZ-treated mice, but was produced in abundance 2 h after the last cerulein treatment in diabetic as well as non-diabetic mice (Fig. 7d–f; not shown for sham group). In order to quantify REG3β, we analysed relative protein concentrations by western blot. After cerulein administration STZ-treated animals showed a significantly lowered level of REG3β production when compared with cerulein-treated non-diabetic animals (Fig. 7g, h). We also evaluated whether administration of REG3β can inhibit the inflammation during pancreatitis. On day 23, we observed a small 5.3% decrease (not significant) in the number of CAE-positive cells in STZ+ REG3β+cerulein-treated mice when compared with STZ + cerulein-treated animals (data not shown).

Progression of pancreatitis in insulin-treated and aged mice In order to evaluate whether the observed phenotype is reversible by treating diabetes with insulin, we implanted insulin-containing pellets s.c. in STZ-treated mice on day 15. Insulin treatment reduced the glucose concentration by day 22 (Fig. 8a). After induction of pancreatitis we observed on day 30 that STZ + insulin + cerulein-treated mice had an increased pancreas/body weight ratio, reduced cell death, reduced expansion of interstitial cells as well as

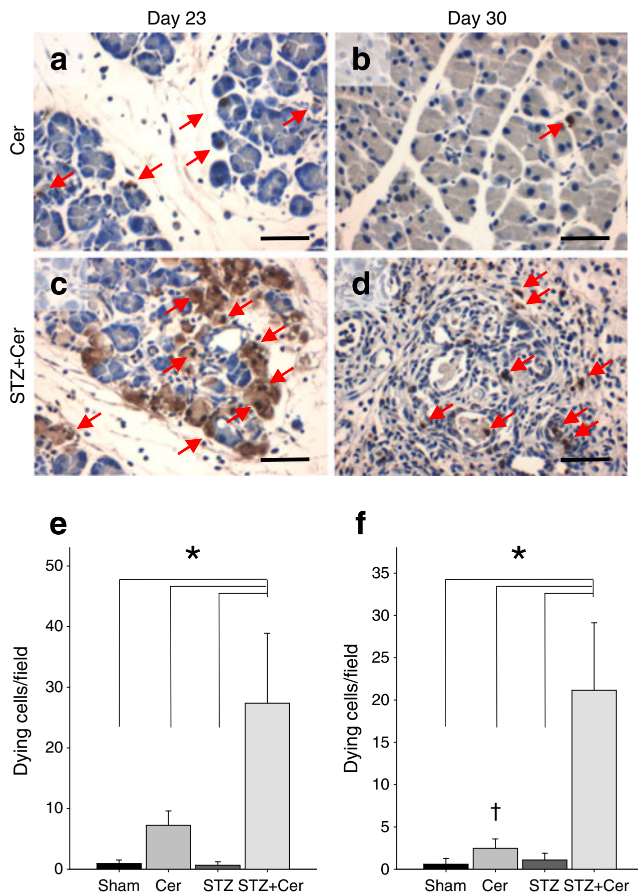

Fig. 5 Detection of cell death. The brown colour depicts cells with DNA double-strand breaks after cerulein (**a**,**b**) and STZ + cerulein (**c**,**d**) treatment on day 23 (**a**,**c**) and day 30 (**b**,**d**). Quantification of the number of cells with DNA double-strand breaks on day 23 (**e**) and day 30 (**f**) reveals increased cell death after STZ + cerulein treatment. Values denote mean±SD (for day 23, sham n=4, cerulein n=4, STZ n=4, STZ + cerulein n=6; for day 30, sham n=5, cerulein n=7, STZ n=5, STZ + cerulein n=8). Symbols denote significant differences between the indicated groups (*), or between cerulein- and sham-treated mice (†). Scale bar, 50 μm. Cer, cerulein

duct cells and enhanced expansion of acinar cells (Fig. 8b–f) when compared with STZ + cerulein-treated mice. In order to evaluate whether diabetes has a similar effect in aged mice, we compared the pancreases of STZ- and STZ + cerulein-treated 12-month-old mice. Also in aged mice, diabetes reduced acinar cell, but increased interstitial cell as well as duct cell expansion, increased the number of CAE-positive cells, reduced the pancreas/body weight ratio and increased cell death on day 30 (ESM Fig. 2a–f).

Discussion

In order to test the hypothesis that diabetes is not only a concomitant phenomenon of AP, but can also aggravate this disease, we compared the progression of pancreatitis after induction of pancreatitis in non-diabetic and diabetic mice. The following observations documented that experimental diabetes caused an aggravation of cerulein-induced AP. Compared with non-diabetic mice, diabetic mice displayed: (1) an enhanced and extended inflammatory response; (2) increased local oedema formation; (3) enhanced cell death; (4) reduced acinar cell expansion but increased duct cell as well as interstitial cell expansion; (5) reduced production of REG3β; and (6) increased activity of lipase and amylase in blood plasma. The increased cell death and inhibited regeneration of acinar cells resulted in almost complete atrophy of the pancreas. Administration of insulin lowered the blood glucose concentration and inhibited the observed aggravation of pancreatitis by diabetes. This suggests that the diabetic metabolic state rather than non-specific cytotoxicity of administered compounds aggravates pancreatitis. The observed protective effect of insulin might also explain why evolutionary pressure caused the dispersion of the endocrine islets of Langerhans within a predominantly exocrine organ [24]. This so-called islet–acinar axis might not only be

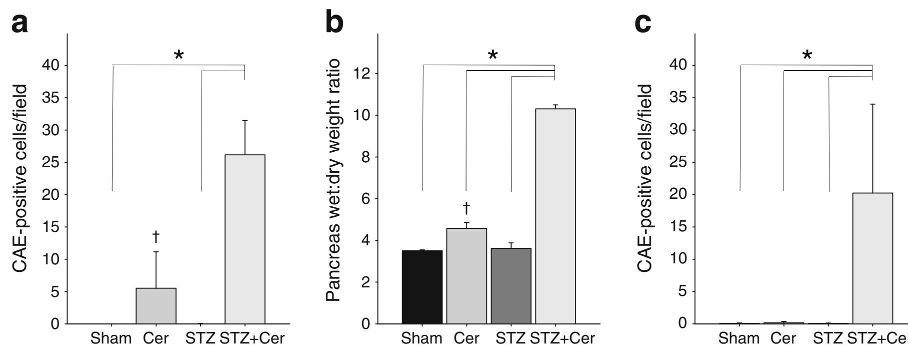

Fig. 6 Quantification of immune cell infiltrates and oedema formation. On day 23 the number of CAE-positive immune cells per field (**a**) and the pancreas wet/dry weight ratio (**b**) is increased in cerulein- and STZ + cerulein-treated mice. On day 30 the number of CAE-positive immune cells per field is increased only in diabetic mice after pancreatitis (**c**). Values denote mean±SD (for day 23, sham n=5, cerulein n=8, STZ n=5, STZ + cerulein n=8; for day 30, sham n=7, cerulein n=7, STZ n=9, STZ + cerulein n=8). Symbols denote significant differences between STZ + cerulein-treated mice in comparison with other groups (*), or between cerulein- and sham-treated mice (†). Cer, cerulein

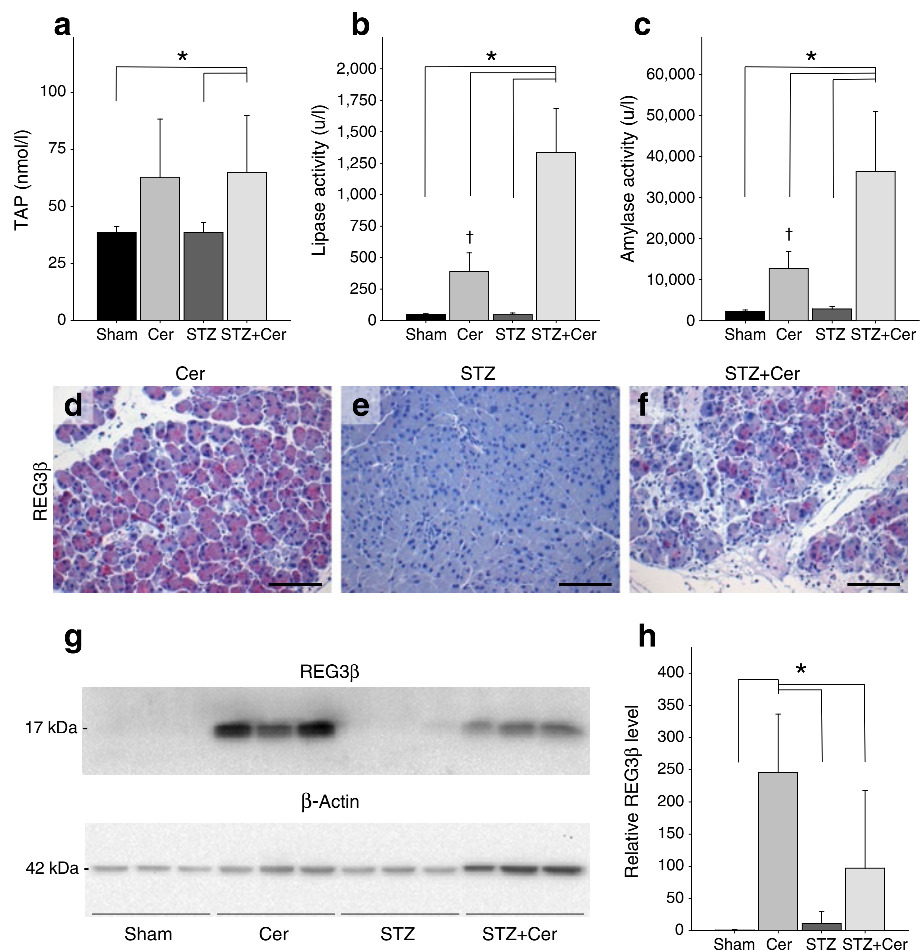

Fig. 7 Quantification of TAP, lipase, amylase and REG3β production. The concentration of TAP in plasma is increased in mice treated with cerulein (n=10) and STZ + cerulein (n=15) when compared with animals treated with sham (n=10) or STZ (n=14) (**a**). The activity of lipase (**b**) and amylase (**c**) is increased in STZ + cerulein-treated mice (n=14) compared with sham- (n=11), cerulein- (n=11) or STZ-treated animals (n=13). **d**–**f** Anti-REG3β immunohistochemistry of mice treated with cerulein (**d**; n=3), STZ (**e**; n=3) and STZ + cerulein (**f**; n=3). Scale bar, 100 μm. **g**,**h** Representative western blot of REG3β and β-actin (**g**) and quantification of REG3β production (sham n=5, cerulein n=12, STZ n=7, STZ + Cer n=14) relative to β-actin (**h**). Values denote mean±SD. Symbols denote significant differences between the indicated groups (*), or between cerulein- and sham-treated mice (†). Cer, cerulein

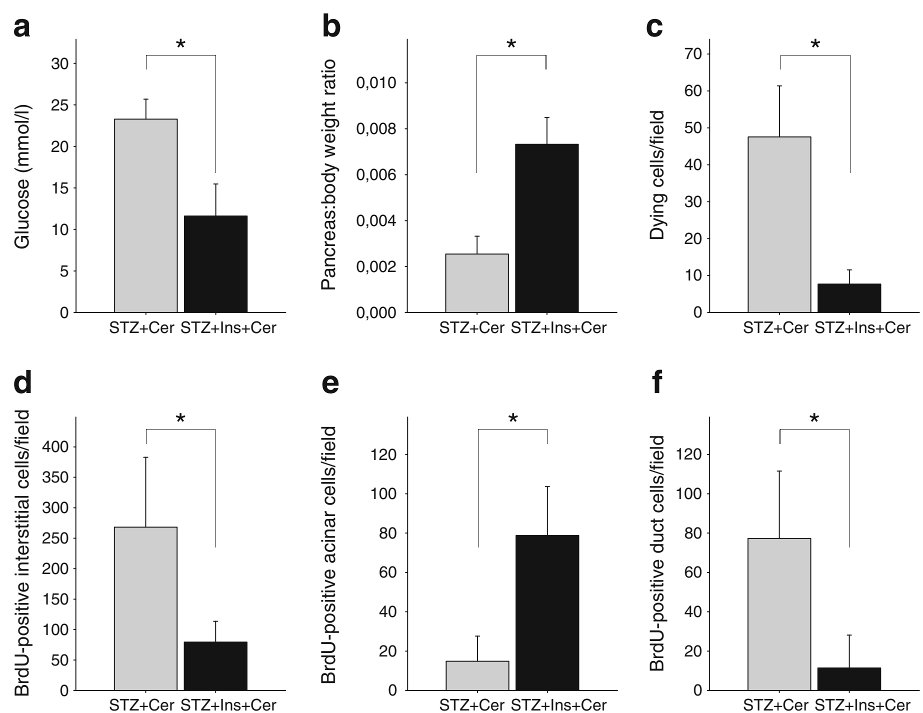

Fig. 8 Influence of insulin administration on pancreatitis induced atrophy. Insulin administration reduces blood glucose concentration (**a**) by day 22. Comparison of STZ + cerulein-treated (n=5) with STZ + insulin + cerulein-treated (n=6) mice on day 30 demonstrates that insulin increases pancreas/body weight ratio (**b**), reduces cell death (**c**), reduces the number of BrdU-positive interstitial cells (**d**), increases the number of BrdU-positive acinar cells (**e**) and decreases the number of BrdU-positive duct cells (**f**). Values denote mean±SD. Significant differences are marked (*). Cer, cerulein; Ins, insulin

relevant for normal physiology of exocrine tissue, but might be especially relevant for distinct pathologies such as pancreatitis.

AP is thought to be caused by the intracellular activation of trypsinogen [23]. However, in our study diabetes had no influence on the concentration of TAP in the plasma of mice 2 h after the last cerulein injection, suggesting that diabetes might influence downstream mechanisms of pancreatitis rather than the activation of trypsinogen. One of these downstream mechanisms might be the induction of the acute emergency programme. REG3β is part of the acute emergency programme for protecting the pancreas and has been shown to inhibit inflammation during pancreatitis [20–22]. We demonstrate that REG3β production is significantly suppressed in diabetic mice during pancreatitis. This finding is surprising, as REG3β production usually correlates with the severity of pancreatitis in animal models as well as in patients [25, 26]. The suppression of REG3β production suggests that the inhibitory mechanism of diabetes on REG3β production overwhelms the stimulatory mechanisms of aggravated pancreatitis. However, we observed only a small non-significant repression of inflammation after administration of REG3β. These data suggest that diabetes significantly alters the induction of the emergency programme during pancreatitis, but that several additional factors in addition to reduced REG3β production might contribute to the aggravation of pancreatitis in diabetic mice. We did not observe an obvious increase in REG3β protein in STZ-treated mice when compared with sham-treated animals, whereas other groups could demonstrate an increase in *Reg3β* RNA level in diabetic animals [27, 28]. These seemingly conflicting results could be explained by assuming that the detection limit for *Reg3β* RNA might be lower than the detection limit for REG3β protein or that results from RNA analysis do not always correlate with the actual protein content.

Differences between diabetic and non-diabetic mice were first observed 2 h after the last cerulein injection and included reduced production of REG3β, increased activity of lipase and amylase in blood plasma, enhanced inflammatory response, increased local oedema formation and increased cell death. Other differences such as reduced acinar cell expansion were observed on day 30, but not on day 23. This observation is consistent with data describing that the main cell expansion of acinar cells occurs during the regenerative, but not the acute, phase of pancreatitis [16, 17]. Interestingly, a significant reduction in BrdU-positive acinar cells was observed in diabetic mice even in the absence of pancreatitis when comparing diabetic mice with sham-treated animals. These data suggest that diabetes might directly inhibit the expansion of acinar cells independent of pancreatitis. In addition, we observed that STZ + cerulein treatment influenced cell expansion in a cell-type-specific manner. Diabetes inhibited the expansion of acinar cells, whereas it induced the expansion of interstitial cells and duct cells after pancreatitis. This stimulatory effect on the expansion of these cells might, however, constitute the response of the tissue to increased tissue damage in diabetic mice.

The distinct proliferative behaviour of acinar and duct cells in response to diabetes may lead to the observed formation of tubular complexes in diabetic mice. Though the significance of tubular complexes is controversial, they have been proposed to be early cancer precursors [29, 30] and are formed by transdifferentiation of acinar cells to duct cells, as well as increased proliferation of duct cells [31]. Possibly, the increased formation of tubular complexes in diabetic mice after pancreatitis may provide an explanation for the observation that diabetes and chronic pancreatitis are major risk factors for pancreatic cancer [32–35].

Up to 50% of patients with AP have an elevated blood sugar level [3, 5, 6]. In these patients hyperglycaemia may be caused by decreased insulin secretion, parenteral nutritional therapy, increased gluconeogenesis and decreased glucose use [3]. These patients, however, are also susceptible to hypoglycaemia, resulting in conservative insulin therapy at intensive care units [3]. During the last 10 years an intensified insulin therapy for critically ill patients has been a major issue of discussion. An intensified insulin therapy has been found to be of benefit or of no benefit to patients, depending on the clinical study [36–38]. Possibly this discrepancy may be resolved by the assumption that an intensified insulin therapy is beneficial to certain subsets of patients, whereas in other subsets of critically ill patients the risk of hypoglycaemia will outweigh beneficial effects. Thus, it is essential to understand whether diabetes can aggravate distinct diseases such as AP or whether diabetes is just a concomitant phenomenon of specific diseases. Additional preclinical as well as clinical studies might, therefore, be expedient to provide evidence for the decision on whether repetitive blood glucose measurements or technological advances such as continuous glucose monitoring systems followed by precise blood glucose control may improve the survival of patients with severe AP.

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Contribution statement DZ was responsible for the conception of the study, analysis and interpretation of data, drafting the article and revising the article critically. MS, AB, NK and AK were responsible for analysis and interpretation of data and revising the article critically. BV was responsible for conception of the study and revising the manuscript critically. All authors approved the final version of the manuscript to be published.

Duality of interest The authors declare that there is no duality of interest associated with the manuscript.

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Anhang - Teil 2

Impact of hyperglycemia and acute pancreatitis on the receptor for advanced glycation endproducts

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Original Article

Impact of hyperglycemia and acute pancreatitis on the receptor for advanced glycation endproducts

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Abstract: Since hyperglycemia aggravates acute pancreatitis and also activates the receptor for advanced glycation endproducts (RAGE) in other organs, we explored if RAGE is expressed in the pancreas and if its expression is regulated during acute pancreatitis and hyperglycemia. Acute pancreatitis was induced by cerulein in untreated and streptozotocin treated diabetic mice. Expression of RAGE was analyzed by Western blot and immunohistochemistry. To evaluate signal transduction the phosphorylation of ERK1/ERK2 was assessed by Western blot and the progression of acute pancreatitis was monitored by evaluation of lipase activity and the pancreas wet to dry weight ratio. RAGE is mainly expressed by acinar as well as interstitial cells in the pancreas. During acute pancreatitis infiltrating inflammatory cells also express RAGE. Using two distinct anti-RAGE antibodies six RAGE proteins with diverse molecular weight are detected in the pancreas, whereas just three distinct RAGE proteins are detected in the lung. Hyperglycemia, which aggravates acute pancreatitis, significantly reduces the production of two RAGE proteins in the inflamed pancreas.

Keywords: Receptor for advanced glycation endproducts (RAGE) isoforms, soluble RAGE, pancreatitis, hyperglycemia, inflammation, ERK1/ERK2 phosphorylation

Introduction

Acute as well as chronic pancreatitis often correlates with hyperglycemia in patients [1-4]. This correlation may be explained by two causal relationships. On the one hand it is well accepted that pancreatitis can cause the development of diabetes mellitus [2]. On the other hand several publications suggest that diabetes causes also the aggravation of pancreatitis [2]. For example, hyperglycemia may predispose patients with acute pancreatitis to systemic organ failure and patients with diabetes have a higher risk for pancreatitis [5-9]. Moreover, blood glucose level is an important criterion for assessing the prognosis of acute pancreatitis by the Ranson score and is also an accurate predictor of the outcome in gallstone pancreatitis [10, 11]. In addition, experiments using an animal model for reversible edematous acute pancreatitis have demonstrated that hyperglycemia indeed aggravates pancreatitis by enhancing inflammation and inducing cell death, which results in ample atrophy of the pancreas [12].

A membrane bound receptor, which has been implicated in regulating inflammation, is the receptor for advanced glycation end products (RAGE) [13, 14]. This receptor is activated by a diverse group of molecules such as S100 proteins, high mobility group box-1 (HMGB1) protein, lipopolysaccharide (LPS) or advanced glycation end products (AGEs) [15]. The association of these ligands with N-terminal domains of RAGE results in the induction of pro-inflammatory intracellular signaling cascades, such as the ERK1/ERK2 MAPK or the NF-κB signaling pathway [13]. Membrane bound RAGE has, therefore, a pro-inflammatory function during various pathologies such as rheumatoid arthritis, atherosclerosis, septic shock and endotoxemia [16-19]. Some truncated isoforms of RAGE, however, do not contain the membrane binding domain, but span the N-terminal extracellular ligand-binding domain. These isoforms

are not membrane bound, but soluble, and have been proposed to have anti-inflammatory function by acting as a decoy for RAGE ligands [20]. Such soluble RAGE isoforms are produced by either proteolytic cleavage of the membrane bound RAGE or alternative splicing of the RAGE pre-mRNA [21-25]. Indeed, administration of soluble RAGE has been demonstrated to inhibit various diseases, such as atherosclerosis, ischemia/reperfusion injury and autoimmune diabetes [17, 26-28]. Thus, different forms of RAGE have pro- and anti-inflammatory functions.

The aim of the present study was to assess i) if RAGE is expressed in the pancreas, ii) if the expression is altered during acute pancreatitis and iii) if the expression correlates with hyperglycemia induced aggravation of acute pancreatitis.

Materials and methods

Animals

8-12 weeks old C57BL/6J mice were grouped into 4 cohorts, which were either sham (Sham), cerulein (Cer), streptozotocin (STZ) or streptozotocin plus cerulein treated (STZ+Cer). Experiments were performed under analgesia as described previously [12]. In brief, diabetes was induced in two cohorts (STZ, STZ+Cer) by intraperitoneal (i.p.) injection of 50 mg/kg streptozotocin (Sigma-Aldrich, St Louis, MO, USA) on 5 consecutive days and diabetes was monitored with the blood glucose meter Contour (Bayer Vital, Leverkusen, Germany) for 3 weeks before pancreatitis was induced in two cohorts (Cer, STZ+Cer). Acute pancreatitis was induced either by administration of six i.p. injections of 50 μg/kg cerulein (Sigma-Aldrich) at a rate of one every hour (analysis: 5.5 hours after the first cerulein injection) or by administration of eight i.p. injections of 50 μg/kg cerulein at a rate of one every hour over 2 consecutive days (analysis: 33 hours after the first cerulein injection). All control mice were sham treated appropriately (0.9% wt/vol. saline solution instead of cerulein, 50 mmol/l sodium citrate pH 4.5 instead of STZ).

Analysis of plasma and tissue

Oedema formation was quantified as pancreas wet to dry weight ratio by dividing the weight of the pancreas after drying (for 48 h at 60°C) by the weight of the native pancreas. Blood samples were taken at the indicated time points after the first cerulein injection. The activity of lipase and amylase in plasma was analyzed using the Cobas c111 spectrophotometer (Roche Diagnostics, Mannheim, Germany). Pancreas and lung tissue for Western blots and immunohistochemistry was preserved at the indicated time points after the first cerulein injection and processed as described previously [12]. Western blots were performed by separating 10 mg pancreas lysate or 0.4 mg lung lysate on 12% (wt/vol.) SDS gels. After transferring the proteins to a polyvinyldifluoride membrane (Immobilon-P; Millipore) the membrane was blocked with 2.5% (wt/vol.) bovine serum albumin (BSA) and incubated overnight at 4°C with primary antibodies. As primary antibodies a goat anti-RAGE antibody raised against an epitope at the N-terminus of RAGE (Santa Cruz Biotechnology, Santa Cruz, USA; code sc8230, 1:1250), a goat anti-RAGE antibody raised against Gln24 to Ala342 of mouse RAGE (R&D Systems, Wiesbaden, Germany, code AF1179, 1:500), or a rabbit anti-phospho-ERK1/2(T202/Y204)/ERK2(T185/Y187) antibody (R&D Systems, code MAB1018, 1:1000) were used. After washing, the membrane was incubated for 2 hours at room temperature with a secondary peroxidase-linked anti-goat-antibody (Santa Cruz Biotechnology, code sc2020, 1:15000) or peroxidase-linked anti-rabbit-antibody (Cell Signaling, Boston, USA, code 7074, 1:5000). For analysis of β-tubulin or ERK1/ERK2 production, membranes were stripped, blocked by 2.5% (wt/vol.) BSA and incubated with a rabbit anti-β-tubulin antibody (Santa Cruz Biotechnology, code sc9104, 1:500) or a mouse anti-ERK1/ERK2 antibody (R&D Systems, code MAB15761, 1:500) followed by incubation of peroxidase conjugated anti-rabbit antibody (Cell Signaling, code 7074, 1:15000) or a peroxidase conjugated anti-mouse antibody (Sigma-Aldrich, code A9044, 1:20000). Proteins were detected by luminol-enhanced chemiluminescence and quantified by densitometry. The signal intensities of phospho-ERK1/ERK2 were corrected with the corresponding signal intensities of ERK1/ERK2. The signal intensities of all other proteins were corrected with the corresponding signal intensities of β-tubulin. Immunohistochemistry for RAGE was performed using a goat anti-RAGE antibody (Santa Cruz Biotechnology, code sc8230, 1:1000) and peroxidase conjugated donkey-anti-goat anti-

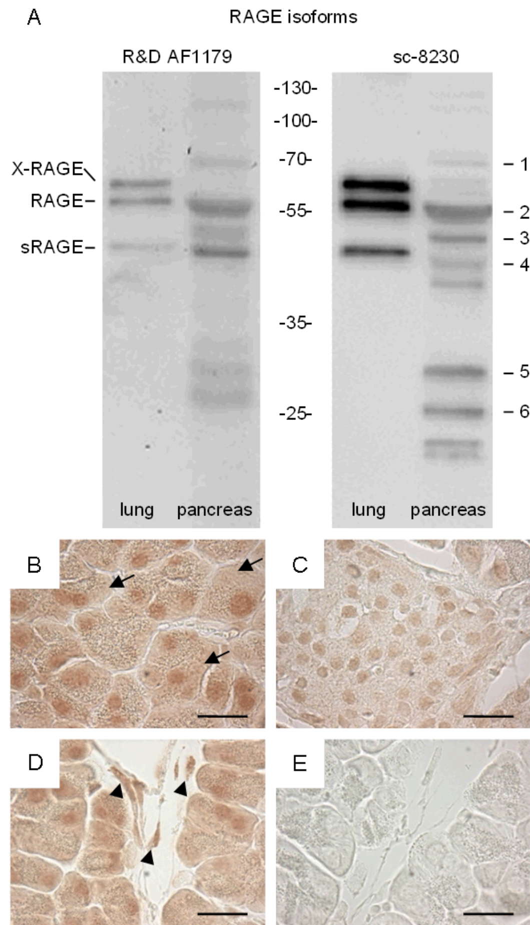

Figure 1. Detection of RAGE proteins in murine lung and pancreas. Western blots using two distinct anti-RAGE antibodies, R&D AF1179 and sc-8230, detected three and six RAGE proteins in murine lung and pancreas, respectively (A). Immunohistochemistry on pancreas tissue using the sc-8230 anti-RAGE antibody detected RAGE in acinar cells (B), in the islets of Langerhans (C), and in interstitial cells (D), while no staining was observed in the negative control when the primary antibody was omitted (E). Bar=20 micrometer.

body (Santa Cruz Biotechnology, code sc2020, 1:100).

