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CD40L is not involved in acute experimental pancreatitis

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ABSTRACT

Recent data suggest that platelets not only control thrombosis and hemostasis but may also regulate inflammatory processes such as acute pancreatitis. However, the specific role of platelet-derived mediators in the pathophysiology of acute pancreatitis is not known. Herein, we examined the role of CD40 ligand (CD40L, CD154) in different models of acute pancreatitis. Acute pancreatitis was induced by repetitive caerulein administration (50 μg/kg, i.p.) or infusion of sodium taurocholate (5%–10 μl) into the pancreatic duct in wild-type C57BL/6 and CD40L-deficient mice. Neutrophil infiltration, myeloperoxidase (MPO), macrophage inflammatory protein-2 (MIP-2) levels, acinar cell necrosis, edema and hemorrhage in the pancreas as well as serum amylase activity and lung levels of MPO were quantified 24 h after induction of acute pancreatitis. Caerulein and taurocholate challenge caused a clear-cut pancreatic damage characterized by increased acinar cell necrosis, neutrophil infiltration, focal hemorrhage, edema formation as well as increased levels of serum amylase and MIP-2 in the pancreas and lung MPO and histological damage. Notably, CD40L gene-deficient animals exhibited a similar phenotype as wild-type mice after challenge with caerulein and taurocholate. Similarly, administration of an antibody directed against CD40L had no effect against acute pancreatitis. Our data suggest that CD40L does not play a functional role in experimental acute pancreatitis. Thus, other candidates than CD40L needs to be explored in order to identify platelet-derived mediators in the pathophysiology of acute pancreatitis.

1. Introduction

The clinical course of acute pancreatitis ranges from a mild and self-limiting condition to a severe and systemic disease. The majority of patients experience a mild form and close to 20% develop severe acute pancreatitis associated with mortality rates ranging between 10 and 30% (Appelros et al., 2001; Mann et al., 1994). The management of patients with acute pancreatitis is mainly limited to supportive care. An improved understanding of the pathogenesis is necessary for developing more effective and specific therapeutic options for patients with acute pancreatitis. Premature activation of trypsinogen followed by subsequent activation of coagulation and inflammatory cascade systems constitute key components in the pathophysiology in acute pancreatitis (Awla et al., 2011; Bhatia, 2009; Saluja et al., 2007). Indeed, increased leukocyte and platelet activation have been well-documented in patients with acute pancreatitis (Bromberg et al., 2009; Dambrauskas et al., 2010; Mimidis et al., 2004).

Beyond their recognized function in thrombosis and wound healing, accumulating evidence suggest that platelets also exert proinflammatory actions, such as supporting tissue infiltration of leukocytes (Asaduzzaman et al., 2009; Laschke et al., 2008; Pitchford et al., 2004; Zarbock et al., 2006). Notably, a recent study demonstrated that platelets play an important role in acute pancreatitis by regulating macrophage inflammatory protein-2 (MIP-2)-dependent neutrophil infiltration into the pancreas (Abdulla et al., 2011). However, the detailed mechanism behind this platelet-dependent pancreatic accumulation of neutrophils in acute pancreatitis is not known. Interestingly, a recent study showed that platelet-derived CD40 ligand (CD40L, CD154) regulates neutrophil activation and recruitment in septic lung injury (Rahman et al., 2009). CD40L is a member of the tumor necrosis factor family and expressed as a transmembrane protein in activated CD4+ T-cells and platelets (Andre et al., 2002; Berner et al., 2000). CD40L exerts several proinflammatory and procoagulant effects, including synthesis of interleukin-1, tumor necrosis factor-α, and tissue factor in monocytes as well as upregulation of adhesion molecules on endothelial cells (Stout and Suttles, 1996). CD40L is also shed from the surface of activated platelets into a soluble form (sCD40L) and increased levels of sCD40L are detected in patients with inflammatory diseases (Chew et al., 2010; Danese et al., 2004; Henn et al., 2001; Vishnevetsky et al., 2004). Thus, it is tempting to hypothesize that CD40L may also be involved in platelet-mediated neutrophil recruitment and tissue damage in the pancreas. However, the literature on the role of CD40L in acute pancreatitis is contradictory. One study (Frossard et al., 2001) has reported that CD40L plays a key role in acute pancreatitis whereas another study (Demols et al., 2000) could not identify any role of CD40L in acute pancreatitis despite similar methodology.

