Die Rolle von Protease-aktivierten Rezeptoren und Augmenter of liver regeneration während der hepatischen Ischämie-Reperfusion

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INHALTSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

ALR	Augmenter of Liver Regeneration
GP	Glycoprotein
I/R	Ischämie-Reperfusion
ICAM1	Intercellular adhesion molecule 1
IL	Interleukin
min	Minuten
PAR	Protease-aktivierter Rezeptor
rALR	Rekombinanter Augmenter of Liver Regeneration
TNF α	Tumornekrosefaktor α

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EINLEITUNG

Die hepatische I/R stellt weiterhin ein relevantes Problem im Rahmen von Leberchirurgischen Eingriffen und vor allem im Rahmen von Lebertransplantationen dar (1). Die Reduktion dieser sterilen Inflammation kann zu einer Verbesserung des Outcomes nach Leberchirurgie führen. Das Ziel der im Rahmen dieser Dissertation durchgeführten Studien bestand in der Evaluation zweier neuartiger Therapieansätze während der hepatischen I/R.

Initiale Mechanismen der hepatischen I/R

Durch das Ausbleiben der arteriellen Gefäßversorgung kommt es im Lebergewebe zur mangelnden Versorgung von Hepatozyten, Kupfferschen Zellen und hepatischen Endothelzellen (2). Die fehlende Sauerstoffzufuhr führt zur intrazellulären Ausbildung von freien Sauerstoffradikalen wie dem Hydroxyl-Radikal und dem Hyperoxid-Anion. Diese freien Radikale können zum einen direkt über Lipidperoxydation zur Zellschädigung führen (3), zum anderen modulieren sie Signaltransduktionswege (4) (5). Durch die Aktivierung von Transkriptionsfaktoren und die Beeinflussung von Redox-sensitiven Enzymen können freie Sauerstoffradikale die Expression von Adhäsionsmolekülen und damit die Rekrutierung von zirkulierenden inflammatorischen Zellen regulieren (4). Zugleich werden auch weitere proinflammatorische Mediatoren durch die aktivierten hepatischen Zellen gebildet (6). Tumor-Nekrose-Faktor-α wird während der hepatischen I/R vermehrt freigesetzt (7) (8) (9) (10) (11) und kann über TNF α Rezeptoren zum einen zur Induktion von Apoptose führen, zum anderen auch über die Modulation der Expression zur Konzentrationssteigerung weiterer Cytokine beitragen (12) (13). Verschiedene Interleukine wie IL-1, IL-6, IL-17 und IL-18 tragen in der Folge durch die Rekrutierung zirkulierender inflammatorischer Zellen zur weiteren Ausbildung des inflammatorischen Schadens bei (14) (15) (16) (17) (18).

Leukozytenrekrutierung

Durch die Expressionssteigerung von Adhäsionsmolekülen auf der Oberfläche von durch die oben genannten proinflammatorischen Mediatoren aktivierten hepatischen

Endothelzellen, kommt es zur Rekrutierung von Leukozyten in den postsinusoidalen Venolen der postischämischen Leber. Hierbei spielen vor allem E-Selektin, P-Selektin und auch das ICAM1 eine entscheidende Rolle (19). Es kommt zum Selektin-vermittelten Leukozytenrolling und schließlich zur festen leukozytär-endothelialen Adhäsion durch ICAM1 (20). Von Hepatozyten sezernierte Chemokine führen zur Extravasation der Leukozyten (21) (22), welche im Gewebe dann durch die Freisetzung von hydrolytischen Enzymen aus ihren Granula zu einem zytotoxischen Schaden der Hepatozyten führen (23) (24) und durch Freisetzung von Sauerstoffradikalen den postischämischen Schaden weiter modulieren (25).

Thrombozytenrekrutierung und Protease-aktivierte Rezeptoren

Blutplättchen sind entscheidend für die Blutgerinnung, da sie nach der Interaktion mit Kollagenen aus dem extravasalen Gewebe im verletzten Gefäß und der anschließenden Bildung eines Clots zur Blutstillung beitragen (26).

Neben der prokoagulatorischen Wirkung spielen Thrombozyten aber auch eine wichtige Rolle während inflammatorischer Prozesse wie der hepatischen I/R. Sie verfügen über verschiedenste proinflammatorische Mediatoren wie platelet-derived-growth-factor, Thromboxan A2, Serotonin und Leukotriene und können hiermit sowohl selbst den postischämischen Schaden modulieren (27), als auch die Aktivierung von Leukozyten direkt (28) oder durch die Aktivierung der hepatischen Endothelzellen indirekt beeinflussen (29) (30).

Die Relevanz von Thrombozyten während der hepatischen I/R drückt sich auch in klinischen Studien aus: Die Applikation von Thrombozytenkonzentraten ist ein unabhängiger negativer Faktor für das Überleben nach Lebertransplantation (31).

Bisher konnte jedoch aufgrund der zu befürchtenden Blutungskomplikationen noch keine Intervention auf Ebene der Thrombozyten etabliert werden, da die in der Klinik gängigen Therapeutika (Acetylsalicylsäure, Tirofiban, Clopidogrel, etc.) zu einer kompletten Inaktivierung der Thrombozyten inklusive der Blutgerinnungsfunktion führen.

Während die Aktivierung von Thrombozyten im Rahmen der Blutgerinnung vornehmlich durch die Interaktion von Kollagen aus dem Extravasalraum mit dem von-Willebrand-Faktor und dem von-Willebrand-Rezeptor stattfindet kommt es bei der hepatischen Ischämie-Reperfusion sowie anderen inflammatorischen

Krankheitsbildern jedoch nicht primär durch die Verletzung des Endothels zu einer Aktivierung (32).

PAR vermitteln in diesem Fall die Aktivierung der Thrombozyten. PAR sind eine Gruppe von G-protein-gekoppelten Rezeptoren und werden in 4 Isotypen unterteilt: PAR1-4. Diese Rezeptorklasse wird von Serinproteasen wie Thrombin und Trypsin durch die Abspaltung des N-terminalen, extrazellulären Endes aktiviert und ist vornehmlich auf Blutplättchen, Endothelzellen, Myozyten und Nervenzellen exprimiert. Im Menschen vermittelt PAR1 nach Spaltung durch Thrombin die Kollagen-unabhängige Aktivierung von Thrombozyten und kann so zur inflammatorischen Antwort beitragen. Beim Nagetier übernimmt diese Rolle PAR4 (33) (34) (35).

Experimentelle Studien konnten zeigen, dass PAR eine wichtige Rolle bei der Ausbildung des postischämischen Schadens spielen: Im Rahmen der kardialen (36), renalen (37) und cerebralen (38) I/R kommt es durch Inhibition bzw. Knockout von PAR zu einer Reduktion des inflammatorischen Schadens. Welche Rolle PARs während der hepatischen I/R spielen blieb jedoch bislang unklar.

Die erste Arbeitshypothese der vorgelegten Dissertation ist, dass durch die Intervention auf Ebene der Thrombin-PAR-Interaktion mittels eines Antikörpers (TcY-NH2) die inflammatorische Antwort und somit der hepatische I/R-Schaden reduziert wird ohne die Blutgerinnung negativ zu beeinflussen, da die Möglichkeit der Aktivierung von Thrombozyten im Sinne der Blutgerinnung erhalten bleibt.

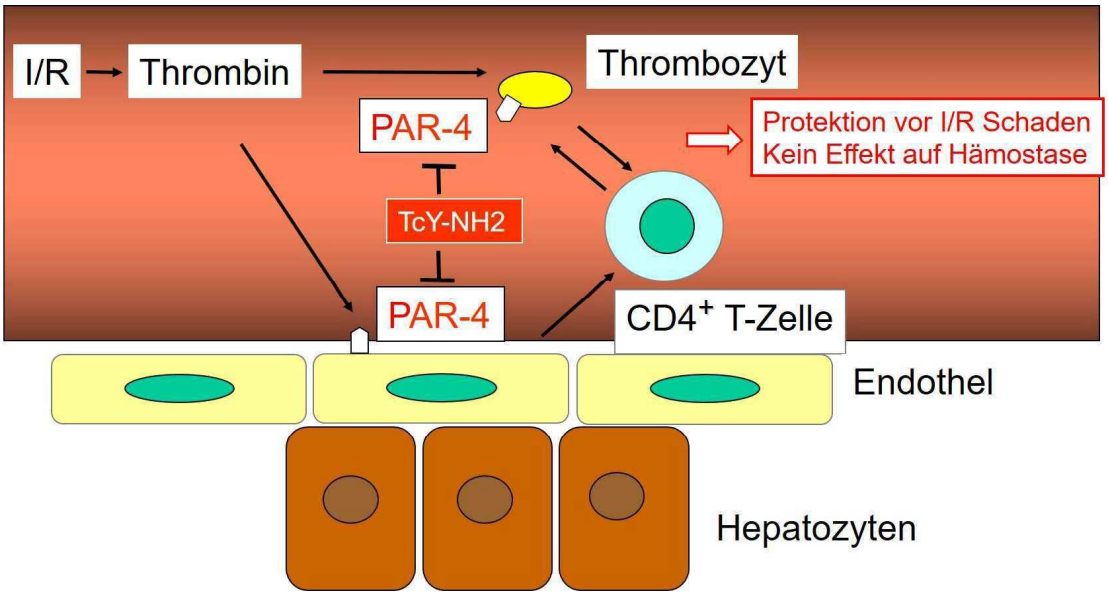

Abb. 1: Darstellung der Arbeitshypothese zum PAR-4 Teilprojekt. Durch die Inhibition der proinflammatorischen Effekte von Thrombin auf Thrombozyten mittels eines spezifischen Inhibitors (TcY-NH2) wird eine Protektion vor dem hepatischen I/R-Schaden bewirkt ohne die Blutgerinnung negativ zu beeinflussen.

CD4+ T-Lymphozyten

Im Rahmen der hepatischen I/R spielen auch CD4+ T-Zellen eine entscheidende Rolle. Unsere Arbeitsgruppe konnte bereits zeigen, dass es hierbei zu einer wechselseitigen Aktivierung von Thrombozyten und CD4+ T-Zellen kommt und dies einen Aktivierungsmechanismus der CD4+ T-Zellen während der hepatischen I/R darstellt (39). Somit könnten Interventionen auf Ebene der Thrombozytenaktivierung auch zu einer Reduktion der Rekrutierung von CD4+ T-Zellen in der postischämischen Leber führen.

Augmenter of liver regeneration

ALR ist ein in der Leber synthetisierter Wachstumsfaktor, welcher zu einer Induktion einer hepatozellulären Regeneration führt und damit mitverantwortlich für die außergewöhnliche regenerative Kapazität der Leber ist (40).

Außerdem sorgt ALR für eine Reduktion des oxidativen Stresses durch die Induktion von antioxidativen Proteinen, welche reaktive Sauerstoffspezies neutralisieren können (41). Diese reaktiven Sauerstoffspezies spielen eine entscheidende Rolle im Rahmen der hepatischen I/R (32).

Daher ist die zweite Arbeitshypothese, dass ALR durch die Reduktion von reaktiven Sauerstoffspezies einen protektiven Effekt auf den hepatischen I/R-Schaden hat und sich positiv auf die Regeneration nach I/R der Leber auswirkt.

Zielsetzung

Die Zielsetzung dieser Dissertation ist, beide Arbeitshypothesen zu überprüfen und den Einfluss von PAR 4 und ALR auf die hepatische I/R zu untersuchen.

