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Liver and Biliary Tract Physiology/Pathophysiology

Emerging role of extracellular vesicles in liver diseases

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Malhi H. Emerging role of extracellular vesicles in liver diseases. *Am J Physiol Gastrointest Liver Physiol* 317: G739–G749, 2019. First published September 23, 2019; doi:10.1152/ajpgi.00183.2019.—Extracellular vesicles (EVs) are membrane-defined nanoparticles released by most cell types. The EVs released by cells may differ quantitatively and qualitatively from physiological states to disease states. There are several unique properties of EVs, including their proteins, lipids and nucleic acid cargoes, stability in circulation, and presence in biofluids, which make them a critical vector for cell-to-cell communication and impart utility as a biomarker. EVs may also serve as a vehicle for selective cargo secretion. Similarly, EV cargo may be selectively manipulated for targeted therapeutic delivery. In this review an overview is provided on the EV classification, biogenesis, and secretion pathways, which are conserved across cell types. Next, cargo characterization and effector cell responses are discussed in the context of nonalcoholic steatohepatitis, alcoholic hepatitis, and acetaminophen-induced liver injury. The review also discusses the potential biomarker and therapeutic uses of circulating EVs.

alcoholic hepatitis; exosome; microvesicle; nonalcoholic steatohepatitis; nanoparticle

INTRODUCTION

In recent years, there has been an explosion in advances in extracellular vesicle (EV) biology driven by improved isolation and characterization techniques and increased understanding of their vital role in intercellular communication (33, 95). EVs are heterogeneous, membrane-defined, nanometer-sized vesicles, primarily released from cells via two distinct biogenesis pathways. It is well recognized that EVs are released from live cells basally, from diseased cells, and from cancer cells, and that their cargo composition may reflect the pathophysiological state of the donor cell (Fig. 1) (85). Cells undergoing apoptosis also release EVs termed apoptotic bodies. Recent studies have expanded understanding of the recipient cell responses activated by EVs and their diverse bioactive cargo (72). EVs may act on recipient cells in an autocrine, paracrine, or endocrine manner, and induce recipient cell responses by engagement of cell surface receptors, internalization, or fusion with the recipient cell membrane (72, 73, 79). EVs may offer advantages over soluble mediators in this regard. Certain cargoes are selectively enriched in EVs—for example, miR-122 in a model of alcoholic liver disease—which could facilitate high-dose delivery of miR-122 to recipient cells (3). Additionally, selective cargoes that function as recipient cell-targeting signals, such as integrins (34), home EVs to particular recipient cells. EVs are also stable in biofluids, which would also ensure stability of bioactive cargoes contained within (44, 93). EVs are present in most bio-fluids, which along with their stability and cargo selectivity, makes them potential disease biomarkers (52). Recent studies have implicated EVs in injury, inflammation, and fibrosis in the liver in response to diverse stimuli (19, 32, 33, 53, 68, 69, 82, 83, 87, 107). In this review, EV classification, biogenesis and secretion pathways, isolation and characterization methods, and relevance to nonalcoholic steatohepatitis, alcoholic hepatitis, and acetaminophen-induced liver injury are discussed. Lastly, this review discusses the biomarker and therapeutic potential of EVs.

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CLASSIFICATION OF EVs

EVs are classified into two groups based on their cellular biogenesis from viable cells: exosomes and microvesicles. Dying cells release a third category of EVs termed apoptotic bodies (Fig. 2). In addition to these well-accepted lipid bilayer enclosed EVs, recently, a smaller nanoparticle isolated by asymmetric flow field-flow fractionation has been described. This nanoparticle is less than 50 nm in diameter and lacks a lipid bilayer (119, 121). This newly characterized nonmembranous particle has been termed "exomere" (121). Though exomeres were found to contain distinct cargoes in comparison with other types of extracellular vesicles, their biogenesis pathways remain to be elucidated, in contrast to exosomes and microvesicles. Exosomes are derived from the endocytic trafficking pathway. They are the intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) trafficked to the plasma membrane and released into the extracellular space. Perhaps limited by the size of the MVB, the diameter of exosomes ranges from 40 to 150 nm, although large MVBs have been described in the context of impaired endolysosomal trafficking (80). Mi-

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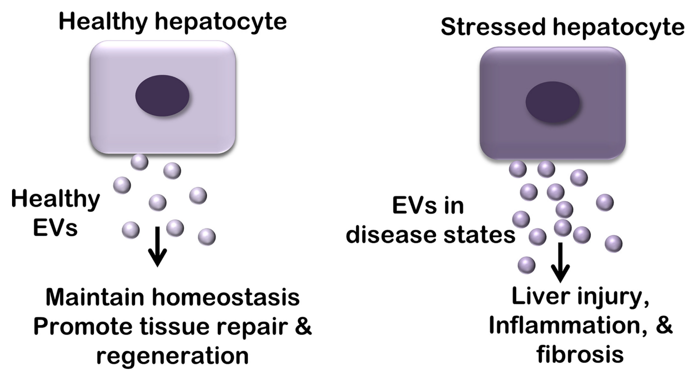