Based on these considerations, we undertook this study to re-evaluate the role of CD40L in acute pancreatitis. For this purpose, we

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used two different experimental models of acute pancreatitis, including taurocholate- and caerulein-induced pancreatitis.

2. Materials and methods

2.1. Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden. Wild-type and CD40L-deficient (B6.129 S2-Cd40lg^{tm1Imx}/J, Jackson Laboratory, Bar Harbor, ME) male C57BL/6 mice weighing 20–26 g (6–8 weeks old) were maintained in a climate-controlled room at 22 °C and exposed to a 12:12-h light-dark cycle. Animals were fed standard laboratory diet, and given water *ad libitum*. Mice were anesthetized by intraperitoneal (i.p.) administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight in 200 µl of saline.

2.2. Experimental models of taurocholate- and caerulein-induced pancreatitis

Through a small (1–2 cm) upper midline incision the second part of duodenum and papilla of vater were identified. Stay sutures were placed 1 cm from the papilla. A small puncture was made through the duodenal wall opposite to the papilla of vater with a 23 G needle. A non-radiopaque polyethylene catheter (inner diameter 0.28 mm) was inserted through the punctured hole in the duodenum and 1 mm into the pancreatic duct via the papilla of vater. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp. Ten µl of either 5% sodium taurocholate (Sigma-Aldrich, Sollentuna, Sweden) or sodium chloride (0.9%) was infused for 5 min at constant speed (2 µl/min) by use of a microinfusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden). Then, the catheter was withdrawn and the common hepatic duct clamp was removed. The duodenal puncture closed by a simple suture (7–0 monofilament). The stay sutures were removed and the abdomen was closed in two layers. Animals were allowed to wake up and given free access to food and water. In separate experiments, acute pancreatitis was induced by seven hourly i.p. injections of a supramaximal dose of the pancreatic secretagogue caerulein (50 µg/kg, C9026, Sigma-Aldrich) in 200 µl of PBS as described previously (DiMagno et al., 2004; Niederau et al., 1985). Animals were sacrificed 24 h after pancreatitis induction. Blood was collected from the tail vein for systemic leukocyte differential counts and determination of serum amylase levels. All pancreatic tissue was harvested from the head of the pancreas because most of the pathological changes occur in the pancreatic head in this taurocholate-based model of pancreatitis (Laukkarinen et al., 2007**).** Tissues were removed and kept in two pieces; one piece was snap frozen in liquid nitrogen for biochemical analysis of myeloperoxidase (MPO) and MIP-2 and the other piece was fixed in formalin for later histological analysis.

2.3. Serum amylase

Serum amylase was used as an indicator of acute pancreatitis. Amylase was quantified in serum with a commercially available assay (Reflotron ®, Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Systemic leukocyte and platelet counts

At the end of the experiments, blood was sampled from the tail vein and diluted 1:500 in Stromatol solution (Mascia Brunelli S.p.A. Viale Monza, Italy) or 1:20 in Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) for quantification of platelets and leukocytes (monomorphonuclear and polymorphonuclear cells), respectively, in a Burker chamber.

2.5. MPO assay

Frozen pancreatic and lung tissue were pre-weighed and homogenized in 1 ml mixture (4:1) with PBS and Trasylol® 10,000 KIE/ml (Aprotinin, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenate was centrifuged (13,148 g, 10 min) and the supernatant was stored at −20 °C. The pellet was mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide and then frozen for 24 h. Next, the sample was sonicated for 90 s and put in a water bath at 60 °C for 2 h, after which the MPO activity of the supernatant was measured spectrophotometrically as previously described (Laschke et al., 2007). Values are expressed as MPO units per gram tissue.