MATERIAL UND METHODEN

Die Experimente wurden am Walter-Brendel-Zentrum für experimentelle Medizin nach Genehmigung des entsprechenden Tierversuchsantrages durch die Regierung Oberbayern im Mausmodell mit 5-7 Wochen alten C57BL/6 Mäusen durchgeführt.
Für die Studien waren mehrere Studienarme jeweils unterteilt in Interventionsgruppe, scheinoperierte Gruppe und Placebo-Gruppe (n=6-7) nötig.
Die Ischämie wurde unter invasiver Blutdruckmessung durch reversibles Abklemmen des linken Leberlappens für 90 Minuten induziert. Die Reperfusionszeit variierte je nach Versuchsansatz zwischen 60 und 240 Minuten.
Zur Blockade von PAR 4 wurde der spezifische Inhibitor TcY-NH2 5 min vor Beginn der Reperfusion appliziert (TcY-NH2 0.6 mg/kg KG in 200 µlphysiologischer Kochsalzlösung), ALR wurde vor Beginn der Ischämie intraarteriell verabreicht (ALR 100 µg/kg KG in 200 µLphysiologischer Kochsalzlösung).
Anschließend wurde mittels intravitaler Fluoreszenzmikroskopie Thrombozyten-Endothelzell-Interaktion, CD4+T-Zell-Rekrutierung, leukozytär-endotheliale Interaktion wie auch die sinusoidale Perfusion analysiert.
Zu diesem Zwecke wurden Thrombozyten aus dem Vollblut syngener Spendertiere isoliert, CD4+ T-Zellen wurden mittels magneticcell-sortingsystem aus den Milzen ebenfalls syngener Spendertiere gewonnen.
Aus dem Serum der Versuchstiere wurden die Leberenzyme GPT und GOT gemessen, aus Gewebeproben wurde die Caspase-3 Aktivität zur Bestimmung der Apoptose ermittelt und Nekrose mittels histologischer Schnitte analysiert.
Ki-67 Färbungen wurden zur Analyse der Leberregeneration angefertigt.
Außerdem wurden Thrombelastographie aus Vollblut und Versuche zur Bestimmung der Blutungszeit durchgeführt.
Zur statistischen Analyse wurden Anova on ranks gefolgt von Student-Newman-Keuls durchgeführt. P-Werte <0,05 wurden als statistisch signifikant angenommen.

ZUSAMMENFASSUNG DER ERGEBNISSE UND DISKUSSION

Targeting platelet migration in the postischemic liver by blocking protease-activated receptor 4

Die entscheidende Rolle von Thrombozyten während der hepatischen I/R konnte in klinischen sowie in experimentellen Studien gezeigt werden (42) (32). Somit wurde auch immer wieder das therapeutische Potential einer Inhibition der postischämischen Thrombozytenrekrutierung in der Literatur diskutiert. Allerdings konnte bislang noch keine Intervention auf Ebene der Thrombozytenaktivierung erfolgreich eine klinische Anwendung finden, da ein hohes Risiko von Blutungskomplikationen zu befürchten war.

Einen möglichen Ansatz zur Inhibition der Thrombozytenaktivierung bieten PARs, da diese die proinflammatorischen Effekte von Thrombin auf Thrombozyten vermitteln, jedoch im Rahmen der Blutgerinnung keine Rolle spielen (43) (44).

Die Ergebnisse unserer in vivo Studie zeigen, dass die Inhibition von PAR4 mittels eines spezifischen PAR4-Inhibitors tatsächlich zu einer Reduktion der thrombozytär-endothelialen Interaktion in der postischämischen Leber führt. In der Interventionsgruppe waren sowohl die Zahlen für adhärente als auch rollende Thrombozyten reduziert.

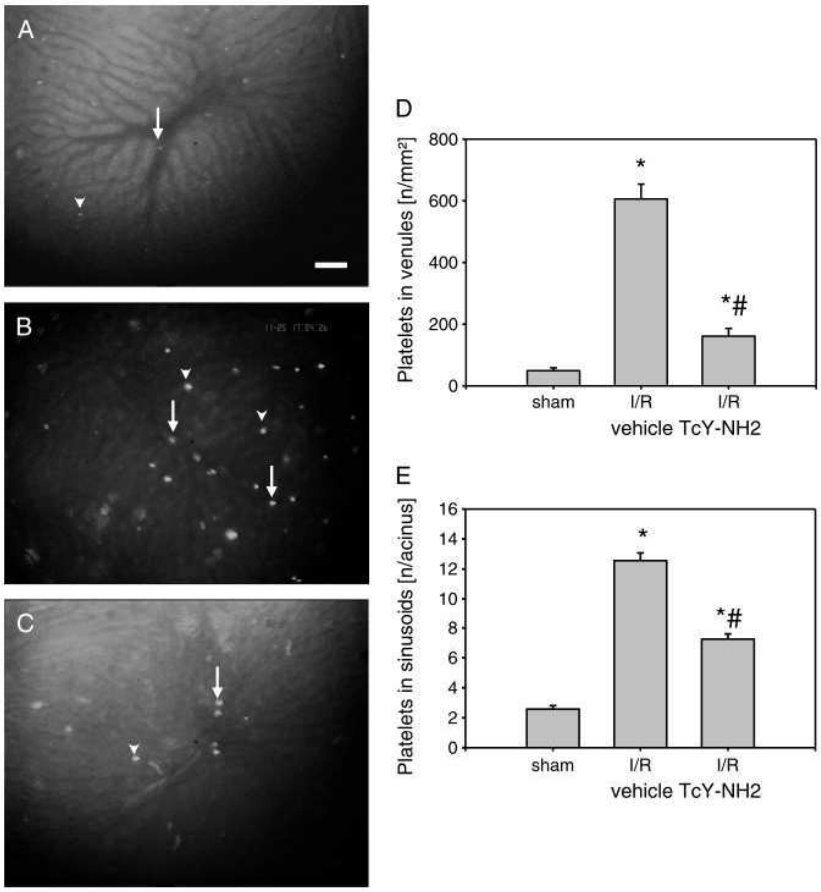

Abb. 2: Rekrutierung von Thrombozyten in vivo. Diese intravitalmikroskopischen Bilder zeigen fluoreszenzmarkierte Thrombozyten in der scheinoperierten Gruppe (A), nach I/R und Applikation von isotoner Kochsalzlösung als Kontrolle (B) und nach I/R und TcY-NH2-Behandlung. Pfeile verweisen auf Thrombozyten in postsinusoidalen Venolen und Pfeilköpfe auf Thrombozyten, welche in Sinusoiden akkumuliert sind. Die quantitativen Daten zu thrombozytär-endothelialer Interaktion in postsinusoidalen Venolen (D) und Sinusoiden (E) werden als Blakendiagramme dargestellt. 500fache Mikroskopvergrößerung. N=7 Tiere pro Versuchsgruppe. *P<0,05 versus scheinoperierte Gruppe, #P<0,05 versus Kontrollgruppe. Der Skalierungsbalken in (A) stellt 50µm dar.
Aus: Targeting Platelet Migration in the Postischemic Liver by Blocking Protease-Activated Receptor 4

Die initiale Interaktion, also das Rollen, wird durch P-Selektin auf der Oberfläche von aktivierten Endothelzellen vermittelt. Zur festen Adhärenz führt das Binden des auf Thrombozyten exprimierten GPIIb/IIIa-Rezeptors mit dem ICAM1, welches auf aktivierten Endothelzellen exprimiert wird. Hierbei fungiert Fibrinogen als Bridgingmolekül (45). Die nachgewiesene Reduktion der thrombozytär-endothelialen

Interaktion kann somit sowohl durch die Unterbrechung der thrombin-abhängigen Aktivierung von Thrombozyten als auch durch die Beeinflussung der endothelialen Aktivierung erklärt werden.
Die Ergebnisse der Messung der Schwanzvenenblutungszeit als auch die der Thrombelastometrie zeigen, dass die Intervention mittels eines spezifischen PAR4-Inhibitors nicht zu einer Beeinträchtigung der Blutgerinnung führen. Hierin zeigt sich der entscheidende Vorteil gegenüber schon in der Klinik eingesetzten Inhibitoren der Thrombozytenaktivierung: Durch die fehlende Beeinträchtigung der nicht über PAR vermittelten Funktionen von Thrombin, also Fibrinformation und Feedback-Aktivierung der Blutgerinnung, kann die Gerinnungsfunktion erhalten werden.
Das humane Äquivalent zum murinen PAR 4 ist PAR 1. Mit Vorapaxar und Atopaxar gibt es bereits heute PAR 1 Inhibitoren, welche in klinischen Phase 3 bzw. Phase 2 Studien im Bereich der Reduktion von kardiovaskulären Ereignissen getestet werden und somit möglicherweise einen vielversprechenden Ansatzpunkt zur Behandlung der hepatischen I/R darstellen (46) (47).

Augmenter of liver regeneration attenuates inflammatory response in the postischemic mouse liver in vivo

Vor dem Hintergrund des wachsenden Bedarfs an Spenderorganen und dem Mangel an Spendern ist es unverzichtbar, die vorhandenen Organe optimal zu nutzen und so lange wie möglich die Funktionsfähigkeit zu erhalten. Da der hepatische Ischämie-Reperfusionsschaden der häufigste Grund für Organdysfunktion nach Lebertransplantation ist (42), werden Strategien, welche die negativen Effekte der Ischämie reduzieren, benötigt. Aufgrund der zunehmenden Notwendigkeit auf marginale Organe zurückzugreifen, ist es entscheidend, sowohl die postischämische inflammatorische Antwort zu reduzieren als auch die Leberregeneration zu stimulieren. Hier ist ALR ein vielversprechender Ansatzpunkt. ALR wird von Hepatozyten produziert und findet sich in Mitochondrien, dem Nukleus und dem Zytosol (48) (49) (50). Im Tiermodel führt die exogene Gabe von ALR zur Beschleunigung der Regeneration nach Leberschädigung (51) (52) (53). Darüber hinaus weisen Studien eine antioxidative und antiapoptotische Wirkung nach (54) (55). Da die hepatische I/R mit einer Ausschüttung von Sauerstoffradikalen einhergeht und die Apoptose die führende Zelltodform während der hepatischen I/R ist, haben wir die Wirkung von ALR während der hepatischen I/R untersucht.

Wir konnten nachweisen, dass die Applikation von rALR den apoptotischen und nekrotischen Zelluntergang bei der hepatischen I/R im Tiermodel reduziert. Dies ist zum einen in der essentiellen Funktion von ALR für die Biogenese und Funktion von Mitochondrien begründet, zum anderen in der antiapoptotischen Wirkung. Außerdem wird angenommen, dass ALR durch die Infiltration und Beeinflussung von Zellen des Immunsystems (infiltrierende inflammatorische Zellen und lokale Makrophagen) eine Reduktion des hepatozellulären Schadens bewirken kann (56). In unserer Studie wurde ein protektiver Effekt der ALR-Applikation auf die Leukozytenmigration, die Migration der CD4+ T-Zellen und den hepatozellulären Schaden nach 240 min Reperfusion nachgewiesen. Hierfür könnte neben der Reduktion des Zellschadens als Auslöser der Zellmigration auch eine Beeinflussung der de novo Expression von Adhäsionsmolekülen (z. B. P-Selektin, ICAM-1) verantwortlich sein.

Auch die Rekrutierung von CD4+-T-Zellen, welche durch reaktive Sauerstoffspezies aktiviert werden können, war in unserer Studie reduziert. Eine Erklärung hierfür ist die antioxidative Wirkung von ALR (54) (55).

Unsere in vivo Daten belegen das therapeutische Potential von ALR im Rahmen der hepatischen I/R. Als Mechanismen sind der direkte antiapoptotische und antinekrotische Effekt und die Reduktion der Rekrutierung von inflammatorischen Zellen anzunehmen.

