Crystal Clots as Therapeutic Target in Cholesterol Crystal Embolism

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Rationale: Cholesterol crystal embolism can be a life-threatening complication of advanced atherosclerosis. Pathophysiology and molecular targets for treatment are largely unknown.

Objective: We aimed to develop a new animal model of cholesterol crystal embolism to dissect the molecular mechanisms of cholesterol crystal (CC)- driven arterial occlusion, tissue infarction, and organ failure.

Methods and Results: C57BL/6J mice were injected with CC into the left kidney artery. Primary end point was glomerular filtration rate (GFR). CC caused crystal clots occluding intrarenal arteries and a dose-dependent drop in GFR, followed by GFR recovery within 4 weeks, that is, acute kidney disease. In contrast, the extent of kidney infarction was more variable. Blocking necroptosis using mixed lineage kinase domain-like deficient mice or necrostatin-1s treatment protected from kidney infarction but not from GFR loss because arterial obstructions persisted, identifying crystal clots as a primary target to prevent organ failure. CC involved platelets, neutrophils, fibrin, and extracellular DNA. Neutrophil depletion or inhibition of the release of neutrophil extracellular traps had little effects, but platelet P2Y12 receptor antagonism with clopidogrel, fibrinolysis with urokinase, or DNA digestion with recombinant DNase I all prevented arterial occlusions, GFR loss, and kidney infarction. The window-of-opportunity was <3 hours after CC injection. However, combining Nec-1s (necrostatin-1s) prophylaxis given 1 hour before and DNase I 3 hours after CC injection completely prevented kidney failure and infarcts. In vitro, CC did not directly induce plasmatic coagulation but induced neutrophil extracellular trap formation and DNA release mainly from kidney endothelial cells, neutrophils, and few from platelets. CC induced ATP release from aggregating platelets, which increased fibrin formation in a DNase-dependent manner.

Conclusions: CC embolism causes arterial obstructions and organ failure via the formation of crystal clots with fibrin, platelets, and extracellular DNA as critical components. Therefore, our model enables to unravel the pathogenesis of the CC embolism syndrome as a basis for both prophylaxis and targeted therapy.

Visual Overview: An online visual overview is available for this article.

Key Words: acute kidney injury ■ endothelial cells ■ extracellular traps ■ fibrin ■ necroptosis

Atherosclerosis is a leading cause of global morbidity and mortality. In advanced atherosclerosis, cholesterol crystal (CC) embolism is a potentially life-threatening complication with an average mortality of 62.8%. Autopsies or tissue biopsies reveal CC inside the arterial lumen surrounded by an undefined biological matrix obstructing the vessel lumen. Little is known about the precise cellular and molecular mechanisms following CC embolism, in part, due to the lack of animal models. We hypothesized that developing a reproducible mouse model of CC embolism would be instrumental to dissect the molecular mechanisms of CC-driven arterial occlusion, tissue infarction, and organ failure.
Novelty and Significance

**What Is Known?**
- Cholesterol crystal embolism is a potentially life-threatening complication of advanced atherosclerosis.
- Lack of reproducible animal model to investigate the mechanism of cholesterol crystal embolism.
- Platelet adhesion promotes thrombus growth.
- Extracellular DNA is an important component of vascular thrombosis.

**What New Information Does This Article Contribute?**
- Established a new animal model to mimic the morphological and functional characteristics of cholesterol crystal embolism in human.
- Not the crystal embolism but the clots forming around the crystals cause arterial obstruction and organ failure.
- Extracellular DNA is a critical element of crystal-induced arterial occlusion and can be a target for therapy.
- Combining preemptive necroptosis inhibition plus delayed DNase I treatment could be a feasible therapy in patients at risk for cholesterol crystal embolism.

Cholesterol crystal embolism (CCE) is a potentially life-threatening complication of advanced atherosclerosis, due to lack of animal models, we know very little about the precise cellular and molecular mechanisms following CCE. Our new animal model mimics the morphological and functional characteristics of CCE in human. We found that clots formed around the crystals cause arterial obstruction and organ failure rather than crystals themselves. Therefore, crystal clots are therapeutic and indeed targeting thrombosis and hemostasis, especially enhancing fibrinolysis or inhibiting platelet purinergic signaling could reduce arterial occlusions and organ failure. Our results suggest that prophylactic necroptosis inhibition with a combination of DNase I therapy could have a synergistic effect on CC induced clot formation in mice and might be a feasible 2-step prophylactic/therapeutic approach in human with a risk for procedure-related CCE.

