Conjugated Bilirubin Triggers Anemia by Inducing Erythrocyte Death

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Hepatic failure is commonly associated with anemia, which may result from gastrointestinal bleeding, vitamin deficiency, or liver-damaging diseases, such as infection and alcohol intoxication. At least in theory, anemia during hepatic failure may result from accelerated clearance of circulating erythrocytes. Here we show that bile duct ligation (BDL) in mice leads to severe anemia despite increased reticulocyte numbers. Bilirubin stimulated suicidal death of human erythrocytes. Mechanistically, bilirubin triggered rapid Ca$^{2+}$ influx, sphingomyelinase activation, formation of ceramide, and subsequent translocation of phosphatidylserine to the erythrocyte surface. Consistent with our in vitro and in vivo findings, incubation of erythrocytes in serum from patients with liver disease induced suicidal death of erythrocytes in relation to their plasma bilirubin concentration. Consistently, patients with hyperbilirubinemia had significantly lower erythrocyte and significantly higher reticulocyte counts compared to patients with low bilirubin levels. Conclusion: Bilirubin triggers suicidal erythrocyte death, thus contributing to anemia during liver disease. (Hepatology 2015;61:275-284)

Liver failure and fibrosis are commonly associated with anemia, a phenomenon which remains ill-defined due to the heterogeneous nature of anemia’s pathogenesis. During liver disease, anemia can occur after blood loss, infection, cancer, or nutritional imbalances. Bleeding through esophageal varices can result in rapid blood loss and consequently anemia in patients with liver failure. Additionally, chronic viral infections can reduce the number of circulating erythrocytes by depletion of progenitor cells in the bone marrow. Hepatitis B and C infections and their treatment regimens are similarly associated with reduced erythrocyte counts. Furthermore, folate and vitamin B$_{12}$ are critical for erythrocyte development, nutritional imbalances may result in reduced erythrocyte production and consequently anemia. Moreover, expression of ferroportin-1 in the liver can impact the development of anemia, as ferroportin-1 is critical for iron metabolism and hematopoiesis during an iron-deficient diet. Although many conditions are associated with anemia, the impact of liver damage on erythrocytes and the associated underlying mechanisms remain poorly understood.

Erythrocytes exhibit a simplified programmed cell death termed eryptosis. Eryptosis shares several key features with apoptosis of nucleated cells, such as cell shrinkage, membrane blebbing, and phosphatidylserine (PS) exposure on the outer cell membrane. However, erythrocytes lack cell organelles participating in the machinery of apoptosis. Eryptosis contributes to...
anemia in several clinical disorders.\textsuperscript{14} In anemia caused by genetic mutations such as sickle cell anemia and thalassemia, erythrocytes may undergo accelerated suicidal death.\textsuperscript{15-18} Furthermore, other diseases including intoxications or nutritional imbalance result in increased eryptosis and consequently anemia.\textsuperscript{19,20}

Eryptosis leads to rapid clearance of affected erythrocytes from circulating blood.\textsuperscript{14,16,21,22} PS receptors on macrophages may recognize PS on the outer cell membrane of apoptotic and eryptotic cells.\textsuperscript{23} The asymmetric organization of PS in the cell membrane is executed by flippases, which translocate PS to the inner leaflet of the cell membrane.\textsuperscript{24} During cell stress, activation of scramblases and flippases can exteriorize PS, thus making apoptotic cells visible to macrophages.\textsuperscript{14} In erythrocytes, PS can be translocated during cell stress such as oxidative stress, hyperosmotic stress, and energy deprivation.\textsuperscript{14,25} Opening of a nonselective cation channel triggers rapid influx of Ca\textsuperscript{2+}, which leads to Ca\textsuperscript{2+}-activated potassium efflux and subsequent cell shrinkage.\textsuperscript{14,26,27} Furthermore, Ca\textsuperscript{2+} influx can induce a scramblase activity, which translocates PS from the inner to the outer cell membrane leaflet. During cell stress, erythrocytes may further activate sphingomyelinase, resulting in ceramide formation and scramblase activation.\textsuperscript{28} Various inhibitors can be used to prevent scramblase activation during eryptosis.\textsuperscript{19} Inhibition of the nonselective cation channel, the Ca\textsuperscript{2+}-sensitive potassium channel or sphingomyelinase, counteracts death of erythrocytes.\textsuperscript{13} Furthermore, erythropoietin (EPO) can limit Ca\textsuperscript{2+} entry through the nonselective channel and prevent the programmed cell death of erythrocytes.\textsuperscript{29} Thus, EPO not only stimulates the production of erythrocytes, but also enhances the life span of circulating erythrocytes.\textsuperscript{29} Taken together, several molecular mechanisms which can modulate eryptosis have been described.