2.6. MIP-2 levels

Tissue levels of MIP-2 were determined in stored supernatants from homogenized pancreatic tissues. MIP-2 levels were assessed using double-antibody Quantikine enzyme linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine MIP-2 as standard. The minimal detectable protein concentration is less than 0.5 pg/ml.

2.7. Histology

Pancreas tissue samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six µm sections were stained (hematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system including edema, acinar cell necrosis, haemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scales as previously described in detail (Schmidt et al., 1992).

2.8. Statistics

Data are presented as median (range) in the text, and as medians and ranges in the tables. Statistical evaluations were performed using Mann Whitney rank sum test unless stated otherwise. $P<0.05$ was considered significant, and n represents the number of animals. SigmaStat® for Windows® version 3·5 software (Systat Software, Chicago, Illinois, USA) was employed.

3. Results

3.1. Role of CD40L in acute experimental pancreatitis

First we examined the role of CD40L in taurocholate-induced pancreatitis representing a model of severe acute pancreatitis. We found that taurocholate increased serum amylase by more than 20-fold as well as caused a clear-cut infiltration of leukocytes in the pancreas and lung (Table 1). Moreover, taurocholate challenge elevated MIP-2 levels and caused significant acinar cell necrosis, edema and hemorrhage in the pancreas (Table 1). Notably, it was found that these changes caused by taurocholate challenge were almost identical in wild-type and CD40L-deficient mice (Table 1), suggesting that CD40L plays no role in taurocholate-induced acute pancreatitis. This observation was also confirmed by use of a monoclonal antibody directed against CD40L in taurocholate-treated animals (not shown). Next, we evaluated the role of CD40L in caerulein-induced pancreatitis, which represents a mild model of acute pancreatitis and was the same method in which the contradictory findings on CD40L function and acute pancreatitis were obtained (Demols et al., 2000; Frossard et al., 2001). As shown in Table 2, caerulein also provoked acute pancreatitis but the increase in serum amylase was 87% less than that in taurocholate-induced acute pancreatitis, confirming the milder nature of the caerulein-based acute pancreatitis model. Moreover, we observed that caerulein provoked

Table 1
Taurocholate-induced pancreatitis.

	Sham	WT + taurocholate	CD40L −/− + taurocholate
Serum amylase (μKat/L, $n=6$)	58 (49–75)	1224 (978–1740)[a]	1275 (556–1740)[a]
MPO in the pancreas (U/g, $n=6$)	0.3 (0.01–0.5)	4.9 (2.3–8.6)[a]	3.8 (1.9–6.6)[a]
MPO in the lung (U/g, $n=6$)	0.9 (0.8–1.2)	3.0 (1.2–5.9)[a]	2.9 (0.9–3.5)[a]
MIP-2 in the pancreas (pg/mg, $n=6$)	0.5 (0.15–5)	9.7 (3.5–17.4)[a]	8.0 (3.3–19.7)[a]
Acinar cell necrosis (Score 0–4, $n=6$)	0 (0–0.5)	1.8 (1–3.5)[a]	2 (1–3.5)[a]
Neutrophil infiltration (Score 0–4, $n=6$)	0.13 (0–0.5)	1.6 (1.5–3)[a]	1.5 (1–2.5)[a]
Edema (Score 0–4, $n=6$)	0.5 (0–1)	2.5 (2–3)[a]	2.5(2–3)[a]
Hemorrhage (Score 0–4, $n=6$)	0.25 (0–0.5)	2.5 (2–4)[a]	3.25 (2–3.5)[a]

Acute pancreatitis was induced by retrograde infusion of 10 μl sodium taurocholate (5%) into the pancreatic duct in wild-type (WT) and CD40L gene-deficient (CD40L −/−) mice. Sham animals underwent infusion of 10 μl saline into the pancreatic duct. Myeloperoxidase (MPO), macrophage inflammatory protein-2 (MIP-2), acinar cell necrosis, edema and hemorrhage as well as serum amylase and MPO in the lung were determined 24 h after infusion. Values represent median (range).
[a] $P<0.05$ versus sham.