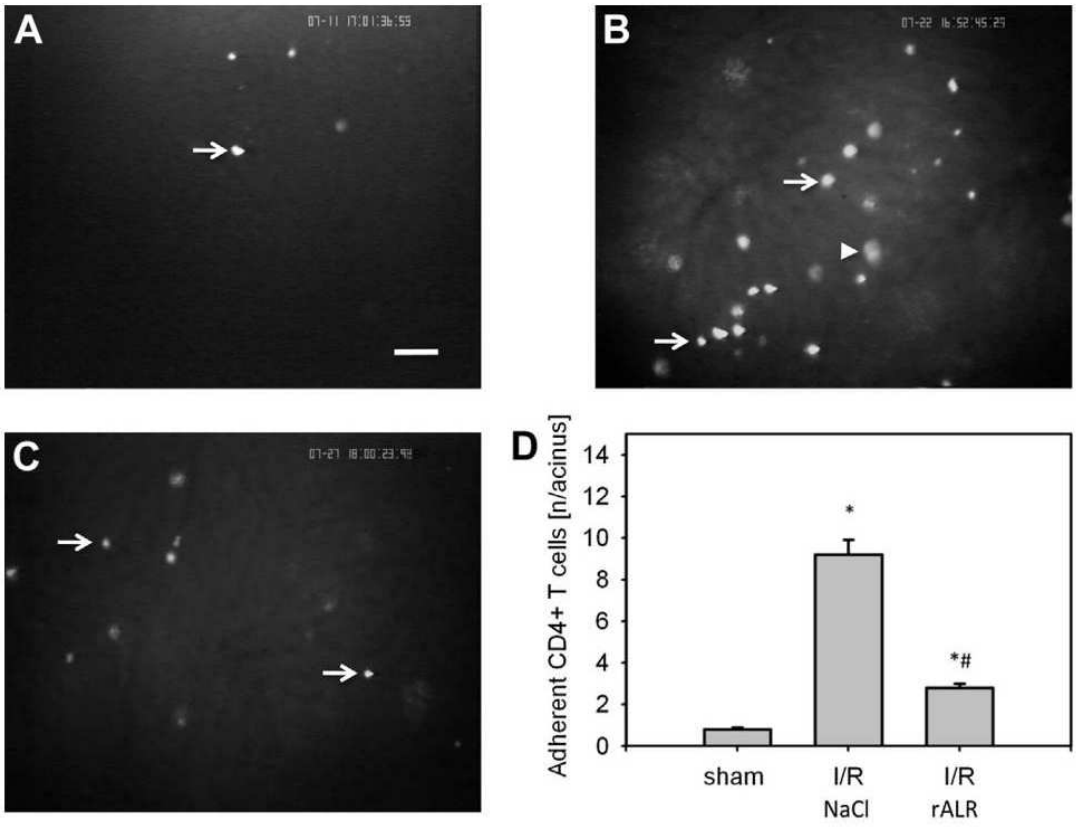

Abb. 3: Rekrutierung von CD4+ T-Zellen. Die intravitalmikroskopischen Bilder zeigen fluoreszenzmarkierte CD4+ T-Zellen in der hepatischen Mikrozirkulation in der Sham-Gruppe (A), nach hepatischer I/R (90/60min) in der Kontrollgruppe (B) und nach I/R mit ALR-Behandlung (C). Pfeile indizieren in Sinusoiden akkumulierte CD4+ T-Zellen, Pfeilköpfe verweisen auf außerhalb der Mikroskopieebene liegende CD4+ T-Zellen, welche nicht in die Auswertung eingingen. Mikroskopvergrößerung 500fach. Der Referenzbalken in (A) stellt 50µm dar. In (D) werden die quantitativen Daten dargestellt: N=6 Tiere pro Versuchsgruppe, *P<0,05 versus scheinoperierte Gruppe, #P<0,05 versus Kotrollgruppe.

Aus “Augmenter of Liverregeneration attenuates inflammatory response in the postischemic liver in vivo”.

ANTEIL DER ARBEIT AM ALR PROJEKT

Im Rahmen des Originalartikels zum Einfluss von ALR auf die hepatische I/R habe ich Herrn Iskandarov in die mikrochirurgischen Techniken unseres Mausmodels eingelernt und ihn in der Durchführung der Versuche unterstützt. Die Versuche mit 240 min Reperfusionszeit wurden vollständig durch mich durchgeführt. Außerdem habe ich Teile der Auswertung der Aufnahmen der Intravitalmikroskopie übernommen sowie Teile der Manuskripterstellung.

ZUSAMMENFASSUNG

Der hepatische I/R-Schaden ist ein relevantes Problem in der Leberchirurgie, insbesondere bei der Lebertransplantation. Aufgrund des wachsenden Bedarfs an Spenderorganen bei sinkender Spendebereitschaft ist es geboten, die vorhandenen Organe möglichst effizient und lange zu nutzen. Da der hepatische I/R-Schaden der häufigste Grund für Organdysfunktion nach Lebertransplantation ist, werden Strategien, welche die negativen Effekte der Ischämie reduzieren, benötigt. Im Rahmen dieser Dissertation wurden zwei Ansatzpunkte zur Reduktion des postischämischen inflammatorischen Schadens untersucht.

Zum einen konnten wir zeigen, dass die Inhibition des PAR-4, eines für die proinflammatorische Antwort aber nicht die prokoagulatorischen Effekte von Thrombin verantwortlichen Rezeptors, ein vielversprechender Ansatzpunkt ist. Es konnten die Migration von inflammatorischen Zelltypen und somit auch der hepatozelluläre Schaden im Tiermodel reduziert werden, ohne die Blutgerinnung negativ zu beeinflussen.

Zu anderen konnten wir die protektiven Effekte von ALR im Rahmen der hepatischen I/R nachweisen. ALR ist ein Wachstumsfaktor, welcher neben Funktion für die Induktion der Regeneration von Hepatozyten auch antiapoptotisch, antinekrotisch und antioxidativ wirkt. In unserem in vivo Model führte die Applikation von ALR während der hepatischen I/R zu einer Reduktion des mikrovaskulären und hepatozellulären Schadens und zur Verminderung der Rekrutierung von proinflammatorischen Zellen.

In unseren Studien konnten wir nachweisen, dass sowohl die Inhibition von PAR-4 als auch die Applikation von ALR vielversprechende Ansatzpunkte zur Reduktion des hepatischen I/R-Schadens sind.

SUMMARY

Hepatic I/R injury is a relevant problem during liver surgery, in particular after liver transplantation. With the increasing need of donor organs and the declining donation rates taken into account it is vital to use the available organs as efficiently and long as possible. Hepatic I/R injury being the most common cause for early organ dysfunction after liver transplantation, we are in deep need of finding strategies to reduce negative effects of Ischemia.

This dissertation analyzes two possible pathways to decrease postischemic inflammatory damage.

On the one hand, we were able to show that inhibition of PAR-4 is a promising approach. PAR-4 is a receptor inducing the proinflammatory effects of thrombin on platelets without interfering in blood coagulation ability. We were able to reduce the migration of inflammatory cell types and consecutively hepatocellular damage in our animal model without hampering blood coagulation.

On the other hand, we proved the protective effects of ALR during hepatic I/R. ALR is a growth factor which induces hepatocellular regeneration and has antiapoptotic, antinecrotic and antioxidative effects. In our in vivo model application of ALR during hepatic I/R lead to reduction of microvascular and hepatocellular damage and decreased recruiting of proinflammatory celltypes.

In our studies we were able to prove inhibition of PAR-4 as well as application of ALR to be promising approaches to reduce hepatic I/R injury.

Targeting Platelet Migration in the Postischemic Liver by Blocking Protease-Activated Receptor 4

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Background. Platelets play a critical role during hepatic ischemia/reperfusion (I/R). Antiplatelet strategies during liver transplantation are, however, limited because of bleeding complications. Thrombin is activated during reperfusion and regulates platelet and endothelial cell function via protease-activated receptor 4 (PAR-4). Interventions at the level of PAR-4, the main platelet receptor for thrombin, are assumed to attenuate the proinflammatory effects of thrombin without affecting blood coagulation. The aim of our study was to analyze the impact of PAR-4 blockade on platelet recruitment and microvascular injury during hepatic I/R.

Methods. C57BL/6 mice undergoing hepatic I/R (90 min/60 min and 240 min) were treated either with a selective PAR-4 antagonist TcY-NH2 or vehicle. Sham-operated animals served as controls. Recruitment of freshly isolated and fluorescence-labeled platelets and $CD4^+$ T cells was analyzed using intravital video fluorescence microscopy. Parameters of tissue injury, regeneration, and blood coagulation were assessed in tissue/blood samples.

Results. Results show that treatment with TcY-NH2 attenuated I/R-induced platelet and $CD4^+$ T-cell recruitment, improved sinusoidal perfusion failure, and reduced apoptotic and necrotic injury. The protective effect of PAR-4 blockade did not suppress hemostasis or liver regeneration.

Conclusion. Our in vivo data suggest PAR-4 as a potential target for future therapeutic strategies against platelet-mediated liver injury on transplantation.

Keywords: Ischemia-reperfusion, Platelets, T cells, Thrombin receptors, Microvascular injury, Regeneration.

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Ischemia/reperfusion (I/R) injury remains the main reason for hepatic dysfunction after liver transplantation and major surgery. It causes up to 10% of early organ failure and can lead to higher incidence of both acute and chronic rejection (*1*). The hepatic microcirculation is considered as the primary target of I/R injury of the liver. Microvascular hepatic I/R injury is initiated by the release and action of proinflammatory cytokines and oxygen radicals, which trigger upregulation of adhesion molecules, intravascular deposition of fibrinogen, as well as interaction of neutrophils, $CD4^+$ T cells, and platelets with the endothelial lining of the hepatic microvasculature. The failure of nutritive sinusoidal perfusion results in the prolongation of focal hypoxia or anoxia and loss of endothelial integrity, which, together, lead to edema formation and oncotic necrosis (*2*).

As previously shown by our group (*3–5*) and other investigators (*6*), platelet recruitment in the hepatic microvasculature plays a central role in the induction of microvascular and hepatocellular injury after warm and hepatic ischemia. We have shown that platelets accumulate in the postischemic hepatic microvasculature already during the early reperfusion phase, release inflammatory mediators, and induce necrotic

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and apoptotic injury. Indeed, immunostaining of liver specimens for platelet accumulation has been suggested as a prognosis-relevant clinical marker of liver injury after transplantation (*7, 8*). Moreover, platelet transfusions are an independent risk factor for survival after liver transplantation (*9*). Recently, we have described a reciprocal interaction between $CD4^+$ T cells and platelets in hepatic sinusoids during hepatic I/R (*10*). Thus, the interaction with platelets represents one of the pathways of T-cell activation and their intravascular recruitment in the postischemic liver. Therefore, interventions at the level of platelet activation would not only attenuate the immediate platelet-induced liver injury but also prevent T-cell recruitment and postischemic liver damage. Otherwise, such blockade might impair liver regeneration because platelets are assumed to be proregenerative by releasing serotonin (*11*).