### Nonstandard Abbreviations and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| α-SMA        | α-smooth muscle actin |
| CC           | cholesterol crystal |
| CRP          | collagen-related peptide |
| ecDNA        | extracellular DNA |
| GFR          | glomerular filtration rate |
| GPVI-ITAM    | glycoprotein VI-immunoreceptor tyrosine-based activation motif |
| MLKL         | mixed lineage kinase domain-like |
| NLRP3        | NOD-like receptor pyrin domain-containing protein 3 |
| PAR          | protease activated receptor |

**METHODS**
The authors declare that all supporting data are available within the article (and in the Data Supplement). A detailed description of the experimental procedures, data analysis, and statistical methods is provided in the Data Supplement. All methods have corresponding literature references.

**RESULTS**

**CC Injection Induces Arterial Occlusions, Tissue Infarction, and Organ Failure in C57BL/6J Mice**

Injecting CC into the left kidney artery (Figure IA and IB in the Data Supplement) was reliable and well-tolerable as assessed by post-interventional scoring (not shown), as this way we avoided discomfort from skin ulcerations, pancreatitis, peritonitis, or uremia. Injecting different amounts of CC resulted in a dose-dependent decline of glomerular filtration rate (GFR) at 24 hours, that is, acute kidney injury (Figure 1A). Ex vivo magnetic resonance imaging displayed tissue defects and perilesional signal enhancement inside kidneys (Figure 1B; Movie I in the Data Supplement). The macroscopic analysis revealed kidney swelling and territorial kidney infarctions, with much higher dose-dependent infarct size variability compared with GFR (Figure 1C and 1D; Figure IC in the Data Supplement). Microscopic analysis revealed kidney swelling and territorial kidney infarctions, with much higher dose-dependent infarct size variability compared with GFR (Figure 1C and 1D; Figure IC in the Data Supplement). Microscopic analysis revealed dose-dependent intravascular CC, wide areas of TUNEL+ (TdT-mediated dUTP-biotin nick-end labeling) corticomedullary necrosis, interstitial edema, neutrophil infiltrates, and loss of CD31+ microvasculature (Figure 1E; Figure 1D through 1G in the Data Supplement). Territorial infarctions were associated with complete or partial occlusions of Aa. interlobares, Aa. Arcuatae, or Aa. interlobulares proximal to the infarct as assessed by α-SMA (α-smooth muscle actin)/fibrin staining to identify intrarenal arteries (Figure 1F through 1F′; Figure VIII A in the Data Supplement). Three-dimensional (3D) reconstructions of arterial contrast-enhanced microcomputed tomography angiography showed partial and complete arterial occlusions with associated blood vessel rarefaction, blood vessel volume change, and vasoconstriction (Figure 1G through 1G′; Movies II and III in the Data Supplement). We selected the CC dose of...
Figure 1. A new model of cholesterol crystal (CC) embolism.

A. Injection of different doses of CC stock solution into the renal artery of C57BL/6J mice induced acute kidney injury as defined by a sudden drop in glomerular filtration rate (GFR) 24 h post-injection (P=0.000000001 for Kruskal-Wallis test for whole group, followed by Dunn post-test).

B. Magnetic resonance imaging (MRI) indicates a large tissue defect, here in the renal medulla in the injected vs the noninjected contralateral kidney (see also Movie I in the Data Supplement).

C. Periodic acid-Schiff (PAS) stained section of control and CC embolism kidney. (Continued)
10 mg/kg for further studies, which would allow the detection of both disease improvement or aggravation. Disease severity at 24 hours was identical in male and female mice (Figure IH and I in the Data Supplement). Time-course analysis showed that after 14 days, GFR recovered back to baseline and infarct size declined (Figure U and IK in the Data Supplement). Thus, intra-arterial CC injection induces arterial occlusions causing acute territorial infarctions, perilesional inflammation, and organ failure.