Statistics

Data are presented as box plots indicating the median, the interquartile range in form of a box, and the 10th and 90th percentiles as whiskers. Graphs were made by using SigmaPlot software version 12.0. The significance of data was assessed by SigmaStat software version 3.5 (SigmaStat, Jandel Corporation, San Rafael, CA, USA). Differences between the groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks with the pairwise multiple comparison procedure as suggested by the software and indicated in the figure legends. The criterion for significance was $p<0.05$.

Results

RAGE expression in the native pancreas

In order to compare RAGE expression in the pancreas to its well characterized expression in lung, the tissue lysates of both native organs were analyzed by Western blot using two distinct anti-RAGE antibodies (**Figure 1A**). In lung three RAGE proteins, the membrane bound X-RAGE, RAGE and a soluble sRAGE, were detected in agreement with previously published data [24, 29]. In pancreas, however, six RAGE proteins were consistently detected by both antibodies (**Figure 1A**). Experiments leaving the pancreas up to 20 minutes at room temperature before lysing the tissue confirmed that the RAGE proteins with lower molecular weight were not degradation products of the RAGE proteins with higher molecular weight (data not shown). Immunohistochemistry revealed strong RAGE expression in acinar and interstitial cells as well as weak expression in the islets of Langerhans (**Figure 1B-E**).

Regulation of RAGE expression by hyperglycemia and pancreatitis

In order to assess if the expression of any of the six RAGE proteins (RAGE 1-6) is altered by hyperglycemia or acute pancreatitis, four distinct cohorts of mice were sham, cerulein, streptozotocin or streptozotocin plus cerulein treated. Repetitive cerulein injections induced acute pancreatitis 33 hours after the first cerulein administration, as characterized by increased lipase activity in the blood (Sham: 31/26-43, cerulein: 657/512-689, STZ: 27/25-31, STZ+cerulein: 1636/1076-1795, median/interquartile range in units/l). Consistent with previously published data, STZ+cerulein treatment caused higher lipase activity compared to cerulein-treated mice [12]. In mice treated with STZ three weeks before tissue asservation, the blood glucose concentration was strongly increased at the time point when pancreatitis was induced (Sham: 6.7/6.1-7.2, cerulein: 6.6/6.0-7.3, STZ: 31.5/28.2-33.3, STZ+cerulein: 29.0/19.2-32.6, median/interquartile range in mmol/l). The expression of RAGE 1 was modestly reduced by application of STZ,

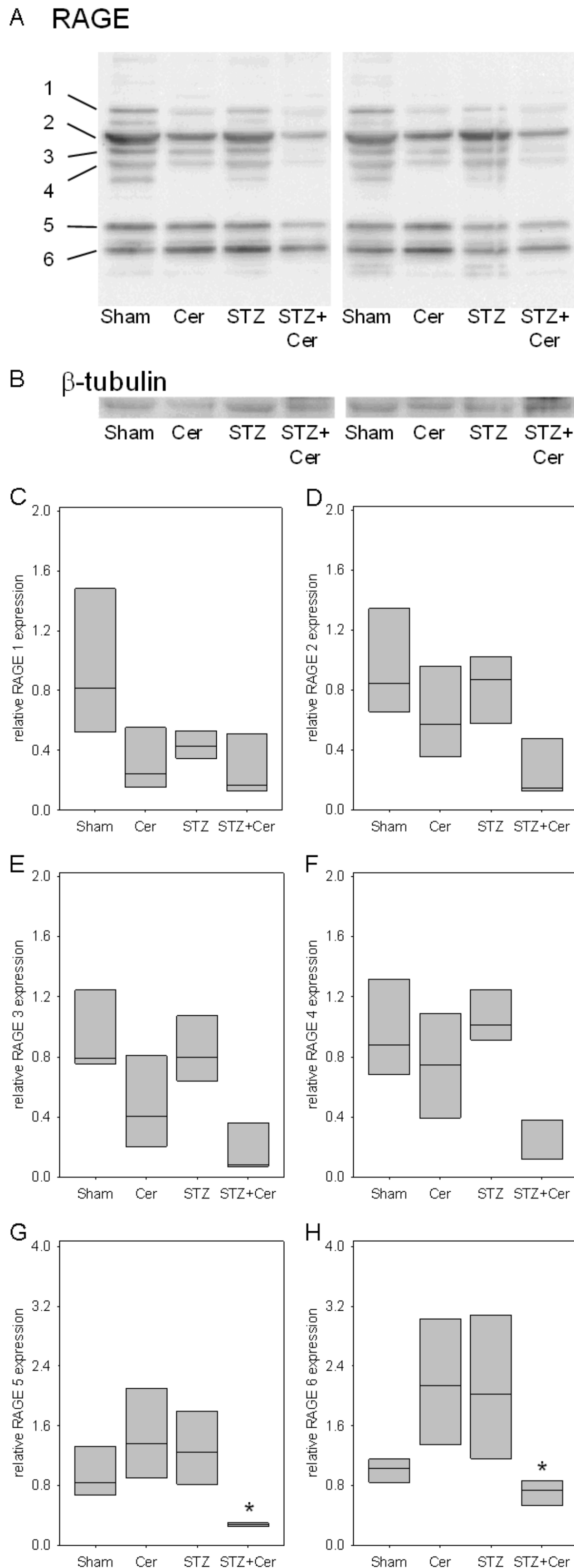

Figure 2. Influence of hyperglycemia and pancreatitis on RAGE proteins in the pancreas 33 hours after the first cerulein administration. Pancreas of control (sham), cerulein (Cer), streptozotocin (STZ) or streptozotocin plus cerulein (STZ+Cer) treated mice were analyzed by Western blots using the sc-8230 anti-RAGE antibody (A) and an anti-β-tubulin antibody as control (B). Densitometry of Western blots compared the expression of RAGE 1-6 (C-H) and revealed a significant reduction of RAGE 5 and 6 in STZ+cerulein treated mice (G and H). Values denote median and interquartile range. *$p \leq 0.038$ versus cerulein treated mice using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey Test for all pairwise comparisons.

cerulein or the application of STZ+cerulein compared to sham-treated mice (**Figure 2A-C**). In contrast, the expression of RAGE 2-6 was reduced mainly by application of STZ+cerulein (**Figure 2D-H**). Especially RAGE 5 and 6 were reduced significantly by application of STZ+cerulein compared to cerulein-treated mice (**Figure 2G** and **2H**). Evaluation of the pancreatic tissue by immunohistochemistry suggests a reduction of RAGE expression in acinar cells during inflammation (**Figure 3A-D**). However, at the same time point infiltrating inflammatory cells express RAGE strongly (**Figure 3A-D**). In summary, these data correlate the reduced expression of RAGE 5 and 6 with the previously published aggravation of acute pancreatitis caused by hyperglycemia [12].

Time course of ERK1/ERK2 activation and RAGE expression

In order to evaluate at which time point a classical RAGE induced signal transduction pathway, the ERK1/ERK2 MAPK pathway, is activated during acute pancreatitis, we evaluated the phosphorylation of ERK1/ERK2 on various time points during the first and second day of repetitive cerulein administration. Cerulein induces the phosphorylation of ERK1/ERK2 within 1.5 hours after the first cerulein injection and the phosphorylation reaches its maximum at 5.5 hours after the first cerulein administration (**Figure 4A-C**). At 5.5 as well as 10 hours after the first cerulein injection the amount of RAGE protein 5 and 6 was

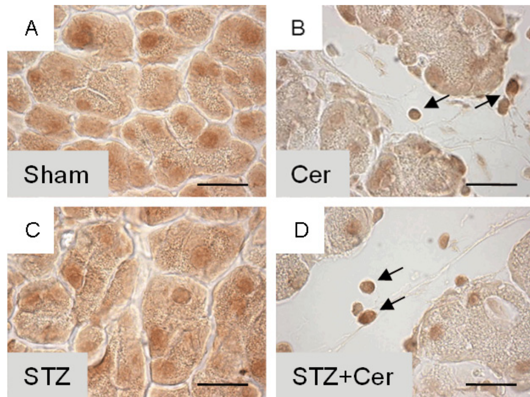

Figure 3. Influence of hyperglycemia and pancreatitis on RAGE in the pancreas 33 hours after the first cerulein administration. Pancreas of sham (A), cerulein (B), streptozotocin (C) or streptozotocin plus cerulein (D) treated mice were analyzed by immunohistochemistry using the sc-8230 anti-RAGE antibody. Infiltrating inflammatory cells produce RAGE indicated by arrows in B and D. Bar=20 µm.

moderately increased (**Figure 4D** and **4E**). These data suggest that phosphorylation of ERK1/ERK2 might be studied best 5.5 hours after the first cerulein injection.

ERK1/ERK2 activation and RAGE expression during the early phase of acute pancreatitis

In order to assess, if the ERK1/ERK2 MAPK signal transduction pathway is altered by hyperglycemia or pancreatitis 5.5 hours after the first cerulein injection, the phosphorylation of ERK1/ERK2 was assessed in sham, cerulein, STZ and STZ+cerulein-treated animals 5.5 hours after the first cerulein administration. Both cerulein as well as STZ+cerulein treatment caused strong phosphorylation of ERK1/ERK2 (**Figure 5A** and **5B**). However, STZ+cerulein administration did not lead to a significantly altered phosphorylation of ERK1/ERK2 compared to cerulein-treated mice (**Figure 5C**). The expression of RAGE 5 and 6 was also almost unchanged when comparing the STZ+cerulein with cerulein-treated mice (RAGE 5: cerulein 1.4/0.9-2.1, STZ+cerulein 1.3/0.9-1.3, RAGE 6: cerulein 1.4/1.2-1.7, STZ+cerulein 1.3/1.1-1.7, median/interquartile range in relative expression intensity). At this early time point cerulein as well as STZ+cerulein application caused increased wet to dry weight ratio of the pancreas (**Figure 5D**). Furthermore lipase as well as amylase activity were increased in

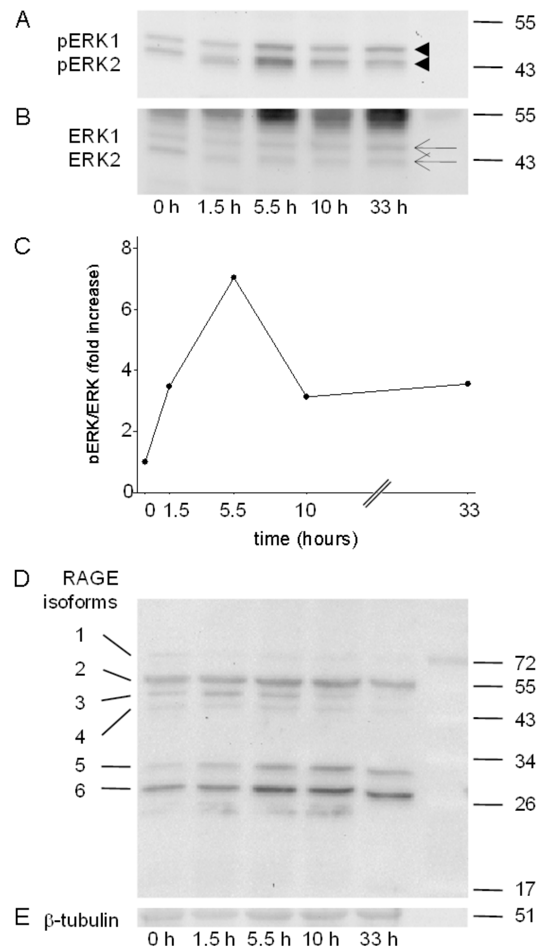

Figure 4. Time course of ERK1/ERK2 activation and RAGE expression during repetitive cerulein administration. The phosphorylation of ERK1/ERK2 was analyzed by Western blots (arrowheads, A) and compared to ERK1/ERK2 expression (arrows, B) at the indicated time points after the first cerulein administration. The phosphorylation intensity of ERK1/ERK2 reveals a maximum of phosphorylation at 5.5 hours after the first cerulein administration (C). No obvious effect on the expression level of any of the six RAGE proteins was observed 5.5 hours after the first cerulein administration (D) compared to β-tubulin expression (E).

the blood plasma compared to sham-treated mice (**Figure 5E** and **5F**). However, STZ+cerulein administration, compared to cerulein-treated mice, did neither lead to a significantly altered wet to dry weight ratio of the pancreas nor to significantly altered lipase or amylase activities at this early time point (**Figure 5D-F**). Hyperglycemia, therefore, has no obvious effect on the early phase of acute pancreatitis in contrast to strong aggravation of pancreatitis at a later time point [12].

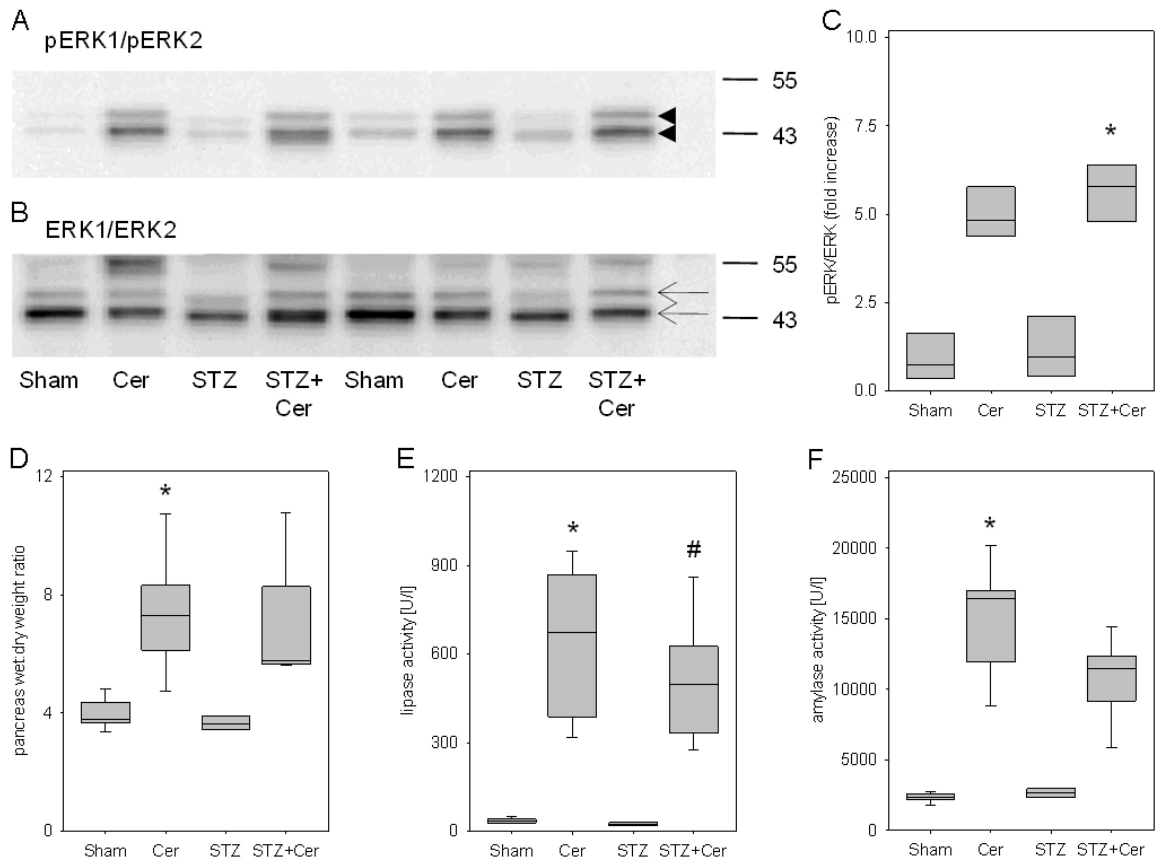

Figure 5. Influence of hyperglycemia and cerulein administration on RAGE proteins and on parameters of pancreatitis 5.5 hours after the first cerulein administration. Pancreas and blood plasma of control (sham), cerulein (Cer), streptozotocin (STZ) or streptozotocin plus cerulein (STZ+Cer) treated mice were analyzed. The phosphorylation of ERK1/ERK2 (arrowheads, A) was compared to ERK1/ERK2 expression (arrows, B) by Western blots. The quantification by densitometry reveals increased phosphorylation of ERK1/ERK2 after cerulein as well as STZ+cerulein treatment (C). Pancreatitis was quantified by pancreas wet to dry weight ratio (D) and lipase (E) and amylase (F) activity in blood plasma. Box plots indicate the median, the interquartile range in the form of a box and the 10th and 90th percentiles as whiskers. *p≤0.01 versus sham treated mice and $^{\#}$p≤0.003 versus STZ treated mice using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey Test (C) or Dunn's Method (D-F) for all pairwise comparisons.

Discussion

The evaluation of RAGE expression in the pancreas demonstrates that i) six RAGE proteins with distinct apparent molecular weight can be detected in the pancreas (**Figures 1A** and **2A**), ii) the expression of two RAGE proteins is significantly reduced in hyperglycemic mice during the late phase of acute pancreatitis (**Figure 2A**) and iii) that this reduction correlates with an aggravation of acute pancreatitis [12].

For the detection of the six RAGE proteins in pancreas, two different RAGE antibodies were used to control for possible unspecific binding of an antibody to other proteins. These two anti-RAGE antibodies were either raised against a peptide mapping directly at the N-terminus of RAGE [29] or were raised against Gln24 to Ala342 of RAGE. Since the N-terminal Ig domain, called V domain (amino acid 34-110 of mouse RAGE), is the primary binding site for most ligands, we reason that all six detected RAGE proteins in the pancreas may bind RAGE ligands [30]. RAGE 2 has a similar molecular weight as the membrane bound RAGE isoform in the lung and might therefore be a membrane bound full length RAGE protein [24, 29]. RAGE proteins with lower molecular weight, such as RAGE 4, 5 and 6, contain the N-terminus of RAGE, but probably not the entire C-terminal domain. Since the membrane binding domain of RAGE is encoded by amino acids 341-361 close to the C-terminus [30], such proteins

would not be membrane bound, but soluble. Such proteins have been described previously in the literature and are generated by a shed-dase which proteolytically processes full length RAGE or by alternative splicing of the pre-mRNA of RAGE [20-25]. Soluble RAGE (sRAGE) has been demonstrated to inhibit inflammation in several pathological processes such as athero-sclerosis, ischemia/reperfusion injury and auto-immune diabetes by serving as decoy receptor for RAGE ligands [17, 26-28]. In pancreas we do observe a RAGE protein with very similar molecular weight to sRAGE (RAGE 4). Its expression is reduced after STZ+cerulein application compared to cerulein treatment (**Figure 2F**). Due to the anti-proliferative function of sRAGE in other distinct diseases, the reduced expression of RAGE 4 in hyperglycemic mice could be partially responsible for the observed aggravation of acute pancreatitis by hyperglycemia [12]. Interestingly, we also observe two additional RAGE proteins with approximate molecular weight of 32 (RAGE 5) and 27 kDa (RAGE 6). The expression of these small RAGE proteins, is significantly reduced after STZ+cerulein application compared to cerulein treatment (**Figure 2G** and **2H**). In analogy to sRAGE, the reduced expression of RAGE 5 and RAGE 6 in hyperglycemic mice could be partially responsible for the observed aggravation of acute pancreatitis by hyperglycemia [12]. RAGE 5 and RAGE 6 might act as decoy receptor for HMGB1, LPS or S100 proteins which stimulate inflammation by binding to receptors such as TLR4 or membrane bound RAGE [13, 31]. Since RAGE has been described to be alternatively spliced, proteolytically processed and N-glycosylated, any of these processes or even a combination of them might result in the six RAGE proteins in the pancreas [20, 21, 23-25, 29, 32]. It has been reported that PNGaseF treated lung extract resulted in deglycosylated RAGE proteins with an apparent molecular weight well above 40 kDa [29]. Interestingly, RAGE 5 and 6 have an apparent molecular weight well below 35 kDa. Thus it is unlikely that a lack of N-glycosylation alone can explain the low molecular weights of RAGE 5 and RAGE 6. It is more likely that these short proteins are generated by alternative splicing or proteolytic processing of RAGE.

The aggravation of acute pancreatitis by hyperglycemia was observed at late time points such as 33 hours after the first cerulein administration and during the regeneration phase of the pancreas [12], but was not observed during the early phase of pancreatitis, 5.5 hours after the first cerulein injection (**Figure 5D-F**). This suggests, that hyperglycemia does not influence early events during pancreatitis, but might have a major influence on the perpetuation of inflammation. Since RAGE has also been suggested to regulate the perpetuation of inflammation in other context [33], it is likely that it has a similar function during pancreatitis. Hyperglycemia might therefore aggravate pancreatitis by increasing the concentration of AGEs or other RAGE ligands and by reducing the expression of soluble RAGE proteins with anti-inflammatory functions.

Based on the correlation of RAGE 5 and RAGE 6 production in the pancreas and the progression of pancreatitis, additional research could focus on the following questions: i) Can the RAGE 5 and RAGE 6 proteins be isolated and the amino acid sequences be defined? ii) Do these two RAGE proteins inhibit inflammation? iii) Are these RAGE proteins more or less potent than sRAGE in inhibiting inflammation? And iv) does RAGE influence pancreatitis?

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Disclosure of conflict of interest

None of the authors have any conflicts of interest.

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Anhang - Teil 3

Diabetes increases pancreatitis induced systemic inflammation but not lung injury

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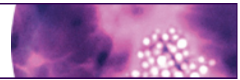

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ORIGINAL ARTICLE

Diabetes increases pancreatitis induced systemic inflammation but has little effect on inflammation and cell death in the lung

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SUMMARY

Acute pancreatitis (AP) can lead to a systemic inflammatory response that often results in acute lung injury and single or multiple organ failure. In a previous study we demonstrated that diabetes aggravates the local pathophysiological process during AP. In this study we explore, if diabetes also increases pancreatitis induced systemic inflammation and causes lung injury. Acute pancreatitis was induced in untreated and streptozotocin-treated diabetic mice by injection of cerulein. Systemic inflammation was studied by IL-6 ELISA in blood plasma and white blood cell count. Lung inflammation and lung injury were quantified by chloroacetate esterase staining, evaluation of the alveolar cellularity index and cleaved caspase-3 immunohistochemistry. In normoglycaemic mice AP increased the IL-6 concentration in plasma and caused lymphocytopenia. Diabetes significantly increased the IL-6 concentration in plasma and further reduced the number of lymphocytes during AP, whereas diabetes had little effect on these parameters in the absence of pancreatitis. However, diabetes only marginally increased lung inflammation and did not lead to cell death of the lung epithelium during AP. We conclude that diabetes increases parameters of systemic inflammation during AP, but that this increase is insufficient to cause lung injury.

Keywords
acute lung injury, chloroacetate esterase staining, cleaved caspase-3, sepsis, systemic inflammatory response syndrome

About 15%–20% of patients with acute pancreatitis (AP) develop severe symptoms, such as pancreatic parenchymal necrosis, a systemic inflammatory response and concomitant single or multiple organ failure (Forsmark & Baillie 2007). The AP-induced inflammatory response often causes acute lung injury (ALI) with a mortality rate of up to 40% (Zhou *et al.* 2010). This is often associated with lymphopenia and an increase in the concentration of the pro-inflammatory cytokine IL-6 in the blood (Takeyama *et al.* 2000; Gregoric *et al.* 2010). IL-6 is not only of prognostic value, but has also been demonstrated to be an essential mediator of pancreatitis-associated lung injury (Zhang *et al.* 2013). The pro-inflammatory milieu during AP leads to activation and infiltration of neutrophil granulocytes in the lung and to an increase in the alveolar cellularity index (Guice *et al.* 1989; Frossard *et al.* 1999; Pastor *et al.* 2006; Tascilar *et al.* 2007). Pulmonary inflammation can cause lung injury, which is often characterized by apoptosis of lung epithelial cells (Yuan *et al.* 2000; Nakamura *et al.* 2003). Although several proteins, such as the cytokine induced neutrophil chemoattractant, the CD40 ligand or the toll-like receptor 4, have been implicated in the pathophysiological process of AP-induced ALI, underlying mechanisms and contributing parameters of this process are still not well understood (Bhatia *et al.* 2000; Frossard *et al.* 2001; Sharif *et al.* 2009; Zhou *et al.* 2010).