In this investigation we report that high bilirubin levels present during liver disease were identified as critical triggers of suicidal erythrocyte death and anemia in vitro and in vivo. Mechanistically, this was linked to both Ca\textsuperscript{2+} influx as well as sphingomyelinase activation.

### Materials and Methods

**Erythrocytes From Patients and Volunteers.** To investigate eryptosis in vivo, heparinized blood was drawn from 27 patients. The patient characteristics are shown in Table 1. For the ex vivo measurements, with hyperbilirubinemic serum, serum specimens were used for incubation of erythrocytes from healthy individuals or the patients' own erythrocytes. For the in vitro experiments with conjugated bilirubin, blood was freshly drawn from healthy volunteers. All patients and volunteers gave informed consent. The study was approved by the Ethics Committees of the University of Tübingen and the University of Duesseldorf. For the blood count correlation, 41 inpatients (Table 1) and 113 outpatients (Supporting Table 1) from the Department of Gastroenterology, Hepatology, and Infectious Diseases gave informed consent. Patients without known bleeding, erythrocyte disease, untreated vitamin B\textsubscript{12} deficiency, and untreated iron deficiency were recruited from inpatient and outpatient clinics.

**Mice.** Experiments were performed on 12-week-old mice. All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. For bile duct ligation (BDL), animals were anesthetized by isoflurane and placed on a heating pad. After intubation and ventilation, the animals were shaved and the skin disinfected. A midline incision in the upper abdomen was made; the common bile duct and the gallbladder were identified, isolated, and ligated with 6–0 coated vicryl Polyglactin fiber from Ethicon (Johnson & Johnson Medical GmbH, Norderstedt, Germany). The fascia and skin of the midline abdominal incision were closed with 5–0 vicryl Polyglactin fiber from Ethicon (Johnson & Johnson Medical GmbH, Norderstedt, Germany). Sham treatment was performed similarly but without ligation of the bile duct and gallbladder. Animals were monitored until recovery and treated with carprofen (5 mg/kg b.w.) after the procedure. Erythrocyte turnover was assessed by injection of CFSE\textsuperscript{\textsuperscript{1}}-labeled erythrocytes from healthy mice injected into the tail vein of either sham or BDL mice.
Table 1. Clinical and Laboratory Characteristics of Patients

| Characteristics   | Cohort A (n=27) | Cohort B (n=5) |
|-------------------|-----------------|----------------|
| Age (years)       | 62 ± 2          | 60 ± 3         |
| Male (female)     | 19 (8)          | 8 (15)         |
| Bilirubin in mg/dL| 8.7 ± 1.9       | 0.50 ± 0.05    |
| MCV in fl         | 95.1 ± 1.4      | 87.2 ± 1.5     |
| MCH in pg         | 30.9 ± 0.4      | 28.8 ± 0.6     |
| Hematocrit in %   | 32.7 ± 1.2      | 37.0 ± 1.1     |
| Erythrocytes in Mio/μL | 3.41 ± 0.1 | 4.29 ± 0.1     |
| Hemoglobin in g/dL| 11.0 ± 0.4      | 12.2 ± 0.4     |
| Reticulocytes in 1,000/μL | 59.3 ± 8.6 | 74.0 ± 8.3     |
| Albumin in g/dL   | 3.8 ± 0.2       | 8.6 ± 0.1     |
| CHILD/CCC         | A(4),B(2),C(5)  | B(1)          |
| HBV               | 4               | 3             |
| HCV               | 1               | 1             |
| PSC, PBC, NASH, AIH, PFIC, BRIC | 2 | 1 |
| Cholestatic disease| 7               | 2             |
| Ethytoxic liver disease | 14 | 1 |
| Spontaneous bacterial peritonitis | 5 | 0 |
| Others            | 6               | 14            |