Blocking Necroinflammation Prevents Kidney Infarction but not Kidney Failure

MLKL (mixed lineage kinase domain–like)-dependent necroptosis and NLRP3 (NOD-like receptor pyrin domain–containing protein 3) inflammasome-dependent sterile inflammation contribute to tubular necrosis and loss of kidney function upon transient artery clamping.13–17 Indeed, injecting 10 mg/kg CC into the renal arteries of Mlkl-deficient mice, mice pretreated with the necroptosis inhibitor Nec-1s (necrostatin-1s) or the NLRP3 inhibitor MCC950 significantly reduced infarct size, kidney injury, and neutrophil infiltration at 24 hours compared with wild-type controls (Figure 2A through 2C). However, none of these interventions had an effect on CC embolism–related loss of GFR (Figure 2E). Perfusion of the viable nephrons is a central determinant of kidney excretory function, and consistently with GFR, interfering with CC embolism–induced necroinflammation had no impact on arterial occlusions at 24 hours (Figure 2D and 2D′ and Figure VIIIB in the Data Supplement). Thus, as nephron perfusion is ultimately required for kidney function, inhibiting necrosis without targeting arterial occlusions does not prevent kidney failure.

CC Occlude Arteries by Forming Crystal Clots—Role of Neutrophils and Platelets

Interestingly, CC were a minor component of vascular occlusions, while vascular obstruction was rather related to the surrounding material staining positive for fibrin, CD61+ platelets, and Ly6B2+ neutrophils (Figure 3A through 3D), the typical components of arterial thrombi.3 Therefore, we named them crystal clots. Neutrophils contribute to arterial thrombosis by releasing extracellular traps from neutrophils,16 a process initiated by activated platelets.19,20 Surprisingly, crystal clots stained weakly positive for histological markers of neutrophils including extracellular DNA (ecDNA), citrullinated histone H3, and cytoplasmic proteins such as elastase or granular proteins such as myeloperoxidase21,22 (Figure 3E). Eosinophils were not found (not shown). To further test the potential contribution of neutrophils to crystal clot formation, we depleted neutrophils with anti-Ly6G IgG (Figure IIIA in the Data Supplement) as confirmed by blood flow cytometry done before CC injection (Figure IIIB in the Data Supplement). At 24 hour after CC injection, neutrophil depletion had completely extinguished the perinfarct neutrophil infiltrates and significantly decreased kidney infarct size compared with IgG control group, but this had no significant effect on arterial obstructions or GFR loss, maybe because mononuclear cells had partially replaced neutrophils in the crystal clots, and ecDNA was still present in crystal clots of the neutrophil depletion group (Figure 3F through 3I; Figures IIIF and IIIF2 and IXA in the Data Supplement). As another approach, Cl-amidine was used to block PAD4 (peptidylarginine deiminase)-dependent neutrophil formation with similar results (Figure IIIG through IIIJ and Figure IXB in the Data Supplement). In contrast, the platelet P2Y12 receptor antagonist clopidogrel completely protected mice from intravascular obstructions, GFR loss, kidney infarction, and perilesional neutrophil infiltrates (Figure IIIC and Figure 3F through 3I). Together, CC occlude arteries by forming crystal clots consisting of fibrin, platelets, and neutrophils, of which platelets but not neutrophils are central for CC embolism–related arterial occlusion, organ failure, and tissue infarction.

CC Occlude Arteries by Forming Crystal Clots—Role of Anticoagulants

The fibrin mesh is a validated target for arterial and venous thrombosis, and fibrin was also present in crystal clots. Hence, we tested the effects of the anticoagulant heparin and the fibrinolytic agent urokinase (Figure IVA in the Data Supplement). At 24 hours, both heparin and urokinase significantly reduced the numbers of arterial occlusions, albeit the crystal component