One parameter that has been described to aggravate AP is diabetes. Several correlative studies in patients have suggested that diabetes leads to a higher risk for pancreatitis (Seicean *et al.* 2006; Noel *et al.* 2009; Girman *et al.* 2010; Xue *et al.* 2012) and that hyperglycaemia may predispose patients with AP to systemic organ failure (Mentula *et al.* 2008). In addi-

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tion, blood glucose level is an accurate predictor of outcome in gallstone pancreatitis and an important criterion for the Ranson score, which is used to assess the prognosis of AP (Ranson *et al.* 1974; Rajaratnam & Martin 2006).

We have previously demonstrated in experimental settings that diabetes indeed aggravates the local pathophysiological process during acute as well as chronic pancreatitis (Zechner *et al.* 2012, 2013, 2014). The purpose of this study was to explore if the aggravation of AP by diabetes leads to increased systemic inflammation, and if adequate alterations in the lung can be observed.

Methods

Animals

Eight to 12-week-old C57BL/6J mice were allowed access to water and standard laboratory chow *ad libitum*. The mice were treated as published previously (Zechner *et al.* 2012). Diabetes was caused by i.p. injection of 50 mg/kg streptozotocin (STZ; Sigma-Aldrich, Steinheim, Germany) on 5 consecutive days (day 1-5), whereas AP was induced on day 22 and day 23 by administration of eight i.p. injections per day of 50 μg/kg cerulein (Sigma-Aldrich) at a rate of one every hour. All control mice were Sham-treated appropriately (0.9% wt/vol. NaCl solution instead of cerulein, 50 mmol/l sodium citrate pH 4.5 instead of STZ). At 2 h before induction of pancreatitis, and up to the time point of tissue preservation, all mice received drinking water containing 800 mg/l metamizol (Ratiopharm, Ulm, Germany) and 1 g/l BrdU (Sigma-Aldrich Chemie GmbH). For sampling blood and tissue the animals were anaesthetized with 75 mg/kg ketamine (Bela-Pharm, Vechta, Germany) and 5 mg/kg xylazine (Bayer Health Care, Leverkusen, Germany). All experiments were executed in accordance with German legislation, the local animal wellfare committee and the EU-directive 2010/63/EU.

Analysis of plasma and tissue

Blood samples were taken 2 hours after the last cerulein injection on day 23. A differential blood cell count was performed with an automated hematology analyzer Sysmex KX 21 (Sysmex Cooperation, Kobe, Japan) as previously published (Bobrowski *et al.* 2013). Concentrations of interleukin (IL)-6 were measured in blood plasma with commercially available enzyme-linked immunosorbent assay (ELISA) kits from Thermo Fisher Scientific (Rockford, IL, USA) following the manufacturer's instructions, and data were plotted as fold induction compared to the average of IL-6 in Sham-treated mice. Tissue samples were taken on day 23 (2 hours after the last cerulein injection) or on day 30 (7 days after the last cerulein injection). Naphthol AS-D chloroacetate esterase (CAE) staining, primarily staining neutrophil granulocytes, or hematoxylin/eosin staining was performed on paraffin-embedded tissue to evaluate lung inflammation and histology. To evaluate the alveolar cellularity index (nuclei/septum), the number of nuclei crossing three gridlines of the integrating eyepiece (using a 100× objective) was divided by the number of septa crossing these gridlines (Tascilar *et al.* 2007;). We analysed 10 randomly chosen microscopic fields from each lung. Cell death was analysed by immunohistochemistry using a rabbit-anti-mouse cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology Inc., Denver, USA, code 9661, dilution 1:500) and a HRP-conjugated goat-anti-rabbit secondary antibody (code P0448; Dako Deutschland GmbH, Hamburg, Germany). Cell proliferation was evaluated by immunohistochemistry using mouse anti-BrdU (clone: Bu20a, dilution: 1:50) and the Universal LSABTM+ Kit/HRP kit (Dako Deutschland GmbH). Quantification of inflammation, BrdU incorporation and cell death was performed on 10 random fields per mouse using a 40× objective.

Statistics

Data are given as means and standard deviation respectively. The significance of data was assessed by SigmaStat 3.5 software (SigmaStat; Jandel Corporation, San Rafael, CA, USA). In all cases where the assumption of normality or the homogeneity of variance across groups failed, the Mann–Whitney rank sum test was performed, including correction of the α-error according to the Bonferroni probabilities for repeated analysis. In other cases, the unpaired Student's *t* test including the correction of the α-error according to the Bonferroni was performed. The criterion for significance was $P < 0.05$ divided by the number of meaningful comparisons.

Results

Diabetes aggravates systemic inflammation during pancreatitis

To evaluate if STZ-induced diabetes has an influence on parameters of systemic inflammation during cerulein-induced AP, the IL-6 concentration in plasma was determined in cerulein-treated diabetic mice (STZ + Cer) and compared to healthy normoglycaemic mice (Sham), cerulein-treated normoglycaemic mice (Cer) and diabetic mice without pancreatitis (STZ). Administration of cerulein in normoglycaemic mice during the acute phase of pancreatitis (on day 23, 2 hours after the last cerulein administration) increased the IL-6 plasma concentrations when compared to healthy mice (Figure 1a). STZ plus cerulein treatment lead to an even more pronounced increase in IL-6 concentration when compared to Sham, cerulein or STZ-treated mice (Figure 1a). Evaluation of the blood cell count revealed that administration of cerulein only slightly reduced the number of leucocytes in normoglycaemic animals (Figure 1b). However, STZ plus cerulein treatment lead to a significant decrease in the number of leucocytes when compared to cerulein-treated mice (Figure 1b). A decrease in

the number of lymphocytes was observed after administration of cerulein in normoglycaemic mice compared to healthy mice (Figure 1c). STZ plus cerulein treatment lead to an even more pronounced decrease in the number of lymphocytes when compared to Sham, cerulein or STZ-treated mice (Figure 1c). The number of monocytes plus granulocytes was increased by cerulein administration, but was not significantly influenced by STZ (Figure 1d).

Diabetes only marginally aggravates lung inflammation during pancreatitis

To evaluate if the observed aggravation of systemic inflammation by diabetes has an influence on lung inflammation, infiltrating inflammatory cells were identified by CAE staining on lung sections on day 23 (Figure 2a–d). Administration of cerulein in normoglycaemic or diabetic mice significantly increased the number of CAE^{+} cells in the lung tissue when compared to Sham or STZ-treated mice (Figure 2e). STZ plus cerulein treatment lead to a slight increase (P = 0.023) in the number of CAE^{+} cells when compared to cerulein-treated mice (Figure 2e). The alveolar cellularity index was marginally increased by cerulein treatment (P = 0.143), but barely influenced by STZ treatment (Figure 2f).

Diabetes does not induce cell death in the lung epithelium

To evaluate if STZ-induced diabetes has an influence on cell death in the lung during the acute phase of pancreatitis (on day 23, 2 hours after the last cerulein administration), cleaved caspase-3^{+} cells were identified by immunohistochemistry (Figure 3a). However, the administration of cerulein, STZ or STZ plus cerulein did not result in an increased number of cleaved caspase-3^{+} cells in the lung when compared to Sham-treated animals (Figure 3b). To evaluate if this lack of cell death in the lung epithelium might be caused by inadequate severity of pancreatitis we evaluated the histology of the pancreas on day 23 and on day 30. Normal histology of the pancreas was observed in Sham and

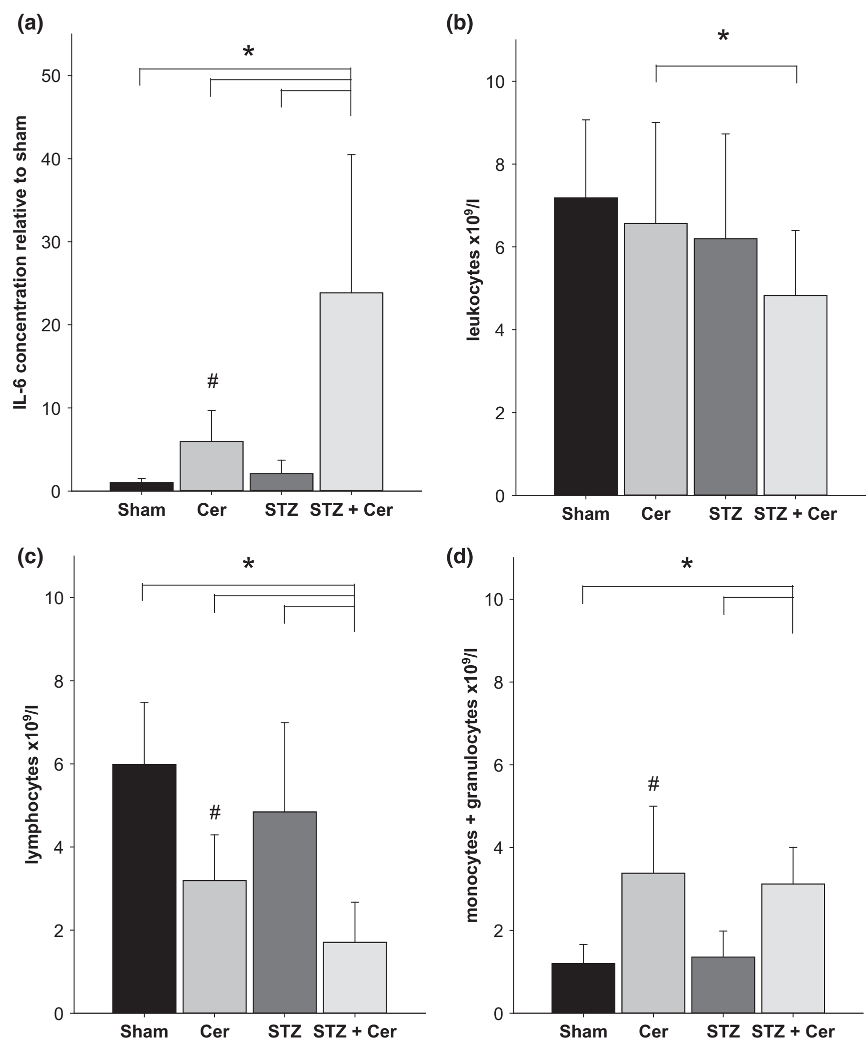
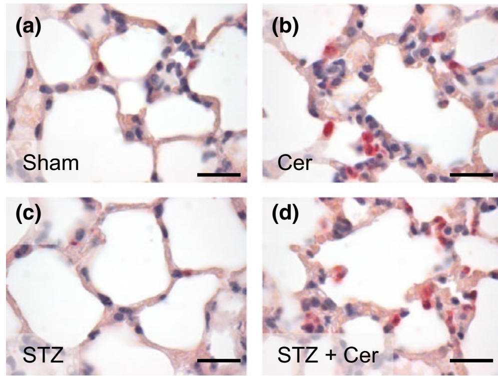


Figure 1 Diabetes aggravates pancreatitis induced systemic inflammation. The concentration of IL-6 in blood plasma (a), and the concentration of leucocytes (b), lymphocytes (c) or monocytes plus granulocytes (d) in the blood was determined on day 23 (2 hours after the last cerulein administration) in control mice (Sham), mice with AP (Cer), diabetic mice (STZ) and diabetic mice with AP (STZ + Cer). Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was n = 8 (Sham), n = 19 (Cer), n = 11 (STZ), n = 21 (STZ + Cer) in panel a and n = 6 (Sham), n = 20 (Cer), n = 7 (STZ), n = 19 (STZ + Cer) in panel b to d. Significant differences between the cohorts are indicated, Mann–Whitney rank sum test: $^{*}P \leq 0.001$ (a, c, d), $^{*}P = 0.008$ (b), $^{\#}P \leq 0.001$ compared to Sham-treated mice (a, c, d).



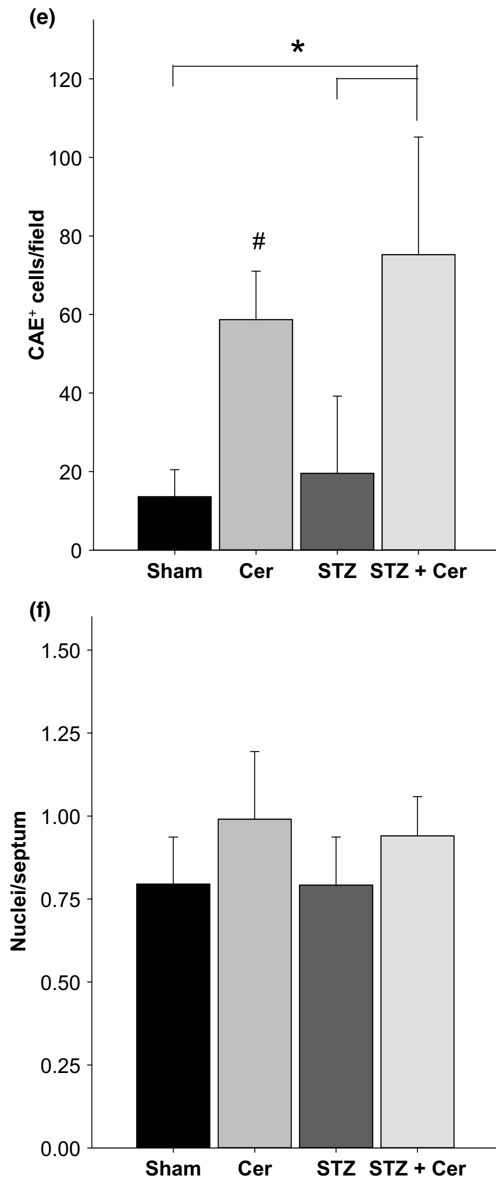
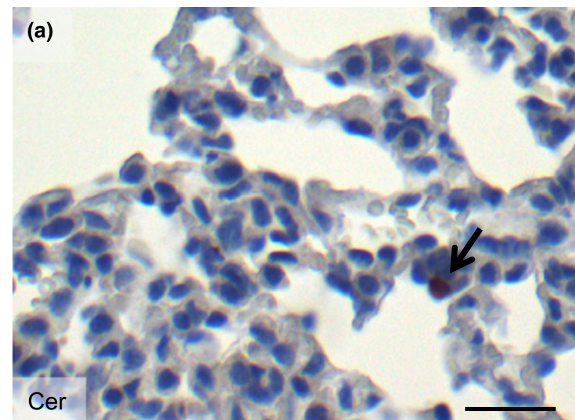


Figure 2 Diabetes and pancreatitis influence lung inflammation and alveolar cellularity on day 23. Representative images of CAE^+ inflammatory cells in the lung in control mice (a), mice with AP (b), diabetic mice (c) and diabetic mice with AP (d). The number of CAE^+ inflammatory cells per field (e) and the alveolar cellularity index (f) was quantified. Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was $n = 9$ (Sham), $n = 17$ (Cer), $n = 12$ (STZ), $n = 17$ (STZ + Cer) in panel e and $n = 4$ (Sham), $n = 5$ (Cer), $n = 4$ (STZ), $n = 6$ (STZ + Cer) in panel f. Significant differences between the cohorts are indicated, Mann–Whitney rank sum test: $*P < 0.001$, $^{\#}P < 0.001$ compared to Sham-treated mice. Bar = 20 μm.



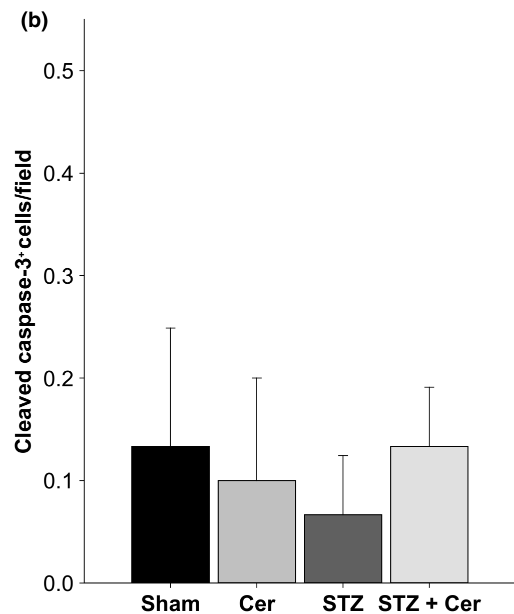


Figure 3 Diabetes does not enhance cell death in the lung epithelium on day 23. Representative image of cleaved caspase-3^+ cells in the lung of a cerulein-treated mouse (a). The number of cleaved caspase-3^+ cells in the lung epithelium per field (b) was quantified. Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was $n = 4$. No significance was observed by Mann–Whitney rank sum test followed by Bonferroni correction for repeated analysis. Bar = 50 μm.

STZ-treated mice (data not shown). In cerulein-treated mice features of AP such as oedema, and tissue-infiltrating inflammatory cells were observed on day 23, but pancreatitis was reversible as judged by histology of the pancreas on day 30

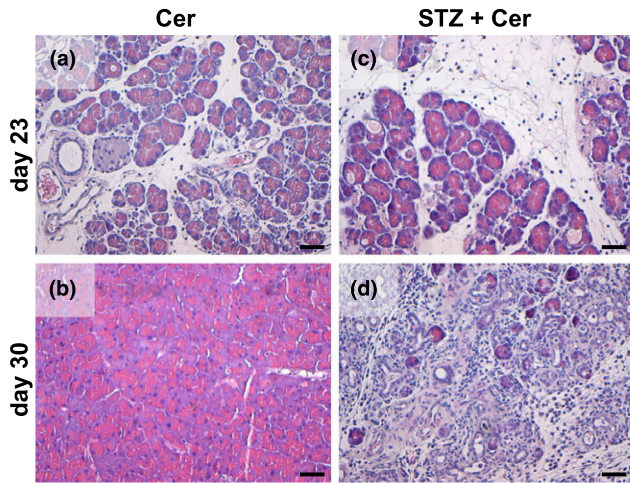


Figure 4 Histology of the pancreas after induction of acute pancreatitis. Representative images of hematoxylin/eosin-stained pancreas sections of cerulein (a, b) or STZ plus cerulein (c, d)-treated mice on day 23 (a, c) and day 30 (b, d). Bar = 50 μm.

(Figure 4a,b). In STZ plus cerulein-treated mice an even stronger induction of oedema and increased infiltration of inflammatory cells was observed in the pancreas on day 23 when compared to cerulein-treated mice (Figure 4a,c). In addition, AP continued until day 30 leading to a massive reduction of acinar cells (Figure 4d). These data suggest that cerulein induces a mild reversible form of pancreatitis, whereas STZ plus cerulein treatment leads to a more severe form of pancreatitis resulting in an impressive difference in the histology of the pancreas on day 30.

Analysis of inflammation and cell death on day 30

On day 30, STZ plus cerulein-treated mice have a marginally increased number of CAE$^+$ cells (P = 0.114) in the lung epithelium when compared to cerulein-treated mice (Figure 5a). In addition, the alveolar cellularity index was also slightly increased (P = 0.114) by STZ plus cerulein treatment when compared to cerulein-treated mice (Figure 5b). However, we observed no increase in the number of cleaved caspase-3$^+$ cells in STZ plus cerulein-treated mice when compared to cerulein-treated mice (Figure 5c). As lung injury can cause proliferation of lung epithelial cells, we also quantified BrdU incorporation into the nuclei of lung epithelial cells during 8 days of AP (day 22–30). We observed no increase in the number of BrdU$^+$ cells in STZ plus cerulein-treated mice when compared to cerulein-treated animals (Figure 5d).

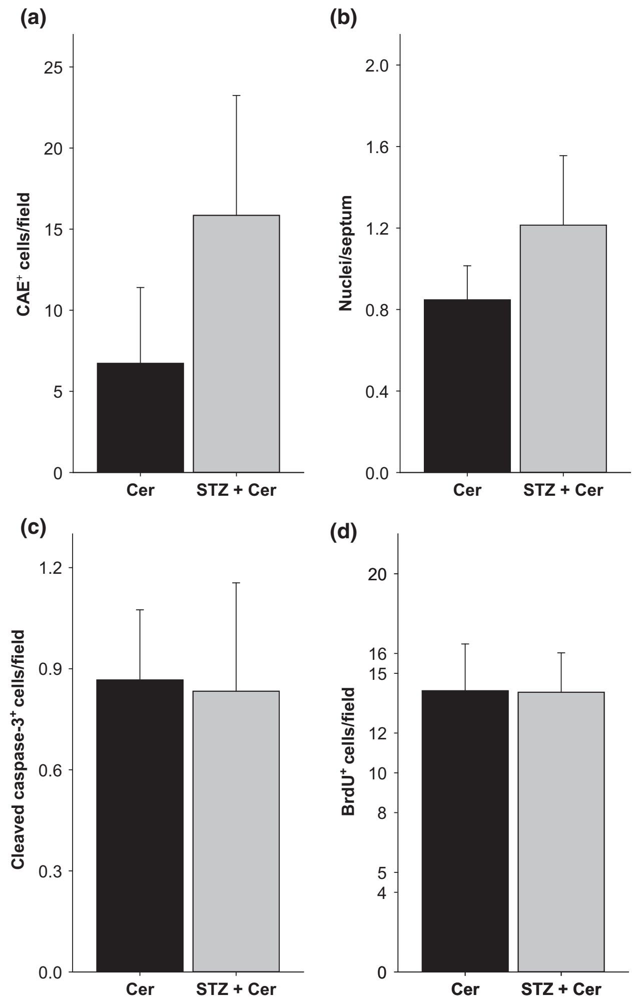


Figure 5 Evaluation of inflammation and cell death in the lung on day 30. The number of CAE$^+$ inflammatory cells per field (a), the alveolar cellularity index (b), the number of cleaved caspase-3$^+$ cells in the lung epithelium per field (c) and the number of BrdU$^+$ cells per field (d) were quantified. Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was n = 4. No significance was observed by Mann–Whitney rank sum test followed by Bonferroni correction for repeated analysis.

Discussion

The presented data demonstrate that diabetes during AP (i) enhances IL-6 concentration in blood plasma while decreasing the number of lymphocytes in the blood, (ii) only marginally increases pancreatitis induced lung inflammation, but (iii) does not lead to major cell death or proliferation in the lung epithelium. We conclude that diabetes has a fundamental influence on the progression of pancreatitis at a local level as published previously (Zechner *et al.* 2012, 2013, 2014) and can also increase systemic inflammatory parameters such as IL-6 concentration in blood plasma. However, these data also suggest that the observed strong aggravation of pancreatitis by diabetes leads neither to strong enhancement of lung inflammation nor to induction of cell death in the lung epithelium.

Redundant administration of cerulein causes an oedematous form of AP, which is associated with lung inflammation and a very mild form of lung injury (Elder *et al.*

2011). This animal model system should be ideal to test if additional parameters such as diabetes aggravate lung injury, but will not detect a possible inhibition of pancreatitis induced lung injury. The seemingly contradictory result, that diabetes worsens AP leading to a severe form of pancreatitis, but does not cause lung injury, could be explained by the following assumption. Possibly diabetes aggravates pancreatitis, but at the same time reduces the risk for lung injury. This conclusion that lung injury is not aggravated but rather reduced by diabetes is supported by clinical as well as experimental studies. For example, diabetes predicts mortality in critically ill patients, but is not associated with ALI (Koh *et al.* 2012). A meta-analysis also suggests that pre-existing diabetes leads to reduced rather than increased risk of lung injury in critically ill patients (Gu *et al.* 2014). Diabetes also does not increase, but reduces the risk for lung dysfunction in patients with sepsis (Esper *et al.* 2009; Yang *et al.* 2011). Experiments in rats demonstrate that sepsis-induced ALI is milder in diabetic rats than in normoglycaemic controls (Filgueiras *et al.* 2012). Although a few studies suggest that in specific model systems, pre-existing diabetes can also increase the risk of lung injury (Hagiwara *et al.* 2011; Xiong *et al.* 2013) a consensus seems to develop that diabetes is protective against lung injury (Honiden & Gong 2009). Our presented data and the above cited literature, therefore, suggest that it might be especially valuable to carefully adjust glucose concentration to avoid local complications during AP, but that hyperglycaemia might not increase the risk of lung injury during AP.

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Conflict of interests

The authors declare that there is no conflict of interest.

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Anhang - Teil 4

Diabetes increases pancreatic fibrosis
during chronic inflammation

Original Research

Diabetes increases pancreatic fibrosis during chronic inflammation

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Abstract

Diabetes and fibrosis can be concurrent processes in several diseases such as cystic fibrosis or chronic pancreatitis. To evaluate whether diabetes can influence fibrosis and thus aggravate the pathological process, the progression of chronic pancreatitis was assessed in diabetic and non diabetic mice. For this purpose, insulin producing beta-cells in C57Bl/6 J mice were selectively impaired by administration of streptozotocin. Chronic pancreatitis was then induced by repetitive administration of cerulein in normoglycaemic and hyperglycaemic mice. Diabetes caused enhanced collagen I deposition within three weeks of the onset of chronic pancreatitis and increased the proliferation of interstitial cells. This was accompanied by an increased number of interlobular fibroblasts, which expressed S100A4 (fibroblast-specific protein-1) and stimulation of α-smooth muscle actin expression of pancreatic stellate cells. In addition, the observed aggravation of chronic pancreatitis by diabetes also led to a significantly enhanced atrophy of the pancreas, increased infiltration of inflammatory chloracetate esterase positive cells and enhanced acinar cell death. We conclude that diabetes has a detrimental influence on the progression of chronic pancreatitis by aggravating fibrosis, inflammation and pancreatic atrophy.