Antiplatelet strategies during liver transplantation or surgery are, however, limited because of bleeding complications. The challenge at present is to develop antiplatelet agents that inhibit platelet-triggered inflammatory cascades but do not affect hemostasis. A promising strategy could be an intervention at the level of the protease-activated receptors (PARs), G protein-coupled receptors that are responsible for the proinflammatory effects of thrombin on platelets, leukocytes, and endothelial cells. PAR-1 antagonists were able to attenuate proinflammatory effects of thrombin but have a lower suppressive impact on blood coagulation than other antiplatelet drugs (*12, 13*). The family of PARs is composed of four members: PAR-1, PAR-2, PAR-3, and PAR-4. PAR-1 is the high-affinity thrombin receptor in humans, whereas that role is taken by PAR-3 and -4 in rodents (*14*). PAR-4 is the major thrombin receptor in murine platelets and is the only PAR that is expressed in both human and murine platelets. Studies using PAR-deficient mice as well as animals undergoing treatment with selective PAR antagonists have shown a clear protective effect on myocardial (*15*), renal (*16*), and cerebral (*17*) I/R injury. From our own studies, we know that thrombin is activated within the first minutes after the onset of

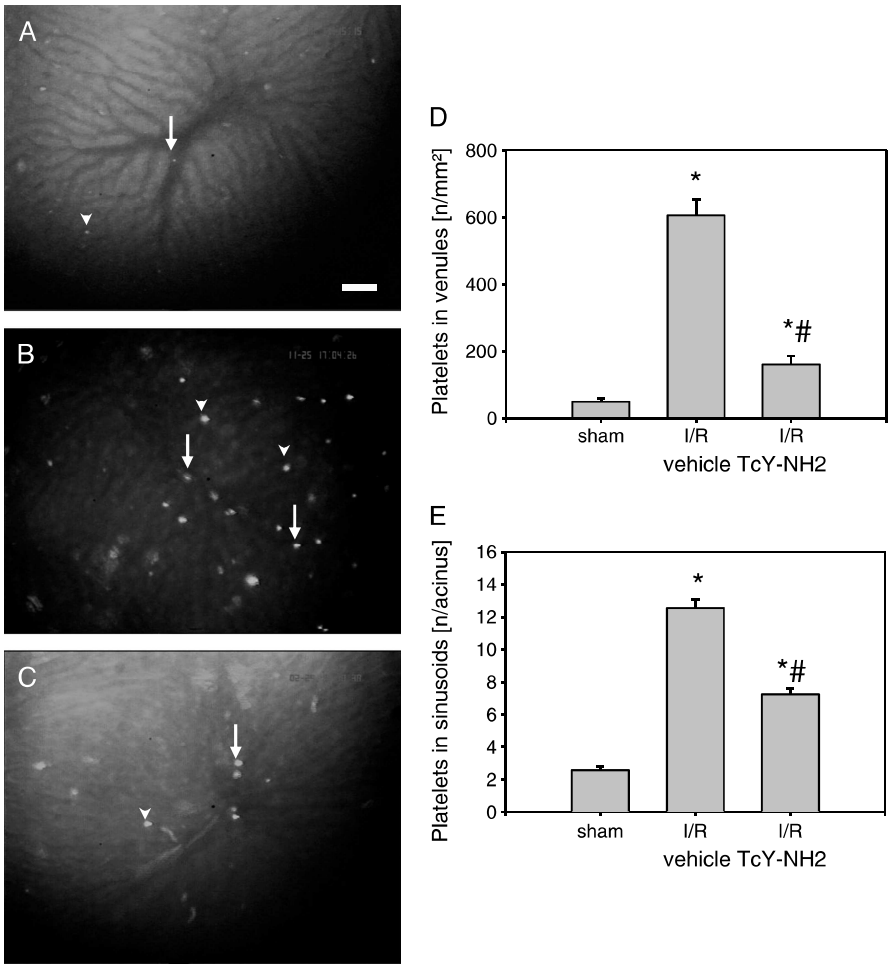

FIGURE 1. Platelet accumulation in vivo. Microphotographs demonstrating rhodamine 6 G-labeled platelets in the hepatic microcirculation of a sham-operated mouse (A), a mouse after I/R (90 min/60 min) treated with saline as vehicle (B), and a mouse after I/R treated with PAR4 inhibitor TcY-NH2 (C) visualized by intravital fluorescence microscopy. *Arrows* depict platelets adherent in postsinusoidal venules; *arrowheads* show platelets accumulated in sinusoids. Also shown are quantitative data on platelet–endothelial cell interactions in postsinusoidal venules (D) and sinusoids (E), respectively. Microscope magnification, ×500. n=7 animals per group, mean±SEM. *P<0.05 versus sham-operated group. #P<0.05 versus I/R+ vehicle. Scale bar, 50 μm.

hepatic reperfusion and that its activity positively correlates with the extent of platelet accumulation in the liver (*18*).

In this study, we tested the hypothesis that inhibition of PAR-4 attenuates platelet and $CD4^+$ T-cell intravascular recruitment, finally leading to protection from I/R injury without increasing the bleeding risk.

RESULTS

Platelet–Endothelial Cell Interactions

Platelets were isolated form syngeneic mice and labeled ex vivo with rhodamine 6 G, and their interactions with the hepatic endothelium were analyzed using intravital microscopy. In sham-operated animals, only few rolling and adherent platelets were observed in postsinusoidal venules (1.5±0.2/mm·sec and 49.3±9.8/mm^2, respectively). Platelet accumulation was also rarely observed in sinusoids of sham-operated mice (2.6±0.2/acinus). Hepatic I/R induced a significant increase in the number of rolling and adherent platelets in venules (14.0±0.9/mm·sec and 606.1±48/mm^2, respectively), as well as of platelets accumulated in sinusoids (12.6±0.5/acinus). In contrast, I/R-induced platelet–endothelial cell interactions were significantly (P<0.05) attenuated in sinusoids (7.3±0.4/acinus) and postsinusoidal venules (4.9±0.6/mm·sec rolling platelets and 161±27.5/mm^2 adherent platelets) of mice treated with PAR-4 inhibitor TcY-NH2 (Fig. 1).

$CD4^+$ T-cell–Endothelial Cell Interactions

The interactions of freshly isolated and fluorescence-labeled $CD4^+$ T cells with endothelial cells were analyzed in hepatic sinusoids in vivo in a separate set of experiments. Hepatic I/R induced a significant increase in the number of $CD4^+$ T cells adherent in hepatic sinusoids (7.1±0.8/acinus)

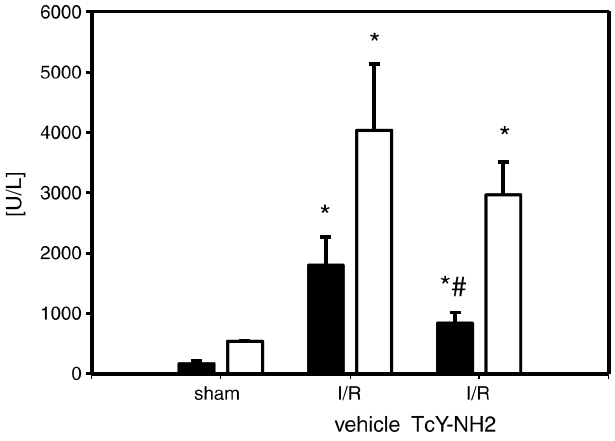

FIGURE 3. Liver enzyme activity. Serum activity of the liver enzymes ALT (*closed bars*) and AST (*open bars*) was determined as a marker of hepatocellular necrotic injury in sham-operated mice, in mice after I/R (90 min/60 min) treated with saline as vehicle, and in mice after I/R treated with PAR4 inhibitor TcY-NH2. n=7 animals per group, mean±SEM. *P<0.05 versus sham-operated group. #P<0.05 versus I/R+vehicle.

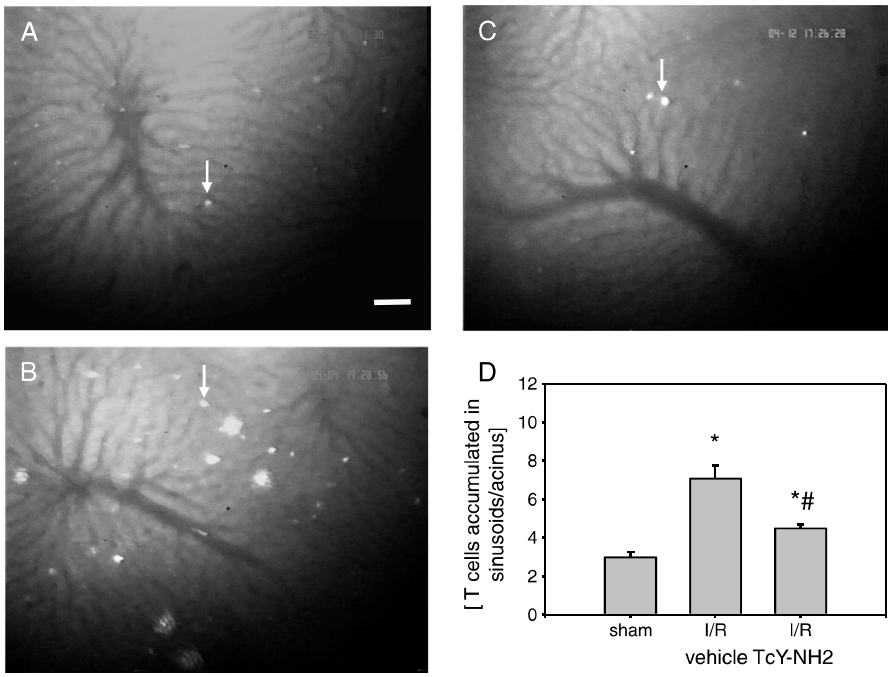

FIGURE 2. Recruitment of $CD4^+$ T cells in vivo. Microphotographs demonstrating CFSE-labeled $CD4^+$ T cells in the hepatic microcirculation of a sham-operated mouse (A), a mouse after I/R (90 min/60 min) treated with saline as vehicle (B), and a mouse after I/R treated with PAR4 inhibitor TcY-NH2 (C) visualized by intravital fluorescence microscopy. *Arrows* depict $CD4^+$ T cells accumulated in sinusoids. Quantitative data on $CD4^+$ T-cell accumulation in sinusoids are shown (D). Microscope magnification, ×500. n=7 animals per group, mean±SEM. *P<0.05 versus sham-operated group. #P<0.05 versus I/R+vehicle. Scale bar, 50 μm.

as compared to the sham-operated group (2.8±0.3/acinus). In contrast, accumulation of $CD4^+$ T cells was significantly lower in the I/R group treated with TcY-NH2 (Fig. 2).

Leukocyte Migration

Since the PAR-4 signaling has been reported to trigger also endothelial activation during inflammation (endothelial cells also express PAR-4), we analyzed leukocyte–endothelial cell interactions in vivo. Leukocytes were labeled by a systemic application of rhodamine 6 G. As shown in Figure S1 (see **SDC**, http://links.lww.com/TP/A889), the number of rolling and firmly adherent leukocytes in postsinusoidal venules was increased after I/R (15.2±2.2/mm·sec and 667.9±55.3/mm^2, respectively) as compared to the sham-operated mice (2.8±0.3/mm·sec and 70.1±18.5 /mm^2, respectively). Treatment with the PAR-4 inhibitor, however, did not affect the postischemic leukocyte–endothelial cell interactions within hepatic venules.

Sinusoidal Perfusion Failure

Microvascular I/R injury was assessed by determining sinusoidal perfusion via intravital microscopy. In the sham-operated group, almost all sinusoids were perfused (8.6%±1.2% nonperfused sinusoids). In contrast, the hepatic I/R led to a significant impairment of sinusoidal perfusion (30.1%±2.1% nonperfused sinusoids). The microvascular I/R injury was, however, significantly improved in mice undergoing PAR-4 blockade (see **Figure S2, SDC**, http://links.lww.com/TP/A889).

Liver Necrosis: Liver Enzyme Activity and Histology

The serum activity of hepatic transaminases was measured as a parameter of necrotic I/R injury. Hepatic I/R (90 min/60 min) increased dramatically the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST; 1798±462 and 4033±1107 U/L, respectively) as compared to the sham-operated group (166±39 and 538±86 U/L, respectively). In the treated I/R group, the activity of ALT was significantly reduced. The AST activity was also lower; however, the changes did not reach the level of significance (Fig. 3). In line with these data, evaluation of hematoxylin-eosin–stained tissue sections revealed a markedly lower extent of necrosis in the TcY-NH2–treated group (see **Figure S3, SDC**, http://links.lww.com/TP/A889).