Fig. 1. Extracellular vesicles (EVs) in health and disease. Salutary properties of healthy hepatocyte-derived EVs in maintaining liver homeostasis by promoting repair and regeneration have been recently described. This is in contrast to EVs originating from stressed hepatocytes in disease states, which activate injury, inflammation, and fibrotic responses in recipient cells.

crovesicles, also known as microparticles or ectosomes, bud directly from the plasma membrane and, therefore, have a broader size range, 50–1,000 nm in diameter. Microvesicles derived from cancer cells are termed oncosomes and may be up to 10 μm in diameter. Therefore, size alone is not a reliable discriminator of exosomes from microvesicles. Similarly, the biophysical properties and density of exosomes and small microvesicles overlap such that most isolation methods cannot separate them (102). A recent comprehensive proteomic characterization further highlighted the heterogeneity of small EVs and the lack of gold standard markers (54). Although certain markers, such as syntenin-1 and TSG101, when present in tetraspanin-expressing EVs, might represent exosomes, and annexin A1 may be a marker for microvesicles (39, 54), these proposed markers need wider validation in order to support their acceptance as gold standard markers for exosomes or microvesicles, respectively. Thus, given overlapping size and density, lack of gold standard markers and standardized isolation methods (discussed later), the term "extracellular vesicle" has been used throughout this review, unless specifically discussing a type of EV. This review does not focus on exomeres, which need further characterization, nor on apoptotic bodies, which are formed during apoptosis by plasma membrane blebbing and range in size from 100 to 5,000 nm (27, 55, 121). Because of the size and cargo overlap with exosomes and small microvesicles, when studying EVs under potentially lethal stress conditions, it is important to exclude the contribution of apoptotic bodies.

BIOGENESIS AND SECRETION PATHWAYS

Exosome and microvesicle biogenesis is distinct on the basis of the membrane-defined organelle from which they arise, exosomes from the multivesicular body, and microvesicles from the plasma membrane. Although the organelle of origin is unique, both utilize conserved cellular machineries with some distinctions, as discussed below (86).

Exosomes. Exosomes are formed as ILVs from inward membrane protrusions within endosomes as they traffic intracellularly (85). An endosome with ILVs is referred to as an MVB or multivesicular endosome (MVE). MVBs may traffic to the trans-Golgi network, the recycling endosome, the plasma membrane, or the lysosome (104). Upon fusion with the plasma membrane the ILVs contained within their lumen are released into the extracellular environment, giving rise to exosomes. On the basis of how ILVs are formed, and by extension, exosome biogenesis is broadly categorized into endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways (35, 40).

The ESCRT machinery comprises several protein complexes classified into four subgroups (0, I, II, and III), which associate in succession depending on their functional role in protein cargo selection, clustering, and membrane fission (13, 40, 116). Cargo selection, in general, is governed by ubiquitination (84). ESCRT 0 complex recognizes and corrals ubiquitylated proteins, ESCRT I, II, and III induce bud formation and scission sequentially (84). Using an RNA interference strategy, several groups reported that silencing of individual ESCRT components or accessory proteins (HRS, STAM 1, TSG101, and CHMP4) leads to a reduction in the production of small EVs, as does silencing of the ATPase vacuolar protein sorting-associated protein 4 (VPS4) (13, 38).

In contrast, ESCRT-independent MVB and exosome generation was observed in cells depleted of key components of the ESCRT machinery, although the resulting MVBs and ILVs were enlarged (97). In this regard, phospholipids and sphingolipids are involved in the formation of exosomes. Following epidermal growth factor (EGF) stimulation, EGF receptor (EGFR) was not sorted into the ILVs of ESCRT-depleted cells, suggesting diversity in MVB formation pathways. In this study the late endosomal lipid marker, bismonoacylglycerophosphate (BMP), which is also known as lysobisphosphatidic acid (LBPA), was found to colocalize with EGF containing MVBs, though other studies have suggested that LBPA-containing MVBs are distinct from EGF containing MVBs after EGFR stimulation (EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation) (49, 113). BMP is found on late endosomes and not on the plasma membrane; therefore, BMP may be a lipid maker for exosomes (49). In a breast cancer cell line, the release of syntenin-enriched exosomes was mediated by ADP-ribosylation factor 6 (ARF6)-dependent activation of phospholipase D2, which catalyzes the breakdown of phosphatidyl choline to phosphatidic

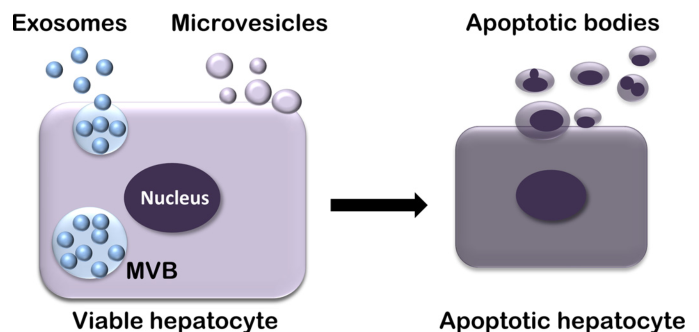

Fig. 2. Extracellular vesicle release along a continuum of stress responses. Healthy and stressed hepatocytes release extracellular vesicles (EVs) into the extracellular space that can be classified into exosomes and microvesicles on the basis of their cellular biogenesis pathways. Exosomes are derived from the intraluminal vesicles of multivesicular bodies (MVB), whereas microvesicles are released by direct budding from the plasma membrane. Apoptotic cells release apoptotic bodies which are formed by plasma membrane blebbing and may contain nuclear material. Cell stress, one feature of which is EV release, can eventually result in apoptosis; thus, both EV release and apoptosis can be viewed as a continuum of responses to cellular stress.