Keywords: Diabetes, chronic inflammation, fibrosis, pancreatic stellate cells, S100A4, proliferation in islets of Langerhans

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Introduction

Fibrosis occurs in many tissues as a result of inflammation or damage and can have a devastating effect on the function of organs, as observed, for example in liver cirrhosis, pulmonary fibrosis or chronic pancreatitis.[1–3] In chronic pancreatitis fibrosis is caused by stimulation of interstitial cells called pancreatic stellate cells.[4] Upon stimulation stellate cells start to express α-smooth muscle actin, which is followed by deposition of extracellular matrix.[4] These cells can be found at the basolateral aspect of acinar cells (periacinar cells) or in between lobuli (interlobular fibroblasts).[5–7]

During some diseases, such as cystic fibrosis or chronic pancreatitis, fibrosis is often accompanied by diabetes.[1,3] In particular, chronic pancreatitis is regularly associated with diabetes.[1] Some patients with beginning chronic pancreatitis may have either type 2 diabetes mellitus mostly due to obesity or long-term type 1 diabetes, whereas patients with longstanding chronic pancreatitis can develop type 3 c diabetes mellitus.[8] The prevalence of diabetes in chronic pancreatitis depends on aetiology, age, genetic predisposition, degree of pancreatic damage, the presence or absence of pancreatic calculi and the duration of the disease.[1] For example, in one prospective cohort study with 500 patients the development of diabetes was observed in 83% of patients with chronic pancreatitis.[9] Chronic pancreatitis causes type 3 c diabetes by reducing the beta-cell mass and possibly by causing a reduced functionality of beta-cells.[10–12] Interestingly, it has also been documented that diabetes is a mortality risk factor for chronic pancreatitis.[13] This suggests that diabetes may also have an influence on the progression of chronic pancreatitis. Surprisingly, no experimental data exist to address the hypothesis if diabetes influences fibrosis during chronic pancreatitis.

In this study, we explored whether diabetes influences main features of chronic pancreatitis such as fibrosis, inflammation and pancreatic atrophy. Our data demonstrate that diabetes has a fundamental influence on the progression of chronic pancreatitis by enhancing collagen I deposition and inducing the proliferation of interstitial cells. In addition, diabetes enhances cell death of acinar cells, increases the number of infiltrating inflammatory cells and aggravates atrophy of the pancreas.

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Materials and methods

Animal husbandry and tissue collection

Eight- to twelve-week-old C57BL/6 J mice were either sham- (Sham), cerulein- (Cer), streptozotocin- (STZ), or streptozotocin plus cerulein- (STZ + Cer) treated (Figure 1). Diabetes was induced in two cohorts (STZ, STZ + Cer) by intraperitoneal injection of 50 mg/kg streptozotocin (Sigma-Aldrich, St Louis, MO, USA) daily on day 1–5 of experimental design. Chronic pancreatitis was then induced in two cohorts (Cer, STZ + Cer) by administration of three intraperitoneal injections of 50 µg/kg cerulein (Sigma-Aldrich) at a rate of one every hour three times a week (thus Monday, Wednesday and Friday) over a period of three weeks (Figure 1). All control mice were sham-treated with appropriate vehicles (0.9% wt/vol. saline solution instead of cerulein; 50 mmol/L sodium citrate pH 4.5 instead of STZ). All four cohorts of mice received drinking water containing 800 mg/L of metamizol to prevent potential pain caused by pancreatitis (Ratiopharm, Ulm, Germany). In addition, all mice received 1 g/L 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) during the entire period of chronic pancreatitis in the drinking water, in order to evaluate cell proliferation. Blood samples for assessing amylase and lipase activity were taken 2 h after the third cerulein injection on day 22, or on day 47, one week after the last cerulein injection. Pancreatic tissue was sampled on day 26, 2 h after the last cerulein administration or on day 47. Blood glucose was measured with the blood glucose metre Contour (Bayer Vital, Leverkusen, Germany) on day 1 before the first STZ injection and on day 22 before the first cerulein injection. For retrobulbar blood sampling and tissue collection, the animals were anaesthetised with 75 mg/kg ketamine (bela-pharm, Vechta, Germany) and 5 mg/kg xylacine (Bayer Health Care, Leverkusen, Germany). After the start of laparotomy, the tissue was isolated within a maximum of 5 min and fixed in 4% (wt/vol.) phosphate-buffered formalin for 2–3 days. In addition, squeezing of the pancreas with tweezers was avoided, in order to minimise tissue damage. All experiments were performed in accordance with German legislation and the principles of laboratory animal care.

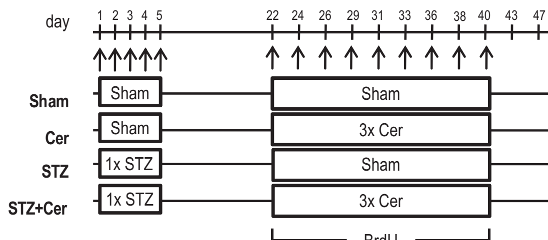

Figure 1 Experimental protocol. In two cohorts (STZ, STZ + Cer) diabetes was induced by intraperitoneal injection of 50 mg/kg streptozotocin on day 1–5 of the experimental paradigm. Control cohorts (Sham, Cer) were sham-treated in the same manner by injection of 50 mmol/L sodium citrate pH 4.5. In two cohorts (Cer, STZ + Cer) chronic pancreatitis was then induced from day 22 to day 40 by administration of three intraperitoneal injections of 50 µg/kg cerulein at a rate of one every hour on Monday, Wednesday and Friday. Control cohorts (Sham, STZ) were sham-treated in the same manner with 0.9% wt/vol. saline. In order to evaluate cell proliferation, all mice received 1 g/L BrdU during the entire period of chronic pancreatitis in the drinking water. The tissue was either collected on day 26 or on day 47

Analysis of plasma and tissue

To assess acinar cell damage, the activity of lipase and amylase in blood plasma was analysed using the Cobas c111 spectrophotometer (Roche Diagnostics, Mannheim, Germany). Pancreatic atrophy was quantified as pancreas to body weight ratio and the pancreas was processed as described previously for histological staining.[14] To evaluate the cellular inflammatory response, which is characterised by infiltration of granulocytes during cerulein-induced pancreatitis,[15] naphthol AS-D chloroacetate esterase (CAE) staining was performed on paraffin embedded tissue. Cell death was analysed using the ApopTag Plus Peroxidase *in situ* detection kit (Millipore, Eschborn, Germany). Cell proliferation or fibrosis was evaluated by immunohistochemistry using mouse anti-BrdU (clone Bu20a, dilution 1:50), rabbit anti-collagen-I (Abcam, Cambridge, UK, code ab 34710, dilution 1:200), goat anti-S100A4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, code sc-19949, dilution 1:50) or rabbit anti-α-smooth muscle actin (Abcam, ab5694, dilution 1:800). All immunohistochemical procedures were performed using the Universal LSAB$^+$ Kit/HRP as source for appropriate secondary antibodies (Dako, Hamburg, Germany). Planimetric analysis of collagen I positive areas in the pancreas was performed on 10 randomly chosen pictures (taken with a 40 x objective) of pancreatic tissue per mouse by using Adobe Photoshop CS5 (Adobe, San Jose, CA, USA).

Statistics

Data presentation and statistics were performed as described previously.[14] The significance of differences was evaluated using a Mann–Whitney rank-sum test, followed by the correction for the accumulation of the α error by considering the number of meaningful comparisons. Differences with $P \leq 0.05$, divided by the number of meaningful comparisons were considered to be significant. Differences with $P < 0.08$, divided by the number of meaningful comparisons, were considered to indicate a tendency.

Results

Quality control of induced diabetes and chronic pancreatitis

At the beginning of the experiment, on day 1, all four cohorts of mice had similar blood glucose concentrations (Sham: 6.8/6.6–8.3, Cer: 7.7/7.0–8.8, STZ: 7.1/6.3–7.9, STZ + Cer: 7.0/6.0–8.2, median/interquartile range in mmol/L). Injection of STZ caused a strong rise in blood glucose concentration in STZ- and STZ plus cerulein-treated cohorts by day 22 when compared to control cohorts (Figure 2a). Thus, the blood glucose concentrations of the STZ versus STZ plus cerulein cohorts were comparable to each other, but were significantly increased in comparison to sham- and cerulein-treated mice. Two hours after the first three consecutive cerulein or sham injections on day 22, lipase and

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amylase activity in blood plasma was assessed. Lipase activity increased significantly in cerulein as well as STZ plus cerulein-treated mice when compared to control cohorts (Figure 2b), verifying the onset of pancreatic tissue injury. The analysis of amylase activity confirmed the lipase activity data, since amylase activity increased significantly in cerulein as well as STZ plus cerulein-treated mice when compared to control cohorts (Figure 2c). On day 47, one week after the last episode of cerulein-induced chronic pancreatitis, both lipase as well as amylase activity returned to physiological levels (data not shown).

Diabetes enhances collagen I deposition and proliferation of interstitial cells

Immunohistochemical analysis of the pancreas on day 47 revealed barely any collagen I deposition in sham-treated or STZ-treated mice, whereas in cerulein and especially STZ plus cerulein-treated mice prominent collagen I deposition was observed (Figure 3a). Planimetric evaluation of the collagen I positive tissue area affirmed a significant increase in collagen I deposition in the pancreas of cerulein-treated mice when compared to sham-treated animals (Figure 3b). Collagen I deposition in STZ plus cerulein-treated mice was increased, when compared to sham, STZ- or cerulein-treated animals (Figure 3b). In order to assess if this increase in collagen deposition correlates with an expansion of interstitial cell populations, the BrdU incorporation in interstitial cells was evaluated on day 26. Proliferation of interstitial cells was increased in the mouse cohort treated with cerulein and a major increase in proliferation of interstitial cells was observed in mice treated with STZ plus cerulein (Figure 3c). Analysis of the percentage of $BrdU^+$ cells in the islets of Langerhans on day 26 revealed reduced proliferation of islet cells during chronic pancreatitis, but increased proliferation in diabetic mice (Sham: 1.45/0.65–2.23, Cer: 0.71/0.00–0.95, STZ: 2.06/1.9–3.23, STZ + Cer: 1.57/0.51–2.32, median/interquartile range in percentage of $BrdU^+$ cells, the differences were not significant). Diabetes, therefore, significantly stimulates the expansion of interstitial cells and enhances collagen I deposition during chronic pancreatitis, but only moderately stimulates the proliferation of islet cells.

Diabetes stimulates activation of pancreatic stellate cells

Collagen can be produced by stimulated fibroblasts. In the pancreas, especially stellate cells have been reported to produce collagen during pancreatitis.[7] Thus, we evaluated the expression of S100A4 (fibroblast-specific protein-1), as general fibroblast marker and α-smooth muscle actin, which is expressed by pancreatic stellate cells only after tissue injury. Immunohistochemical analysis of the pancreas revealed that interlobular fibroblasts express S100A4 (fibroblast-specific protein-1) independent of diabetes or pancreatitis (Figure 4a). In cerulein and especially STZ plus cerulein-treated mice, however, more S100A4 positive interlobular cells could be observed on day 26 (Figure 4a). The expression of α-smooth muscle actin was observed in azinar as well as interlobular stellate cells only after cerulein and STZ plus cerulein treatment, whereas in all animals α-smooth muscle actin positive blood vessels could be noticed (Figure 4b). The intensity of α-smooth muscle actin staining of periacinar cells as well as interlobular stellate cells was increased in STZ plus cerulein-treated mice in comparison to cerulein-treated mice. This suggests that diabetes enhances the activation of stellate cells during chronic pancreatitis.

Diabetes enhances pancreatic atrophy and alters pancreas histology

Analysis of the pancreas on day 47 revealed a distinct atrophy of the pancreas in cerulein-treated mice compared to sham-treated animals (Figure 5a). This atrophy was even more pronounced in STZ plus cerulein-treated mice, when compared to sham-, STZ- or cerulein-treated animals (Figure 5a). Haematoxylin/eosin staining of sections on day 47 revealed no pathological features in the exocrine tissue in sham- and STZ-treated mice, whereas cerulein and especially STZ plus cerulein-treated animals had fields of acinar cells interrupted by interstitial cells (Figure 5b).

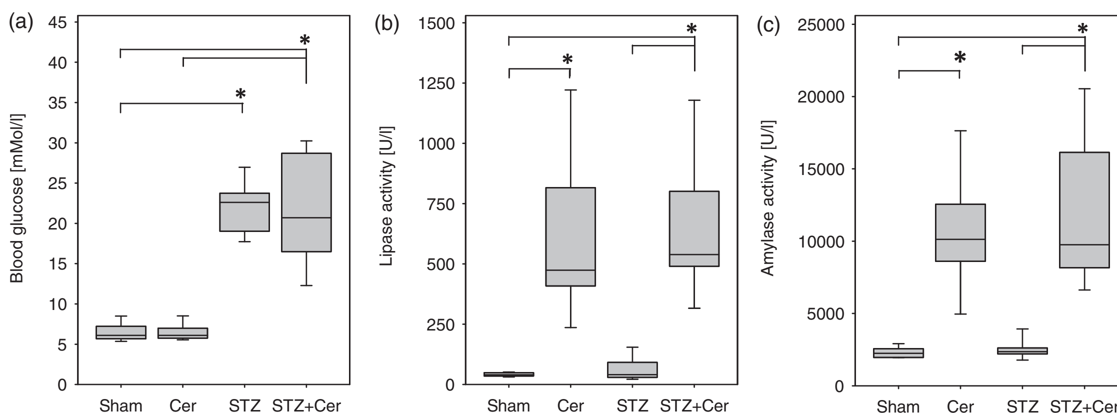

Figure 2 Analysis of blood glucose concentration, lipase and amylase activity on day 22. Blood glucose concentration (a) was measured in the morning before any injection, whereas blood samples for lipase activity (b) and amylase activity (c) were taken 2 h after the last cerulein or sham injection in control (Sham), cerulein- (Cer), streptozotocin- (STZ) or streptozotocin plus cerulein (STZ + Cer)-treated mice. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles in the form of whiskers. Significant differences between the cohorts are indicated, $^*P \leq 0.001$

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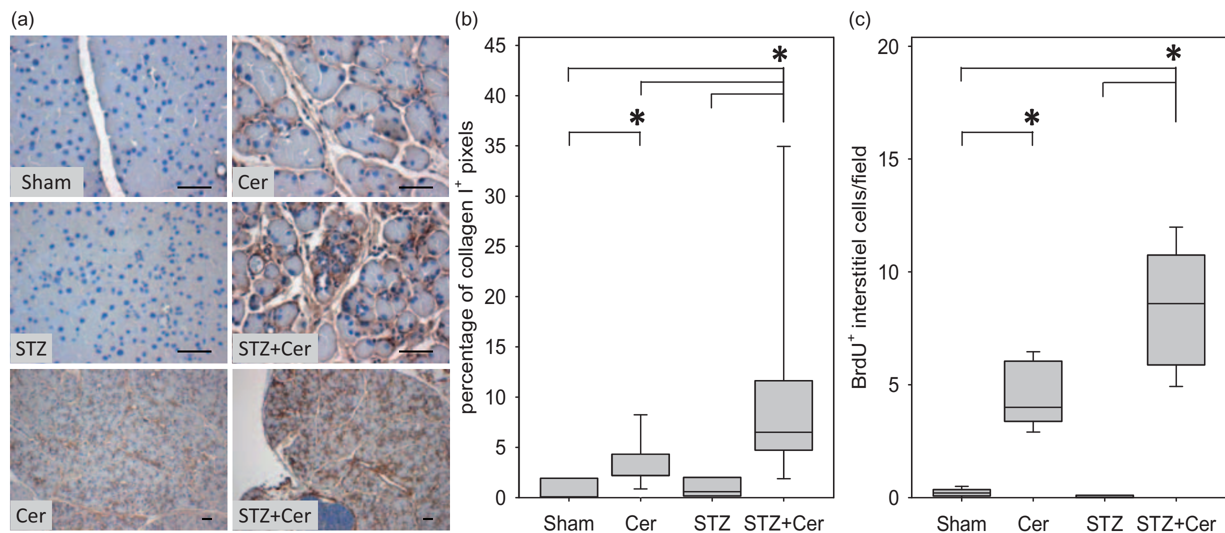

Figure 3 Diabetes increases collagen I deposition and proliferation of interstitial cells. Indicated parameters were assessed in control mice (Sham) and cerulein (Cer), streptozotocin- (STZ) or streptozotocin plus cerulein- (STZ + Cer) treated cohorts. Deposition of collagen I was determined in the pancreas on day 47 by immunohistochemistry and counterstaining with haematoxylin (a). The relative area of collagen I deposition was quantified as percentage of collagen I positive pixels per high power field on day 47 (b). Proliferation of interstitial cells in the pancreas was evaluated by determining the number of BrdU positive interstitial cells per field on day 26 (c). Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles in the form of whiskers. Significant differences between the cohorts are indicated, $*P \leq 0.01$, bar = 50 μm. (A color version of this figure is available in the online journal.)

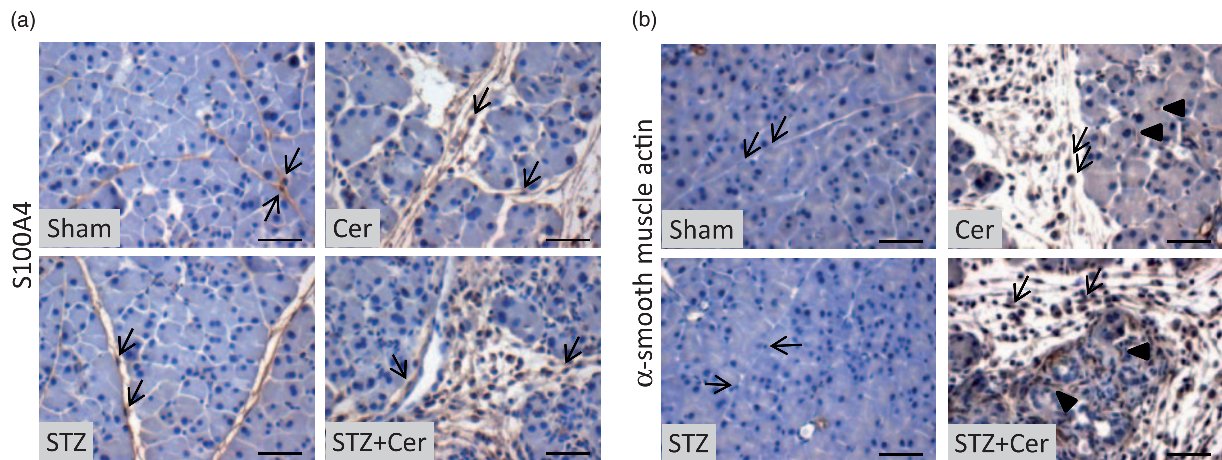

Figure 4 Diabetes activates stellate cells. Expression of S100A4 (a) or α-smooth muscle actin (b) was detected by immunohistochemistry and counterstaining with haematoxylin in interlobular control mice (Sham) and cerulein- (Cer), streptozotocin- (STZ) or streptozotocin plus cerulein- (STZ + Cer) treated cohorts on day 26. Interlobular stellate cells are marked by arrows, whereas arrowheads mark stimulated α-smooth muscle positive periacinar stellate cells. Bar = 50 μm. (A color version of this figure is available in the online journal.)

In addition, beginning acinar to ductal metaplasia was often observed in STZ plus cerulein-treated mice (Figure 5b).

Diabetes enhances inflammation and cell death

On day 26, a significantly increased number of CAE positive infiltrating inflammatory cells were observed in the pancreas of cerulein-treated mice when compared to sham-treated animals (Figure 6a). STZ plus cerulein-treated mice showed an even stronger increase in the number of CAE^{+} inflammatory cells when compared to sham-, STZ- or cerulein-treated animals (Figure 6a). Cell death of acinar cells was modestly increased in the mouse cohort treated with cerulein, whereas a major increase in dying acinar cells was observed in mice treated with STZ plus cerulein (Figure 6b).

Discussion

The presented data demonstrate that diabetes (i) enhances collagen I deposition, (ii) increases proliferation of interstitial cells, (iii) stimulates the expression of α-smooth muscle actin in stellate cells, (iv) aggravates inflammation and (v) induces cell death during chronic pancreatitis. Diabetes leads, therefore, to a detrimental increase in fibrosis and pancreatic atrophy within three weeks of chronic pancreatitis. Thus, diabetes fundamentally aggravates the progression of chronic pancreatitis.

The observations in this study correlate well with a clinical study describing that diabetes is a mortality risk factor for chronic pancreatitis.[13] A detrimental influence of diabetes has also been discussed in the context of acute pancreatitis.[15] For example, patients with diabetes have a

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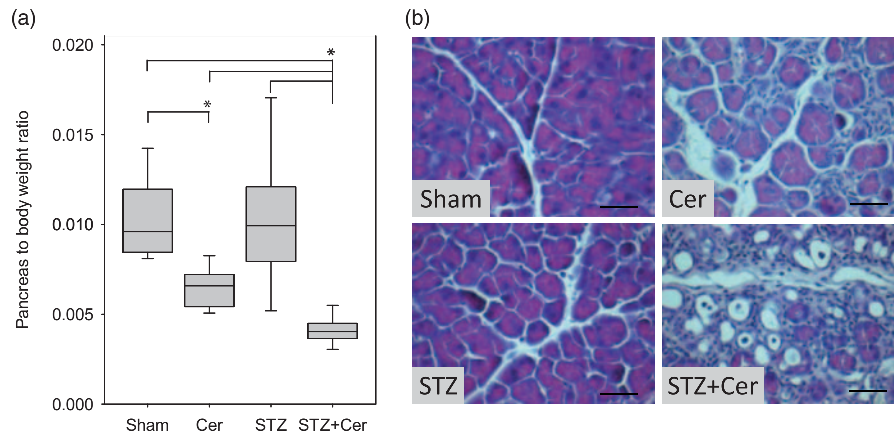

Figure 5 Diabetes aggravates pancreatic atrophy, and alters the histology. Pancreas to body weight ratio was determined in control (Sham) and cerulein- (Cer), streptozotocin (STZ) or streptozotocin plus cerulein- (STZ + Cer) treated mice (a) on day 47. Histology was evaluated by haematoxylin/eosin staining of pancreas sections in mice of the indicated cohorts on day 47 (b). Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles in the form of whiskers. Significant differences between the cohorts are indicated, *$P \leq 0.003$, bar = 50 µm. (A color version of this figure is available in the online journal.)

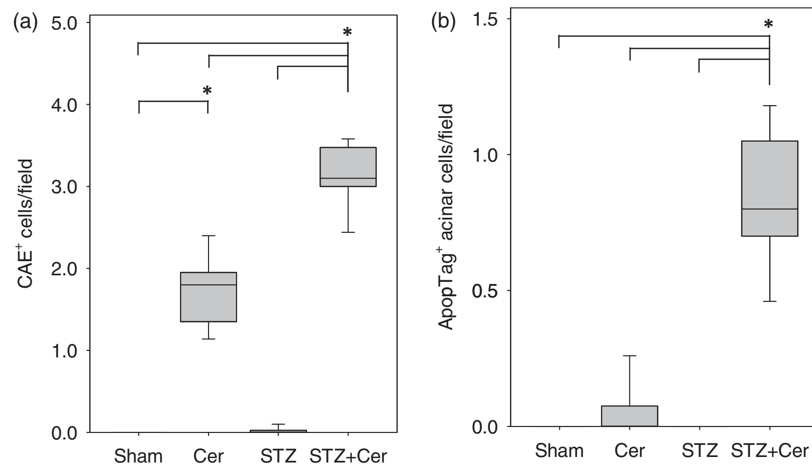

Figure 6 Diabetes activates inflammation and cell death. Indicated parameters were assessed in control mice (Sham) and cerulein- (Cer), streptozotocin- (STZ) or streptozotocin plus cerulein- (STZ + Cer) treated cohorts on day 26. The number of CAE^+ inflammatory cells per field was quantified (a) and cell death was evaluated by determining the number of ApopTag positive acinar cells per field (b). Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles in the form of whiskers. Significant differences between the cohorts are indicated, *$P \leq 0.003$

higher risk of acute pancreatitis and hyperglycaemia may predispose patients with acute pancreatitis to systemic organ failure.[16–19] In addition, blood glucose level is an accurate predictor of outcome in gallstone pancreatitis and an important criterion for assessing the prognosis of acute pancreatitis by the Ranson score.[20,21] However, a definite cause and effect relationship between diabetes and pancreatitis cannot be evaluated in these clinical studies, but needs to be addressed in an experimental setting.

Only few experimental data are available that address the question whether diabetes influences pancreatitis. For example, hyperglycaemia correlates with increased inflammation during chronic pancreatitis in CCR2 loss of function mice.[22] In addition, we demonstrated in a previous study that diabetes increases tissue damage and reduces regeneration in the pancreas after acute pancreatitis.[23] Both publications are consistent with this study and support the hypothesis that diabetes has a major influence on the exocrine compartment during pancreatitis.

The observed aggravation of chronic pancreatitis by diabetes raises the question whether diabetes has a direct effect on acinar cells and stellate cells. A direct effect of diabetes on acinar cells has been described previously and has been summarised as so called endocrine to exocrine axis hypothesis.[1,24] For example, numerous publications document that diabetes reduces the secretion of digestive enzymes such as amylase.[25–27] These observations might partially explain exocrine deficiency that can be observed in some diabetic patients.[28] However, it seems to be counterintuitive that the aggravation of pancreatitis by diabetes could be explained by exocrine insufficiency of acinar cells. It is more likely that diabetes has a profound influence on pancreatitis through

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other mechanisms such as modulation of the inflammatory response or the aggravation of cell death.