Apoptosis Assays: TUNEL and Caspase-3 Tissue Activity

TUNEL was performed in paraffin sections in an attempt to assess the apoptotic tissue injury. After 60 min of

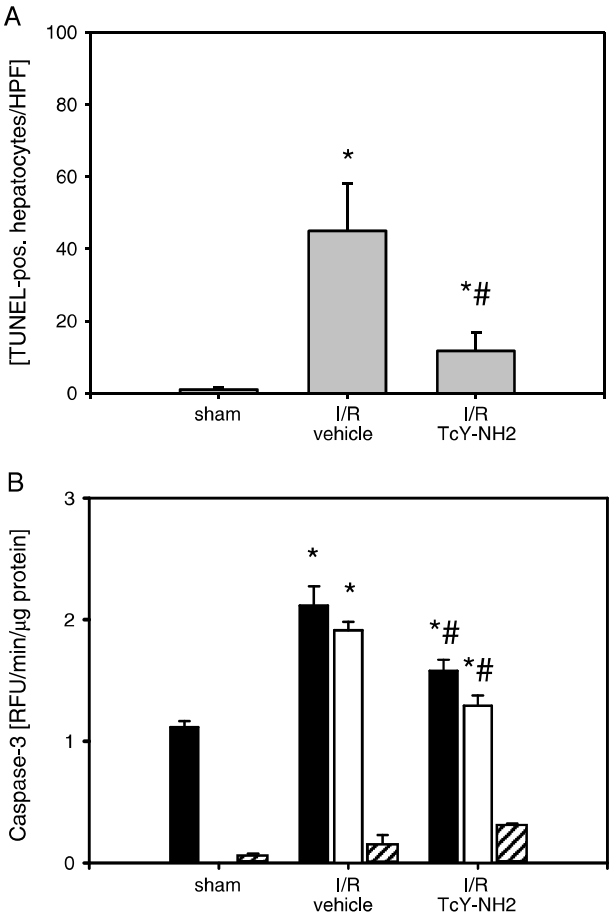

FIGURE 4. Apoptosis assays. (A) number of apoptotic hepatocytes in tissue sections of sham-operated mice, in mice after I/R (90 min/240 min) treated with saline as vehicle, and in mice after I/R treated with PAR4 inhibitor TcY-NH2. (B) quantitative data on caspase-3 activity in the same groups after 60 min (*closed bars*) and 240 min (*open bars*) of reperfusion as determined in shock-frozen tissue specimens. *Dashed bars* show additional controls on incubation of tissue homogenates with caspase-3, -7, and -8 inhibitor Ac-DEVD-CHO. n=7 animals per group, mean±SEM. *P<0.05 versus sham-operated group. #P<0.05 versus I/R+vehicle.

TABLE 1. Effect of PAR-4 blockade on blood coagulation

Parameter/group	I/R vehicle	I/R TcY-NH2
ROTEM		
Clotting time (sec)	170.7± 25.3	94.3±18.8
Clot formation time (sec)	75.7±9.3	58.0±4.2
Maximum clot firmness (mm)	59.8±1.0	59.2±1.1
α angle (°)	75.3±1.7	79.0±0.7
Tail bleeding time (sec)	165.2±22	170.8±23

In two independent sets of experiments, blood coagulation was evaluated either by thrombelastography (ROTEM) in whole blood or by tail bleeding assay. Mice undergoing I/R (90/60 min) were treated either with TcY-NH2 or vehicle. Mean±SEM. N=7 each group.

reperfusion, almost no apoptotic hepatocytes were detected in all experimental groups (sham: 0±0 /HPF, I/R+vehicle: 0.5±0.8 /HPF, I/R+TcY-NH2: 0±0 /HPF). After 240 min of reperfusion, however, the number of apoptotic hepatocytes was dramatically increased (45.0±11.8/HPF) in the vehicle-treated group. As shown in Figure 4A, postischemic apoptosis was significantly attenuated in the group treated with TcY-NH2.

Caspase-3 activity was measured in liver tissue after 60 and 240 min of reperfusion. Upon ischemia, we observed a significant increase in the caspase-3 tissue activity at both reperfusion times (by about twofold). In the treated group, however, caspase-3 activity was significantly lower (Fig. 4B).

Ki-67 Staining

Liver specimens were stained for Ki-67, a well-recognized marker of liver regeneration. As demonstrated in Figure S4 (see **SDC**, http://links.lww.com/TP/A889), liver regeneration was rather suppressed in both postischemic groups after 60 min of reperfusion (sham: 2.8±0.7, I/R+vehicle: 1.6±0.6, I/R+TcY-NH2: 2.0±0.4 Ki-67–positive cells/HPF). After a prolonged reperfusion time (240 min), the regeneration capacity was recovered as demonstrated by the increased number of Ki-67–positive hepatocytes (I/R+vehicle: 5.6±0.6 and I/R+TcY-NH2: 4.6±0.8 Ki-67–positive cells/HPF). No significant difference was observed between the I/R groups treated either with vehicle or with the PAR-4 inhibitor.

Blood Coagulation

In two separate sets of experiments, we analyzed the effect of PAR-4 blockade on blood coagulation. In the first set, whole blood was harvested by heart puncture from animals undergoing hepatic I/R and treatment either with vehicle or with TcY-NH2 infused intra-arterially before reperfusion. Blood coagulation was assessed by thrombelastography (ROTEM). In the second set, tail bleeding time was determined in the experimental groups. As shown in Table 1, the blockade of PAR-4–dependent platelet–endothelial cell interactions did not significantly suppress blood coagulation. All parameters measured were comparable between both I/R groups.

DISCUSSION

Experimental and clinical studies have suggested a critical role of platelets for hepatic I/R injury. Although a therapeutic potential of inhibition of postischemic platelet recruitment during liver surgery or transplantation has been frequently discussed in the literature, this approach could not find a way into the clinical routine because of the assumed high risk of bleeding complications. A potential target for intervention of platelet activation during hepatic I/R represent the PARs, which are responsible for the proinflammatory effects of thrombin but not required for blood coagulation.

Our in vivo results show that PAR-4 inhibition with the specific inhibitor TcY-NH2 leads to a significant attenuation of postischemic platelet-endothelial cell interactions in the liver. Both I/R-induced platelet rolling and firm adherence were reduced in the treated group. The mechanisms of platelet recruitment during hepatic I/R have been described by our group previously. Platelets become activated because of the initial contact with activated endothelial cells. Whereas the initial interaction (platelet rolling) is mediated by endothelial P-selectin (*3*), their permanent adherence occurs because of the binding of GPIIb/IIIa receptor on platelets to Intercellular Adhesion Molecule 1 (ICAM-1) on hepatic endothelial cells. Fibrinogen serves here as a bridging molecule (*4*). Based on these data, we suggest that PAR-4 blockade can influence platelet migration in the liver i) by interrupting the thrombin-dependent pathway of platelet activation and ii) by affecting the endothelial activation, which occurs in a PAR-4–dependent manner. Taken together, our in vivo study demonstrates for the first time the role of PAR-4 for platelet recruitment during I/R of the liver.

As shown by thrombelastography as well as by tail bleeding time assay, treatment with the PAR-4 inhibitor was not associated with inhibition of blood coagulation in animals undergoing hepatic I/R. PAR-4 is the sole thrombin receptor capable of transducing a signal sufficient for platelet activation in mice (*19*). Indeed, $PAR4^{-/-}$ mice are protected against several experimental models of thrombosis, yet they do not exhibit spontaneous bleeding (*19–22*). Thus, a key advantage proposed of PAR antagonists over anticoagulants is that they may preserve hemostatic function by sparing the non-PAR functions of thrombin—most notably that of fibrin formation—but also feedback activation of coagulation.

The next important finding of the study is that blockade of the PAR-4–dependent platelet recruitment attenuates $CD4^+$ T-cell migration. We, as well as other groups, have demonstrated that $CD4^+$ T cells become activated during alloantigen-independent hepatic I/R. Such activation is associated with a rapid recruitment of $CD4^+$ T cells in hepatic sinusoids already during early (30 min) reperfusion followed by their migration through the endothelial barrier to injured areas (*10*). The mechanisms of $CD4^+$ T-cell activation and migration during alloantigen-independent hepatic I/R are not fully understood. In our previous work, we have described a colocalization and a reciprocal activation of platelets and $CD4^+$ T cells in the postischemic hepatic microvasculature. We suggest, therefore, a role of platelets as an alternative mechanism of T-cell activation via P-selectin–PSGL-1 as well as CD40-CD40L binding. In addition, PARs can directly regulate functions of T cells. Recently, Rullier et al. (*13*) have reported that PAR-1 knockout reduces experimentally induced liver fibrosis. There was also a significant decrease in T lymphocyte infiltration in PAR-1–deficient mice. The authors suggest that the profibrogenic effects of thrombin are independent of its effects on blood coagulation and, in fact, are caused by direct effects on fibrogenic cells and possibly on T lymphocytes. The effect of PAR-4 blockade on T-cell migration might get major clinical relevance for liver transplantation. Recent overviews propose that activation of elements of the innate immune system, triggered as a consequence of tissue injury sustained during I/R, can initiate and amplify the adaptive response leading to transplant rejection (*23, 24*). Therefore, a reduction of the I/R-mediated, alloantigen-independent T-cell response might decrease the rejection rate upon liver transplantation.

Because PAR-4 is also expressed on endothelial cells, we assumed that an interruption of thrombin-dependent endothelial activation by PAR-4 inhibitor would also attenuate neutrophil recruitment. Recruitment of neutrophils is mediated by translocation selectins on activated endothelial cells and their ligands on the surface of neutrophils (rolling),

by interactions between endothelial ICAM-1 and neutrophil integrins (firm adherence), and, finally, by cell contact receptors junctional adhesion molecule A and endothelial cell–selective adhesion molecule (transmigration) (*25, 26*). We observe, however, that neutrophil migration is independent of PAR-4 signaling. A possible explanation is that the postischemic expression patterns of endothelial selectins or ICAM-1 are independent on PAR-4 stimulation.

A major aim of our study was to prove whether the treatment with the PAR-4-inhibitor is protective against I/R injury. Such an effect seems plausible because both platelets and T cells are known to mediate necrotic and apoptotic injury in the postischemic liver. They also play a critical role for the development of sinusoidal perfusion failure. The mechanisms by which both cell types mediate I/R injury have been discussed in the recent literature (*27–30*). Although platelets can induce tissue injury directly by the release of inflammatory mediators and free oxygen radicals, $CD4^+$ T cells are not cytotoxic and contribute to I/R injury by modulating activation and function of other cells, such as platelets, endothelial cells, and, most likely, hepatic stellate cells. Our results show a clear protective effect of PAR-4 blockade on apoptotic injury as well as on sinusoidal failure. AST/ALT levels were measured as a marker of hepatocellular integrity and liver necrosis. While AST is localized in the cytoplasm and mitochondria, ALT is localized in the cytoplasm only. Activity of both enzymes was reduced in the treated group. The protective impact of PAR-4 blockade on liver necrosis is also supported by the histologic analysis.

In addition to their proinflammatory role, platelets seem to play a role for liver regeneration. The data from the literature are, however, controversial. Lesurtel et al. (*11*) suggested that platelet-derived serotonin is involved in the initiation of liver regeneration. In a mouse model of liver regeneration, thrombocytopenia or impaired platelet activity resulted in the failure to initiate cellular proliferation in the liver. In contrast, TGF-β released by activated platelets strongly suppresses liver regeneration (*31*). Our data demonstrate that initial liver regeneration is slightly suppressed after 60 min of reperfusion in both postischemic groups irrespective of PAR-4 blockade. After prolonged reperfusion time (240 min), the liver recovers from the initial injury and we have seen a strong increase in the number of Ki-67–positive hepatocytes in mice treated with vehicle as well as with PAR-4 inhibitor. There was no difference between the treated and nontreated groups. We conclude from these findings that a therapeutic interruption of platelet recruitment via PAR-4 does not negatively affect liver regeneration after hepatic I/R, at least during the first hours of reperfusion.