Figure 1 Continued. C′: Infarct (asterisk) demarcation (tubular damage, bleeding, neutrophils). C′′: Diffuse (tubular) ischemic damage and arterial crystal (asterisk). D, Triphenyl tetrazolium chloride (TTC)-stained kidney slices allowed to distinguish viable from infarcted tissue and calculation of infarct area (P=0.000000001 for Kruskal-Wallis test for whole group, followed by Dunn post-test). E, CC-injected kidney showing crystal inside artery. F, Schematic depiction of kidney vasculature. G, Immunostaining for α-SMA (α-smooth muscle actin) and fibrin displays partial or complete arterial occlusions. Arrows pointing to CC. F′′: Quantitative results for obstructed intrarenal arteries of different sizes. We set 6 different parameters to analyze the ratio of obstructed arteries (Details see Figure VIIIA in the Data Supplement): partially and completely interlobar, partially and completely arcuate, partially and completely interlobular. F′ only shown the mean value of each parameters in each group. G′, Three-dimensional reconstructions of arterial contrast micro computed tomography displays peripheral vascular rarefaction (Movies II and III in the Data Supplement). G′′, Blood vessel volume change in CCE group. G′′′, CCE induced vasoconstriction. All quantitative data are means±SD. G′ and G′′ used t-test.
Figure 2. Role of necroinflammasome in cholesterol crystal (CC) embolism.

We injected 10 mg/kg CC stock solution into the renal arteries of wild type (WT), Mlkl⁻/⁻ mice on C57BL/6J background. A, Infarct size was determined from 2,3,5-triphenyltetrazolium chloride (TTC)-stained kidney slices and shown as percentage of kidney mass in WT, Mlkl⁻/⁻, Nec-1s (necrostatin-1s), and MCC950 treatment groups. All those treatments significantly reduced kidney infarct size (P=0.000000055 for Kruskal-Wallis test for whole group, followed by Dunn post-test). B, Representative periodic acid-Schiff (PAS) stained sections of CC-injected kidneys in WT, Mlkl⁻/⁻ mice, Nec-1s or MCC950 treated groups, arrows show tubular damage. Mlkl⁻/⁻ mice, Nec-1s or MCC950 treated groups show significantly less injury (P=0.00000008 for Kruskal-Wallis test for whole group, followed by Dunn post-test). C, Perilesional neutrophil infiltrates; * indicates crystals. Mlkl⁻/⁻ mice and treated groups had less neutrophils (P=0.0003 for Kruskal-Wallis test for whole group, followed by Dunn post-test). D, Representative α-SMA (α-smooth muscle actin)/Fibrin stained section. D’, Occlusions of renal arteries of various sizes, same analysis as Figure 1F’ (details see Figure VIIIB in the Data Supplement). E, Glomerular filtration rate (GFR) was measured at 24 hour in WT, Mlkl⁻/⁻ mice, Nec-1s, or MCC950 treated groups to assess any drop compared to baseline GFR, an indicator of kidney failure. Mlkl⁻/⁻ mice and treated groups had no effects on kidney function (P=0.00000004 for Kruskal-Wallis test for whole group, followed by Dunn post-test). All quantitative data are means±SD.
Figure 3. Components of intraarterial crystal clots (CC)—role of neutrophils and platelets.

A. Periodic acid-Schiff (PAS) staining shows that intravascular crystals are surrounded by a biological mass. Immunostaining of crystal clots with prominent positivity CC for fibrinogen (B), platelets (C), and neutrophils (D). E, neutrophil extracellular trap (NET) formation illustrated by extracellular positivity of citrullinated histone H3 and MPO (myeloperoxidase). F, Representative image of Ly6B2+ stained kidney section in control, anti-Ly6G and clopidogrel groups, neutrophil depletion, and platelet antagonist significantly reduced neutrophil infiltration into the kidney (P=0.0000043 for Kruskal-Wallis test for whole group, followed by Dunn post-test). G, Neutrophil depletion had no significant effect on CC injection-induced loss of glomerular filtration rate (GFR; P=0.00000065 across groups, 1-way ANOVA, followed by Dunnett post-test). H, Neutrophil depletion significantly reduced infarct size (P=0.000000009 for Kruskal-Wallis test for whole group, followed by Dunn post-test), and platelet antagonist had a complete protection on GFR loss (G) and infarct size (H). The latter indicated by the percentage of kidney mass as compared with mice injected with control IgG. I, Representative αSMA (α-smooth muscle actin)/fibrin stained section in neutrophil depletion and platelet inhibition group. I', Occlusions of renal arteries of various sizes in neutrophil depletion and platelet inhibition group. Same analysis as Figure 1F' (details see Figure IXA in the Data Supplement). All quantitative data are means±SD.
Persisted (Figure 4A and 4A'); Figure XA in the Data Supplement). This was associated with complete protection from GFR loss, a significant reduction in kidney infarct size, kidney injury, neutrophils infiltration, vascular injury as well as kidney cell death as indicated by TUNEL positivity compared with vehicle-treated mice.
Figure 5. Neutralization of extracellular DNA protects mice from tissue infarction and organ failure. 
A. Representative DAPI (4',6-diamidino-2-phenylindole)-stained sections in cholesterol crystal embolism (CCE) kidneys showing crystals clots without and with extracellular DNA (ecDNA; arrows). B. Quantification of arteries containing crystal clots with ecDNA in healthy, recombinant DNase I or NaCl treated mice kidney after CC injection. DNase I treatment significantly decreased the percentage of arteries with ecDNA positive clots (P=0.0002 for Kruskal-Wallis test for whole group, followed by Dunn post-test). C. Occlusions of renal arteries of varying sizes were identified and quantified. Recombinant DNase I significantly reduced the number of completely or partially obstructed interlobar, arcuate, and interlobular arteries. We set 6 different parameters to analyze the ratio of obstructed artery (Figure XB in the Data Supplement); (Continued)
CC Occlude Arteries by Forming Crystal Clots—Role of Extracellular DNA

