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Summary

Previous studies in mouse models of self-limited viral hepatitis showed that platelets contribute to acute liver damage by promoting the intrahepatic accumulation of virus-specific CD8 T cells and, secondarily, virus-nonspecific inflammatory cells. Built on these observations, a recent preclinical study took advantage of a previously established hepatitis B virus (HBV) transgenic mouse model of immune-mediated chronic hepatitis that progresses to hepatocellular carcinoma (HCC) to demonstrate that clinically achievable doses of the anti-platelet drugs aspirin and clopidogrel - administered continuously after the onset of liver disease - can prevent hepatocarcinogenesis and greatly improve overall survival. These outcomes were preceded by and associated with reduced hepatic accumulation of virus-specific CD8 T cells and virus-nonspecific inflammatory cells, reduced hepatocellular injury and hepatocellular proliferation, and reduced severity of liver fibrosis. The observation that anti-platelet therapy inhibits HCC development identifies platelets as key players in the pathogenesis of HBV-associated liver cancer and supports the notion that a sustained immune-mediated necroinflammatory liver disease is sufficient to trigger HCC. The results abovementioned and their clinical implications are discussed in this report.

Key Point Box

1. Platelets promote the intrahepatic accumulation of virus-specific CD8 T cells in mouse models of acute HBV infection.

2. Virus-specific CD8 T cells induce liver injury by killing HBV-replicating hepatocytes and by secondarily recruiting pathogenic virus-nonspecific inflammatory cells.

3. The anti-platelet drugs aspirin and clopidogrel inhibit the intrahepatic accumulation of virus-specific CD8 T cells and virus-nonspecific inflammatory cells.

4. Sustained administration of aspirin and clopidogrel in mouse models of chronic HBV infection reduces the severity of persistent liver injury and prevent HCC development.

5. Anti-platelet therapy may represent a novel therapeutic option in patients with chronic HBV infection.

HCC is the fifth most frequently diagnosed cancer worldwide and the third leading cause of cancer-related death [1]. Poor prognosis of HCC-bearing patients is linked to the relative resistance of this cancer to radio- or chemo-therapy and the limited success of surgical (tumor resection or liver transplantation), percutaneous (ethanol injection or radiofrequency thermal ablation) and transarterial (embolization or chemoembolization) interventions [2].

HBV is a noncytopathic DNA virus whose host range is limited to man and chimpanzees and whose tropism is limited to the parenchymal cell of the liver, i.e. the hepatocyte [3]. HBV can cause persistent infection, fibrosis and cirrhosis of the liver and it is globally responsible for more than 50% of HCC cases [4]. Although the pathogenic mechanisms whereby HBV causes HCC may involve viral factors (e.g. the deregulation of procarcinogenic genes due to HBV DNA integration and/or the expression of HBV-derived procarcinogenic polypeptides), host factors seem to play a predominant role in this process [3]. The interval between viral infection and HCC is typically several decades, indicating that HBV is not acutely oncogenic. Further, almost all HCC cases occur after many years of immune-mediated chronic hepatitis [3]. Chronic hepatitis B (CHB) is characterized by a functionally inefficient virus-specific CD8 T cell response that fails to eliminate HBV from the liver but maintains continuous cycles of low-level hepatocellular injury, promoting hepatocellular proliferation and exposing proliferating hepatocytes to inflammatory mutagens [3]. Over time, the persistence of necrosis, regeneration and inflammation coupled with compromised repair functions are thought to trigger random genetic and chromosomal alterations ultimately leading to HCC development [3].

Prevention of HBV infection by universal infant vaccination is starting to produce a positive impact on HCC incidence [4]. Termination of chronic HBV infection by available antiviral therapies has also been associated with reduced occurrence of HCC [5, 6]. Unfortunately, the majority of CHB patients do not respond to these therapies with sustained virus elimination (because of limited efficacy, dose-limiting side effects, high costs and the emergence of drug-resistant mutants [7]). As such, a huge burden of cancer risk remains in store for more than 350 million people chronically infected with HBV [4]. Based on these considerations, it becomes apparent that new drugs reducing the risk of HCC in these individuals will meet a pressing need. Relevant to this, a recent preclinical study of ours suggested that aspirin and clopidogrel – two anti-platelet drugs widely used in man for the prevention of arterial thrombosis [8] – inhibit the development of HCCs emerging from a context of immune-mediated chronic liver injury [9]. The research that led to this preclinical study, the mechanisms whereby anti-platelet drugs may exert anti-HCC potential and the clinical implications of these findings are discussed below.