In conclusion, these in vivo data show that inhibition of PAR-4 attenuates platelet as well as $CD4^+$ T-cell recruitment in the postischemic liver and ameliorates sinusoidal perfusion failure and apoptotic cell death without adverse effects on blood coagulation and postischemic liver regeneration. Since PAR antagonists are currently tested in clinical trials (phase 3) in cardiology (*32*), a potential usage in the liver surgery seems to be possible in the future.

MATERIALS AND METHODS

The methods are described in more detail in the Supplemental Digital Content (see **SDC**, http://links.lww.com/TP/A889). Briefly, in C57BL/6 mice, a catheter was inserted into the carotid artery for measurement of mean arterial pressure and application of fluorescence dyes. A warm reversible ischemia of the left liver lobe was induced for 90 min as described previously (*4*). All experiments were carried out according to the German legislation on protection of animals.

In Vivo Imaging of Cell Migration in the Hepatic Microcirculation

Intravital video fluorescence microscopy was performed as described previously (*3, 4*). Leukocyte (neutrophil)–endothelial cell interactions were visualized in postsinusoidal venules after an intra-arterial application of rhodamine 6 G. Sinusoidal perfusion was analyzed after plasma labeling with FITC-dextran. Platelets were isolated by density gradient centrifugation from syngeneic donor animal's whole blood and labeled with rhodamine 6 G as described (*4*). A total of 1×10^8 platelets stained with rhodamine 6 G were infused intra-arterially after 60 min of reperfusion. $CD4^+$ T cells were isolated from spleens of syngeneic mice using a magnetic cell sorting system (miniMACS) and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described previously (*10*). A total of 1×10^7 $CD4^+$ CFSE-labeled cells were infused after 60 min of reperfusion and visualized in hepatic sinusoids.

Assessment of Tissue Injury and Regeneration

Blood and tissue samples were taken at the end of the experiment. Serum AST and ALT activities were determined at 37°C using standardized test systems (HiCo-GOT, HiCo-GPT; Roche). For the assessment of apoptosis, paraffin sections were stained by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling (TUNEL). Hematoxylin-eosin staining of paraffin sections was performed in an automatized manner for histologic examination of necrotic damage. Caspase-3 activity was determined as a parameter of apoptosis in homogenates of frozen liver tissue by using the selective substrate for caspase-3, -7, and -8, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid 7-amino-4-methylcoumarin (Ac-DEVD-AMC) in the presence or absence of the specific caspase-3, -7, and -8 inhibitor acetyl- L-aspartyl- L-glutaminyl- L-threonyl- L-aspart-1-al (Ac-DEVD-CHO) (PeptaNova). The effect of PAR-4 blockade on hepatocellular proliferation was assessed by Ki-67 staining of paraffin sections with a commercially available kit (Dako).

Experimental Protocols

Effect of PAR-4 Blockade on Platelet/Leukocyte Recruitment, Liver Regeneration, and I/R Injury

PAR-4 blockade was achieved by using the selective water-soluble inhibitor TcY-NH2 (*trans*-Cinnamoyl-YPGKF-NH^2; Tocris Bioscience, Ellisville, MO). The inhibitor was applied intra-arterially 5 min before the onset of reperfusion. In pilot dose-finding experiments, we found that the dose of 0.6 mg/kg body weight has an optimal effect in our model (data not shown). The same dose of the inhibitor was also effective in a model of pleural inflammation in mice (*33*).

Platelet– and leukocyte–endothelial cell interactions as well as sinusoidal perfusion were quantified using intravital microscopy in sham-operated mice and mice after I/R (90 min/60 min) treated with saline (200 μL) as a vehicle (n=7 per group). In an additional I/R group (n=7), PAR-4 was blocked with TcY-NH2 (0.6 mg/kg bodyweight in 200 μL of saline). The intravital microscopy was performed after 60 min of reperfusion. Thereafter, tissue and plasma samples were taken at the end of the experiment. Measurement of the liver enzyme activity, TUNEL, and Ki-67 staining was performed as described above.

Effect of PAR-4 Blockade on $CD4^+$ T-cell Migration

In a separate set of experiments (n=7 each group), $CD4^+$ T-cell migration was analyzed in a sham-operated group, as well as in two I/R groups treated with either saline or TcY-NH^2 (0.6 mg/kg body weight). $CD4^+$ T cells were isolated and labeled as described above. Intravital microscopy was performed after 60 min of reperfusion.

Effect of PAR-4 Blockade on Blood Coagulation

In two independent sets of experiments (n=7 each group), we assessed the question of whether PAR-4 inhibition affects blood coagulation:

I) ROTEM: In two I/R groups (I: 90 min, R: 60 min), thrombelastography was performed in whole blood withdrawn from mice treated with either TcY-NH2 or saline (vehicle) as described above (n=7 each group).
II) Tail bleeding time: In two I/R groups (90 min/60 min), 2 mm of the tip of the tail was cut, and the tail was immersed in 37°C saline immediately. The time to stopping of bleeding was defined as tail bleeding time (n=7 each group).

Statistics

ANOVA on ranks followed by Student-Newman-Keuls methods were used for the estimation of stochastic probability in intergroup comparison. Mann-Whitney test was used for two-group comparison for the analyses of blood coagulation (SigmaStat, Jandel Scientific, Erkrath, Germany). Mean values±SEM are given. $P<0.05$ was considered significant.

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Augmenter of liver regeneration attenuates inflammatory response in the postischemic mouse liver *in vivo*

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ABSTRACT

Background: Augmenter of Liver Regeneration (ALR), a protein synthesized in the liver is suggested to be protective against oxidative stress–induced cell death. Hepatic ischemia–reperfusion (I/R) injury is triggered by reactive oxygen species. Here, we tested the hypothesis that ALR attenuates hepatic I/R injury *in vivo*.

Methods: C57BL6 mice were subjected to warm hepatic ischemia for 90 min. Either recombinant ALR (100 μg/kg) or vehicle were administered to mice prior ischemia. During reperfusion, neutrophil and CD4+ T cell migration and sinusoidal perfusion were analyzed using intravital microscopy. Alanine aminotransferase–aspartate aminotransferase (plasma) and caspase-3 (tissue) activities were determined as markers of hepatocellular necrotic and apoptotic injury.

Results: Hepatic I/R led to dramatic enhancement of neutrophil and CD4+ T cell recruitment in hepatic microvessels, sinusoidal perfusion failure, and strong elevation of aspartate aminotransferase–alanine aminotransferase and caspase-3 activities. During early reperfusion (60 min), the pretreatment with ALR improved postischemic perfusion failure ($P < 0.05$) and attenuated liver enzyme activities. Recruitment of CD4+ T cells, but not of neutrophils was attenuated. After 240 min of reperfusion, the protective effect of ALR was stronger, since the liver enzyme activity, perfusion failure, and leukocyte influx were significantly attenuated. As shown by the measurement of caspase-3 activity, postischemic apoptosis was reduced in the ALR-treated group.

Conclusions: Our *in vivo* data show that ALR has a therapeutic potential against postischemic liver injury. As a mechanism, we suggest a direct protective effect of ALR on apoptotic and necrotic death of hepatocytes and an attenuation of inflammatory cell influx into the postischemic tissue.

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1. Introduction

Hepatic ischemia–reperfusion (I/R) injury is the main reason for graft dysfunction on liver transplantation. The postischemic inflammatory response is primarily initiated by oxidative burst within minutes after reoxygenation. The mechanisms of hepatic I/R injury include intravascular recruitment of inflammatory blood cells, such as neutrophil granulocytes, CD4+ T cells, platelets in the hepatic microcirculation, activation of Kupffer cells, and local release of inflammatory mediators. These pathways together lead to edema formation, deterioration of the nutritive sinusoidal perfusion and, finally, cell death via apoptosis and necrosis (reviewed in [1]).

In this study, we investigated the role of Augmenter of Liver regeneration (ALR) on the inflammatory response on hepatic I/R *in vivo*. ALR, a 22-kDa protein encoded by the growth factor erv1-like gene in humans, is a physiologically apparent growth factor, which is expressed in the liver and in other tissues such as heart, brain, spleen, lung, skeletal muscle, kidney, and testis [2,3]. In the liver, ALR is found ubiquitously, but exclusively in hepatocytes [4]. ALR supports liver regeneration and is currently discussed as a possible hepatoprotective factor in clinical settings. In addition to its strong proregeneratory effect, ALR has been shown to attenuate hepatocellular apoptosis induced by ethanol, TRAIL, anti-Apo, transforming growth factor β, and actinomycin D *in vitro* [5]. Moreover, intracellular ALR was found to be a survival factor as its depletion causes rapid mitochondrial dysfunction and apoptotic and necrotic death of hepatocytes [6]. Noteworthy, unlike the growth factors hepatocyte growth factor and epidermal growth actor, ALR acts in a liver-specific manner [5,7]. The recent findings have shown that ALR [8] can also act as a reductant for cytochrome C that is imported via the Mia40-independent pathway [9,10]. The abundance of cytochrome C in mitochondrial intermembrane space argues for its function as a potential oxidant for ALR *in vivo* [8].

The ability of ALR to attenuate oxidative stress–induced inflammatory mechanisms in combination with antiapoptotic and proregeneratory effects suggests this protein as a promising therapeutic agent against I/R-induced liver injury. The impact of ALR during hepatic I/R, however, has not been investigated, so far. In the present study, we tested the hypothesis that ALR attenuates the inflammatory response after hepatic I/R injury by affecting inflammatory cell trafficking and reducing apoptotic and necrotic cell death *in vivo*.

2. Materials and methods

2.1. Animals

For experiments, 5- to 7-wk-old female C57BL/6 wild-type mice (Charles River, Sulzfeld, Germany) were used. All experiments were carried out according to the German legislation on protection of animals.

2.2. Surgical procedure and experimental protocol

The surgical procedure was described elsewhere [11]. Briefly, animals received buprenorphine analgesia (0.1 mg/kg body weight, subcutaneously) 30 min before surgery. Thereafter, under inhalation anesthesia with isoflurane-N_2O, a catheter was inserted into the left carotid artery for measurement of mean arterial pressure and application of fluorescence dyes as described previously. A warm (37°C) reversible ischemia of the left liver lobe was induced for 90 min by clamping the supplying nerve vessel bundle using a microclip. Three separate sets of experiments were performed.

Reperfusion time was 60 min in the first set of experiments. A sham-operated group and two I/R groups were analyzed ($n = 6$ each): an I/R group treated with saline (200 μL, intra-arterially 15 min before ischemia) and an I/R group treated with ALR (100 μg/kg body weight in 200 μL saline, intra-arterially before ischemia). Recombinant ALR (rALR) was prepared in our laboratory as described previously [12]. In pilot dose-finding experiments, we found that the dose of 100 μg/kg body weight has an optimal effect in our model (data not shown). In the second set of experiments, the effects were assessed after a prolonged reperfusion time (240 min) in a sham-operated group and in two I/R groups treated either with vehicle or ALR ($n = 6$ each). In the third set of experiments, migration of CD4+ T cells was analyzed after 60 min of reperfusion in a sham-operated group, in a vehicle-treated I/R group, and in an ALR-treated I/R group ($n = 6$ each group). Sham-operated animals underwent short (3 s) clamping of the left liver lobe and afterward were monitored under anesthesia for a total time of 150 min (90 + 60 min) in the first and the third sets of experiments, and for 330 min (90 + 240 min) in the second set.