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acid and choline (26). Thus, membrane microdomain-specific generation of phosphatidic acid may mediate the formation of exosomes in a cell-specific manner.

A second class of lipids more clearly implicated in ESCRT-independent ILV formation is ceramides (103). Using the trafficking of a candidate cargo, proteolipid protein, it was demonstrated that silencing of ESCRT components did not alter its trafficking to ILVs or secretion in exosomes. Rather, this was dependent on the synthesis of ceramide via the salvage pathway. The de novo synthesis of ceramide also underlies the release of EVs from palmitate-treated hepatocytes (42). Ceramides can generate the signaling sphingolipid, sphingosine 1-phosphate (S1P) (48). The continuous compartmental activation of S1P receptors on MVBs was demonstrated to select candidate cargoes into exosomes; this process could be inhibited by the neutral sphingomyelinase inhibitor GW4869, which decreases ceramide formation (41).

Cargo sorting into MVBs is an area of intense interest (35). Observations with candidate cargoes suggest that selective cargo sorting rather than passive loading occurs with ILVs destined to be released as exosomes, although the determinants of cargo selection remain incompletely understood (9). Overexpression of proteins, such as MHCII in ALIX-silenced cells, was noted to lead to increase in MHCII-containing exosomes. Tetraspanins, such as CD63, CD81, and CD9, and the compartmental generation of sphingosine 1-phosphate have been demonstrated to lead to cargo sorting into MVBs (41, 106). KRAS-MEK, major vault protein, and Y-box binding protein 1 have been recently defined as determinants of microRNA sorting into exosomes (9, 92, 101).

MVB trafficking to the plasma membrane is partly regulated by Rab GTPases, several of which can be detected in exosomes and a few of which have been shown to impact MVB trafficking and exosome release (96). The impact of Rab11 on MVB trafficking is well studied in several cell lines. Its overexpression stimulated exosome secretion, and inhibition decreased exosome secretion, in the K562 erythroleukemia cell line (89). In these cells, Rab11 promoted MVB docking and fusion in a calcium-dependent manner. Rab11 also plays a role in endocytic trafficking of ligated p75 nuerotrophin receptor to a CD63-positive MVB compartment in neuronal cells and the protein evenness interrupted (or Evi) and its binding partner wingless-containing exosome release in *Drosophila* (5, 20). Rab35 has been shown to regulate exosome release from oligodendrocytes (23). Silencing of Rab27a or Rab27b in HeLa cells led to reduced exosome secretion without any alterations in the protein content of the exosomes (8). Rab27a knockdown reduced exosome secretion for breast cancer cell lines (8). The variable role of Rabs across different cell types may be a reflection of cell type specificity for particular Rabs (7). MVB-plasma membrane fusion is regulated by SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) proteins (111). Using a combination of live total internal reflectance microscopy and dynamic correlative light-electron microscopy and tetraspanin-based pH-sensitive reporters to visualize single MVB-plasma membrane fusion events, Verweij et al. (108) demonstrated that G protein-coupled receptor activation by histamine led to an increase in an MVB-plasma membrane fusion event mediated by the phosphorylation of SNAP23.

Microvesicles. Microvesicles arise from the outward protrusion and scission of the plasma membrane and share many molecular mediators with exosomes, although the exact pathways leading to the formation of microvesicles are less well understood (75). Microvesicles are only shed from specific plasma membrane domains enriched in specific proteins and lipids, which may facilitate membrane curvature, as discussed below. Plasma membrane budding occurs as a result of rearrangements in the protein components due to lateral or vertical trafficking to the plasma membrane, which may lead to higher-order oligomerization (22), lipid aggregation, loss of asymmetry of membrane phospholipids (phosphatidylserine on the cell surface outer leaflet), and rearrangement of actin cytoskeleton that occur via the action of translocases, scramblases, and calcium signaling, which leads to vesicle pinching (32, 83). Similar to exosome biogenesis, ESCRT components and ceramides play a role in the formation of microvesicles, and microvesicles display diverse cargoes, pointing to inherent heterogeneity within this type of extracellular vesicle.