Accumulation of ecDNA in the fibrin clot can be an important component of vascular thrombosis.\(^2\)\(^,\)\(^3\) Using DAPI (4',6-diamidino-2-phenylindole) staining, we noticed positivity for ecDNA in crystal clots (Figure 5A) and recombinant DNase I treatment significantly decreased the percentage of CC clots (Figure 5B). To test its functional contribution for arterial occlusions, we treated C57BL/6J mice with recombinant DNase I (Figure VA in the Data Supplement). After 24 hours of DNase I treatment, intraarterial ecDNA had disappeared together with a reduction in the number of arterial occlusions, and the fibrin and cellular components of crystal clots, while the crystal component, persisted (Figure 5C and 5C' Figure XB in the Data Supplement). This effect was confirmed by 3-dimensional micro-computed tomography imaging (Figure 5D; Movies IV and V in the Data Supplement). Preventing arterial occlusions was associated with a complete protection from GFR loss, a significant reduction in kidney infarct size as well as kidney cell death as indicated by less TUNEL positivity, neutrophil infiltrates, and vascular rarefaction (Figure 5E through 5G; Figure VB through VD in the Data Supplement). Thus, ecDNA is another nonredundant component of CC embolism-related arterial obstruction, tissue infarction, and organ failure.

CC Directly and Indirectly Induce DNA Release From Several Cellular Sources

Because DNase I treatment was superior to neutrophil depletion in protecting the kidney, ecDNA should derive from multiple sources. We addressed this by a series of in vitro studies. Exposing CC or supernatant of CC-activated platelets to human neutrophils induced neutrophil necrosis and neutrophil-like chromatin release and free DNA in the cell-culture supernatant (Figure 6A through 6C). Exposure to increasing doses of CC also induced renal endothelial cells to undergo necrosis and to release DNA in 2D as well as 3D culture (Figure 6D through 6I, Figure VIA and VIB and Movies VI and VII in the Data Supplement). Pretreated GEnC with Nec-1s, MCC950, or Cl-amidine had no effect on DNA release (Figure VIC and VID in the Data Supplement). Exposure of human platelets to CC also induced the release of minor amounts of ecDNA from their mitochondria (Figure VIE in the Data Supplement). Thus, in vitro studies support that CC and platelet-dependent ecDNA release from neutrophils and endothelial cells could be a contributing factor in CC-induced arterial obstruction.