2.3. Leukocyte (neutrophil)-endothelial cell interactions and sinusoidal perfusion failure

Leukocytes were stained *in vivo* by rhodamine 6G (0.05%, 100 μL, intra-arterially, Sigma-Aldrich, Taufkirchen, Germany) and visualized in hepatic postsinusoidal venules using intravital fluorescence microscopy as described previously [13]. Thereafter, the plasma marker fluorescein isothiocyanate–conjugated dextran (MW 150000; 0.1 mL, 5%, Sigma) was infused and sinusoidal perfusion was analyzed using an I2/3 filter block in sinusoids within seven to 10 acini. Intravital microscopy was started either after 60 min or after 240 min (second set of experiments) and lasted about 20 min.

All videotaped images were quantitatively analyzed offline in a blinded fashion using Capimage software (Zeintl, Heidelberg, Germany). Rolling leukocytes were defined as cells crossing an imaginary perpendicular through the vessel at a velocity significantly lower than the centerline velocity in the microvessel. Their numbers are given as cells per second per vessel cross-section. Leukocytes firmly attached to the endothelium for more than 20 s were counted as permanently adherent cells and were quantified as the number of cells per square millimeter endothelial surface calculated from the diameter and length of the vessel segment observed. The sinusoidal perfusion failure was calculated as the percentage of nonperfused sinusoids of all sinusoids visible.

In a separate set of experiments, migration of CD4+ T cells was analyzed in the hepatic microcirculation *in vivo*. CD4+ T cells were isolated from spleens of syngeneic mice using

a magnetic cell sorting system (miniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as described previously [14]. A total of 1×10^7 $CD4^+$ CFSE-labeled cells were infused after 60 min of reperfusion and visualized using intravital microscopy in hepatic sinusoids. Adherent CD4+ T cells were counted in five to seven acini per experiment, their numbers are given as cells per acinus.

2.4. Liver enzymes

Blood samples were taken from the carotid artery at the end of the experiment, immediately centrifuged at 2000*g* for 10 min and stored at −80°C. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined at 37°C with an automated analyzer (Hitachi 917; Roche-Boehringer, Mannheim, Germany) using standardized test systems (HiCo GOT and HiCo GPT; Roche-Boehringer).

2.5. Caspase activity

Frozen liver tissue was homogenized with 1 mL of ice cold lysis buffer per 100 mg of liver tissue containing 25 mM HEPES, pH 7.5, 5 mM MgCl2, 0.1% CHAPS, 0.1 mM ethylenediaminetetraacetic acid. After filtration, the homogenates were centrifuged, and the protein concentration of the supernatant was determined using the bicinchoninic acid protein assay kit (Pierce Protein Biology Products, Rockford, IL). Twenty microgram of protein were used in a final volume of 200 μL in a 96-well plate with 50 μM of the selective substrate for caspase-3, -7, and -8, Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid 7-amino-4-methylcoumarin (PeptaNova, Sandhausen, Germany) in the presence or absence of 10 μM of the specific caspase-3, -7, and -8 inhibitor Acetyl-L-aspartyl-L-glutaminyl-L-threonyl-L-aspart-1-al (PeptaNova) in substrate buffer including 50 mM HEPES, pH 7.5, 1% sucrose, 0.1 % CHAPS, and 10 mM dithiothreitol. The amount of the fluorescent AMC released was measured by fluorometry (Infinite F200; Tecan, Männedorf, Switzerland) with 360 nm excitation and 430 nm emission filters. Data are expressed as change in fluorescence (ΔF) per minute per microgram.

2.6. Statistics

Analysis of variance on ranks followed by Student–Newman–Keuls methods were used for the estimation of stochastic probability in intergroup comparison (SigmaPlot 12; Systat Software, Erkrath, Germany). Mean values ± standard error of the mean are given. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Leukocyte-endothelial cell interactions

Leukocyte (neutrophil)-endothelial cell interactions were analyzed in postsinusoidal venules as a sign of inflammation after hepatic I/R. Leukocytes were labeled by systemic application of rhodamine 6G. As shown in Figure 1, the number of

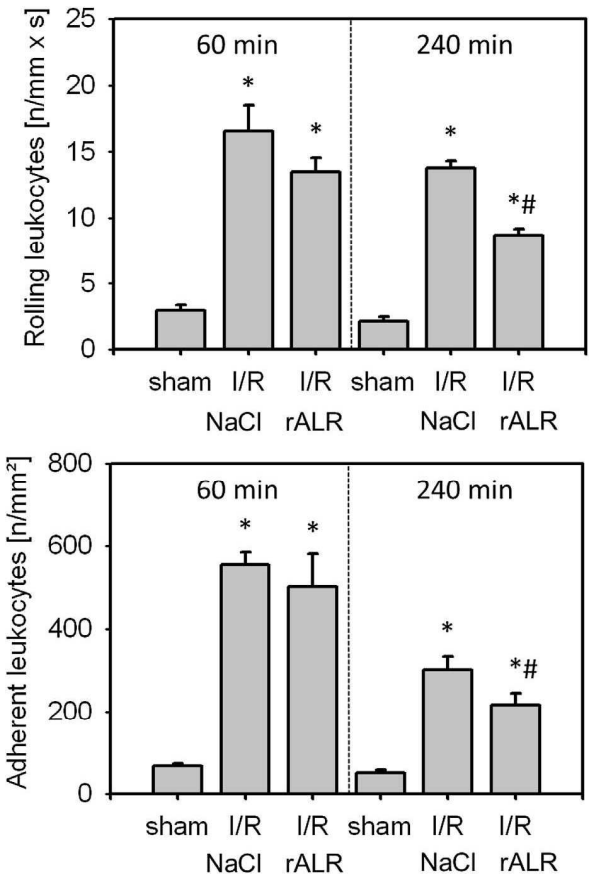

Fig. 1 – Leukocyte-endothelial cell interactions. Leukocyte rolling and adherence were quantified using intravital microscopy in postsinusoidal venules of sham-operated mice, mice after I/R (90/60 or 240 min) treated with saline as vehicle, and of mice after I/R treated with rALR. N = 6 animals per group, mean ± standard error of the mean, *P < 0.05 *versus* sham-operated group, #P < 0.05 *versus* I/R + vehicle.

rolling and firmly adherent leukocytes in postsinusoidal venules was dramatically enhanced after 90 min of ischemia followed by 60 min (16.5 ± 1.6/mm·sec and 554.4 ± 24.7/mm^2, respectively) and 240 min (13.8 ± 0.5/mm·sec and 318.1 ± 21.3/mm^2, respectively) of reperfusion compared with the sham-operated mice. On application of ALR, however, leukocyte migration was not affected after 60 min of reperfusion. In contrast, leukocyte recruitment was significantly attenuated (rolling by about ~37%, adherence ~33%) after a prolonged reperfusion time (240 min).

3.2. Sinusoidal perfusion failure

Sinusoidal perfusion failure was determined using intravital microscopy as a recognized parameter of microvascular I/R injury. Hepatic I/R resulted in a severe deterioration of the sinusoidal perfusion after 60 min (32.9 ± 4.5% nonperfused sinusoids) and 240 min (29.3 ± 0.4%) of reperfusion (Fig. 2). In the ALR-treated groups, the perfusion failure was significantly improved after 60 min and after 240 min of reperfusion.

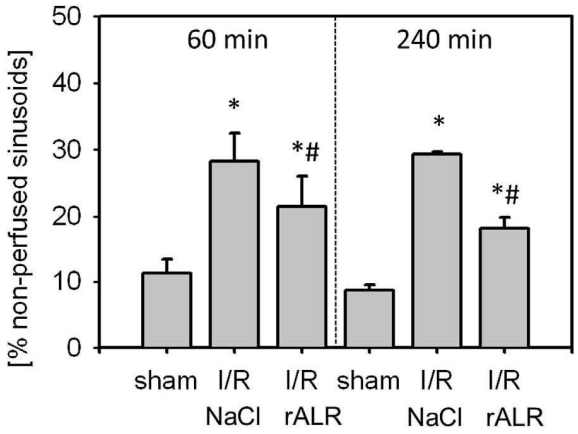

Fig. 2 – Sinusoidal perfusion failure. Sinusoidal perfusion failure (=percentage of nonperfused sinusoids) was measured using intravital microscopy as a parameter of microvascular hepatic injury in sham-operated mice, mice after I/R (90/60 or 240 min) treated with saline as vehicle, and of mice after I/R treated with rALR. N = 6 animals per group, mean ± standard error of the mean, *P < 0.05 *versus* sham-operated group, #P < 0.05 *versus* I/R + vehicle.

3.3. *CD4+ T cell migration*

CD4+ T cells play a critical role during hepatic I/R. In a separate set of experiments, we answered the question whether ALR influences T cell migration in the postischemic liver. As shown in Figure 3, the number of accumulated CD4+ T cells was very low in the sham-operated group (0.8 ± 01 per acinus), whereas it was significantly increased in the vehicle-treated group after I/R (9.2 ± 0.7 per acinus). Adherent CD4+ T cells were found predominantly in sinusoids and were almost absent in postsinusoidal venules. In ALR-treated animals, CD4+ T cell recruitment was significantly attenuated (by about ~70%).

3.4. *Apoptosis*

During the early reperfusion phase (60 min), apoptosis was almost absent in all experimental groups (data not shown). In contrast, the caspase-3 activity was significantly increased in the vehicle-treated I/R group after prolonged reperfusion time (125.8 ± 4.5 RFU/min/μg protein). Treatment with ALR, however, led to a significant protection from apoptotic damage after 240 min of reperfusion (Fig. 4).

3.5. *Liver enzyme activity*

The serum activity of hepatic transaminases was measured as a parameter of necrotic I/R injury. Hepatic I/R (90/60 min) dramatically increased the activity of AST and ALT (3938 ± 610 and 1615 ± 457 U/L, respectively) compared with the sham-operated group. After 60 min of reperfusion, the liver enzyme activity was lower (AST significant and ALT not significant) in the ALR-treated group (Fig. 5). After 240 min of reperfusion, the protective effect of ALR on necrotic injury was much stronger, because both ALT and AST were significantly lower than that in the vehicle-treated controls.

4. Discussion

In response to the steeply rising demand for transplantation, both the number of transplant centers and the number of patients on waiting lists have grown rapidly. Because of a shortage of organ donation, each year a greater number of patients die while awaiting donor organs. This increases interest to maximize and optimize the use of potential organs (e.g., partial liver transplantation or usage of marginal organs). Hepatic I/R is the most common cause for organ dysfunction and failure after liver transplantation and, therefore, strategies to minimize the negative effects of ischemia are now at the forefront of clinical and experimental studies [15]. The increasing usage of marginal organs and undersized grafts requires a therapeutic strategy with a potential (1) to stimulate liver regeneration and (2) to attenuate postischemic inflammatory response.

A promising candidate is ALR. ALR is a protein secreted constitutively by hepatocytes and is present in the intermembrane space of mitochondria, in the cytosol and the nucleus [4,16,17]. Importantly, hepatic inflammation is associated with a decreased expression of ALR in hepatocytes and its exogenous substitution (e.g., as rALR) is protective. As shown previously, treatment with ALR accelerated liver regeneration in animal models of liver damage by diverse toxic agents (e.g., carbon tetrachloride, D-galactosamine, acetaminophen, ethanol, and cadmium) and after extended liver resection [18–20,20,21]. Recent *in vitro* studies provide new aspects and suggest that ALR might attenuate oxidative stress and inhibit apoptotic cell death [22,23]. As known, hepatic I/R injury is triggered by oxidative burst from activated endothelial cells, Kupffer cells, and inflammatory blood cells (e.g., neutrophils and platelets). Apoptosis is the main form of cell death on hepatic I/R [24,25]. Here, we tested the hypothesis that ALR exerts a protective effect on hepatic I/R injury *in vivo*.