Of the ESCRT components, I, II and III are implicated in microvesicle formation (35, 77), as is the Ras-related GTPase ADP-ribosylation factor 6 (ARF6), which also may participate in cargo selection (74). In cancer cell lines, ARF6 regulated the release of microvesicles by myosin light chain-dependent membrane abscission (74). Interactions between TSG101 and arrestin domain-containing protein 1 (ARRDC1) leads to the relocation of TSG101 from endosomes to the plasma membrane and release of microvesicles, which contain TSG101 and ARRDC1, but lack the endosomal marker CD63 (76). The release of these microvesicles also required the ATPase activity of the ESCRT component VPS4 (38). Membrane curvature may also be induced by protein-protein crowding, which may explain how higher-order oligomers of proteins facilitate membrane budding (22, 94). In addition to proteins, changes in lipid composition affect membrane curvature and bending. For example, some microvesicles are enriched in ceramides, sphingolipids, and cardiolipins, depending on the cell of origin (29). Ceramides are cone-shaped lipids, and select phospholipids may induce a conical shape of the plasma membrane, leading to vesicle budding (29). In glioma cells that have been stimulated with ATP, microvesicle release is dependent upon acid sphingomyelinase, which relocates to the outer plasma membrane, likely leading to local ceramide synthesis (6). In keeping with this observation, microvesicles from some cell types demonstrate enrichment of sphingolipids, and microvesicles from other cell types do not (29). This is consistent with heterogeneity in microvesicle biogenesis pathways.

Regulation of EV release. Release of both types of EVs is an active energy-dependent process, for example, the ATPase VPS4 is necessary for release of exosomes and microvesicles (38). Both appear to be regulated, although how the biogenesis and release are regulated is not fully understood. Serum deprivation decreases exosome release from many cell types, suggesting that growth factors induce the release of exosomes (30). Exosome release increases in cells experiencing stress, such as hepatocytes exposed to lipotoxic endoplasmic reticulum stress, under hypoxic stress, or genotoxic stress from chemotherapeutic drugs (42, 46, 47, 117). Exosomes carry stress-induced danger signals, such as danger-associated molecular patterns (or DAMPs), which may serve to convey cell stress in the microenvironment or mitigate cell stress and

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restore cellular homeostasis (16, 110). Another level of regulation of exosome formation is MVB trafficking to the lysosome versus the plasma membrane remain, the determinants of which remain undefined. However, exosome formation is linked to endosomal trafficking and autophagy. Inhibition of endosomal trafficking impairs exosome release, and inhibition of autophagy increases exosome release, perhaps by inhibiting MVB fusion to the lysosome. Conversely, induction of autophagy impairs exosome release by targeting MVBs to the lysosome (21). Similarly, microvesicle release is regulated and responsive to the pathophysiological state of the cell of origin, though much needs to be understood about regulatory mechanisms (75, 105). When cells are serum starved, and thus deprived of growth factors, microvesicle release is attenuated; conversely, growth factor-stimulated cells can release microvesicles (1, 75). Furthermore, microvesicle cargo signature differs from parental cells, suggesting regulated targeting of specific cargoes to microvesicles (29).

Isolation and characterization of EVs. Currently, there is no gold standard method for EV isolation or characterization, and comprehensive and transparent reporting of EV isolation methodology is encouraged to increase reproducibility and interpretation of data (14, 102, 115). There has been a proliferation of EV isolation methods, and these continue to evolve with technological advances. Choice of method for EV isolation and sample preparation are commonly informed by the intended downstream application of isolated EVs. Most EV isolation methods are based on using the biophysical properties of EVs, size, affinity capture, or polymer-based precipitation, and carry the risk of coisolating proteins and lipoproteins. Differential ultracentrifugation remains a popular method for the isolation of EVs (14). The reader is referred to EV isolation methods, which have been reviewed elsewhere in detail for understanding the advantages and shortcomings of available isolation methods (51, 70, 118). Suffice it to say that most methods isolate a heterogeneous population of EVs, and any EV isolate should be well characterized quantitatively and qualitatively (102). Furthermore, the efficiency of isolation across techniques is highly variable (100), making the establishment of normative levels challenging. Recognizing these caveats, a recent position paper of the International Society for Extracellular Vesicles has published guidelines on the minimal information for studies of EVs, with the objective of increasing the rigor of EV-related research (102). These guidelines highlight the importance of quantitative assessment of isolated EVs and qualitative bulk and single EV characterization of isolated particles.