DNase I Abrogates CC-Induced Platelet Activation and Fibrin Formation

Given the efficacy of clopidogrel in inhibiting crystal clot formation, we considered that ATP release and purinergic receptor P2Y12 signaling are directly involved in this process.\(^24\) We exposed washed mouse platelets to thrombin in the presence or absence of CC and quantified fibrin formation with a turbidity assay at 405 nm. CC did not influence turbidity in resting platelets, but upon thrombin activation, turbidity decreased in CC-treated platelets (Figure 6J). Thus, CC enhances fibrinogen release from platelet alpha (\(\alpha\)) granules, which further promotes fibrin clot formation. CC exposure also induced ATP secretion from dense (\(\delta\)) granules, but co-incubation with DNase I strongly reduced these extracellular ATP levels to the levels of untreated platelets (Figure 6K). Next, we stimulated platelets with thrombin and CRP (collagen-related peptide) to activate PAR-Gq (protease activated receptor) and GPVI-ITAM (glycoprotein VI-immunoreceptor tyrosine-based activation motif) signaling, respectively, and degranulation was monitored by an \(\alpha\)-granule marker P-selectin. In the presence of CC, a significant increase of P-selectin exposure was detected, while DNase I treatment could inhibit this process in the presence or absence of CC (Figure 6L and 6M). This indicated a crucial role of ecDNA in platelet degranulation, thereby inhibiting fibrinogen and ATP secretion and subsequent fibrin formation and P2Y12 receptor signaling, respectively.

Finally, a known element in local platelet adhesion and thrombus growth in CC embolism is vascular collagen matrix.\(^26\) To model this process in vitro, we tested collagen-driven platelet aggregation in the presence or absence of CC. Results showed that collagen I triggered massive platelet aggregation in 5 minutes in the presence of CC, and DNase I treatment normalized this accelerated aggregation response (Figure 6N and 6O). Thus, DNase I can attenuate CC-induced platelet activation, aggregation, and fibrin clot formation.
Figure 6. In vitro studies with endothelial cells, platelets, and neutrophils.

A–C, Supernatants of platelets exposed to cholesterol crystal–induced neutrophil extracellular trap (NET) formation in neutrophils (A, confocal microscopy with Sytox Green ecDNA staining), cell death (B, $P<0.000000001$ across groups, 1-way ANOVA, followed by Dunnett post-test) and DNA release (C, $P=0.000000001$ across groups, 1-way ANOVA, followed by Dunnett post-test) of human neutrophil.

D–F, Cholesterol crystal (CC) induced mouse glomerular endothelial cells to release DNA (D, confocal microscopy with Sytox Green, $P=0.000000001$ across groups, 1-way ANOVA, followed by Dunnett post-test), to undergo cell death (E, $P=0.000000001$ across groups, 1-way ANOVA, followed by Dunnett post-test), and to release DNA into the cell culture supernatant, where it was measured by Pico green Kit (Continued)
A 2-Step Strategy to Improve Outcomes of CC Embolism

As cardiac or aorta surgeries preclude the use of anti-coagulants or fibrinolytic agents, we considered recombinant DNase I as a possible alternative to attenuate CC clot formation by inhibiting fibrin formation, ecDNA accumulation, and ATP signaling. First, we tested the therapeutic window-of-opportunity and administered recombinant DNase I 3, 6, and 12 hours after CC injection (Figure VIIA in the Data Supplement). While DNase I given at 6 and 12 hours lacked effects on any of the end points, DNase I treatment given 3 hours after CC embolism showed trends toward improved outcomes albeit not in a consistent manner (Figure 7A through 7D; Figure VIIIB through VIIID in the Data Supplement). To further optimize outcomes in the setting of a cardiovascular procedure-related CC embolism, we tested a regimen combining a preemptive single dose of the necroptosis inhibitor Nec-1s with therapeutic recombinant DNase I given 3 hours after intraarterial CC injection (Figure VIIIE in the Data Supplement). This approach could be feasible as a prophylaxis given to all patients at risk, while DNase I would be only given to those with signs of CC embolism into the kidney, for example, an early decline of urinary output. This dual strategy resulted in a significant protection from GFR loss and kidney infarction in almost all animals together with a significant reduction in vascular occlusions by crystal clots (Figure 7E through 7G’; Figure VIIIF through VIIH in the Data Supplement).

DISCUSSION

Crystal induced diseases involve shared and unique pathomechanisms. We had hypothesized that developing a reproducible model of CC embolism would help to dissect the pathophysiology underlying CC embolism-driven arterial occlusion, tissue infarction, and organ failure. Indeed, our novel mouse model not only mimics all local aspects of human atheroembolic kidney disease, but our data also provide a new pathophysiological concept for CC embolism (Figure 8) and identify novel molecular targets for prophylaxis and therapy.