A direct effect of diabetes on stellate cells is supported by some *in vitro* experiments. For example, high glucose concentration has been reported to induce proliferation and synthesis of extracellular matrix proteins in interstitial cells which were isolated from the pancreas.[29–31] However, since STZ-treated hyperglycaemic mice did not have any obviously increased collagen I deposition or activation of stellate cells, higher glucose concentration alone seems to be insufficient to induce fibrosis *in vivo*. Only in the context of chronic pancreatitis we observed that diabetes increased collagen deposition and activation of stellate cells. This suggests that diabetes does not cause, but aggravates inflammation-induced fibrosis. However, we cannot determine if diabetes stimulates stellate cells directly or indirectly, for example, via modulation of inflammation. Nevertheless the characterised aggravation of fibrosis by diabetes might be of clinical relevance, since some clinical studies support this conclusion. For example, enhanced fibrosis was observed *post mortem* in the pancreas of patients with type 2 diabetes.[32] In addition, enhanced fibrosis was also observed in other organs, in diabetic patients with hepatitis C virus-infected liver and in patients suffering from idiopathic pulmonary fibrosis.[33,34]

As a secondary finding, we observed that diabetes moderately increased proliferation of islet cells. This is consistent with previously published data, describing increased proliferation of β-cells as well as α- and δ-cells in islets after application of STZ.[35–37] To our surprise application of supraphysiological levels of cerulein, an analogue of cholecystokinin, did not increase, but rather reduced the proliferation of islet cells. This is not consistent with previous publications, which describe increased proliferation of islet cells after application of moderate levels of cholecystokinin.[38,39] We assume that the inflammatory micromilieu caused by supraphysiological levels of cerulein has the opposite effect than administration of moderate concentrations of cholecystokinin.

Recently, an intensified insulin therapy for patients with pancreatitis as well as for critically ill patients in general has been widely discussed.[40–42] Since the danger of hypoglycaemia in patients with pancreatitis is high, a conservative insulin therapy is usually pursued.[8,43,44] However, studies also report that a more intensified careful insulin therapy can be applied to patients with chronic pancreatitis without increasing the incidence of hypoglycaemic events.[42] Thus, if diabetes had a similar strong negative effect on pancreatitis in humans as observed in mice, a more intensified insulin therapy could be beneficial to some patients.

Author contribution: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; NK, DZ, TR and BG conducted experiments.

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Anhang - Teil 5

Risk factors for pancreatic ductal adenocarcinoma specifically stimulate pancreatic duct glands in mice

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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Risk Factors for Pancreatic Ductal Adenocarcinoma Specifically Stimulate Pancreatic Duct Glands in Mice

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Diabetes mellitus type 2 and chronic pancreatitis are regarded as risk factors for pancreatic cancer. Pancreatic duct glands (PDGs) were recently described as a new compartment of the major duct in humans and mice. To evaluate the influence of diabetes and chronic pancreatitis on PDGs, cerulein was injected i.p., repetitively over 10 weeks, in mice exhibiting obesity and a type 2 diabetes–like syndrome (B6.V-Lep$^{ob/ob}$) and in lean littermates. By using 5-bromo-2′-deoxyuridine (BrdU), a label-retaining cell population was characterized in PDGs. Cerulein administration led to more BrdU^{+} cells in PDGs of obese mice compared with lean mice. The observed increase was specific to PDGs, because BrdU incorporation in cells of the pancreatic duct was not increased. In addition, the expression of distinct tumor markers in PDGs was characterized by Muc5ac, S100P, regenerating islet–derived 3β, 14-3-3 σ, and prostate stem cell antigen immunochemistry. Type 2 diabetes–like syndrome, accompanied by chronic pancreatitis, enhanced nuclear localization of S100P. Both risk factors for pancreatic cancer also induced the production of Muc5ac and the nuclear localization of S100P. These results demonstrate that diabetes and chronic pancreatitis jointly enhance BrdU incorporation and production of pancreatic cancer–specific proteins in PDGs. The observed alterations suggest that pancreatic tumors might originate from the newly discovered histomorphological structures, called PDGs, which could represent a target for future anticancer therapies. *(Am J Pathol 2013, 182: 965–974; http://dx.doi.org/10.1016/j.ajpath.2012.11.016)*

The survival of patients with pancreatic cancer could only be improved modestly during the past 30 years, resulting in a 5-year survival rate of approximately 6% in the United States.[1] Basic knowledge about precancerous lesions might improve our understanding about tumor progression; as a long-term goal, this may allow the detection of pancreatic cancer at a stage at which intervention could substantially improve the survival rate (eg, in breast cancer).

Diabetes, obesity, and chronic pancreatitis are well-characterized risk factors for this malignant disease. Diabetes mellitus type 2 increases the risk for pancreatic cancer by 80%, and patients with pancreatic cancer who have diabetes have a worse prognosis and a shorter survival time than those without diabetes.[2,3] Hyperinsulinemia itself also increases the cancer risk up to 100%.[4,5] Obesity is also associated with a slightly increased risk of pancreatic cancer, 19% in obese people, per a meta-analysis of 14 studies.[6] Chronic pancreatitis is a major risk factor for the development of pancreatic cancer. The lifetime cumulative probability of developing pancreatic cancer in the overall population is up to 1%.[7] Ten years after the diagnosis of pancreatitis, the cumulative risk of pancreatic cancer is up to 1.8%;[8] in hereditary pancreatitis, the cumulative risk of developing pancreatic cancer is up to 44%.[9]

Concerning the histopathological characteristics, >80% of the pancreatic tumors are classified as pancreatic ductal adenocarcinoma (PDA). Another tumor entity in the pancreas with increasing importance during the past few years is the intraductal papillary mucinous neoplasm (IPMN). IPMNs account for 3% to 5% of pancreatic tumors.[10] IPMNs arise from the pancreatic main duct or its major branches and display different degrees of mucin secretion, cystic dilatation, and invasiveness. They are classified into benign, borderline, and malignant noninvasive and invasive lesions. Approximately

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40% of all resected IPMNs are invasive carcinoma.[11,12] The advanced-stage invasive IPMN has a similar poor prognosis, such as PDA.[13] However, IPMNs themselves are also discussed as precursors of PDAs.[10,14,15]

There are many sites in the pancreas considered as the origin for pancreatic cancer. The best-characterized precursor lesion for PDAs is pancreatic intraepithelial neoplasia (PanIN). PanINs are histologically characterized by a papillary or flat (<5-mm) epithelial, noninvasive lesion, containing mucins. They usually arise in small ducts (<5 mm).[15] PanINs might originate from the pancreatic duct epithelium or acinar cells by acinar-to-ductal metaplasia (ADM).[16−18]

Recently, new compartments of the pancreatic duct, the pancreatic duct glands (PDGs), were described in humans and mice.[19] PDGs are blind-ending, mucin-producing out pouches, which are primarily located in the proximal ductal system near the ampullary region. PDGs might be a distinct compartment with possibly stem cell−like properties, because they express developmental markers, such as Shh, Pdx-1, and Hes-1.[19] For this reason, PDGs are discussed as the precursor lesion for PDA, PanINs, or IPMNs.[19−21]

The most established marker for pancreatic cancer is carbohydrate antigen 19-9, which can predict resectability and survival but fails in the screening of asymptomatic subjects.[22,23] Thus, for the characterization of precursor lesions, other proteins, such as Muc5ac, S100P, regenerating islet−derived 3β (Reg3β), 14-3-3 σ (stratifin), and prostate stem cell antigen (PSCA), have been analyzed. The production of Muc5ac characterizes gastrointestinal metaplasia and is typical for benign and malignant transformation of pancreatic duct epithelium.[24] Muc5ac production was observed in PDA, PanINs, and IPMNs.[25−27] S100P belongs to the S100 family of calcium-binding proteins and is produced in PDA, PanINs, and IPMNs.[28,29] It can be detected in the nucleus when interacting with S100PBPR.[28] The expression of Reg3β (or pancreatitis-associated protein) is observed in PDA.[30,31] Furthermore, proteins overexpressed in PDA and PanINs are 14-3-3 σ, which influence different signal transduction pathways, and the cell surface glycoprotein, PSCA.[32−34]

In this study, we demonstrate that risk factors for pancreatic cancer, such as diabetes, obesity, and pancreatitis, specifically stimulate PDGs. These risk factors increase the number of 5-bromo-2′-deoxyuridine (BrdU) label−retaining cell population in PDGs and induce characteristic features of pancreatic tumors, such as nuclear localization of S100P or the production of Muc5ac.

Materials and Methods

Animals

Male B6.V-Lep$^{ob/ob}$ mice were compared with male lean B6.V-Lep$^{+/?}$ littermates. The strain was originally purchased from The Jackson Laboratory (Bar Harbor, ME). The average ± SD age of mice between the cohorts was similar on day 71 of the experimental protocol (lean sham, 151 ± 116 days; lean cerulein (Cer), 154 ± 59 days; obese sham, 194 ± 84 days; obese Cer, 171 ± 75 days), and no correlation between age and the percentage of BrdU$^+$ cells or nuclear S100P staining in PDGs was observed. Animals were kept on water and standard laboratory chow ad libitum. All experiments were authorized by the local animal welfare committee and were realized in accordance with the German legislation and the principles of laboratory animal care (NIH publication 85-23, revised 1985).

Induction of Chronic Pancreatitis and Analysis of Blood Samples

Chronic pancreatitis was induced over 10 weeks by administration of three i.p. injections of 50 μg/kg cerulein (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 3 days a week, at a rate of one every hour per day. Control mice were sham treated appropriately with 0.9% saline solution instead of cerulein. For pain relief, 800 mg/L metamizol (Ratiopharm GmbH, Ulm, Germany) was added to the drinking water from day 1 to 71. The BrdU label−retaining technique was used to characterize cells that incorporate BrdU and retain this analog of thymidine until day 71, by adding BrdU to the drinking water from day 1 to 7 (1 g/L; Sigma-Aldrich Chemie GmbH). For blood samples and organ extraction, animals were anesthetized with 75 mg/kg ketamine (bela-pharm, Vechta, Germany) and 5 mg/kg xylazine (Bayer Health Care, Leverkusen, Germany).

Blood samples were taken on day 5, 2 hours after the last cerulein injection, and on day 71. The concentration of c-peptide in plasma was quantified on day 5 with the BioVendor Mouse C-Peptide ELISA Kit (BioVendor, Shibukawa, Japan). The activity of amylase and lipase in plasma was analyzed on days 5 and 71 using the cobas c111 spectrophotometer (Roche Diagnostics GmbH, Mannheim, Germany). A differential blood cell count was performed on day 71 with an automated hematology analyzer Sysmex KX 21 (Sysmex Cooperation, Kobe, Japan). For the monitoring of diabetes, the blood glucose concentration was measured weekly with the blood glucose meter Contour (Bayer Vital GmbH, Leverkusen, Germany).

Histological and Immunofluorescent Staining

On day 71 (ie, 72 hours after the last cerulein injection), the proximal half of the pancreas was placed lengthwise in a biopsy cassette, fixed in 4% phosphate-buffered formalin, and embedded in paraffin; sections (4 μm thick) of the pancreatic main duct were prepared. Serial sections in H&E stain were obtained from one animal. To assess the mucin production, PAS staining was performed. BrdU incorporation was quantified with antibodies detecting mouse bromodeoxyuridine (clone, Bu20a; dilution, 1:50) with an Alcian Blue counterstain for the detection of PDGs. Immunochemistry for Muc5ac was performed using the goat anti-mouse

Muc5ac antibody (sc-16903; dilution, 1:600; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For 14-3-3 σ immunochemistry, a goat anti-mouse antibody (ab77187; dilution, 1:800; Abcamplc, Cambridge, UK), for PSCA immunochemistry, a rabbit anti-mouse antibody (251249; dilution, 1:600; Abbiotec, LLC, San Diego, CA), for Reg3β immunochemistry, a rat anti-mouse antibody (MAB5110; dilution, 1:800; R&D Systems Inc., Minneapolis, MN), and for S100P immunochemistry, a rabbit anti-mouse antibody (ab86877; prediluted; Abcamplc), were used. DNA damage was assessed with the ApopTag Plus Peroxidase *in situ* detection kit (Millipore, Eschborn, Germany). Immunofluorescence was performed with a mouse anti-proliferating cell nuclear antigen (PCNA) antibody (ab29; dilution, 1:1000; Abcamplc), a mouse anti-S100P antibody (ab86877; dilution, 1:5; Abcamplc), and TOPRO-3 (Invitrogen, Carlsbad, CA).

The histological evaluation was performed using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan), and the images were taken with cell D 2.5 (Olympus Soft Imaging Solutions GmbH, Hamburg, Germany). All immunohistochemical quantifications of nuclei, cytoplasm, or cell number in PDGs were performed with a 100× objective. Immunofluorescence was evaluated on scanned sections (2 μm thick) with a Zeiss LSM 780 confocal laser scanning microscope using a 63× objective (Carl Zeiss Microscopy GmbH, Jena, Germany).

Statistical Analysis

Data are presented as box plots indicating the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. Significance tests and evaluations of correlations were performed with SigmaStat software version 3.5 (Jandel Corporation, San Rafael, CA). Differences between groups were calculated using a Mann-Whitney rank-sum test, followed by the correction for the accumulation of the α error by considering the number of meaningful comparisons. Differences with $P < 0.05$, divided by the number of meaningful comparisons, were considered to be significant. Differences with $P < 0.08$, divided by the number of meaningful comparisons were considered to indicate a tendency.

Results

We compared obese mice (B6.V-Lep$^{ob/ob}$) with lean littermates after the induction of chronic pancreatitis by

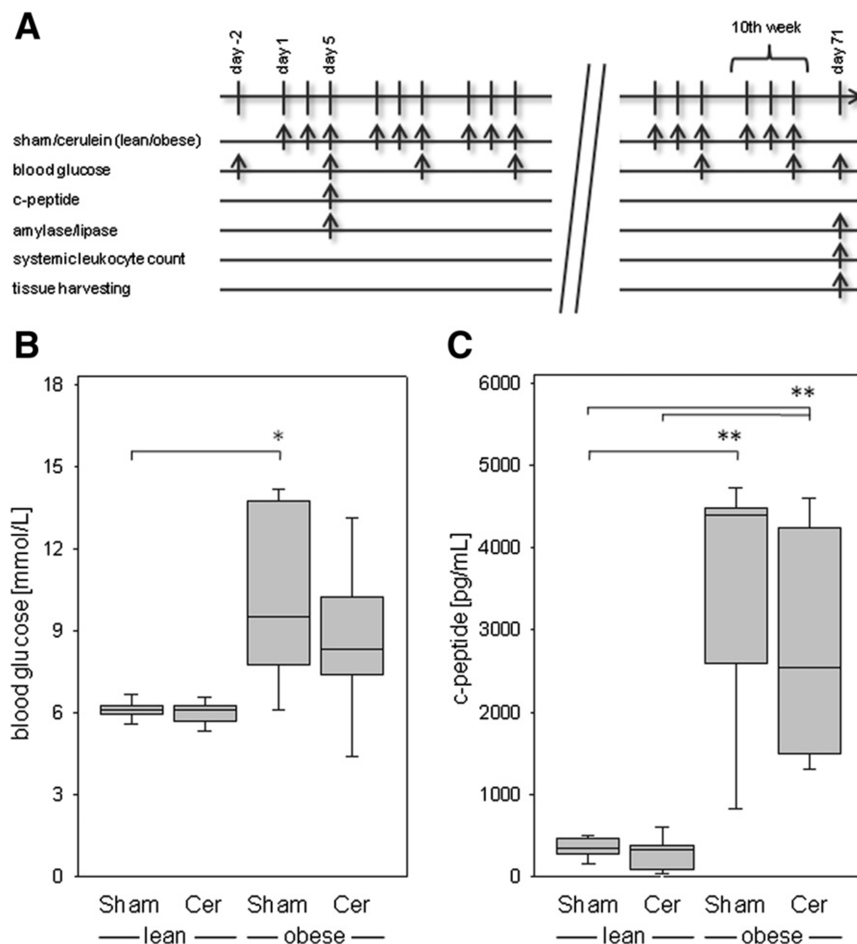

Figure 1 Experimental protocol and characterization of the diabetes type 2–like condition of obese mice. **A:** Chronic pancreatitis was induced by i.p. injection of cerulein in lean B6.V-Lep$^{+/?}$ (lean Cer) and B6.V-Lep$^{ob/ob}$ (obese Cer) cohorts of mice, three times a day, at a rate of one every hour on the indicated days. Control lean (lean Sham) and obese (obese Sham) mice received 0.9% saline solution each time instead of cerulein. The blood glucose concentration was measured once a week. Amylase and lipase activities were analyzed on day 5 at 2 hours after last cerulein injection and on day 71. Systemic leukocyte count and tissue harvesting were performed on day 71. **B:** The average blood glucose concentration of the cohorts from day −2 to 71 is given. **C:** The c-peptide concentrations in plasma on day 5 at 2 hours after the last cerulein injection are shown. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers (lean Sham, $n = 10$; lean Cer, $n = 10$; obese Sham, $n = 10$; obese Cer, $n = 10$). Significant differences between the cohorts are indicated, $^{*}P < 0.01$ (**B**); $^{**}P = 0.002$ (**C**).

cerulein or sham treatment (Figure 1A). The diabetes type 2−like condition of obese mice was characterized by a higher blood glucose concentration compared with lean littermates (Figure 1B) and increased insulin production measured as c-peptide concentration in blood plasma (Figure 1C).

As quality control for the induction of chronic pancreatitis, we evaluated amylase activity, lipase activity, number of leukocytes in the blood, and pancreas atrophy. Amylase activity on day 5 was significantly higher in cerulein-treated mice than in sham-treated animals, but normalized on day 71 (Figure 2A). We also noticed higher basal amylase activity in obese mice on days 5 and 71 when compared with lean littermates. Lipase activity was also significantly higher in cerulein-treated mice than in sham-treated animals on day 5 (Figure 2B). The number of leukocytes in blood decreased in cerulein-treated mice compared with sham-treated animals, possibly because of enhanced migration of leukocytes into the inflamed pancreas (Figure 2C). The pancreatic mass was also significantly reduced by cerulein treatment (Figure 2D).

Representative serial sections stained by H&E highlight that PDGs are out pouches of the pancreatic duct and not just branching ducts (Supplemental Figure S1). In addition, mucin secretion as one of the characteristics of PDGs was assessed by PAS staining. PAS^+ PDGs were observed close to the pancreatic main duct in obese and lean mice. However, the intensity of PAS^+ staining was increased in cerulein-treated mice (Figure 3A). Interestingly, PAS^+

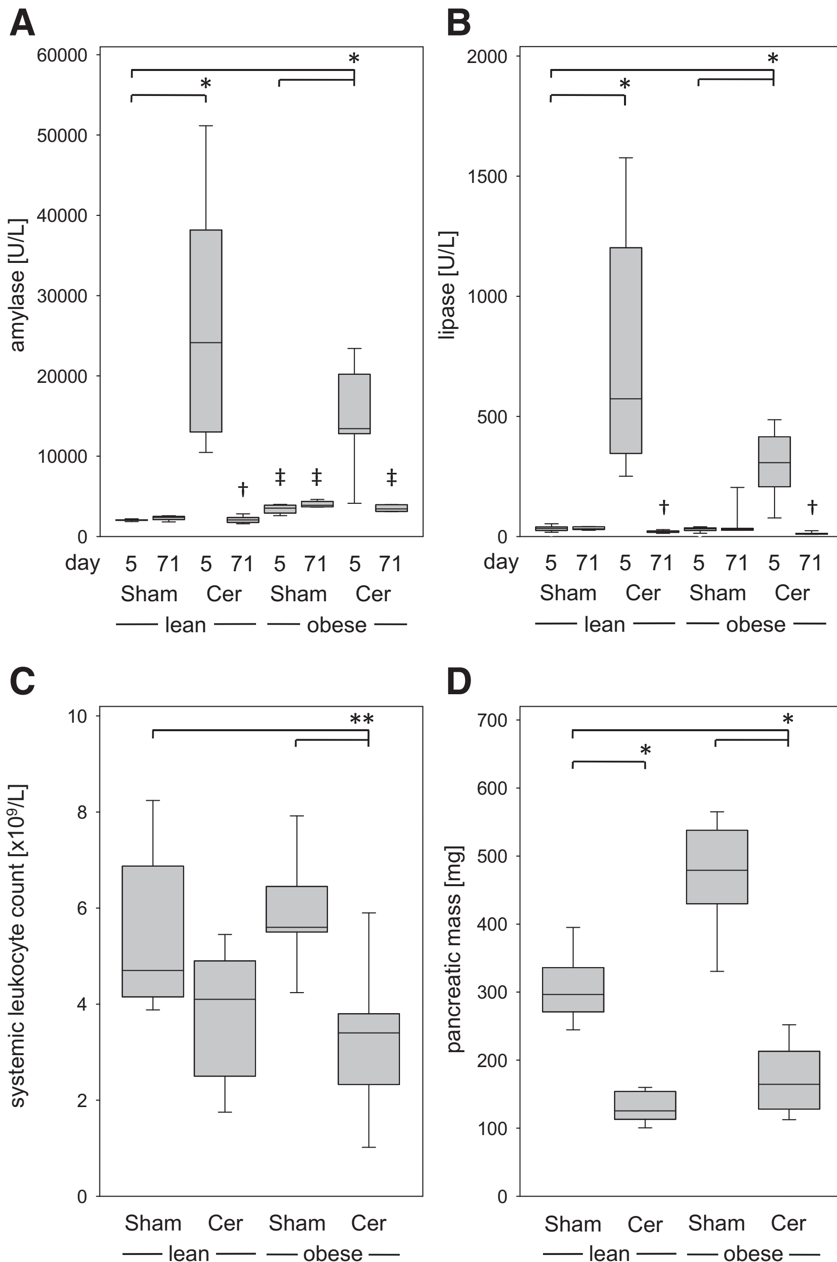

Figure 2 Repetitive cerulein administration leads to chronic pancreatitis. **A** and **B**: Amylase (**A**) and lipase (**B**) activities were determined on days 5 and 71 ($n = 10$ for each cohort). The observed decrease in enzyme activities after 10 weeks of pancreatitis is typical for chronic pancreatitis. **C**: On day 71, cerulein treatment decreased the systemic leukocyte count (lean Sham, $n = 9$; lean Cer, $n = 10$; obese Sham, $n = 8$; obese Cer, $n = 8$). **D**: Cerulein treatment decreased the pancreatic mass on day 71 ($n = 10$ for each cohort). Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. Significant differences between the indicated cohorts are noted $*P \leq 0.001$ (**A**, **B**, and **D**); $**P \leq 0.009$ (**C**); $^{\dagger}P \leq 0.001$ between days 5 and 71 of the same cohort; $^{\ddagger}P = 0.002$ between obese mice and identically treated lean mice on the same day.

structures were also observed distal to the pancreatic main duct. Cerulein-treated mice also had PAS^{+} out pouches in small peripheral ducts (Figure 3B). In addition, PAS^{+} distal structures, which look histologically different from PDGs, were noticed (Figure 3C). These structures could be the result of ADM. In cerulein-treated mice, we observed more peripheral PAS^{+} PDGs and ADMs when compared with sham-treated mice (Figure 3, D and E).

Some cells, such as adult stem cells, often proliferate slowly *in vivo* and, thus, retain certain incorporated labels over a long period.[35,36] To evaluate whether PDGs contain a pool of label-retaining cells, BrdU incorporation during the first week of chronic pancreatitis was quantified on day 71 (Figure 4, A and B). The percentage of BrdU^{+} cell nuclei was significantly higher in cerulein-treated mice than in sham-treated animals. Interestingly, BrdU incorporation after cerulein treatment was significantly higher in obese mice than in lean mice (Figure 4C). In contrast, the rate of BrdU incorporation in epithelial cells of the main duct was independent of genotype or treatment of the mice (Figure 4D). BrdU incorporation might be caused by proliferation or DNA damage, followed by DNA repair during the first week of chronic pancreatitis. Evaluation of DNA damage by an ApopTag kit, however, did not reveal DNA damage in PDGs, although DNA damage was readily detected in cells adjacent to PDGs (data not shown).

Some BrdU^{+} PDGs also produced Muc5ac, which characterizes beginning metaplasia (Supplemental Figure S2). The detection of Muc5ac by immunochemistry revealed some Muc5ac^{+} PDGs independent of genotype or treatment of mice (Figure 5A). However, an increased percentage of Muc5ac^{+} PDGs ($P = 0.015$) was observed in cerulein-treated obese

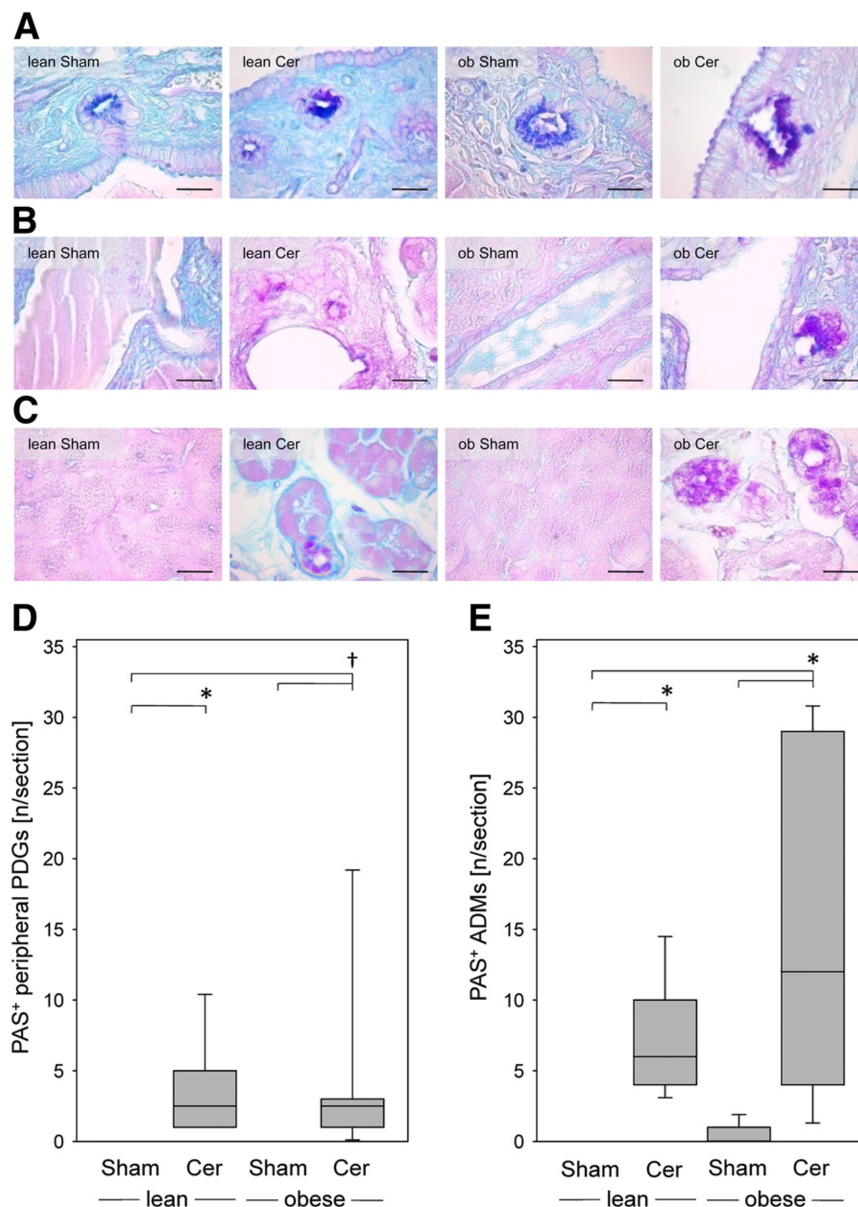

Figure 3 PAS^{+} structures demark higher mucin production in cerulein-treated mice. **A:** PAS^{+} PDGs adjacent to the pancreatic main duct are shown. The intensity increases with cerulein treatment. **B:** PAS^{+} PDGs in small peripheral ducts could be found only in cerulein-treated mice. **C:** PAS^{+} acinar structures are mainly observed in cerulein-treated mice. These structures do not resemble PDGs, but have similar histological characteristics to ADM. **D:** The number of PAS^{+} PDGs located at small peripheral ducts is only detectable in cerulein-treated mice. **E:** The number of PAS^{+} ADMs is significantly increased in cerulein-treated obese (ob) mice compared with sham-treated obese mice. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers ($n = 6$ for each cohort). Significant differences between the indicated cohorts are noted, $^{*}P \leq 0.004$. Tendentious differences between the indicated cohorts are also noted, $^{\dagger}P = 0.015$. Scale bar = 20 μm.