EVs IN LIVER DISEASES

In parallel with the global increase in interest in EVs in health and disease, there has been a significant growth in the understanding of EVs in liver diseases (18, 33, 98). Several groups have demonstrated an increase in circulating EVs in animal models of liver disease and in human subjects as well. This includes a variety of chronic and acute liver diseases, such as nonalcoholic steatohepatitis, alcoholic liver disease, viral hepatitides, drug-induced liver injury, ischemia-reperfusion injury, and hepatobiliary malignancies (3, 19, 32, 37, 42, 43, 57, 71, 82, 83, 88, 90, 120). This review discusses EVs in nonalcoholic steatohepatitis, alcoholic liver disease, and acetaminophen-induced liver injury in detail. Many of these studies have demonstrated an increase in circulating EVs without identifying the cell of origin. Increasingly, with technological advances such as nanoscale flow cytometry, the use of reporter mouse models, and other immune affinity-based-methods, the literature related to hepatocyte-derived EVs in various liver diseases is emerging. In a recent study, it was demonstrated that hepatocyte-derived EVs, although elevated in a murine dietary model of nonalcoholic steatohepatitis, are far less abundant than platelet-derived, macrophage-derived, and neutrophil-derived EVs in NASH, and that hepatocyte-derived EVs constitute a very small percentage of total circulating EVs (0.005–0.02%) (61). These initial observations will have to be validated by other independent research groups and techniques. It is also conceivable that the concentration of hepatocyte-derived EVs is much higher within the hepatic microenvironment and may further increase in the microenvironment and decrease in circulation in cirrhosis due to capillarization of liver sinusoids (81). The turnover and concentration of endogenous cell type-specific EVs are difficult to assess in vivo at this time because of technical limitations, such as the resolution of intravital microscopy. However, additional mechanistic studies with isolated hepatocyte-derived EVs support a role for EV-mediated intercellular communication in vivo in various models of liver diseases, and EV transplantation experiments in animal models lend support to these mechanistic observations (15, 42, 63, 68, 83, 107).

Nonalcoholic steatohepatitis. Circulating EVs are increased in rodent models of nonalcoholic steatohepatitis (NASH) as well as human NAFLD samples where they have been implicated in injury, inflammation, fibrosis and found to correlate with histologic features of NASH (4, 42, 82, 83, 112). Various dietary NASH mouse models demonstrate an increase in EVs in keeping with the concept that EV release can occur as a cellular stress response (42, 83). EV composition changes in NASH, and specific proteomic, lipidomic and microRNA alterations have been described along with their cellular targets (37, 42, 52, 82). Furthermore, not only are hepatocyte-derived EVs increased in NASH models, a recent report has characterized EVs originating from macrophages, neutrophils, and platelets in murine NASH and platelet-, endothelium-, and leukocyte-derived EVs in human NASH with advanced fibrosis (61, 112). These studies and others have reported that EV levels are dynamic; interestingly, in an EV mouse study, macrophage-derived EVs were significantly elevated at a time that hepatic inflammation was evident histologically, and in the human study, CD14- and CD16-positive EVs, representing leukocyte-derived EVs, were inversely correlated with fibrosis (61, 112).

Examination of the pathological consequences of EVs in NASH has demonstrated a robust role for paracrine effects and correlation with histology. In choline-deficient L-amino acid (CDAA) diet-fed experimental NASH, liver-derived and total circulating EVs were elevated, correlated with hepatocyte apoptosis, neoangiogenesis, and fibrosis (82). NASH EVs possessed a unique proteome, which could distinguish NASH from control EVs, and the NASH EV proteome represented signaling processes activated in NASH, including inflammatory responses and cytoskeleton remodeling. Hepatocyte-derived miR-122 was also enriched in NASH EVs in CDAA-diet-fed mice. Plasma mitochondrial DNA (mtDNA) was dem-

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onstrated to be elevated in human NASH samples, and high-fat diet-fed mouse plasma, and found to be preferentially contained in hepatocyte-derived EVs. EV mtDNA activated Toll-like receptor 9 (TLR9) (25). Mice lacking TLR9 globally and in lysozyme-expressing myeloid cells were protected from diet-induced NASH, suggesting that hepatocyte-derived mtDNA might be a ligand for TLR9 activation on myeloid cells, leading to liver injury and inflammation in NASH. In keeping with these observations, adoptive transfer of EVs from HFD-fed mice led to an increase in hepatic and circulating immature myeloid cells (15). Garcia-Martinez et al. (25) demonstrate that mtDNA is contained within microvesicles. The mechanism by which mtDNA within a microvesicle would activate TLR9 was not explored in this article, although they confirm that TLR9 activation is lost in microvesicle-depleted plasma. TLR9 is located within the endolysosomal compartment and presumably mtDNA-containing microvesicle uptake via the endocytic pathway by recipient cells would activate endosomal TLR9 (10).