Table 2
Caerulein-induced pancreatitis.

	Sham	WT + Caerulein	CD40L −/− + Caerulein
Serum amylase (μKat/L, $n=4$)	55 (45–60)	143 (99–184)[a]	160 (98–178)[a]
MPO in the pancreas (U/g, $n=4$))	0.03 (0.02–0.04)	2.2 (1.6–4.8)[a]	3.2 (2.9–4.3)[a]
MPO in the lung (U/g, $n=4$)	0.8 (0.7–1.1)	1.6 (1.4–2.6)[a]	1.9 (1.1–2.5)[a]
MIP-2 in the pancreas (pg/mg, $n=4$)	0.04 (0.03–0.04)	0.2 (0.1–0.3)[a]	0.2 (0.2–0.40)[a]
Acinar cell necrosis (Score 0–4, $n=4$)	0 (0–0)	2.6 (1.5–4)[a]	2.75 (2.5–3)[a]
Neutrophil infiltration (Score 0–4, $n=4$)	0 (0–0)	2.3 (2–3.5)[a]	2.5 (2.5–3)[a]
Edema (Score 0–4, $n=4$)	0.25 (0–0.5)	2.5 (1.5–3)[a]	2.5 (2.5–3)[a]
Hemorrhage (Score 0–4, $n=4$)	0.25 (0–0.5)	3 (1.5–3.5)[a]	2.5 (2.5–3.5)[a]

Acute pancreatitis was induced by seven hourly i.p. injections of the pancreatic secretagogue caerulein (50 μg/kg) in wild-type (WT) and CD40L gene-deficient (CD40L −/−) mice. Sham animals received phosphate buffered saline (PBS). Myeloperoxidase (MPO), macrophage inflammatory protein-2 (MIP-2), acinar cell necrosis, edema and hemorrhage as well as serum amylase and MPO in the lung were determined 24 h after the first i.p. injection. Values represent median (range).
[a] $P<0.05$ versus sham.

leukocyte recruitment into the pancreas and lung (Table 2). Also, there was a clear-cut increase in cell necrosis, edema formation, hemorrhage and MIP-2 production in the pancreas in response to caerulein challenge (Table 2). However, we found that CD40L gene-deficient mice were not protected against caerulein-induced acute pancreatitis and showed close to identical changes as those observed in wild-type animals (Table 2), suggesting that CD40L plays no role in caerulein-induced acute pancreatitis. Systemic leukocyte counts were decreased in all groups of animals with pancreatitis while platelet counts were similar in all the groups (Tables 3 and 4).

4. Discussion

The role of CD40L in acute pancreatitis is controversial with opposite findings in the literature. The present study re-evaluated the role of CD40L in two different models of acute pancreatitis and found that there is no significant role of CD40L in experimental pancreatitis. Thus, our findings indicate that CD40L is likely not a useful target in the treatment of acute pancreatitis.

Exaggerated activation of trypsin provokes excessive tissue damage, which in turn, may cause systemic inflammation and remote organ injury in acute pancreatitis (Bhatia, 2009). Several studies have reported increased leukocyte and platelet activation in experimental and clinical acute pancreatitis (Bromberg et al., 2009; Dambrauskas et al., 2010; Hackert et al., 2007; Mimidis et al., 2004; Uhlmann et al., 2007). A recent study showed that depletion of platelets reduces leukocyte infiltration in the pancreas and protects against acute pancreatitis in mice, suggesting a direct role of platelets in acute pancreatitis (Abdulla et al., 2011). In search for the mechanisms behind this platelet-dependent leukocyte recruitment in the pancreas, we hypothesized herein that CD40L could be a potential link between platelets and leukocyte activation in acute pancreatitis as has been shown to be the case in abdominal sepsis and pulmonary injury (Rahman et al., 2009). Two previous studies have reported diametrically contradictory findings related to the role of CD40L in caerulein-induced acute pancreatitis. Thus, one study reported that CD40L-deficient mice are protected from caerulein-provoked acute pancreatitis (Frossard et al., 2001) whereas another investigation showed that CD40L plays no role in the same model of acute pancreatitis (Demols et al., 2000), which was the basis of our re-evaluation of the function of CD40L in acute pancreatitis. However, we found that CD40L gene-deficient mice were not protected against caerulein-induced pancreatitis, which is in line with the study by Demols et al. (2000). Moreover, it was found herein that CD40L-deficient animals were neither protected against taurocholate-provoked acute pancreatitis. In