Clinical parameters of cohort A (patients with liver disease and anemia, n = 27), and cohort B (patients with and without liver disease, n = 5) are presented. Individual parameters represent the mean ± SEM. P value indicates statistical analysis (t test) between patients from cohort B with bilirubin levels <1 mg/dL and bilirubin levels ≥3 mg/dL.

Blood Chemistry, Blood Count, and Isolation of Murine Erythrocytes. For determination of serum bilirubin concentration and blood count, blood was drawn from the retroorbital plexus. Bilirubin concentration was determined using a clinical chemical analyzer (Spotchem, Wackersdorf, Germany). For blood count, ethylenediamine-tetraacetic acid (EDTA) blood was analyzed using an electronic hematology particle counter (scil Vet abc, Weinheim, Germany).

Solutions, Chemicals, and Incubations. Erythrocytes were incubated in vitro at a hematocrit of 0.4% in either plasma obtained from patients or in Ringer’s solution containing (in mM): 125 NaCl, 5 KCl, 1 MgSO4, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl2; pH 7.4 at 37°C for 6 or 48 hours. Where indicated, conjugated (ditaurate conjugate, disodium salt) bilirubin (Merck Millipore, Darmstadt, Germany, or Echelon Biosciences, Salt Lake City, UT) was added at the indicated concentrations.

Flow Cytometric Analysis of Annexin V-binding. After incubation at the indicated experimental conditions, cells were washed in Ringer’s solution containing 5 mM CaCl2 and then stained with Annexin-V-PE (immunoTools, Friesoythe, Germany) for 15 minutes in the dark.

Measurement of Intracellular Ca2+. Erythrocytes were labeled with Fluo3-AM-Ester during incubation in Ringer’s solution containing 5 μM Fluo3-AM-Ester for 1 hour. Cells were then incubated in Ringer’s solution with or without the indicated concentrations of bilirubin.

Determination of Ceramide Formation. Cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and stained for 1 hour at 37°C with an anticeramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% BSA. After washing twice with PBS-BSA, cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat antimouse immunoglobulin G (IgG) and IgM-specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA.

CFSE Labeling of Mouse Erythrocytes. Whole blood was drawn from the retroorbital plexus of healthy mice and washed three times with PBS. Erythrocytes (500 μL) were stained in 9.5 mL PBS containing CFSE (2.25 μM) for 10 minutes at 37°C. After staining, cells were washed three times in RPMI medium containing 10% fetal calf serum and resuspended in PBS and directly injected into BDL or sham mice at day 21 after surgery. At the indicated timepoints, blood was retrieved from the retro-orbital plexus of the mice, and CFSE+ erythrocytes were detected by flow cytometric analysis. The percentage of CFSE-positive erythrocytes was calculated as the percentage of total labeled fraction determined 6 hours after injection.

Statistics. Data are expressed as arithmetic means ± SEM, and statistical analysis was made using paired
analysis of variance (ANOVA) with Tukey’s test as a posttest, linear regression or t test, as appropriate.

Results

**BDL Causes Rapid Development of Anemia in Mice.** As liver disease is often associated with anemia,\(^1\)\(^,\)\(^30\) we tested whether acute jaundice induces anemia using an animal model of BDL. As expected,\(^31\)\(^,\)\(^32\) mice exhibited a rapid increase in serum bilirubin levels after ligation of the bile duct (Fig. 1A). Interestingly, erythrocyte count rapidly declined within 3 weeks after the surgical procedure (Fig. 1B). Consequently, a decrease of hematocrit (Fig. 1C) and hemoglobin levels (Fig. 1D) occurred in the BDL group as compared to the corresponding sham controls. Moreover, a significant correlation was observed in hematocrit levels and bilirubin concentrations between BDL animals and sham controls.