Additional mechanistic studies using isolated hepatocytes and mouse models have demonstrated that the stress kinase, mixed lineage kinase 3 (MLK3), mediates the release of EVs from hepatocytes treated with the lipotoxic lipid, lysophosphatidylcholine (LPC). These LPC-stimulated EVs are enriched in C-X-C motif ligand 10 (CXCL10), which, in turn, can activate macrophage chemotaxis. *Mlk3* knockout mice had lower plasma EVs and lower CXCL10 expression in plasma and on EVs. It was not determined whether the reduction in EVs is due to an attenuated NASH phenotype in the *Mlk3* knockout mice; regardless, EVs correlated with NASH in this study. Furthermore, *Cxcl10* knockout mice also developed attenuated liver inflammation in NASH implicating CXCL10 signaling in liver inflammation in NASH. This may occur due to reduced CXCL10 containing EV-mediated macrophage recruitment into the liver. CXCL10 activates the plasma membrane receptor CXC receptor 3 (CXCR3) (64). CXCR3 is more potently activated by oligomeric CXCL10 than monomeric forms. Thus, a potential explanation for the potency of CXCL10-enriched EVs, in comparison with soluble CXCL10, in activating macrophage chemotaxis would be a high local concentration of CXCL10 on EVs The immunogold electron micrographs in the article by Ibrahim et al. (37) suggest that, indeed, CXCL10 is abundant on the surface of EVs. Lipotoxic EVs are also enriched in the ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which engaged TRAIL receptor on bone marrow-derived macrophages to activate proinflammatory signaling. The release of EVs from hepatocytes was dependent on TRAIL receptor and rho-associated, coiled-coil-containing protein kinase 1 (ROCK1). Recently, it was demonstrated that lipotoxic EVs are enriched in integrin β_1, which, in turn, promotes monocyte adhesion to sinusoidal endothelial cells, perhaps forming an early step in the recruitment of proinflammatory monocyte-derived macrophages into the liver (28). It is possible that ligand concentration, oligomerization, or stability, when contained in EVs, contribute to EV-mediated signaling effects (25, 34, 37). Recipient cell responses may also be governed by EV homing (34), such that EVs could deliver high-dose payloads effectively to target cells. This possibility is supported by some empirical data but deserves wider testing.

In reductionistic cell culture models, utilized to elucidate the mechanisms of lipotoxic EV formation, several signaling pathways have been identified (Fig. 3). It was demonstrated that in palmitate-treated hepatocytes lipotoxic EV release was mediated by the unfolded protein response sensor inositol-requiring protein 1-α (IRE1α) (42). The release of lipotoxic EVs was dependent on de novo ceramide biosynthesis, and EVs were enriched in bioactive lipids, including ceramides and sphingosine 1-phosphate (S1P). This study established a link between endoplasmic reticulum stress and hepatic inflammation in lipotoxicity via the release of ceramide-enriched EVs and demonstrated an enrichment of C16:0 ceramide in circulating EVs in mouse and human NASH plasma. Furthermore, in a follow-up study, macrophage migration toward S1P-enriched EVs was mediated by macrophage S1P receptor 1, suggesting that lipid mediators on lipotoxic EVs are important in macrophage-mediated inflammation observed in lipotoxic disorders (63). The canonical ceramide trafficking protein, steroidogenic acute regulatory protein-related lipid transfer domain 11, is also necessary for release of lipotoxic EVs, as recently demonstrated (24). This may be due to efficient trafficking of newly formed ceramide from the endoplasmic reticulum to the MVB in palmitate-treated hepatocytes. Palmitate-stimulated EVs had several features of exosomes, whereas, LPC-stimulated EVs had features of microparticles, highlighting the heterogeneity of EVs released by cells and observed in circulating EVs.

Cultured cells have also been used to demonstrate the effects of lipotoxic EVs on recipient cells. Lipotoxic mouse or human hepatocyte-derived EVs induced proangiogenic endothelial cell migration and tube formation via their cargo protein, Vanin-1 (83). MicroRNA cargo is altered in palmitate-stimulated EVs, with an increase in miR-122 and miR-192. MiR-192-containing EVs and miR-192 by itself activated fibrogenic signaling in hepatic stellate cells (58). Gut dysbiosis and adipose tissue inflammation are also linked to the multifactorial pathogenesis of obesity-associated NAFLD. In this context,

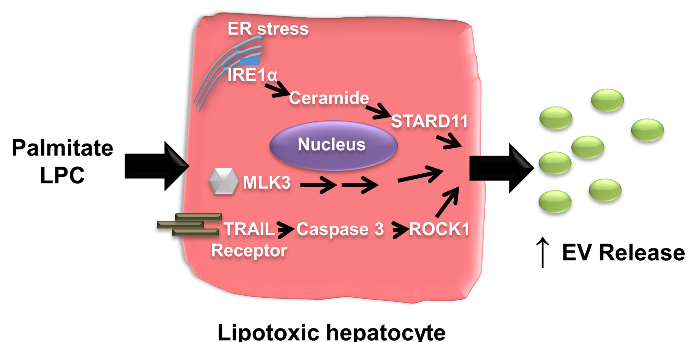

Fig. 3. Mechanism of lipotoxic extracellular vesicle (EV) release. Saturated fatty acids, including palmitate and the phospholipid lysophosphatidyl choline (LPC) stimulate the release of heterogeneous EVs from hepatocytes. The endoplasmic reticulum (ER) stress sensor inositol requiring enzyme 1α (IRE1α) is activated by palmitate, leading to the transcriptional upregulation of de novo ceramide synthesis and the release of ceramide enriched EVs. The ceramide transport protein StAR-related lipid transfer protein 11 (STARD11) mediates efficient trafficking of ceramide from the ER to the multivesicular bodies (MVB) leading to EV release. The stress kinase mixed lineage kinase 3 (MLK3) and tumor necrosis factor-like apoptosis inducing ligand receptor (TRIALR) also mediate transmission of lipotoxic stress signals into EV release via caspase 3 activation and rho-associated protein kinase 1 (ROCK1) activation.