Table 3
Systemic platelet and leukocyte differential counts.

	Platelets	MNL	PMNL	Total
Sham ($n=6$)	1475 (1375–1950)	9.4 (7.2–12)	1.2 (0.6–1.4)	10.4 (7.8–13)
WT + taurocholate ($n=6$)	1737 (1425–1950)	4.2 (3.6–9)[a]	0.6 (0.2–1)[a]	4.8 (3.2–7)[a]
CD40L (−/−) + taurocholate ($n=6$)	1845 (1750–1900)	4.1 (1.6–8.8)[a]	0.6 (0.2–1.2)[a]	4.7 (1.8–9.8)[a]

Acute pancreatitis was induced by retrograde infusion of 10 μl sodium taurocholate (5%) into the pancreatic duct in wild-type (WT) and CD40L gene-deficient (CD40L −/−) mice. Sham animals underwent infusion of 10 μl saline into the pancreatic duct. The number of platelets, polymorphonuclear (PMNL) and mononuclear (MNL) leukocytes were counted using a standard hematocytometer. Values represent median (range).
[a] $P<0.05$ versus sham.

Table 4
Systemic platelet and leukocyte differential counts.

	Platelets	MNL	PMNL	Total
Sham ($n=4$)	1747 (1625–1850)	12.2 (9.4–13.2)	1.5 (1–2.4)	13.7 (11.8–14.2)
WT + Caerulein ($n=4$)	1925 (1600–1960)	5.2 (4.4–5.6)[a]	0.6 (0.4–0.8)[a]	5.8 (4.8–6.2)[a]
CD40L (−/−) + Caerulein ($n=4$)	1950 (1720–2050)	5.6 (5.4–5.8)[a]	0.8 (0.6–0.8)[a]	6.4 (6–6.6)[a]

Acute pancreatitis was induced by seven hourly i.p. injections of the pancreatic secretagogue caerulein (50 μg/kg) in wild-type (WT) and CD40L gene-deficient (CD40L −/−) mice. Sham animals received phosphate buffered saline (PBS). The number of platelets, polymorphonuclear (PMNL) and mononuclear (MNL) leukocytes in the blood were counted using a standard hematocytometer. Values represent median (range).

[a] $P<0.05$ versus sham.

fact, none of the included proinflammatory or pathological parameters were different in wild-type and CD40L-deficient animals undergoing pancreatitis induced by taurocholate or caerulein, suggesting that our data exclude a significant role of CD40L in experimental acute pancreatitis. This notion was also supported by our findings that a monoclonal antibody against CD40L had no effect against taurocholate-induced acute pancreatitis. Thus, CD40L is not a likely candidate for mediating the platelet-dependent leukocyte activation and tissue injury in acute pancreatitis as recently reported (Abdulla et al., 2011). In this context, it should be noted that platelets contain several compounds, including interleukin-1 (Kaplanski et al., 1993), platelet activating factor (Weber and Springer, 1997), platelet factor-4 (Deuel et al., 1981), and adenosine-5′-triphosphate (Wu et al., 2009), all of which have the ability to exert proinflammatory actions, such as chemokine formation and neutrophil migration. Further studies are needed to define the mechanisms behind platelet-regulated inflammation in acute pancreatitis.

5. Conclusions

Taken together, our findings strongly suggest that CD40L is not involved in the pathophysiology of experimental acute pancreatitis. Thus, we conclude that targeting CD40L may not be a rational choice in the management of acute pancreatitis.

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