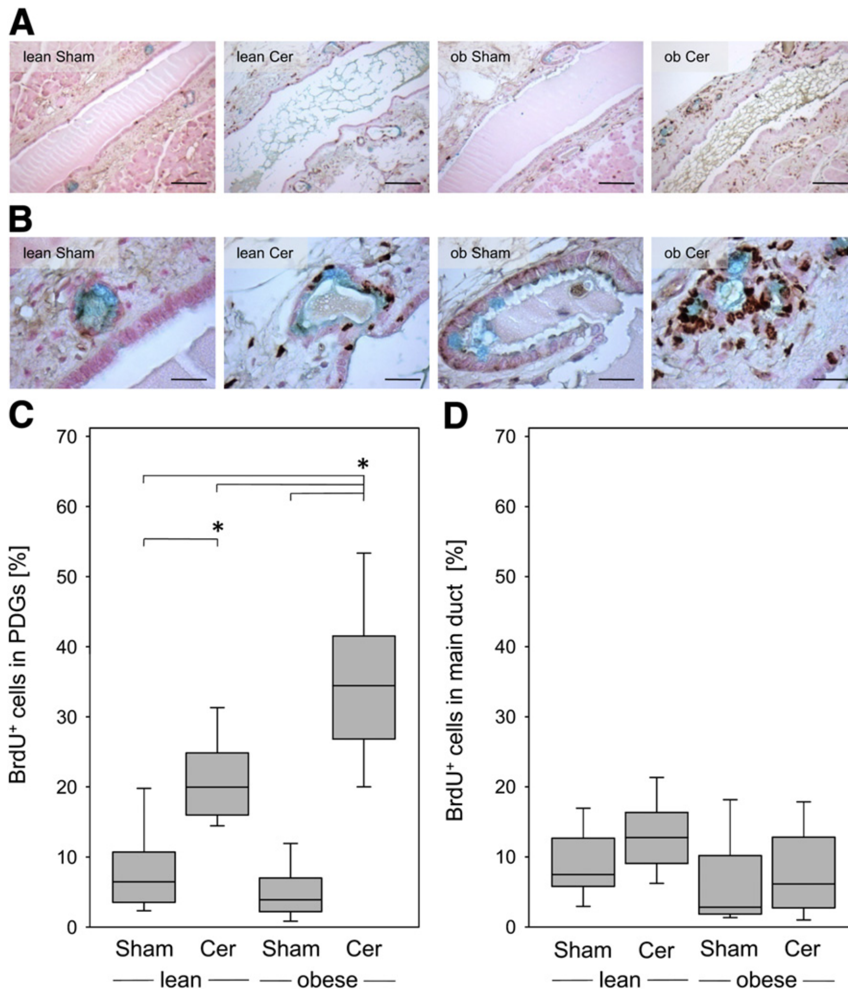

Figure 4 Diabetes and chronic pancreatitis specifically induce BrdU incorporation in PDGs. BrdU incorporation in nuclei was evaluated with antibodies detecting BrdU. The PDGs were identified with Alcian Blue counterstain. **A:** An overview over the pancreatic main duct with PDGs displays modest BrdU incorporation. Scale bar = 100 μm. **B:** In contrast to the duct, PDGs show an increased incorporation of BrdU after cerulein treatment. Scale bar = 20 μm. **C:** Quantification of the percentage of $BrdU^+$ cell nuclei in all PDGs adjacent to the main duct. **D:** Quantification of the percentage of $BrdU^+$ cell nuclei in the epithelium of the main duct. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers [lean Sham, $n = 9$; lean Cer, $n = 10$; obese (ob) Sham, $n = 10$; obese Cer, $n = 10$]. Significant differences between the indicated cohorts are noted, $^*P \leq 0.009$.

mice compared with cerulein-treated lean mice (Figure 5B). Moreover, cerulein-treated obese mice have a significantly higher percentage of $Muc5ac^+$ PDGs than sham-treated lean or sham-treated obese mice (Figure 5B).

To evaluate the expression of pancreatic cancer–specific genes in PDGs, we performed immunochemical analysis. 14-3-3 σ localized at a similar intensity in the cytoplasm of all cohorts (Figure 6A), and PSCA production also did not differ between the cohorts (data not shown). Reg3β was produced in the cytoplasm of PDGs, especially in cerulein-treated mice (Figure 6B). A modest increase in the percentage of Reg3β-producing cells in PDGs was observed in cerulein-treated animals (Figure 6D). In addition, cerulein-treated mice had more $S100P^+$ nuclei in PDGs compared with sham-treated animals. Moreover, a higher intensity of S100P staining was noticed in cerulein-treated mice (Figure 6C). The percentage of $S100P^+$ nuclei in PDGs of cerulein-treated obese mice was significantly higher compared with sham-treated obese mice and with sham- or cerulein-treated lean littermates (Figure 6E). To evaluate whether nuclear localization of S100P and proliferation are part of a coordinated response to risk factors for PDA, we analyzed the colocalization of S100P with the PCNA in PDGs. No significant difference in the percentage of PCNA-positive cells was observed between distinct cohorts, possibly because of the few PCNA-positive cells detected. Some, but not all, of these PCNA-positive cells had S100P-positive nuclei (Supplemental Figure S3, A–D). Consequently, no correlation was observed between the expression of these two proteins (Supplemental Figure S3E).

Discussion

Understanding of pancreatic cancer precursor lesions is important because early detection of such lesions could provide new therapeutic options at a time point when this disease is still curable. We observed that the newly discovered PDGs are stimulated by several established risk factors for pancreatic cancer. Chronic pancreatitis and type 2 diabetes–like syndrome in obese mice induced BrdU incorporation specifically in PDGs, but not in the epithelium of the pancreatic main duct. Furthermore, both risk factors induced characteristic features of pancreatic tumors, such as nuclear localization of S100P and production of Muc5ac in PDGs. However, we cannot differentiate if diabetes, obesity,

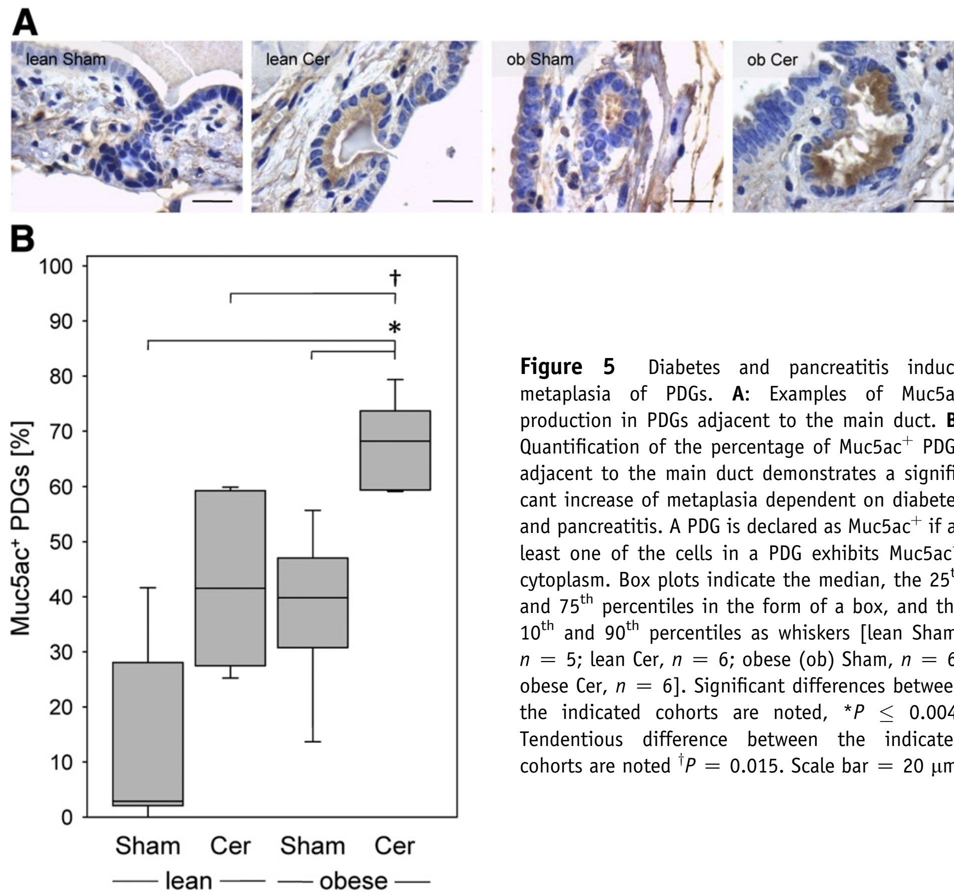

Figure 5 Diabetes and pancreatitis induce metaplasia of PDGs. **A:** Examples of Muc5ac production in PDGs adjacent to the main duct. **B:** Quantification of the percentage of $Muc5ac^+$ PDGs adjacent to the main duct demonstrates a significant increase of metaplasia dependent on diabetes and pancreatitis. A PDG is declared as $Muc5ac^+$ if at least one of the cells in a PDG exhibits $Muc5ac^+$ cytoplasm. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers [lean Sham, $n = 5$; lean Cer, $n = 6$; obese (ob) Sham, $n = 6$; obese Cer, $n = 6$]. Significant differences between the indicated cohorts are noted, $^*P \leq 0.004$. Tendentious difference between the indicated cohorts are noted $^†P = 0.015$. Scale bar = 20 μm.

lipotoxicity, or a combination of these conditions alters the physiological characteristics of PDGs in obese mice. In addition, the *Lep*$^{ob/ob}$ genotype alone had no major influence on the physiological characteristics of PDGs. Compared with lean littermates, Muc5ac expression, BrdU incorporation, and nuclear localization of S100P were observed in PDGs of obese mice primarily after stimulation with cerulein. Moreover, we observed increased BrdU incorporation, when applying BrdU during the first week of chronic pancreatitis, but no increased number of $PCNA^+$ cells on day 71 of chronic pancreatitis. This result might be explained by modest proliferation of epithelial cells, which requires several days of BrdU labeling to detect differences in the proliferation capacity, or proliferation not stimulated by inflammatory stimuli any more on day 71. Such a burnout phenomenon of the pancreas is also supported by the analysis of lipase and amylase activity, as presented in Figure 2, A and B. Thus, we identified a BrdU label−retaining cell population in PDGs that either proliferates slowly, similar to adult stem cells in other tissues, or even becomes postmitotic after the first week of cerulein treatment.[35,36] Alternatively, it might also be feasible that BrdU might be incorporated in these cells by DNA repair during the first week of pancreatitis. In summary, these data suggest that a population of BrdU label−retaining cells within PDGs is influenced by risk factors for pancreatic adenocarcinoma. Pancreatic tumors might, therefore, derive from these histomorphological structures, called PDGs.

However, it is not known if PDGs are the origin for PDA or IPMNs.[19−21] The following reasons underline that PDGs could produce IPMNs. IPMNs are similar to PDGs predominantly associated with the pancreatic main duct or its branches.[15] The expression of sonic hedgehog seems to be an important step in the development of IPMNs[37,38] and can also be induced in PDGs.[19] Thus, PDGs might be precursor lesions for IPMNs. However, we interpret our data that PDGs are more likely the origin for PDA rather than IPMNs for the following reasons: i) our study demonstrates the expression of 14-3-3 σ, a protein typically expressed in PDA, in PDGs, ii) Reg3β, another protein typically produced in PDA, is induced in PDGs, and iii) diabetes is a risk factor mainly for PDA.[2] Because Muc5ac and S100P are detected in IPMNs and PDA, both proteins indicate first progression toward neoplasia, but cannot be used to distinguish between these two tumor entities. However, only specific lineage-tracing studies in mice could finally reveal if PDGs develop toward PDA or IPMNs.

It is widely accepted that PanINs are precursor lesions for PDA. Several facts support this hypothesis. For example, PanINs display metaplastic, hyperplastic, or dysplastic alterations similar to histological alterations in PDAs.[39] In addition, genetically engineered mouse models (eg, using *Pdx1-Cre;LSL-Kras*^{G12D} mice) develop first PanINs and, at a later time point, PDA, suggesting a PanIN to PDA model of pancreatic cancer progression.[16] Moreover, patients with

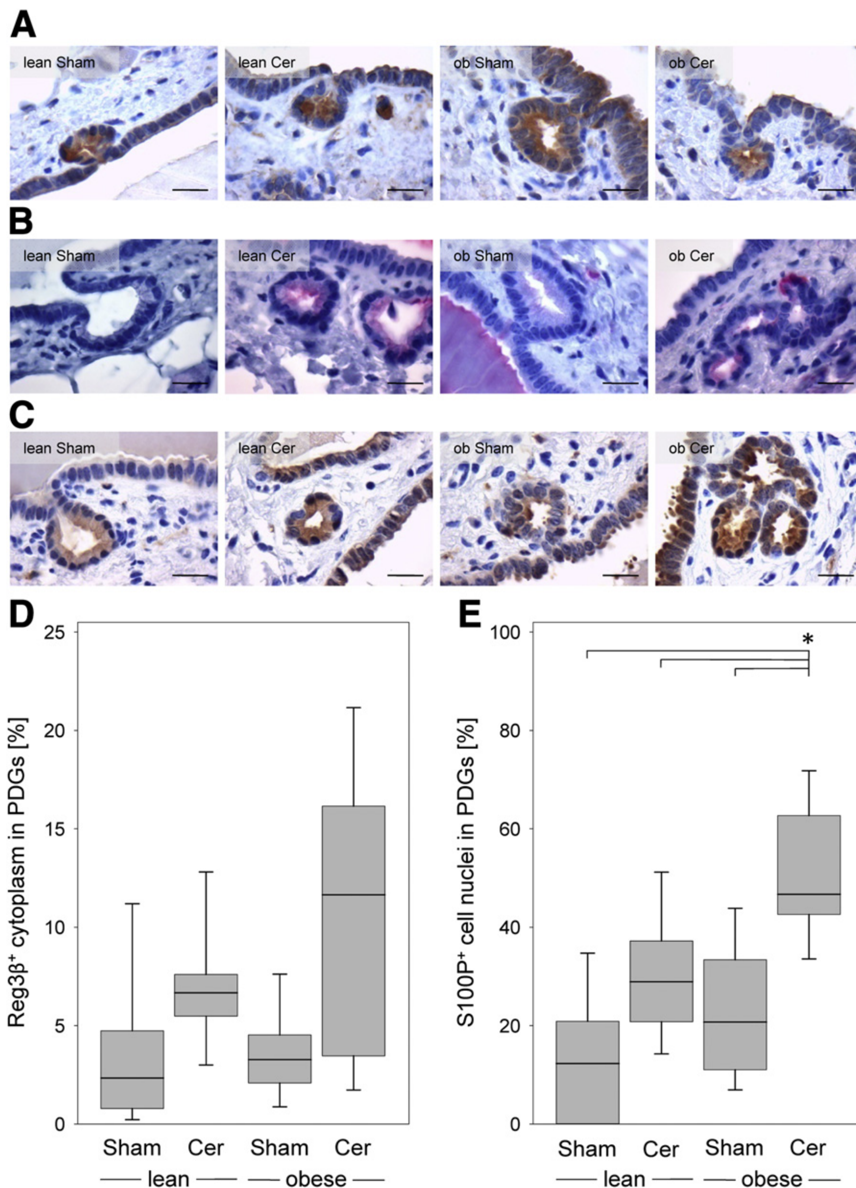

Figure 6 Production of tumor-specific proteins in PDGs. **A:** The PDGs of all cohorts produce 14-3-3 σ mainly in the cytoplasm and rarely in the nuclei. **B:** Reg3β localization in the cytoplasm was increased in cerulein-treated lean or obese (ob) mice. **C:** Diabetes and chronic pancreatitis increase S100P localized in the nuclei of PDGs. **D:** Quantification of the percentage of $Reg3\beta^+$ cytoplasm in PDGs adjacent to the main duct. **E:** Quantification of the percentage of $S100P^+$ cells in PDGs adjacent to the main duct. A cell was declared $S100P^+$ if the nucleus was $S100P^+$. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers (lean Sham, $n = 9$; lean Cer, $n = 10$; obese Sham, $n = 9$ to 10; obese Cer, $n = 10$). Significant differences between the indicated cohorts are noted, $^*P \leq 0.006$. Scale bar = 20 μm.

a strong family history for pancreatic cancer show PanIN lesions before some of them develop cancer.[40]

In contrast, several studies cast doubt on the hypothesis that pancreatic tumors originate from PanINs. The pure histomorphological ductal phenotype of the PDA and the expression of ductal markers do not allow a conclusion to a ductal origin. In approximately 80% of patients with small invasive carcinoma, high-grade PanINs are only observed within 10 mm from the infiltrating carcinoma. This suggests that high-grade PanINs might be just intraductal extensions of the tumor, rather than a residual precursor lesion.[41] The interpretation that PanINs could be the response of the duct epithelium to a process of neoplasia in the pancreas, rather than a precursor lesion, is also supported by the circumstance that PanINs are observed in patients with pancreatic endocrine tumors or acinar cell carcinomas.[42] Moreover, because PanIN-1 is regularly detected in young individuals, it might not be associated with an increased risk of developing pancreatic cancer.[43] A correlation between the PDA risk factor, pancreatitis, and the development of high-grade PanINs was only observed in two studies, whereas most studies could not confirm this observation.[39] Furthermore, there is also no correlation between the PDA risk factors, smoking, coffee consumption, and diabetes, and the incidence of PanINs.[43]

These arguments raise doubt if PDA originates exclusively from PanINs. In contrast to PanINs, we could demonstrate that PDGs are susceptible to diabetes and pancreatitis. In addition, PDGs are predominantly localized in the head or neck of the pancreas, where 80% of PDA is localized.[19,44] Thus, these circumstances suggest that PDA might be derived from the ductal compartment called PDGs. This hypothesis is also supported by the observation that cells with stem cell character may reside within the duct epithelium.[45] PDGs seem to have features of stem cell niches, such as

expression of Shh, Pdx-1, and Hes-1,[19–21] which are characteristic during embryonic development of the pancreas. Because the epithelium of PDGs can show PanIN-like features, such as pseudostratification, loss of polarity, and micropapillary architecture,[20] we cannot exclude that PanINs or PDGs might not represent mutual exclusive origins of PDA. Thus, a progression from PDG to PanIN-like structures to PDA might also be feasible.

Lately, multiple studies have focused on the question if treatment with distinct antidiabetic medication correlates with increased risk for pancreatic cancer. The administration of insulin or other oral antidiabetic drugs has increased cancer risk.[46] Interestingly, our study demonstrates increased BrdU incorporation and metaplasia of PDGs in hyperinsulinemic obese mice. This is consistent with the hypothesis that insulin might increase cancer risk by stimulating PDGs. This conclusion is also supported by another recently published study demonstrating that exendin-4, a glucagon-like peptide-1 analog, stimulates PDGs toward dysplasia.[20] Thus, it is crucial to pay attention on focal alterations in PDGs to evaluate adverse drug effects, such as increased cancer risk.

Early diagnosis and timely treatment of precursor lesions before their progression to invasive cancer seems feasible, because 5 years pass from the start of the parental, nonmetastatic founder cell, until the acquisition of metastatic ability.[47] For example, the observed pathological changes in PDGs, such as Reg3β production, might provide the option to diagnose pancreatic cancer at an early stage. In this context, a novel positron emission tomographic/computed tomographic diagnostic option with Et-[^{18}F]FDL was developed for the detection of Reg3β overexpression in early pancreatic carcinomas.[48] Such screening methods could be rationally applied in older individuals with new-onset diabetes, because up to 80% of patients with a pancreatic cancer diagnosis experience diabetes or impaired glucose tolerance, and most of these patients have just recently developed diabetes.[14,49]

In summary, PDGs represent a promising histomorphological structure that might be the origin for pancreatic tumors. The characterization of PDGs and the evaluation of progression toward malignancy might lead to a better understanding and, in the long run, to timely diagnostic options for pancreatic cancer.

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Supplemental Data

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Anhang - Teil 6

Impact of diabetes type II and chronic inflammation
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Impact of diabetes type II and chronic inflammation on pancreatic cancer

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Abstract

Background: We explored if known risk factors for pancreatic cancer such as type II diabetes and chronic inflammation, influence the pathophysiology of an established primary tumor in the pancreas and if administration of metformin has an impact on tumor growth.

Methods: Pancreatic carcinomas were assessed in a syngeneic orthotopic pancreas adenocarcinoma model after injection of 6606PDA cells in the pancreas head of either B6.V-Lep$^{ob/ob}$ mice exhibiting a type II diabetes-like syndrome or normoglycemic mice. Chronic pancreatitis was then induced by repetitive administration of cerulein. Cell proliferation, cell death, inflammation and the expression of cancer stem cell markers within the carcinomas was evaluated by immunohistochemistry. In addition, the impact of the antidiabetic drug, metformin, on the pathophysiology of the tumor was assessed.

Results: Diabetic mice developed pancreatic ductal adenocarcinomas with significantly increased tumor weight when compared to normoglycemic littermates. Diabetes caused increased proliferation of cancer cells, but did not inhibit cancer cell necrosis or apoptosis. Diabetes also reduced the number of Aldh1 expressing cancer cells and moderately decreased the number of tumor infiltrating chloracetate esterase positive granulocytes. The administration of metformin reduced tumor weight as well as cancer cell proliferation. Chronic pancreatitis significantly diminished the pancreas weight and increased lipase activity in the blood, but only moderately increased tumor weight.

Conclusion: We conclude that diabetes type II has a fundamental influence on pancreatic ductal adenocarcinoma by stimulating cancer cell proliferation, while metformin inhibits cancer cell proliferation. Chronic inflammation had only a minor effect on the pathophysiology of an established adenocarcinoma.

Keywords: Cancer stem cells, Cancer heterogeneity, Cancer cell plasticity, Aldh1, CD133

Background

Pancreatic cancer is one of the most lethal malignancies. The 5-year survival rate is despite therapeutic improvements still only 6% [1]. More than 80% of the pancreatic tumors are classified as pancreatic ductal adenocarcinoma (PDA). Novel therapies, but also the knowledge about pathophysiological factors influencing the progression of this malignant disease might help to find combinations of treatments to improve the survival rate. Key pathophysiological processes of cancer such as recurrence after chemotherapy and metastasis have been suggested to depend on cancer cell plasticity [2]. A prominent albeit controversial hypothesis, describing one form of cancer cell plasticity, is the concept of the existence of cancer stem cells (CSC) [2]. Cancer stem cells (CSC) are assumed to proliferate slowly, to have the capacity to renew themselves but also to give rise to distinct cell populations [3,4]. In PDA these cells have been reported to express specific genes such as Aldh1 or CD133 [5-9].

Much is known about factors increasing the likelihood to develop PDA. Identified risk factors include among others chronic pancreatitis, long lasting diabetes, and obesity [10]. Patients with chronic and especially hereditary pancreatitis have a very high relative risk of developing

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pancreatic cancer of 13.3 and 69.0, respectively [11]. Patients with diabetes and obesity have a moderately increased relative risk of 1.8 and 1.3 [12,13]. These studies indicate that a substantial number of patients with PDA also suffer from local inflammation or diabetes [10,14].

While some experimental studies exist that demonstrate that pancreatitis and diabetes influence potential precursor lesion of PDA such as PanINs or pancreatic duct glands [15-18], it is not known, if these factors also influence the pathophysiology of established carcinomas.

In order to evaluate if diabetes type II and inflammation influence the pathophysiology of PDA, we established a syngeneic orthotopic tumor model in mice and addressed the questions, if pancreatitis or diabetes type II influence cancer cell proliferation, cancer cell death, tumor-stroma interaction or the cancer stem cell compartment in these carcinomas.