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EVs have been implicated in tissue cross talk where the liver or hepatocytes are EV recipient cells. For example, gut-derived EV HMGB1 is implicated in communicating dysbiosis from the gut to the liver (11). Visceral adipose tissue derived EVs can activate fibrogenic pathways in cultured hepatocytes and hepatic stellate cells (50).

Alcoholic hepatitis. There has been a recent increase in research into the role of EVs in AH in parallel with the increased interest in EVs in NASH. Several human and mouse studies have demonstrated an increase in EV numbers, characterized signaling cargoes on bioactive EVs, and examined recipient cell responses (19, 68, 69). Total EVs were elevated in alcohol-fed mice in comparison with pair-fed mice and enriched in seven microRNAs: miR-122, miR-192, miR-30a, miR-744, miR-124b, miR-30b, and miR-130a. EVs were elevated in human AH samples and enriched in miR-192 and miR-30a (69). This group further demonstrated the functional role of hepatocyte-derived EV miR-122 in proinflammatory macrophage responses by transferring miR-122 to myeloid cells. In recipient myeloid cells, miR-122 inhibited heme oxygenase 1, sensitized to lipopolysaccharide and increased proinflammatory signaling (68). Mechanistically, alcohol-induced disruption of autophagic flux led to an increase in EV release from hepatocytes and macrophages. Alcohol treatment increased miR-155 levels, which, in turn, inhibited several regulators of autophagy, including mTOR, LAMP1, and LAMP2 (2). Alcohol can also stimulate monocyte EV release; these monocytes are enriched in miR-27a, which, in turn, induces an M2-like functional polarization of naïve monocytes (87). Unlike hepatocytes, monocytes lack alcohol-metabolizing enzymes; therefore, alcohol-induced monocyte EV release is likely mediated by distinct pathways compared with hepatocytes.

Ethanol-stimulated EVs also contain unique proteomic cargoes. Ethanol-treated hepatocytes released EVs enriched in CD40 ligand (CD40L) in a caspase-dependent manner (107). EV CD40L-induced proinflammatory responses in macrophages, such that mice lacking CD40 were protected from alcohol-induced liver inflammation. CD40L-enriched EVs were increased in human samples with AH. Interestingly, alcohol-induced EVs in human subjects with AH and in mouse AH models are enriched in cytochrome P 450 (CYP) enzymes, with the greatest enrichment in CYP2E1 (56). The toxicity of alcohol toward monocytes was enhanced in the presence of CYP2E1-enriched EVs These studies demonstrate a deleterious effect of alcohol-stimulated EVs mediated by the release of the alcohol-metabolizing enzyme CYP2E1 by hepatocytes. There also has been interest in the lipidomic cargo of AH EVs. We have recently examined the concentration and sphingolipidomic composition of EVs from subjects with AH, in comparison with heavily drinking individuals with no liver injury and normal controls (91). Circulating EV concentration was significantly higher in plasma samples from subjects with alcoholic hepatitis, in comparison with healthy controls and heavy drinking controls (36 subjects each). EV concentration correlated with MELD-based disease severity. The EV sphingolipid cargo in AH was significantly enriched in C16:0 ceramide, sphingosine 1-phophate, C20:0 ceramide, C22:0 ceramide, C24:1 ceramide, and C24:0 ceramide when compared with both control groups. Furthermore, this enrichment was unique to EVs and not seen in paired plasma samples. We found that an EV cutoff has high sensitivity and specificity in diagnosing AH and predicting AH-related mortality.

Acetaminophen-induced liver injury. Hematopoietic stem cell-derived EVs characterized by CD133 were elevated in a mouse model of acetaminophen (APAP)-induced acute hepatotoxicity. The hematopoietic stem cell ectonucleotidase CD39 played a role in release of CD133 containing EVs, as there was no increase in APAP-induced CD133-positive EVs in *Cd39* knockout mice (90). CD39 and CD133-containing EVs were elevated in human plasma samples from subjects with acute liver injury and acute-on-chronic liver injury. EV miR-122 was significantly elevated in a rat model of APAP-induced acute liver injury (71). As a significant proportion of circulating extracellular microRNA is not within EVs, in this study, they compared serum levels of candidate miRs with EV levels. EV miR-122 was superior to serum miR-122 levels in diagnosing liver injury, whereas, serum levels of miR-192, miR-193a, and miR-194 were superior to EV levels compared with EV miR levels (71). The metabolic activity of hepatocyte-derived EVs in APAP-induced acute liver injury was also demonstrated in serum, suggesting that these metabolically active EVs could exacerbate or alleviate APAP toxicity—a question that warrants empirical testing.