As mentioned earlier, virus-specific CD8 T cells are key triggers of hepatic immunopathology during HBV infection [3]. Previous work from our laboratory has also outlined a role for platelets in this process. Indeed, we used two mouse models of self-limited acute viral hepatitis in which hepatocellular damage is resolved in a few days (HBV transgenic mice transferred with HBV-specific CD8 T cells or wild-type mice infected with hepatotropic adenovirus) to show that platelets are present at sites of organ damage; their depletion reduces the number of intrahepatic virus-specific CD8 T

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cells (and the number of intrahepatic virus-nonspecific inflammatory cells that CD8 T cells secondarily recruit into the liver) ameliorating disease severity; and this positive outcome is reversed by reconstituting thrombocytopenic animals with normal but not dysfunctional platelets [10]. In an ongoing effort to explain mechanistically why platelets are required to support the intrahepatic accumulation of virus-specific CD8 T cells, we found that this function is influenced by two specific inhibitors of platelet activation pathways, aspirin that blocks thromboxane (TX) A_2 production and clopidogrel that blocks the $P2Y_{12}$ ADP receptor [8]. Treating adenovirus-infected mice with aspirin, clopidogrel, or a combination of the two attenuates the acute accumulation of virus-specific CD8 T cells in the liver and the associated organ damage [11].

Considering that oral administration of aspirin and clopidogrel can be used for long-term treatment of patients at risk of thrombosis, we then took advantage of a previously established mouse model of immune-mediated CHB [12] to evaluate if and how the sustained inhibition of platelet activation may impact the severity of chronic liver injury and its complications. Treating these mice with clinically relevant doses of aspirin and clopidogrel - beginning after the induction of hepatitis and continuing indefinitely - limited the intrahepatic accumulation of both virus-specific CD8 T cells and virus-nonspecific inflammatory cells and the consequent hepatocellular injury, reduced compensatory hepatocellular proliferation, diminished the severity of liver fibrosis, prevented HCC development and, more importantly, improved overall survival [9]. These results indicate that platelets influence the chronic stages of a pathogenic mechanism triggered by virus-specific CD8 T cells, and strongly imply that immune-mediated inflammatory reactions are the predominant cause of HCC transformation during chronic HBV infection.

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The ability of aspirin/clopidogrel to counteract the intrahepatic accumulation of virus-specific CD8 T cells may be sufficient to explain the outcome of our study. In contrast, long-term anti-platelet treatment had no effect on the phenotype of HBV-specific CD8 T cells, which remained similar to that of chronically infected patients [13] with retention of target cell killing activity but low expression of IFN-γ capable of inhibiting HBV replication [14]. If a functional link exists between platelets and CD8 T cells, it is still undefined. We have proposed that activation-dependent expression of platelet CD40 ligand may contribute to the expansion phase of virus-specific CD8 T cells resulting in their accumulation at sites of infection [15]. In fact, evidence obtained by others indicates that platelet CD40 ligand modulates multiple aspects of adaptive immunity [16]. Whether the functional connection between platelets and T cells depends on direct intercellular contacts remains to be fully demonstrated; recent experiments utilizing intravital microscopy, however, are telling us that platelet-T cell interactions do occur within the hepatic microcirculation.

An alternative explanation for the pathogenic role of platelets in aggravating viral hepatitis has been based on findings in mice acutely infected with lymphocytic choriomeningitis virus (LCMV) [17], a virus that - unlike HBV - in the liver infects primarily resident macrophages. In this model, vasoactive serotonin released from activated platelets appears to promote liver damage by perturbing the liver microcirculation, thus delaying the intrahepatic influx of CD8 T cells mediating LCMV clearance. It remains to be ascertained whether serotonin-dependent hemodynamic

disturbances can also influence HBV-related immunopathology in our transgenic hepatitis model. Recently, it has been shown that platelet-derived serotonin supports HCC development in mice chronically exposed to carbon tetrachloride (CCl_4) [18], a situation in which carcinogenesis is independent of adaptive immune responses. Of note, our recent study indicated that HCC development in mice similarly treated (i.e. chronically exposed to CCl_4) is not influenced by anti-platelet therapy [9]. Although indirectly, this may argue against the hypothesis that the aspirin/clopidogrel-dependent amelioration of HCC observed in mice undergoing immune-mediated chronic hepatitis reflects the inhibition of serotonin-dependent pathways. The lack of efficacy of anti-platelet therapy in CCl_4-treated mice [9] is also an argument against the possibility that platelet-derived angiogenic factors thought to be involved in the development of CCl_4-induced HCC [19, 20] may be targets of aspirin/clopidogrel treatment. Direct evidence for or against a possible role of platelet-derived serotonin and growth factors in immune-mediated chronic hepatitis is yet to be obtained.