Methods

Cell lines and cell culture

The cell lines, 6606PDA, 6606l and 7265PDA were a kind gift from Prof. Tuveson, Cambridge, UK. The 6606PDA and 6606l cell lines were originally isolated from a pancreatic adenocarcinoma or the respective liver metastasis of a mouse with C57BL/6J background, which expressed the $KRAS^{G12D}$ oncogene in the pancreas (p48-cre induced expression of the oncogene) [19]. The 7265PDA cell line was isolated from a pancreatic adenocarcinoma of a mouse, which expressed the $KRAS^{G12D}$ oncogene and in addition the $p53^{R172H}$ allele in the pancreas (Pdx1-creER induced expression of the two alleles). All cell lines were maintained in DMEM high glucose medium with 10% fetal calf serum. For the injection of 6606PDA cells, subconfluent cultures of cells were trypsinized and the trypsinization was stopped by medium. After centrifugation the cells were resuspended in PBS, the suspension was mixed with an equal volume of Matrigel (BD Bioscience, San José, Calif., USA, Nr: 354248) and kept on ice (at a concentration of $1.25x10^7$ cells/ml) until injection [20]. For re-isolation of cells from carcinomas, tumors were isolated and cut up into small pieces. The pieces and outgrowing cells were cultivated in DMEM high glucose medium with 10% fetal calf serum.

Evaluation of cells

Western blots were performed by separating cell lysate on SDS polyacryl gels and transferring the proteins to a polyvinyldifluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). The membranes were blocked with 2.5% (wt/vol.) BSA or 5% (wt/vol.) milk powder (for the analysis of CD133) and incubated overnight at 4°C with a rabbit anti-ALDH1a1 (Cell Signaling, Boston, USA, code 12035, 1:1000), rat anti-CD133 (eBioscience Inc., San Diego, USA, code 14-1331, 1:500) or goat anti-GFAP (Abcam, Cambridge, UK, code ab53554,1:2000) antibody followed by incubation with a secondary peroxidase-linked anti-rabbit antibody (Cell Signaling, code 7074, 1:1000), anti-rat antibody (Santa Cruz Biotechnology, Santa Cruz, USA, code sc3823, dilution 1:10,000), or anti-goat (Santa Cruz Biotechnology, sc-2020, 1:20.000). For analysis of β-actin production, membranes were stripped, blocked by 2.5% (wt/vol.) BSA and incubated with mouse anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, code A5441, dilution 1:20000) followed by peroxidase-linked anti-mouse antibody (Sigma-Aldrich, USA; code A9044, dilution 1:60,000). Protein production was visualized by luminol-enhanced chemiluminescence (ECL plus; GE Healthcare, Munich, Germany) and digitalised with Chemi-Doc XRS System (Bio-Rad Laboratories, Munich, Germany). Signals were densitometrically assessed and corrected with the signal intensity of β-actin (Quantity One; Bio-Rad Laboratories).

For the analysis of CD133 mRNA by PCR total RNA from cells or kidney was isolated using a RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer‘s instructions. After a quality control of the isolated RNA by agarose gel electrophoresis first strand cDNA was synthesized by reverse transcription of 2 μg of total RNA using oligo(dT)18 primer (Biolabs, Frankfurt am Main, Germany) and Superscript II RNaseH-Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). After heat inactivation of the reverse transcriptase 1/20 of the cDNA was amplified (27 cycles: 94°C for 30, 68°C for 40, 72°C for 60 seconds) using CD133 specific primers (forward primer: CCCTCCAGCAAACAAGCAAC, reverse primer: ACAGCCGGAAGTAAGAGCAC) and the PCR product of 325 bp was visualized by agarose gel electrophoresis.

For the quantification of cell proliferation rates, cells were plated on 96 well plates, so that the cells were 20% confluent, when BrdU was added to the medium. The BrdU incorporation was measured after 24 hours of incubation by the colorimetric cell proliferation assay as specified by the manufacturer (Roche Applied Science, Penzberg, Germany).

Animals

For this study male B6.V-$Lep^{ob/ob}$ mice (obese mice) were compared with male B6.V-$Lep^{+/?}$ littermates (lean mice). The therapy with metformin was performed on male C57BL/6J mice. The mouse strains were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our local animal facility. For defining the border between carcinoma and the desmoplastic reaction, carcinoma cells were injected in the pancreas of C57BL6-$Tg^{ACTB-eGFP1Osb/J}$ mice (with a corresponding phenotype to lean B6.V-$Lep^{+/?}$ mice) [21]. Animals were kept on water and standard laboratory chow ad libitum. All experiments were executed in accordance with the

EU-directive 2010/63/EU and approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (7221.3-1.1-069/12).

Syngeneic orthotopic carcinoma model

For injection of carcinoma cells general anesthesia was induced in 93 ± 32 day old mice (average ± standard deviation) by 1.2-2.5% isoflurane. Perioperative analgesia was ensured by sc injection of 5mg/kg carprofen (Rimadyl, Pfizer GmbH, Berlin, Germany) and eyes were protected by eye ointment. After shaving and disinfection of the skin, the abdominal cavity was opened by transverse laparotomy and the head of the pancreas was identified. Duodenum and pancreas was gently lifted by tweezers and 20 μl cell suspension containing $2.5x10^5$ carcinoma cells were injected slowly into the head of the pancreas using a precooled ga22s 710 RN 100 ul syringe (Hamilton Syringe, Reno, Nev., USA). The pancreas was placed back into the abdominal cavity and the cavity was closed by a coated 5-0 vicryl suture (Johnson & Johnson MEDICAL GmbH, Norderstedt, Germany). The skin was then closed by a 5-0 prolene suture (Johnson & Johnson MEDICAL GmbH). On day 8 after the injection of carcinoma cells, chronic pancreatitis was induced over 2 weeks by administration of three ip injections of 50 μg/kg cerulein (Sigma-Aldrich Chemie GmbH), 3 days a week, at a rate of one every hour per day. Control mice were sham treated appropriately with 0.9% saline solution instead of cerulein and tissues were analyzed on day 20. For the evaluation of the impact of metformin on cancer pathophysiology 250 mg/kg 1,1-dimethylbiguanide hydrochloride (Sigma-Aldrich, code 150959) was ip injected daily from day 8 to day 15 followed by daily injection of half of this dose from day 16 to day 29 and analysis of the tumor on day 29 (3-6 hours after the last metformin administration). Control mice were sham treated appropriately with PBS instead of metformin and tumors were analyzed on day 29. For pain relief, 800 mg/L metamizol (Ratiopharm GmbH, Ulm, Germany) was added to the drinking water during the entire timespan of all in vivo experiments. In order to assess cell proliferation 50 mg/kg 5-bromo-2-deoxyuridine (BrdU) was injected ip 2.5 hours before tissue asservation. For blood samples and organ harvest, animals were anesthetized with 90 mg/kg ketamine (bela-pharm, Vechta, Germany) and 7 mg/kg xylazine (Bayer Health Care, Leverkusen, Germany).

Analysis of the blood

Blood glucose concentrations were measured with the blood glucose meter Contour (Bayer Vital, Leverkusen, Germany) on day 0 before injection of carcinoma cells and on day 20 before the first cerulein injection of this day. Blood samples for assessing lipase activity were taken two hours after the third cerulein injection on day 8. The activity of lipase in blood plasma was analysed using the Cobas c111 spectrophotometer (Roche Diagnostics, Mannheim, Germany).

Evaluation of tissue

The pancreas and tumor weight was measured after careful separation of the carcinoma from the pancreas. Evaluation of CD133 expression was performed on 7 μm cryo-sections. These sections were fixed with 4% paraformaldehyde in PBS for 15 min, reactive groups were then quenched in 50 mM NH4Cl for 10 min and the cell membranes were permeabilised with 0.3% saponin in PBS for 15 min, before CD133 immunohistochemistry was performed. All other data were obtained on 4μm paraffin sections after fixing the tissue in 4% (wt/vol.) phosphate-buffered formalin for 2–3 days. Histology was evaluated after staining paraffin sections with haematoxylin and eosin (H/E). Planimetric analysis of necrotic areas was performed on 10 randomly chosen pictures (taken with a 20x objective) of each carcinoma by using Adobe Photoshop CS5 (Adobe, San Jose, CA, USA). Apoptosis was analysed using the ApopTag Plus Peroxidase in situ detection kit (Millipore, Eschborn, Germany). To evaluate the cellular inflammatory response to cerulein injection, naphthol AS-D chloroacetate esterase (CAE) staining was performed on sections. Cell proliferation, chronic pancreatitis, and desmoplastic reaction were evaluated by immunohistochemistry using mouse anti-BrdU (Dako, Hamburg, Germany, clone Bu20a, dilution 1:50), rabbit anti-collagen-I (Abcam, code ab 34710, dilution 1:200), or rabbit anti-α-smooth muscle actin (Abcam, code ab5694, dilution 1:800) antibody. To verify desmoplastic reaction by the host, carcinoma cells were assessed in GFP expressing mice with goat anti-GFP antibody (Gene Tex, San Antonio, Texas, USA, GTX26673, 1:500). Cancer cells were further characterized by immunohistochemistry using rabbit anti-ALDH1a1 (Cell Signaling, code 12035, 1:800), goat anti-GFAP (Abcam, code ab7260,1:2000) or rat–anti CD133 (a generous gift by Denis Corbeil, Dresden, Germany, 1:200). Additional immunohistochemistry was performed using rat-anti-cytokeratin 19 (The Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, USA, clone TROMA-III, dilution 1:50), rat anti-F4/80 (AbD Serotec, Oxford, UK, MCA497, 1:10) or goat anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, USA, sc7557, dilution 1:50) antibody. The following secondary antibodies were used: the Universal $LSAB^+$ Kit/HRP (Dako) for primary goat, rabbit or mouse antibodies or alkaline phosphatase conjugated anti-rat (Santa Cruz Biotechnology, sc2021, 1:200) antibody for primary rat antibodies. All quantifications of cells or of necrotic areas were performed 120 to 270 μm from the tumor margin.

Statistics

Data presentation and statistics were performed as described previously [15]. The significance of differences was evaluated using a Mann-Whitney rank-sum test, followed by the correction for the accumulation of the α error by considering the number of meaningful comparisons. Differences with $P \leq 0.05$, divided by the number of meaningful comparisons, were considered to be significant.

Results

Characterisation of the syngeneic orthotopic carcinoma model

To test whether diabetes, chronic pancreatitis or a combination of both influence the pathophysiology of a fully established PDA, we injected 6606PDA cells into the head of the pancreas in either diabetic mice (obese) or normoglycemic (lean) littermates (Figure 1A and B). Administration of cerulein (Cer) or saline (Sham) in both genotypes allowed us to compare pathophysiological parameters in carcinoma during pancreatitis (lean, Cer), diabetes (obese, Sham), or diabetes with concurrent pancreatitis (obese, Cer) to carcinoma in animals without diabetes or pancreatitis (lean, Sham). We observed that independent of treatment or genotype 100% of mice developed a carcinoma within 20 days. Histological analysis of the carcinomas revealed vital tissue with partial epithelial morphology, but also necrotic areas within the tumor (Figure 1C). Obese mice had significantly increased blood glucose concentrations, when compared to lean littermates (Figure 2A). Successful induction of pancreatitis by cerulein administration was verified by increased lipase activity and reduced pancreas weight in cerulein treated obese as well as lean mice when compared to sham treated controls (Figure 2B and C). In addition, cerulein administration causes the deposition of collagen I (Figure 2D) and the expression of α-smooth muscle actin in periacinar stellate cells (Figure 2E) of lean as well as obese mice when compared to sham treated animals. The induction of α-smooth muscle actin in cerulein treated lean mice, however, was weaker when compared to cerulein treated obese mice (Figure 2E).

Diabetes increases tumor size and proliferation of carcinoma cells

Within three weeks after the injection of adenocarcinoma cells in the pancreas diabetic obese mice developed

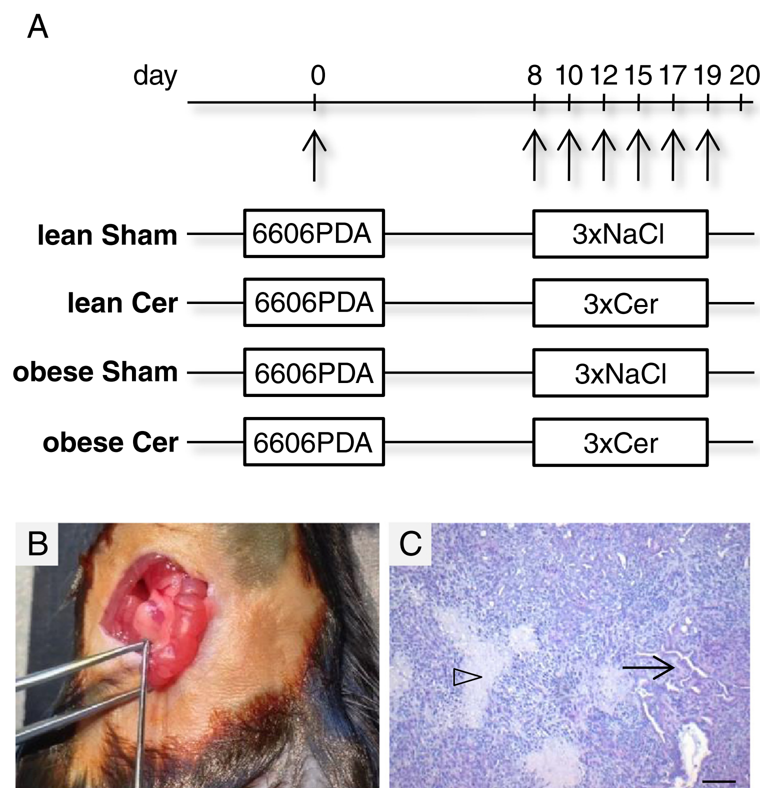

Figure 1 Characterisation of the syngeneic orthotopic PDA model. (A) 6606PDA cells were injected on day 0 into the head of the pancreas of non-diabetic (lean) or diabetic (obese) mice. Chronic pancreatitis was induced by ip injection of cerulein in non-diabetic (lean Cer) and diabetic (obese Cer) cohorts of mice three times a day on the indicated days, whereas control non-diabetic (lean Sham) and diabetic (obese Sham) mice received 0.9% saline solution. Tissue samples were analyzed on day 20. **(B)** The correct injection of carcinoma cells could macroscopically be verified. **(C)** A representative histology of a PDA reveals necrotic areas (arrowhead), but also vital cells with partially epithelial morphology (arrow). Bar = 50 μm.

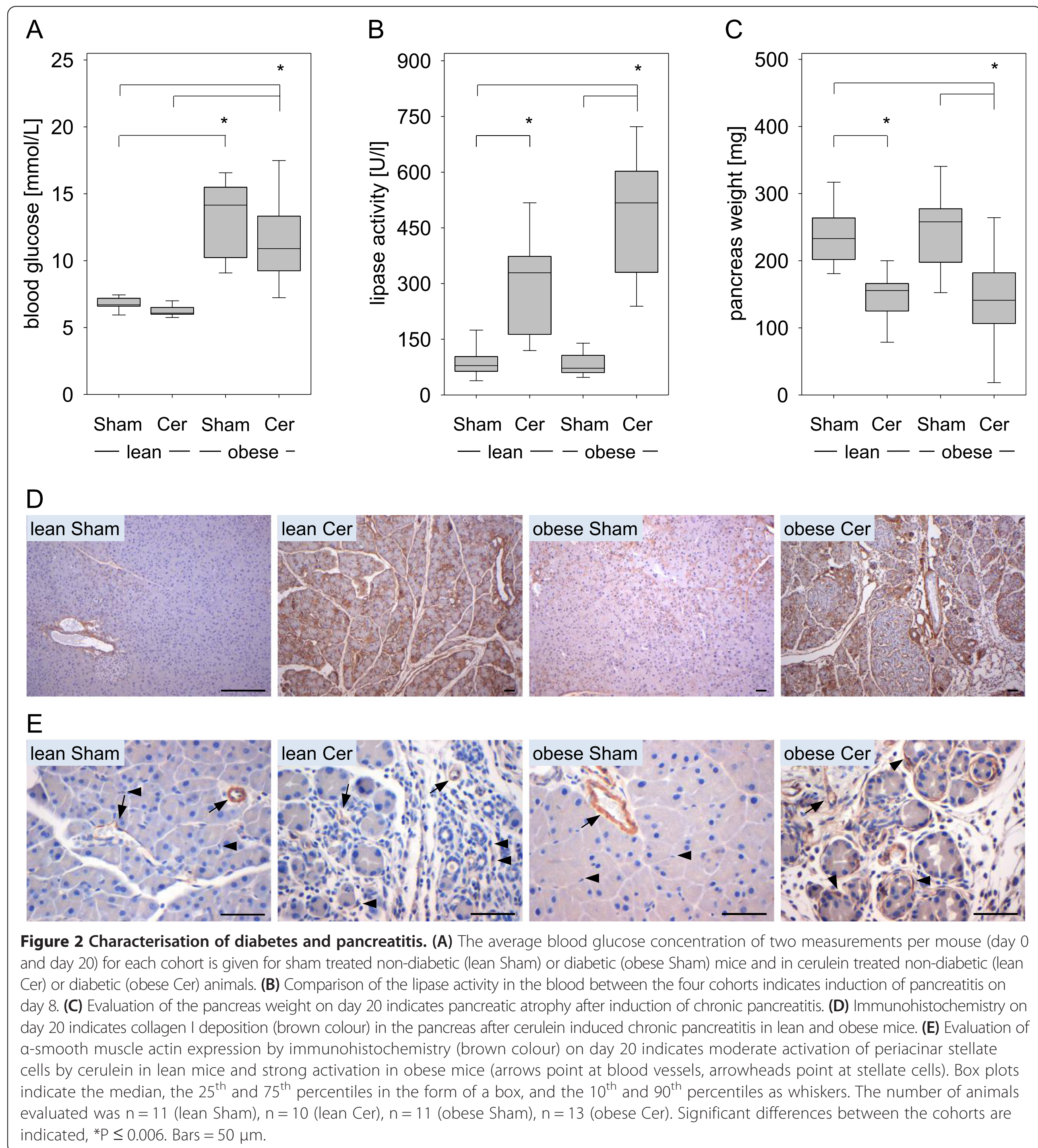

Figure 2 Characterisation of diabetes and pancreatitis. (A) The average blood glucose concentration of two measurements per mouse (day 0 and day 20) for each cohort is given for sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. **(B)** Comparison of the lipase activity in the blood between the four cohorts indicates induction of pancreatitis on day 8. **(C)** Evaluation of the pancreas weight on day 20 indicates pancreatic atrophy after induction of chronic pancreatitis. **(D)** Immunohistochemistry on day 20 indicates collagen I deposition (brown colour) in the pancreas after cerulein induced chronic pancreatitis in lean and obese mice. **(E)** Evaluation of α-smooth muscle actin expression by immunohistochemistry (brown colour) on day 20 indicates moderate activation of periacinar stellate cells by cerulein in lean mice and strong activation in obese mice (arrows point at blood vessels, arrowheads point at stellate cells). Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 11 (lean Sham), n = 10 (lean Cer), n = 11 (obese Sham), n = 13 (obese Cer). Significant differences between the cohorts are indicated, *P ≤ 0.006. Bars = 50 μm.

tumors, which were obviously larger than the tumors in normoglycemic lean littermates (Figure 3A). Measuring the tumor weight revealed significantly larger carcinomas in sham treated obese mice, when compared to sham treated lean littermates (Figure 3B). Increased tumor weight was also observed in cerulein treated obese mice when compared to cerulein or sham treated lean littermates (Figure 3B). Only a moderate increase in tumor weight was observed in cerulein treated obese or lean mice when compared to the same genotype of mice, which received sham treatment (Figure 3B).

To evaluate if diabetes modulates proliferation of cancer cells, the number of BrdU$^+$ cells within the carcinoma were evaluated (Figure 3C). Proliferation of cancer cells was significantly increased in sham treated obese mice when compared to sham treated lean littermates (Figure 3D).

Increased proliferation was also observed in cerulein treated obese mice when compared to cerulein or sham treated lean littermates (Figure 3D). These data suggest that in mice with a diabetes type II like syndrome carcinoma cells have a higher proliferation rate resulting in increased tumor size. In order to evaluate, if the intrinsic growth ability of cancer cells changes permanently in obese mice, we re-isolated the cancer cells from carcinomas in lean and obese mice and compared their proliferation rate in vitro. Carcinoma cells, which were isolated from lean mice, had a very similar proliferation rate to carcinoma cells, which were isolated from obese mice (lean: 1.09/1.06-1.14, n = 3; obese: 1.06/0.96-1.21, n = 6), or 6606PDA cells, which were never injected in any animal (1.05/0.99-1.21, n = 7; median/interquartile range of BrdU incorporation measured by ELISA). Thus,

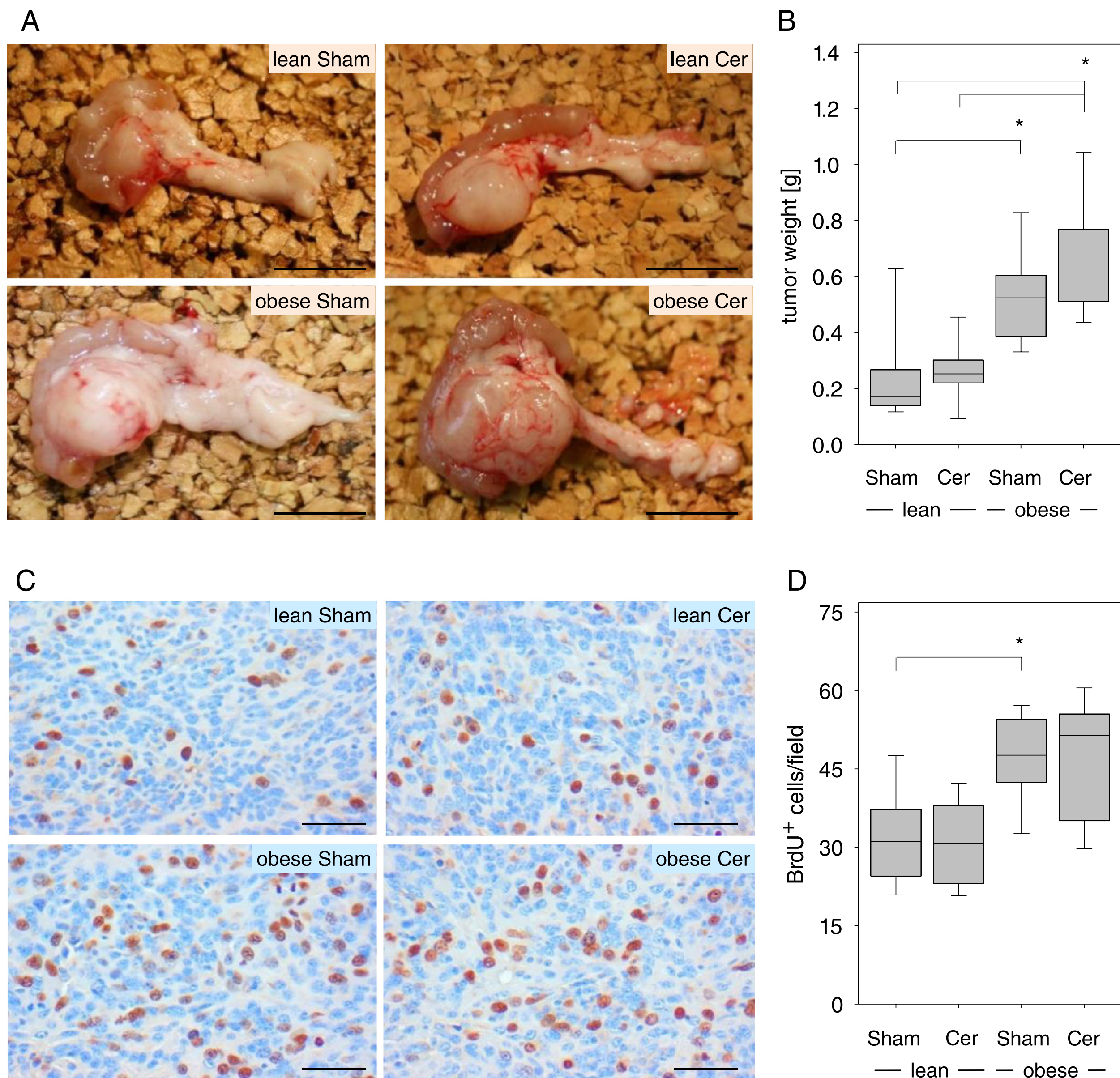

Figure 3 Diabetes leads to increased tumor weight and enhanced cancer cell proliferation on day 20. (A) Representative images of isolated pancreas with a carcinoma shows obvious differences in tumor size in sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. **(B)** Quantification of the tumor weight in the indicated mouse cohorts. **(C)** Representative images of histological sections after BrdU immunohistochemistry. **(D)** Quantification of $BrdU^+$ nuclei within the carcinoma reveals increased proliferation of cancer cells in diabetic mice. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 11 (lean Sham), n = 10 (lean Cer), n = 11 (obese Sham), n = 13 (obese Cer). Significant differences between the cohorts are indicated, *P ≤ 0.002 (B), *P = 0.005 (D). Bar =1 cm (A) or 50 μm (C).

diabetes does not (e.g. via epigenetic mechanisms) permanently change the proliferative capacity of tumor cells.

Diabetes does not decrease cell death in carcinomas

In order to evaluate apoptosis, Apoptag^{+} cells were quantified within carcinomas. No obvious decrease in the number of Apoptag^{+} cells in diabetic mice could be observed when compared to nondiabetic littermates (Figure 4A and B). Planimetric analysis of H/E stained histological sections revealed that diabetes did also not reduce the relative area of necrosis within the carcinomas (Figure 4C and D).