Fig. 1. Mice suffer from anemia following bile duct ligation. (A-D) Mice with BDL were compared to sham controls. (A) Serum bilirubin concentration was measured (each point indicates one animal, \(n = 3-11\)). Arithmetic means ± SEM of (B) red blood cells, which were quantified in the blood of BDL and sham-operated mice (\(n = 5-10\)). (C) Hematocrit was determined after BDL and compared with corresponding sham controls (\(n = 5-10\)). (D) Total hemoglobin levels after BDL and in corresponding sham controls were analyzed (\(n = 5-10\)). (E) Hematocrit is shown as a function of bilirubin concentrations of individual BDL mice and sham controls 21 days postoperation (\(n = 24\)). \(R^2 = 0.7095; P < 0.001\) indicates slope significantly nonzero.
(Fig. 1E). Notably, bile acids were significantly increased after BDL and this increase also correlated with hematocrit decrease (data not shown). Furthermore, bilirubin concentrations correlated negatively with red blood cell numbers and hemoglobin levels taken from individual BDL mice and sham controls (Supporting Fig. 1A,B). In conclusion, these data suggest that BDL induces anemia in mice.

Erythrocytes Show Accelerated Turnover by Enhanced Suicidal Erythrocyte Death Following BDL. A reduction of erythrocyte count may result from either limited production or increased turnover of erythrocytes. Our data shows that the proportion of reticulocytes was increased after BDL, while sham control reticulocyte levels remained constant (Fig. 2A). Consistently, absolute numbers of reticulocytes increased after BDL in comparison to sham controls (Fig. 2B). A significant correlation was observed between absolute reticulocyte number and bilirubin concentration of individual BDL and sham-treated mice (Fig. 2C). We hypothesized that the erythrocyte turnover may be increased after BDL. We thus transferred CFSE\(^+\)-labeled red blood cells from healthy animals into BDL and sham-treated mice and quantified their abundance from circulating blood. The half-life of labeled erythrocytes was significantly shorter in BDL-treated animals than in sham controls (Fig. 2D). These data indicate that the turnover of erythrocytes is increased during liver cell damage and jaundice. Within the first 2 weeks, the accelerated loss of erythrocytes can be compensated by enhanced erythrocyte production. Turnover of erythrocytes can be triggered by eryptosis, which can be detected by PS exposure on the outer leaflet of the cell membrane. PS binds to annexin V, which can be used to identify suicidal erythrocytes. Interestingly, more erythrocytes collected from BDL mice exhibited annexin V binding than erythrocytes from sham controls (Fig. 3A). In addition, exposure of erythrocytes to cell stress such as hypertonic shock or glucose deprivation resulted in a higher induction of annexin V-bound erythrocytes from BDL mice compared to sham...

![Fig. 2. Reticulocyte increases and enhanced erythrocyte turnover in mice following BDL.](image-url)

(A) Percentage of reticulocytes after BDL and in corresponding sham controls was measured over time (n = 6-14). (B) Absolute reticulocyte count in BDL animals and sham controls 7 (D7) and 21 (D21) days postoperation is illustrated (n = 12-14). (C) Absolute reticulocyte count is presented in relation to the bilirubin concentration in individual BDL mice and sham controls 21 days postoperation (n = 24) \( R^2 = 0.7733; P < 0.001 \) indicates slope significantly nonzero. (D) CFSE\(^+\)-labeled red blood cells were injected into BDL animals and sham controls followed by monitoring of transferred erythrocytes. Data are presented as percent of CFSE\(^+\) erythrocytes 6 hours after transfer (n = 8-10).
controls (Fig. 3B). Moreover, treatment with the Ca\(^{2+}\) ionophore ionomycin resulted in elevated annexin V-bound erythrocytes in BDL mice when compared to their controls (Fig. 3B). These data indicate that after BDL the erythrocyte turnover and suicidal erythrocyte death is increased compared to control animals.