LIQUID BIOPSY FOR LIVER DISEASES

The biophysical and compositional properties of EVs coupled with their presence in bio-fluids make them an attractive target for blood-based liquid biopsy, which may even reduce or replace more invasive and risky procedures, such as liver biopsy (12, 59). This would permit not only ease-of-testing but also repeated measurements over time. Several EV cargoes have been identified that could potentially serve as biomarkers. These include miR-122, miR-192, TRAIL, Vanin-1, CD40L, CXCL10, and sphingolipids, including S1P. As discussed above, these markers remain nonspecific and have been reported to be altered in several liver diseases. On the other hand, a broader "omics" approach may establish disease signatures, as demonstrated in a NASH mouse model (83). Particular attention needs to be paid to microRNAs as EV-based biomarkers. A significant proportion of circulating microRNA may be nonvesicular, as recently demonstrated in human plasma samples (39). In this study, a major proportion of RNA binding proteins, such as Argonautes, were absent from exosomes. However, redistribution of circulating miR-122 from the exosome-rich fraction in alcoholic liver disease to the protein-rich fraction in drug-induced liver injury models suggests that EV microRNA may serve as biomarkers for liver disease. Regardless, any such approach would need rigorous validation. Overall, EVs remain an attractive target for biomarker discovery, with the challenge of establishing disease-specific EV cargo signatures and the most reliable and representative bio-fluid from which to isolate EVs. An additional technological challenge remains in the rapid and reliable detection of EV signatures.

EV-BASED THERAPIES

The therapeutic capacity of EVs has been explored in several diverse categories, including tissue repair and regeneration, immunomodulation, antimicrobial therapy, targeted drug delivery, and anticancer therapy (17, 31, 59, 62, 66). Native and

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bioengineered EVs have been utilized for preclinical and proof-of-concept clinical studies. Mesenchymal stem cell (MSC)-derived EVs are protective in various models of liver injury (99). In carbon tetrachloride-induced liver injury in mice, administration of MSC-EVs led to an attenuation of injury and augmentation of hepatocyte proliferation (99). Liver fibrosis was also attenuated by EV administration (62). Human-induced pluripotent stem cell (hiPS)-derived MSC EVs were tested in a model of ischemia-reperfusion liver injury. In this model, hiPS-MSC EVs were salutary via multifaceted reduction of liver injury and improved hepatocyte proliferation (17). EVs from primary hepatocytes also attenuate liver injury in models of ischemia-reperfusion and promote liver regeneration after partial hepatectomy, as do EVs from human liver stem cells (31, 78). These beneficial effects appear to be mediated by S1P (78) or Argonaute-bound mRNA (31); thus, researchers should focus on identifying molecules that could be exogenously enriched in engineered EVs as a therapeutic strategy. The therapeutic efficacy of bioengineered EVs has also been demonstrated in several experimental models and early human trials (60, 65, 66, 114). EV cargoes that can be modified include nucleic acids, proteins, and lipids. EVs can also be engineered for drug delivery. This, coupled with immunomodulatory properties, stability, and cellular homing, make EVs an attractive therapeutic shuttle.

EVs in disease states are implicated in liver injury, inflammation and fibrosis. Thus, in disease states strategies targeting inhibition of EV release may be beneficial. In mouse NASH models, pharmacological or genetic approaches to inhibit EV release, for example, fasudil to inhibit ROCK1 or the genetic deletion of MLK3, led to a reduction in liver injury, inflammation, and attenuated NASH (32, 37). Similarly, inhibiting S1P signaling with the pharmacologic inhibitor FTY720 also attenuated NASH (67). Although these studies may target other pathways in addition to EV release, inhibition of EV release by stressed cells in disease states remains a therapeutic option. In this regard, drugs that specifically inhibit EV release will need to be developed and tested, rather than the broader strategies employed heretofore.

CONCLUSIONS

There are several unanswered questions regarding the physiological relevance of EVs, including their kinetics—which would encompass biogenesis rates, secretion, half-life, and elimination—and their physiological roles—which would address important questions of how recipient cells recognize EVs and how EVs communicate with target recipient cells. There have been recent reports of tracking benign and tumor EVs in live zebrafish models that answer some of these questions and demonstrate uptake of EVs by patrolling macrophages and endothelial cells (36, 109). However, in this regard, there needs to be better understanding of "eat me" signals, such as phosphatidyl serine externalization versus "don't eat me" signals and "find me" signals that are transmitted by EVs (45). Regardless, EVs are present in the circulation at high concentrations under steady-state conditions. This along with the reported salutary effects of normal EVs would suggest an important role in tissue cross-talk in health. In recent years, that has been an exponential growth in understanding the relevance of EVs in both benign and malignant liver diseases. This has uncovered the role of novel EV cargoes that mediate downstream recipient cell responses and opened the field to EV-based diagnostics and therapeutics. EV-based liquid biopsy is a potential biomarker, especially with technological advances that can permit capture and quantification of cargo-defined EVs originating from specific cells of interest. Similarly, drugs that inhibit EV release present a therapeutic opportunity and will need to be tested in preclinical models and in clinical trials. In spite of the technical challenges of EV isolation and characterization and conceptual challenges of EV heterogeneity and what that means in terms of cargo selection and recipient cell responses, the field continues to grow and illuminate more pieces of EV biology and their relevance to liver diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

AUTHOR CONTRIBUTIONS

H.M. prepared figures; H.M. drafted manuscript; H.M. edited and revised manuscript; H.M. approved final version of manuscript.

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