Characterisation of the cancer stem cell compartment

Cytokeratin 19 and vimentin expression was analysed in the carcinomas in order to evaluate if injected cancer cells can give rise to distinct cell types. In tumors, cells with epithelial morphology expressed the epithelial marker cytokeratin 19 (Figure 5A), whereas non-epithelial cells expressed the mesenchymal marker vimentin (Figure 5B). These data suggest that injected cancer cells can differentiate into at least two different cell types, and that a pluripotent cell population might be present within the injected cancer cells. To evaluate if pancreatic cancer cell lines express cancer stem cell markers such as Aldh1 we compared the expression of Aldh1a1 in pancreatic cancer cell lines such as Panc02, 7265PDA and 6606PDA with the liver metastasis cell line 6606l. The Aldh1a1 protein was readily observed with an apparent molecular weight of 55 kDa in all cell lines as well as in kidney cell extract, used as a positive control (Figure 5C). In some cell lines the antibody also detected another protein with an apparent molecular weight of 58 kDA, which is possibly Aldh1a3 or another Aldh family member (Figure 5C). In carcinomas few cells specifically expressed Aldh1 as evaluated by immunohistochemistry (Figure 5D). The number

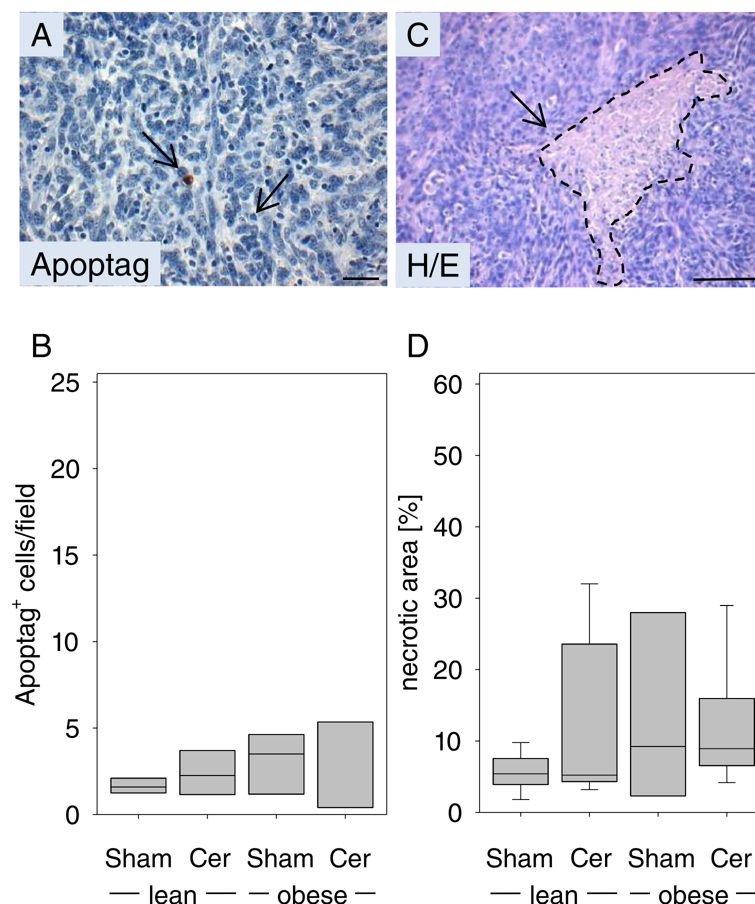

Figure 4 Diabetes does not inhibit cell death in PDA on day 20. **(A)** Representative image of an Apoptag^{+} cell. **(B)** Quantification of apoptotic cell death in the carcinomas of sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in the carcinomas of cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. **(C)** Representative image of a necrotic area. **(D)** Comparison of the percentage of necrotic tissue area in the carcinomas of the indicated mouse cohorts. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 4 (lean Sham), n = 4 (lean Cer), n = 3 (obese Sham), n = 4 (obese Cer) in panel B and n = 7 (lean Sham), n = 7 (lean Cer), n = 3 (obese Sham), n = 6 (obese Cer) in panel D. Differences between the cohorts were not significant. Bar = 50 μm.

of Aldh1$^+$ cells was moderately decreased in sham treated obese mice when compared to sham treated lean littermates (Figure 5E). A significantly decreased number of Aldh1$^+$ cells was also observed in cerulein treated obese mice when compared to cerulein treated lean littermates (Figure 5E).

We also characterized the expression of an additional cancer stem cell marker, CD133. This protein was not detected in the Panc02, 7265PDA and 6606PDA cell lines by Western Blotting, but was highly expressed in the 6606l cell line and kidney (Figure 6A). However, since a low level of CD133 mRNA could be detected in 7265PDA and 6606PDA cells by PCR (Figure 6B), we evaluated if a few CD133$^+$ cells could be observed in 6606PDA cell derived carcinomas. CD133 expression could be easily observed on the apical membrane of epithelial cells lining the proximal tubuli of the kidney as published previously (Figure 6C) [22]. CD133$^+$ cells could also be observed in few cells of 6606PDA derived carcinomas (Figure 6D). The number of CD133$^+$ cells was moderately increased in cerulein treated lean mice when compared to sham treated lean littermates (Figure 6E). A moderately increased number of CD133$^+$ cells was also observed in cerulein treated obese mice when compared to cerulein treated lean littermates (Figure 6E). We also analyzed the expression of GFAP, a protein expressed by glioblastoma and neural stem cells. GFAP was easily detected by Western Blotting in Panc02, 7265PDA, 6606PDA, 6606l cells and brain, but only elusive expression was observed in 6606PDA cell derived carcinomas by immunohistochemistry (data not shown).

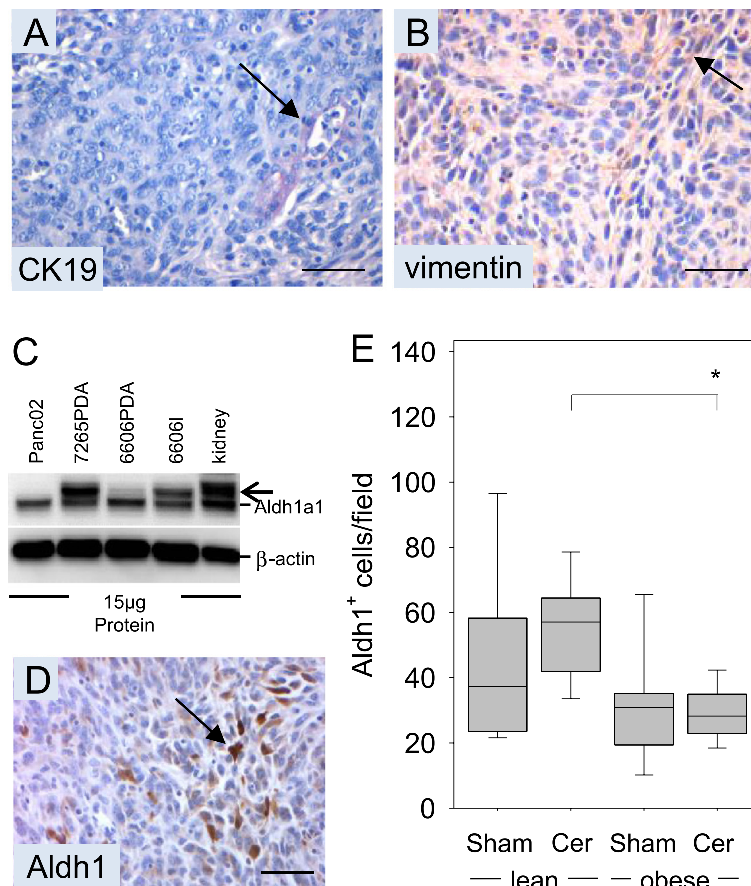

Figure 5 Analysis of CK19, vimentin and Aldh1a1 expression. (A) Representative images of epithelial cells expressing cytokeratin 19 and **(B)** of non-epithelial cells expressing vimentin in 6606PDA derived carcinomas. **(C)** Analysis of Aldh1a1 expression in cultured PDA cell lines and kidney by Western Blotting. An additional band (arrow) is observed in some cell lines and kidney cell extract and might represent another Aldh family member; e.g. Aldh1a3. **(D)** Immunohistochemistry of 6606PDA derived carcinomas reveals expression of the cancer stem cell marker, Aldh1, in some cancer cells. **(E)** Quantification of Aldh1$^+$ cells in the carcinomas of sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in the carcinomas of cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 9 (lean Sham), n = 9 (lean Cer), n = 9 (obese Sham), n = 10 (obese Cer). Significant differences between the cohorts are indicated, *P = 0.003. The Western Blot results were reproduced by three independent experiments. Bars = 50 µm.

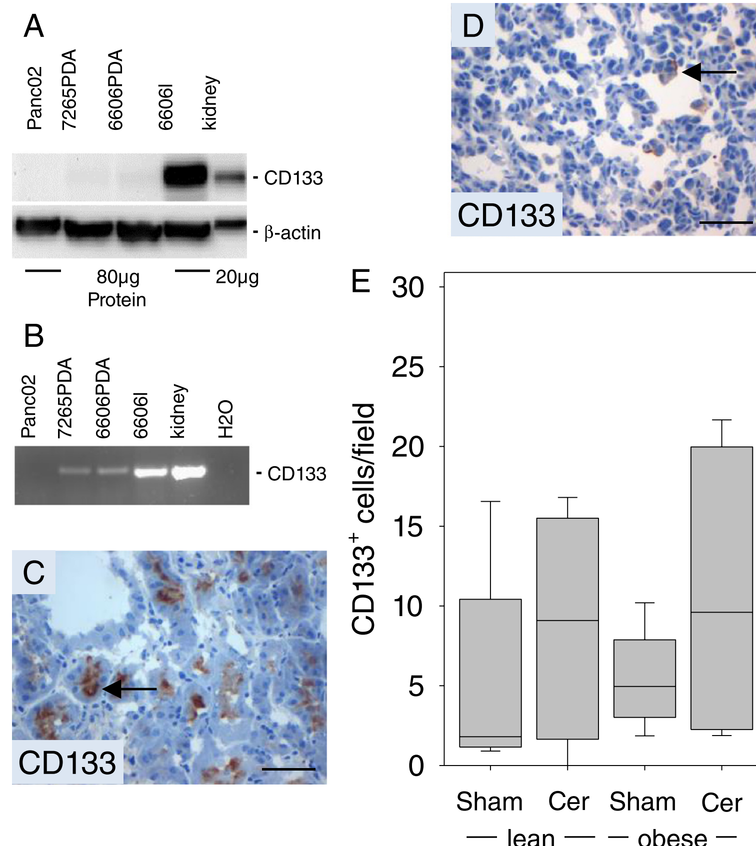

Figure 6 Analysis of CD133 expression. (A) Analysis of CD133 expression in cultured 6606PDA cells and kidney by Western Blotting. **(B)** Analysis of CD133 expression in cultured PDA cell lines and kidney by PCR. **(C)** The positive control for CD133 immunohistochemistry reveals expression of CD133 (arrow) in epithelial cells of proximal tubuli. **(D)** Immunohistochemistry of 6606PDA derived carcinomas reveals expression of CD133 (arrow) in some cancer cells. **(E)** Quantification of $CD133^+$ cells in the carcinomas of sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in the carcinomas of cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 5 (lean Sham), n = 4 (lean Cer), n = 6 (obese Sham), n = 6 (obese Cer). Differences between the cohorts were not significant. The Western Blot results were reproduced by three independent experiments. Bars = 50 µm.

Evaluation of inflammation and the desmoplastic reaction

Since surprisingly little influence of pancreatitis on the pathophysiology of PDA was observed in our study, we evaluated, if pancreatitis lead to more infiltrating inflammatory cells in the carcinoma. Because cerulein induced pancreatitis is characterized mainly by infiltrating neutrophil granulocytes, the number of CAE^+ cells was evaluated (Figure 7A and B). Indeed, a moderately increased number of CAE^+ cells was detected in the carcinomas of cerulein treated mice compared to sham treated animals (Figure 7B). Diabetes, however, caused a small reduction in the number of tumor infiltrating CAE^+ granulocytes. The observed differences were not significant. Similarily, a moderately increased number of $F4/80^+$ mahrophages was detected in the carcinomas of cerulein treated mice compared to sham treated animals (data not shown). This suggests that strong inflammation in the pancreas did not automatically lead to a major increase in the number of inflammatory cells in the tumor. To verify, if a desmoplastic reaction by the host might shield the carcinomas, we injected the 6606PDA cells in C57BL6-$Tg^{ACTB\text{-}eGFP1Osb/J}$ mice expressing GFP ubiquitously. We observed that carcinomas were surrounded by GFP^+ fibroblast like cells (Figure 7C). Quantification of the thickness of the α-smooth muscle actin positive desmoplastic reaction surrounding the carcinomas, revealed a moderate increase in the thickness of the desmoplastic reaction in cerulein treated mice when compared to sham treated animals (Figure 7D). In diabetic mice this desmoplastic reaction was moderately reduced (Figure 7D).

Metformin decreases tumor size and proliferation of carcinoma cells

In order to evaluate if the antidiabetic drug, metformin, has an effect on PDA, we injected 6606PDA cells into the head of the pancreas on day 0. From day 8 to 29 one

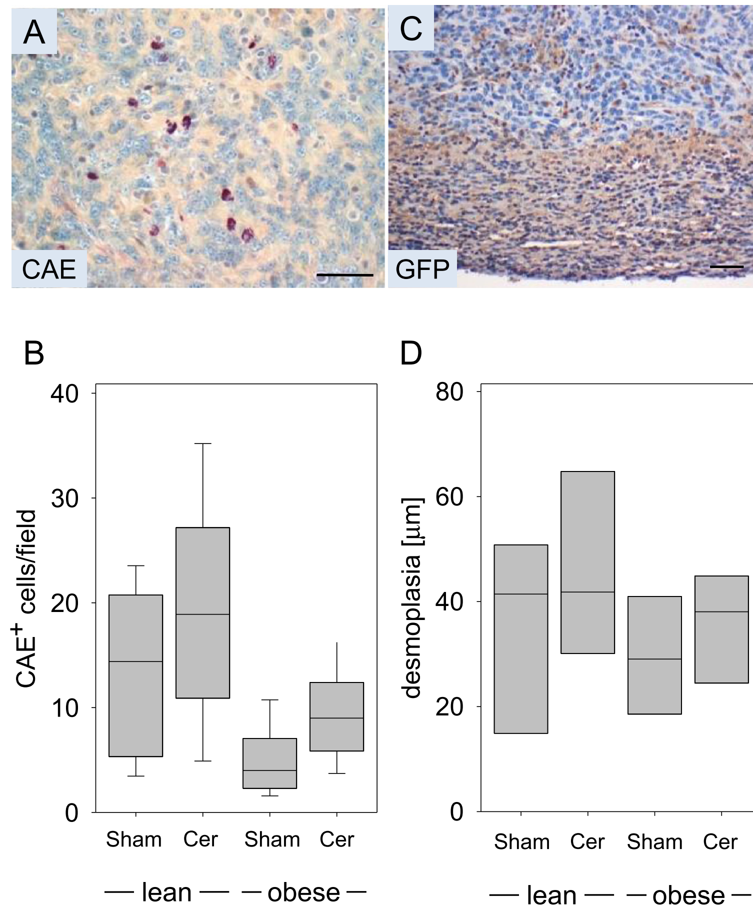

Figure 7 Analysis of inflammation and desmoplasia on day 20. (A) Representative image of CAE+ inflammatory cells in PDA. **(B)** Quantification of CAE+ cells in the carcinomas of sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in the carcinomas of cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. **(C)** Desmoplastic reaction visualized by anti-GFP immunohistochemistry in a C57BL6-Tg$^{ACTB\text{-}eGFP1Osb/J}$ mouse, which ubiquitously expresses GFP. **(D)** Quantification of α-smooth muscle+ desmoplastic reaction surrounding the carcinomas in sham treated non-diabetic (lean Sham) or diabetic (obese Sham) mice and in the carcinomas of cerulein treated non-diabetic (lean Cer) or diabetic (obese Cer) animals. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 11 (lean Sham), n = 9 (lean Cer), n = 9 (obese Sham), n = 12 (obese Cer) in Panel B and n = 4 for each cohort in Panel D. Bar = 50 µm.

mouse cohort was sham treated, whereas the other cohort was treated with metformin (Figure 8A). Measuring the tumor weight on day 29 revealed significantly smaller carcinomas in metformin treated mice, when compared to sham treated littermates (Figure 8B). The proliferation of cancer cells was also significantly decreased in metformin treated mice when compared to sham treated littermates (Figure 8C). These data suggest that metformin reduces the proliferation rate of carcinoma cells resulting in smaller tumors.

Discussion

The presented data demonstrate that a diabetes type II like syndrome i) increases the weight of PDA, ii) stimulates the proliferation of cancer cells, iii) does not inhibit the cell death of cancer cells and iv) reduces the number of Aldh1+ cells within the tumor. We observed, however, no major influence of chronic pancreatitis on the pathophysiology of PDA. In addition presented data demonstrate that the antidiabetic drug metformin i) decreases the weight of PDA and ii) reduces the proliferation of cancer cells.

The observed major effect in B6.V-Lep$^{ob/ob}$ mice on the pathophysiology of PDA might be caused by distinct features of these mice such as hyperinsulinaemia, hyperglycaemia or by adipositas. These features are typical for the early stage of type II diabetes. Alternatively, hyperglycaemia and adipositas are also associated with the metabolic syndrome. Indeed, this mouse strain has been used as model system for both diseases [23,24]. Nevertheless, we favor the idea, that this mouse strain is a model for type II diabetes rather than for the metabolic disease, since B6.V-Lep$^{ob/ob}$ mice do have increased high-density

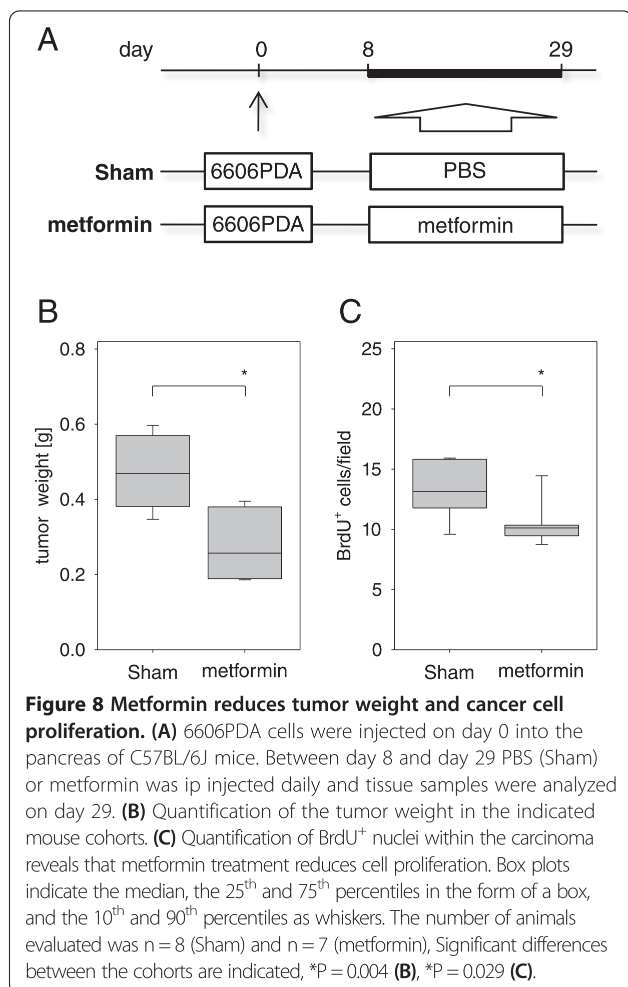

Figure 8 Metformin reduces tumor weight and cancer cell proliferation. **(A)** 6606PDA cells were injected on day 0 into the pancreas of C57BL/6J mice. Between day 8 and day 29 PBS (Sham) or metformin was ip injected daily and tissue samples were analyzed on day 29. **(B)** Quantification of the tumor weight in the indicated mouse cohorts. **(C)** Quantification of $BrdU^+$ nuclei within the carcinoma reveals that metformin treatment reduces cell proliferation. Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 10th and 90th percentiles as whiskers. The number of animals evaluated was n = 8 (Sham) and n = 7 (metformin), Significant differences between the cohorts are indicated, *P = 0.004 **(B)**, *P = 0.029 **(C)**.

lipoprotein concentrations and are thus protected from diet-induced atherosclerosis [23]. Since this mouse strain is characterized by a 10-fold higher C-peptide level but by only about a 2-fold higher glucose concentration in the blood [15], it is likely that hyperinsulinaemia rather than hyperglycemia stimulates cancer cell proliferation and increases the tumor weight. This hypothesis is supported by experimental studies indicating that streptozotocin induced type I like diabetes, which is characterized by higher blood glucose concentration but lower insulin concentration, does not increase tumor weight in pancreatic carcinomas [25]. In addition, the observation that human pancreatic cancer cell lines express insulin receptor and that the proliferation of these cell lines is induced by insulin also supports this conclusion [26,27]. High concentrations of insulin can also activate the IGF-1 receptor [28]. This receptor and its adaptor proteins IRS-1 and IRS-2 are expressed in pancreatic cancer cell lines as well as in human PDA [29-31]. These data underscore the importance of these signaling pathways in promoting pancreatic cancer cell proliferation and suggest that blocking the IGF-I receptor might be a valuable approach for targeted therapy of pancreatic cancer. In fact, Ganitumab, a monoclonal IGF-1 receptor antibody, inhibited growth of pancreatic carcinoma xenografts in mice and showed tolerable toxicity and trends toward an improved 6-month survival rate in patients with metastatic pancreatic cancer [32,33].

Our data indicate that diabetes type II like syndrome increases tumor weight, but at the same time decreases the number of $Aldh1^+$ cells. Since Aldh1 expression is a feature of cancer stem cells in pancreatic carcinoma [7-9,34,35], it is tempting to speculate that diabetes induces the differentiation of $Aldh1^+$ quiescent cancer stem cells into fast proliferating $Aldh1^-$ cells, which might contribute to increased tumor weight. This interpretation is consistent with the concept of quiescence of cancer stem cells and some adult stem cells [36,37]. Interestingly, insulin/IGF receptor signaling has been reported to abrogate the quiescent state of stem cells, which underlines our hypothesis that hyperinsulinemia increases cell proliferation in PDA [38]. However, it is also possible that $Aldh1^+$ cells might not be true cancer stem cells in this animal model and that the reduced number of $Aldh1^+$ cells in diabetic mice reflects how diabetes influences cancer cell plasticity in a cancer stem cell independent manner. It is also worth noticing that another so called tumor stem cell marker, CD133, can be observed in the carcinomas, but that the quantification of $CD133^+$ cells does also not correlate well with tumor size. Since it has been suggested that chemotherapy resistance might be caused by cancer stem cells, we compared the expression of CD133 and Aldh1 between three gemcitabine resistant 6606PDA clones and the original gemcitabine sensitive 6606PDA cell line. These gemcitabine resistant clones did not express higher levels of Aldh1 or CD133 (data not shown). Possibly, Aldh1 or CD133 expression does not in all cases directly correlate with tumor size or chemoresistance and might also not always define cells with stem cell properties [39]. This interpretation is supported by the fact that these proteins are readily expressed by fully differentiated cells. For example, CD133 is expressed in epithelial cells of proximal tubuli in the kidney and Aldh1 is highly expressed in the epithelium of the intestine, in liver and in pancreas [22,40].

In contrast to diabetes type II, chronic pancreatitis had little influence on the pathophysiology of the carcinomas. This result is surprising considering the published consensus that chronic inflammation is a major risk factor for the development of PDA [10,11,14]. The following interpretations may explain this discrepancy: i.) Chronic inflammation might promote cancerogenesis at an early stage of PDA development, but might have little influence on advanced adenocarcinomas. This interpretation is consistent with data indicating that chronic inflammation increases the risk for developing precancerous lesions and PDA in humans as well as in genetically modified

mice [10,16,17]. This hypothesis is also supported by publications, which demonstrate that anti-inflammatory drugs delay the progression of pancreatic cancer precursor lesions [41], but fail to have any benefit in the therapy of PDA [42]. ii.) Alternatively, chronic pancreatitis had little influence on the pathophysiology of the carcinomas, because of limitations of our animal model. Although we were able to induce a strong chronic pancreatitis by redundant administration of cerulein (Figure 2 B-E), we observed some local inflammation adjacent to the carcinoma and a strong desmoplastic reaction, independent of the induction of pancreatitis (data not shown and Figure 7). Possibly, this desmoplastic reaction shields the carcinomas from local inflammation or the observed peritumoral pancreatitis may have blunted the effects of cerulein. If the pathophysiology of a fully established pancreatic adenocarcinoma is influenced by pancreatitis or intra- and peritumoral inflammation, which is detected in most PDAs, is currently of intellectual as well as of clinical interest [14]. Our data indicate that a strong inflammatory milieu does not automatically lead to major changes in cancer cell proliferation, cell death or tumor size. However, a similar study with genetically modified mouse models of PDA needs to be pursued in order to exclude the possibility, that the observed effects are mouse model specific.

Conclusion

In conclusion, these experiments provide support for the concept that a diabetes type II like syndrome promotes growth of PDA, whereas strong inflammation does not have a major influence on the pathophysiology of advanced PDA. Our data also demonstrate that an anti-diabetic medication such as metformin has anti-tumorigenic properties, which is consistent with recently published data on human PDA cells [43,44]. If this anti-tumorigenic effect will also be observed in clinical trials, which are currently pursued, remains to be seen (https://clinicaltrials.gov/) [45]. In addition, modulation of inflammation for the therapy in pancreatic cancer is a goal in several clinical and preclinical studies [14]. However, our data support the idea that modulation of cell metabolism might be more promising than modulation of inflammation for the treatment of PDA [14,46].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. All authors read and approved the final manuscript.

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Eidesstattliche Erklärung

Hiermit erkläre ich die vorliegende Habilitationsschrift selbständig und ohne unerlaubte fremde Hilfe angefertigt zu haben. Ich habe keine anderen als die im Literaturverzeichnis angeführte Quellen benutzt und sämtliche Textstellen die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen wurden, sowie alle Angaben, die auf mündliche Auskünfte beruhen, als solche kenntlich gemacht. Ich versichere weiterhin, dass diese Arbeit nicht vorher und auch nicht gleichzeitig bei einer anderen als der Medizinischen Fakultät der Universität Rostock zur Eröffnung eines Habilitationsverfahrens eingereicht worden ist.

Rostock, den 17.03.2015

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