This inducible model of dose-dependent CC embolism in male and female mice mimics the morphological and functional characteristics of CC embolism syndrome in humans, rabbits, and rats. Different ways of CC preparation may result in CC of different shapes, that is, plate or needle; however, morphologically the shape of crystals found in our model were identical to those found in patients. Still, the ex vivo CC preparation may differ from the lipid material dislocating in human diseases. Organ-specific effects have to be kept in mind as rapid spontaneous revascularization was reported selectively for pulmonary artery CC embolism and rabbit muscle arteries can lack crystal clot formation. Functionally, in our model, the GFR normalized within 14 days, most likely due to compensatory hypertrophy of the contralateral kidney and the nonaffected parts of the cholesterol crystal embolism kidney. In contrast to previous attempts, our model allows functional studies in genetically modified mice. Specifically abrogating necroptosis, a form of regulated necrosis well-known to mediate post-ischemic kidney necrosis as well as NLRP3-dependent sterile inflammation, that is, necroinflammation, prevented CC embolism-induced kidney infarction, but not kidney failure. This dissociation between tissue viability and function was already obvious from the different variabilities of CC embolism-induced GFR loss versus infarct size. This is because, in contrast to other organs, kidney function directly depends on the perfusion of arteries afferent to the glomerular filters, while kidney infarction depends on numerous other factors such as collateral perfusions of tubules, inflammation, and complex stress responses contributing to kidney cell death. Indeed, this finding confirms our choice for GFR as primary end point and infarct size as only a secondary end point in the clinically relevant acute kidney injury context.

In 1973, Warren and Vales reported human atheromatous plaque material injections into rabbit kidneys...
Figure 7. A 2-step strategy prevents cholesterol crystal embolism (CCE)-induced tissue infarction and organ failure. 

A–D. Recombinant DNase I treated on mice after CC injection different time points effect on glomerular filtration rate (GFR) loss (A, P=0.000084 for Kruskal-Wallis test for whole group, followed by Dunn post-test), infarct size (B, P=0.0000081 for Kruskal-Wallis test for whole group, followed by Dunn post-test), vascular occlusions (C, details see Figure XIA in the Data Supplement), and kidney injury (D, P=0.00000045 for Kruskal-Wallis test for whole group, followed by Dunn post-test).

E–G. Dual therapy involving preemptive treatment with Nec-1s+recombinant DNase I given 3 h after CC injection significantly protected from GFR loss (E, P=0.00000076 for Kruskal-Wallis test for whole group, followed by Dunn post-test), infarct size (F, P=0.000017 for Kruskal-Wallis test for whole group, followed by Dunn post-test), and vascular occlusions (G). G′, Ratio of obstructed artery (Details see Figure XIB in the Data Supplement). All quantitative data are means±SD.
Figure 8. Human tissue and schematic illustration of cholesterol crystal (CC) embolism and its therapeutic targets.

A. Periodic acid-Schiff (PAS) staining of human kidney and spleen obtained from autopsy tissue of a patient that died shortly after cholesterol crystal embolism (CCE). Crystal clefts (shown by arrows) indicate the presence of crystals in a thrombotic mass. B. Feulgen stained human kidney and spleen. Extracellular DNA (ecDNA) is indicated by arrows. C. Inside arterial vessel. D. CC triggers the formation of crystal clots composed of activated platelets, neutrophils, fibrin, and extracellular DNA. E. Targeting this crystal clot with either clopidogrel, heparin, uPA inhibitor (urokinase), or recombinant DNase I can resolve the clot component. As the remaining crystal component alone does not account for a relevant vascular obstruction, such interventions can prevent tissue infarction and organ failure.
and brains and found thrombotic material around the embolus.28–30 Our studies extend these observations by showing that CC not only triggers diffuse clot formation but also the crystal clot and not the CC emboli alone account for vascular occlusions, infarct, and kidney failure. This finding is important as it renders the forming clot as putative target for therapeutic intervention to improve outcomes.