**Bilirubin Induces Suicidal Erythrocyte Death by Activation of Ca\(^{2+}\) Influx and Sphingomyelinase.** To investigate the mechanism by which erythrocytes undergo accelerated cell death during BDL, we exposed human erythrocytes *in vitro* to bilirubin. Bilirubin exposure induced suicidal erythrocyte death, as indicated by increased percentage of annexin V-bound erythrocytes (Fig. 4A). Mechanistically, eryptosis can be induced by both Ca\(^{2+}\) influx as well as ceramide formation.\(^{26,28}\) When erythrocytes were labeled with the Ca\(^{2+}\)-sensitive dye Fluo3-AM and exposed to bilirubin, we detected rapid influx of Ca\(^{2+}\) (Fig. 4B). However, eryptosis following exposure to the Ca\(^{2+}\) ionophore ionomycin was similarly enhanced in BDL erythrocytes when compared to sham controls (Fig. 3B). If Ca\(^{2+}\) influx was the sole contributor of eryptosis, we would not have expected additional eryptosis following treatment with ionomycin in bilirubin-exposed erythrocytes. Thus, additional mechanisms likely contributed to suicidal erythrocyte death.
following bilirubin exposure. As illustrated in Fig. 4C, exposure to bilirubin increased ceramide formation in erythrocytes, an observation pointing to sphingomyelinase activation. Next, we wondered whether cell death of erythrocytes can be prevented by blocking the influx of Ca$^{2+}$ and activation of sphingomyelinase. Sphingomyelinase activation can be inhibited by high concentrations of urea. As illustrated in Fig. 5A, the suicidal erythrocyte death could be significantly blunted by removal of Ca$^{2+}$ from the solution or addition of urea or both. Furthermore, the presence of albumin reduced the toxic effects of bilirubin on red blood cells in vitro, suggesting that bilirubin exhibits toxic effects when present in a concentration of several mg/dL. Taken together, these data indicate that bilirubin induces death of erythrocytes and contributes to anemia in liver disease.

**Bilirubin Levels in Sera of Human Patients Correlate With Induction of Suicidal Erythrocyte Death.** Next, we investigated whether suicidal death of erythrocytes could be observed in the serum of human patients with liver disease. Blood samples from patients suffering from liver disease were taken (Table 1). Annexin V binding of either healthy or hepatic patients’ own erythrocytes after incubation in the serum was assessed. A significant positive correlation was observed between the percentage of annexin V-bound erythrocytes and the bilirubin plasma levels obtained from patients with liver disease (Table 1, cohort A, Fig. 6A). Furthermore, exposure of erythrocytes from healthy blood donors to the serum of patients suffering from liver disease increased the percentage of annexin V-bound erythrocytes in accordance with increased bilirubin plasma levels (Fig. 6B), indicating that bilirubin containing blood serum was toxic to erythrocytes in vitro. To investigate whether these data are translationally relevant in vivo, we analyzed erythrocyte numbers of 41 patients (Table 1, cohort B). Patients with increased bilirubin concentration exhibited significantly reduced erythrocyte counts (Table 1, Fig. 6C). Notably, erythrocyte counts did not decrease in dependency of other parameters indicative of liver damage (Supporting Fig. 2A-C). Similar to the data obtained from BDL mice, human patients exhibited increased reticulocyte numbers when bilirubin levels were increased (Table 1). These investigations were also confirmed in 113 outpatients, who comprised a lower percentage of patients with high bilirubin levels (Supporting Table 1, Supporting Fig. 3A, B). Notably, many patients with low bilirubin levels also suffered from anemia and enhanced reticulocyte counts, indicating that there are additional pathological factors driving anemia (Fig. 6C, Supporting Fig. 3A, B). However, increased bilirubin levels clearly predisposed patients to reduced erythrocyte counts. Since the presence of albumin could reduce the toxicity of bilirubin against erythrocytes in vitro, we next determined plasma albumin levels. Notably, increased bilirubin concentrations correlated negatively with the albumin concentration in plasma, which is consistent with progressing liver disease (Fig. 6D). Consequently, the albumin concentration correlated positively with the
numbers of red blood cells (Fig. 6E). These data suggest that albumin can bind bilirubin and thus reduce its toxic effects. However, if bilirubin concentrations reach a critical level, or albumin concentrations decrease through the course of liver disease, bilirubin may induce suicidal erythocyte death and consequently cause anemia.