Furthermore, the observation that mice undergoing immune-mediated CHB showed presence of small platelet aggregates only within hepatic necroinflammatory foci and absence of liver infarction [9] contradicts the possibility that anti-platelet therapy suppresses liver disease by inhibiting the formation of vessel-occluding clots. This is consistent with what observed in models of acute hepatitis, where vessel-occluding clots do not develop and warfarin-based anticoagulant therapy (preventing fibrin deposition) does not affect the capacity of virus-specific CD8$^+$ T cells to cause liver damage [10].

It is noteworthy that aspirin alone - at a dosage known to markedly inhibit the release of serotonin and other small molecules from dense granules - has no effect on the release of α-granule content - where proteins and peptides are stored [21]. This is relevant in relation to the potential role of platelet-derived proteins in the functional cross-talk with lymphocytes; for example, CD40 ligand and P-selectin translocate to the platelet membrane surface in association with the activation-induced α-granule release reaction [22]. Unlike aspirin, clopidogrel has been found by different investigators to downregulate the expression of markers of inflammation, including CD40 ligand and P-selectin, on the surface of activated platelets, an effect through which it inhibits heterotypic platelet-leukocyte interactions linking vascular injury to inflammation [23]. Therefore, the beneficial effect of dual anti-platelet therapy in ameliorating the course of immune-mediated chronic hepatitis may involve distinct pharmacologic effects of the two administered drugs. In synergy, they may reduce platelet contribution to a complex pathogenic mechanism in which multiple pathways modulate intrahepatic virus-specific CD8 T cell accumulation in the liver. In this regard it is relevant to note that, at the low dose used, aspirin has almost exclusively platelet-specific effects with minimal anti-inflammatory properties [8]; in keeping with this, we found that the hepatic expression COX-2 (a pro-inflammatory enzyme that is not produced by platelets [8]) and its relative activity were not reduced in mice treated with dual anti-platelet therapy (unpublished observation).

Altogether, the results presented in [9] indicated that the anti-platelet drugs aspirin and clopidogrel effectively prevent HCC and improve survival in a relevant mouse model of

CHB, supporting the concept that platelets promote hepatic immunopathology following activation events that require normal production of TX-A$_2$ and normal response to ADP. With the limitation that this study could not assess the impact of anti-platelet therapy on HBV replication and viremia, the results also reinforce the notion that a detrimental CD8 T cell response is both necessary and sufficient to induce liver cancer during chronic viral hepatitis and they suggest that drugs targeting platelet function may be a therapeutic option in patients with chronic HBV infection.

A concern about such a treatment could be increasing bleeding risk in individuals with compromised coagulation associated with impaired liver function. However, as pointed out in a recent review [24], excessive bleeding that is prevalent in the advanced stage of liver cirrhosis may be a lesser risk than thrombosis in some subgroups of patients with CHB. The presence of a pro-coagulant imbalance in these patients suggests that anti-platelet therapy might be beneficial at preventing both thrombosis and HCC development. Notably, a large observational study recently indicated that the use of aspirin reduces the risk of HCC in patients suffering from chronic liver disease of unspecified etiologies [25]. Although caution should be taken before evaluating observational studies that lack pretreatment patient stratification and randomized control group inclusion, these results coupled with our preclinical observations strengthen the rationale of designing future clinical trials aimed at specifically evaluating the impact of anti-platelet therapy in CHB patients. Prospective studies in CHB patients will require careful patient selection (for instance initially excluding patients with advanced liver disease), extended follow-up, frequent monitoring of viral parameters and, possibly, the concomitant use of nucleoside inhibitors inhibiting HBV. The design of these studies

could also benefit from additional preclinical work in woodchucks experimentally infected with WHV. WHV is a HBV-related hepadnavirus inducing transient or persistent immune-mediated liver diseases that resemble those observed in HBV-infected humans [26]. There, the evaluation of the impact that anti-platelet therapy will have on bleeding risk, WHV viremia and overall liver disease severity may provide useful information, particularly on the possibility that the anti-platelet therapy-dependent inhibition of intrahepatic T cell accumulation may result on viral reactivation.

Acknowledgements

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