The main result of our study is that the exogenous administration of rALR attenuates apoptotic and necrotic hepatocellular I/R injury *in vivo*. Apoptosis was determined by the measurement of caspase-3 activity in tissue. Plasma activity of the liver enzymes ALT and AST was used as a parameter of liver necrosis. The protective effect was most pronounced after 4 h of reperfusion. Via which mechanisms are ALR able to attenuate hepatocellular injury? First, ALR serves as a survival factor for hepatocytes and is essential for the biogenesis of mitochondria, normal mitochondrial morphology, and stable maintenance of mitochondria [26,27]. Inhibition of ALR synthesis with ALR–messenger RNA antisense oligonucleotide caused rapid (within hours) apoptotic and necrotic death of hepatocytes [28]. Liver inflammation or injury could lead to a mitochondrial ALR deficiency, which causes apoptosis and necrosis of hepatocytes [6]. Second, ALR attenuates exogenously induced apoptosis in liver-specific manner [5]. Although not fully understood, the antiapoptotic effect of ALR seems to be mediated by the antioxidative properties of ALR and by increasing the ratio of Bcl-2-to-Bax

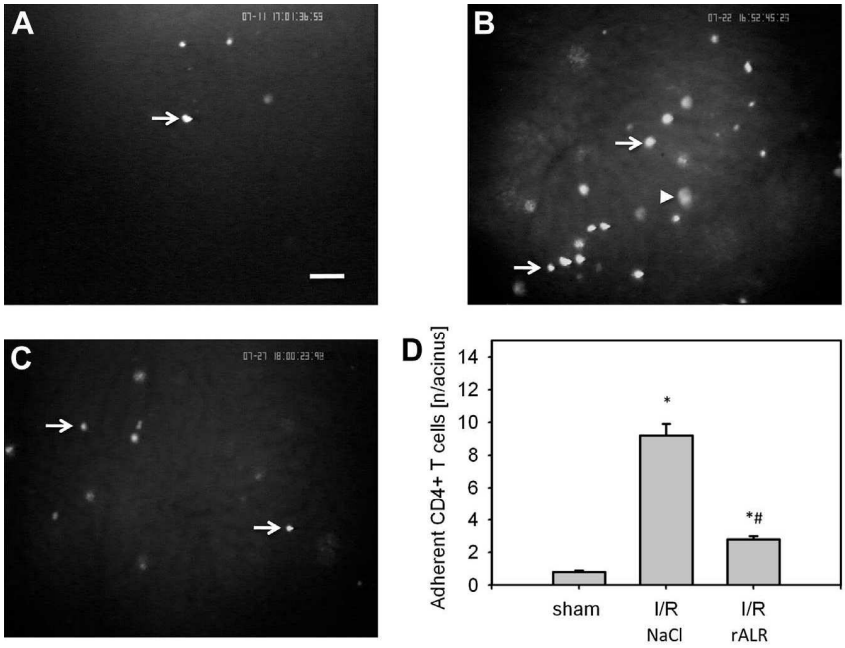

Fig. 3 – CD4+ T cell recruitment. Intravital microscopic images demonstrate CFSE-labeled CD4+ T cells in the hepatic microcirculation in sham-operated mice (A), mice after I/R (90/60 min) treated with saline as vehicle (B), and in mice after I/R treated with rALR (C). Arrows depict CD4+ T cells accumulated in sinusoids, arrow head shows a CD4+ T cell localized out of focus in another tissue layer (not counted). Microscope magnification ×500. Scale bar 50 μm. The quantitative data are presented in chart (D) N = 6 animals per group, mean ± standard error of the mean, *P < 0.05 *versus* sham-operated group, #P < 0.05 *versus* I/R + vehicle.

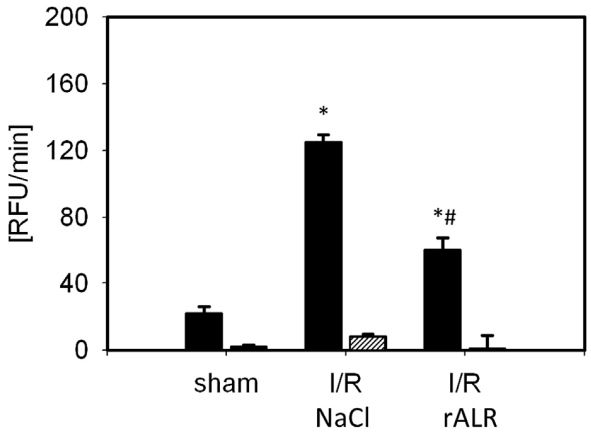

Fig. 4 – Caspase-3 activity. Caspase-3 activity was determined as a parameter of apoptosis in tissue homogenates of sham-operated mice, mice after I/R (90/240 min) treated with saline as vehicle, and of mice after I/R treated with rALR. N = 6 animals per group, mean ± standard error of the mean, *P < 0.05 *versus* sham-operated group, #P < 0.05 *versus* I/R + vehicle. Dashed bars show additional controls upon incubation of tissue homogenates from both I/R groups with caspase-3, -7, and -8 inhibitor Ac-DEVD-CHO. N = 6 animals per group, mean ± standard error of the mean, #P < 0.05 *versus* I/R + vehicle.

[22,29]. Because Bcl-2 has also been shown to be activated by the extracellular signaling-regulated kinase, this is in line with our *in vitro* findings, which demonstrated in primary human liver cells a strong effect of rALR on the phosphorylation of extracellular signaling-regulated kinase already 10 min after treatment [7]. Third, ALR is assumed to prevent hepatocyte injury by affecting cells of the immune system, infiltrating inflammatory cells, and resident macrophages [6]. Indeed, I/R-induced leukocyte migration was attenuated by ALR in our study.

A hallmark feature of hepatic inflammation during I/R is recruitment of various types of leukocytes to the afflicted site. Inflammatory stimuli activate endothelial cells to express adhesion molecules and chemokines that physically engage circulating leukocytes and promote their adhesion to the vessel wall. The initial interaction of neutrophils with hepatic endothelium (rolling) is mediated by P-selectin, whereas the firm adhesion is triggered by the interaction between beta2-integrins of leukocytes and intercellular adhesion molecule 1 on endothelial cells [16,30]. The next step is transendothelial and interstitial migration toward the stimuli from the damaged cells [31,32]. In our study, we analyzed recruitment of neutrophil granulocytes in the hepatic microvasculature using intravital microscopy. We observed a dramatic increase in the number of rolling and adherent leukocytes after as soon as 60 min of reperfusion. Treatment with ALR did not affect leukocyte migration after 60 min, whereas a significant

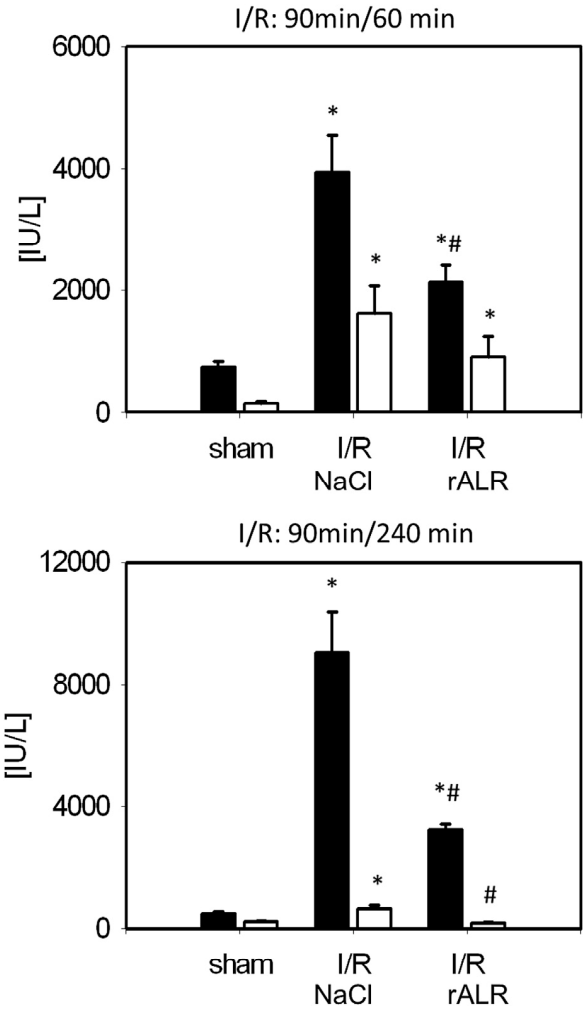

Fig. 5 – Liver enzyme activity. Serum activity of the liver enzymes AST (closed bars) and ALT (open bars) was determined as a marker of hepatocellular necrotic injury in sham-operated mice, mice after I/R (90/60 min) treated with saline as vehicle, and of mice after I/R treated with rALR. N = 6 animals per group, mean ± standard error of the mean, *P < 0.05 *versus* sham-operated group, #P < 0.05 *versus* I/R + vehicle.

reduction was measured after 240 min of reperfusion. The results are in line with the data on hepatocellular injury. The observed reduction of leukocyte influx in our study is probably a consequence of lower cell damage (stimulus for cell migration) on treatment with rALR. In addition, ALR might affect *de novo* expression of relevant adhesion molecules (e.g., P-selectin), which normally needs about 4 h after stimulation.

CD4+ T cells have been shown to play a critical role during hepatic I/R. They accumulate rapidly (30 min of reperfusion) in hepatic sinusoids that are able to activate platelets intravascularly and can influence Kupffer cell functions [14,36]. Moreover, CD4+ T cells contribute to cell death by releasing T cell immunoglobulin mucin-1 and via STAT-dependent pathways [33–35]. The mechanisms of CD4+ T cell activation during alloantigen-independent hepatic I/R are not fully understood. In our previous work, we demonstrated *in vivo* and *in vitro* that CD4+ T cells are activated by reactive oxygen species [36]. Both the free radical scavenger glutathione and a Kupffer cell depletion hampered postischemic T cell recruitment in the liver. We, therefore, assume that the antioxidative effect of ALR could be responsible for the attenuation of CD4+ T cell recruitment in the hepatic microvessels.

Next, our data show that rALR improved postischemic sinusoidal perfusion. Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction after I/R [37]. The impaired nutritive blood flow accompanied by reduced oxygen availability decreases cellular levels of high-energy phosphates and contributes to early and late hepatocellular injury and dysfunction. The postischemic shutdown of the hepatic microcirculation is triggered by sinusoidal narrowing caused by endothelial cell edema [38], stellate cell–mediated vasoconstriction [39,40], or by activated Kupffer cells. In addition, inflammation- and injury-associated adherence of leukocytes in outflow venules may alter sinusoidal perfusion due to an increase of blood viscosity [41] and, hence vascular resistance [42]. Furthermore, perfusion failure in sinusoids is thought to be caused by sluggish blood flow, intravascular hemoconcentration, and procoagulant conditions [1]. Although the impact of ALR on inflammatory activation of hepatic endothelial cells and stellate cells remains unclear and is the focus of our ongoing studies, Ghandi *et al.* [43] described the effect of ALR on function of rat Kupffer cells. Moreover, the reduction of leukocyte accumulation observed in our study supports the data on sinusoidal perfusion.

5. Conclusions

Our *in vivo* data show that ALR has a therapeutic potential against postischemic liver injury. As mechanisms, we suggest a direct protective effect of ALR on apoptotic and necrotic death of hepatocytes and attenuation of inflammatory cell influx into the postischemic tissue.

Acknowledgment

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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