**Discussion**

The present study reveals that hyperbilirubinemic patients with hepatic failure suffer from accelerated suicidal erythrocyte death. Exposure of erythrocytes from healthy individuals to either serum from hyperbilirubinemic patients or to Ringer’s containing conjugated bilirubin was followed by increases in annexin V binding, reflecting erythrocyte membrane scrambling. Moreover, excessive annexin V binding and anemia despite increased reticulocyte numbers were observed in mice following BDL. We observed that albumin could reduce the toxic effect of bilirubin on red blood cells, which may also be a critical factor during progressive liver disease and development of anemia.

A significant correlation was observed between the eryptotic effect of the serum and the bilirubinemia of the respective patients. This observation suggests that bilirubin in the serum of patients with hepatic failure triggers eryptosis. In theory, however, the correlation
could have been due to another eryptosis-triggering component in the patients’ serum, which accounts for the in vitro stimulation of eryptosis, or due to the in vivo increase in erythrocyte turnover, which would be expected to increase heme degradation and thus plasma bilirubin concentration. The effects observed in blood from hyperbilirubinemic patients and the experiments using bilirubin in Ringer’s, however, strongly suggest that enhanced plasma bilirubin concentration at least in part contributes to the stimulation of eryptosis by serum from hyperbilirubinemic patients. Moreover, reduced albumin concentrations in the sera of patients suffering from liver disease and/or reduced liver function may enhance the toxicity of the increased bilirubin levels. Taken together, the toxic effects on red blood cells may contribute to development of anemia during liver disease above a critical threshold of bilirubin (>3 mg/dL). The observation that bilirubin may trigger suicidal erythrocyte death reveals a potential vicious cycle, as death of erythrocytes is followed by heme degradation with formation of bilirubin. Thus, accelerated eryptosis augments plasma bilirubin, which in turn stimulates eryptosis.

The present observations may shed additional light on the effects of bilirubin on nucleated cells. Bilirubin is an antioxidant which, at lower concentrations, protects against bile acid-induced apoptosis of hepatocytes and apoptosis of renal cells during pyelonephritis. On the other hand, bilirubin stimulates PS exposure of erythrocytes and apoptosis of neuronal and glial cells as well as immune cells. Moreover, bilirubin augments radiation-induced apoptosis of various blood cells. Signaling involved in the toxic effects of bilirubin include stimulation of Ca\(^{2+}\) influx and stimulation of p38 MAP kinase. It is noteworthy that eryptosis is regulated by several kinases, including p38 kinase. Moreover, our data indicate that bilirubin induces ceramide formation. The nature of the bilirubin effects on apoptosis seems to be highly concentration-dependent and likely occurs above a threshold of several mg/dL.

Eryptotic erythrocytes are trapped largely in the spleen and are thus rapidly cleared from circulating blood. In a variety of clinical conditions, eryptosis precedes and thus prevents hemolysis of the defective erythrocytes. Hemolysis may lead to release and subsequent glomerular filtration of cellular hemooglobin. The filtered hemooglobin may precipitate in renal tubular fluid and thus occlude the renal tubular lumen and may contribute to kidney fibrosis observed after BDL. The clearance of eryptotic erythrocytes from circulating blood may lead to anemia, as long as the accelerated loss of circulating erythrocytes is not matched by similarly accelerated formation of new erythrocytes.

In conclusion, we demonstrate that bilirubin stimulates Ca\(^{2+}\) entry and ceramide formation, which in turn triggers cell membrane scrambling and thus suicidal death of human erythrocytes. The enhanced eryptosis fosters the development of anemia despite stimulation of erythropoiesis.

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