Current recommendations for the management of patients with CC embolism rely on retrospective reports of single case or small single-center patient series; no randomized controlled interventional trials have thus far performed.5,24 Partially contradictory outcomes have been reported for the use of steroids or anticoagulants, probably because beyond the peripheral lesions, outcomes also depend on the stability of aortic plaques, intraplaque hemorrhages, and the risk for repetitive episodes.3,34–39 Our model allows dissecting these 2 different aspects of pathophysiology, and it clearly demonstrates that using a platelet antagonist or anticoagulants, by interfering with crystal clot formation, can prevent peripheral tissue necrosis and organ failure. This, however, does not exclude effects on plaques to potentially impact on outcomes the opposite way.

In this context, our data on the role of ecDNA in crystal clot-related arterial obstructions are of potential interest. ecDNA has recently been identified as a critical component of thrombosis, either by employing mice mutant for DNases or by using recombinant DNase I.31,32,40,41 These studies documented neutrophils as the source of ecDNA. We considered the same source also for crystal clots, but neutrophil depletion or PAD4 inhibition had little effects on vascular obstructions and acute kidney injury compared with DNase I injection, probably as due to the rather small contribution of neutrophils to intravascular DNA release in our model. Although also platelets could be a source of ecDNA release from their mitochondria,32,43 this process may have a minor role in CC-induced fibrin clot formation in our in vivo mouse model. Surprisingly, DNase I can inhibit platelet degranulation and ATP release, and strongly inhibits CC- and collagen-induced aggregation responses, which may be independent from its effects on ecDNA. Whether DNase I directly hydrolyzes ATP and thereby inhibits purinergic signaling in platelets and other cell types remains to be studied. As another mechanism, CC directly damage the surface of endothelial cells in vitro and ex vivo.44 Indeed, our data suggest that endothelial cell injury could be the major source of locally accumulated ecDNA within the CC fibrin clot, rather than DNA released by neutrophils or platelets. Although we could detect CC and platelet-dependent neutrophil formation in vitro, the origin of DNA was difficult to ultimately prove in our in vivo mouse model. It is important to note that small amounts of CC cannot damage the endothelial cell layer, and therefore, CC not always induces fibrin clot formation and endovasular obstruction.31 However, it has been shown that CC can attack endothelial cells in various ways including complement activation, plasma membrane destabilization, and Src homology region 2 domain-containing phosphatase-2 signaling35–39 but our data suggest that necroptosis, inflammasome or PAD4-dependent pathways are not involved.

In summary, not CC by itself but the fibrin clots forming around CC obstruct peripheral arteries causing tissue infarction and organ failure. Hence, crystal clots represent the primary target for therapeutic interventions. Among the possible molecular targets in thrombosis and hemostasis, especially enhancing fibrinolysis or inhibiting platelet purinergic signaling could reduce arterial occlusions, infarction, and organ failure albeit with a relatively short window-of-opportunity up to 3 hours. Our results suggest that prophylactic necroptosis inhibition with a combination of DNase I therapy could have a synergistic effect on CC induced clot formation in mice and might be a feasible 2-step prophylactic/therapeutic approach in human patients with a risk for procedure-related CC embolism.

**ARTICLE INFORMATION**

Received June 28, 2019; revision received February 5, 2020; accepted February 20, 2020.

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**Acknowledgments**

James. M. Murphy and Warren Alexander, Cell Signalling and Cell Death Division, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia supplied the MK824 mice. We thank Dr Elmina Mammadova-Bach, Institute of Experimental Biomedicine, University Hospital and Rudolf Virchow Center, University of Würzburg, Germany, for her support with platelet experiments.

**Sources of Funding**

This work was funded by the Deutsche Forschungsgemeinschaft (AN372/16-2, 20-2, 24-1) to H.-J. Anders, SFB/TRR57 to T. Lammers, and SFB/TRR57, SFB/TRR219, BO3755/3-1, and BO3755/6-1 to P. Boor; and 374031971-TRR240/A09 to A. Braun. The German Ministry of Education and Research (BMBF; STOP-FSGS-01GM1901A) supported P. Boor. The European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme supported P. Romagnani (grant agreement number 648274) and T. Lammers (grant agreement number 309496). The Ministero dell’Istruzione, dell’Università e della ricerca, PRIN 2017 supported P. Romagnani.

**Disclosures**

None.
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