Carcinogenic Liver Fluke Secretes Extracellular Vesicles That Promote Cholangiocytes to Adopt a Tumorigenic Phenotype

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**Background.** Throughout Asia, there is an unprecedented link between cholangiocarcinoma and infection with the liver fluke *Opisthorchis viverrini*. Multiple processes, including chronic inflammation and secretion of parasite proteins into the biliary epithelium, drive infection toward cancer. Until now, the mechanism and effects of parasite protein entry into cholangiocytes was unknown.

**Methods.** Various microscopy techniques were used to identify *O. viverrini* extracellular vesicles (EVs) and their internalization by human cholangiocytes. Using mass spectrometry we characterized the EV proteome and associated changes in cholangiocytes after EV uptake, and we detected EV proteins in bile of infected hamsters and humans. Cholangiocyte proliferation and interleukin 6 (IL-6) secretion was measured to assess the impact of EV internalization.

**Results.** EVs were identified in fluke culture medium and bile specimens from infected hosts. EVs internalized by cholangiocytes drove cell proliferation and IL-6 secretion and induced changes in protein expression associated with endocytosis, wound repair, and cancer. Antibodies to an *O. viverrini* tetraspanin blocked EV uptake and IL-6 secretion by cholangiocytes.

**Conclusions.** This is the first time that EVs from a multicellular pathogen have been identified in host tissues. Our findings imply a role for *O. viverrini* EVs in pathogenesis and highlight an approach to vaccine development for this infectious cancer.

**Keywords.** extracellular vesicles; *Opisthorchis viverrini*; cholangiocarcinoma; liver fluke; cancer.

The liver fluke *Opisthorchis viverrini* is classified as a group 1 carcinogen by the International Agency for Research on Cancer and is a major public health problem in many parts of Southeast Asia. In northeast Thailand alone, >8 million people harbor the parasite [1], owing to traditional dietary preferences for eating uncooked fish that harbor the infective stage of the fluke [2]. Upon ingestion of infected fish, the metacercariae excret in the duodenum and migrate to the bile ducts of the definitive host, where they feed on the biliary epithelia. Infection is associated with a spectrum of hepato-biliary abnormalities, including bile duct cancer or cholangiocarcinoma (CCA) [2, 3]. The incidence of CCA in northeast Thailand is substantially higher than elsewhere in the world and associates strongly with the prevalence of *O. viverrini* infection [4]. The mechanisms involved in liver fluke-driven tumorigenesis are multifactorial, with apparent roles for (1) mechanical damage caused by parasites grazing on...
the biliary epithelium, (2) chronic immunopathologic processes that are dominated by proinflammatory cytokines, such as interleukin 6 (IL-6) [1], and (3) the active release of parasite-derived excretory/secretory (ES) products into the bile ducts that drive unchecked cell proliferation [2, 5–7]. Intriguingly, some of these ES products have been identified inside cholangiocytes of experimentally infected hamsters [6, 8, 9], akin to the intracellular presence of the CagA protein from another carcinogenic pathogen, *Helicobacter pylori* [10]. Until now, the mechanisms by which liver fluke ES proteins are internalized by cholangiocytes and the ramifications of this process for the host cell have remained unknown.

Recent reports have highlighted the presence of secreted extracellular vesicles (EVs) from parasites of both unicellular [11, 12] and multicellular (helminth) [13–15] origin, and they provide a plausible explanation for the abundance in helminth ES products of apparently “intracellular” proteins [16]. EVs are small, membrane-enclosed structures that are released by many different cell types [17, 18]. EVs from unicellular parasites have been shown to influence host physiological processes, including immunomodulation, and adherence and communication between host and parasite [12, 19, 20]. A recent report described the uptake of parasitic platyhelminth EVs by host cell lines in vitro, using low-resolution fluorescence microscopy [13], but the molecular impact of EV uptake on the recipient cell and the detection of helminth EVs in vivo have, until now, not been reported.

Here we show that *O. viverrini* secretes EVs that induce a proinflammatory/tumorigenic phenotype in human cholangiocytes. We also show that cellular uptake of *O. viverrini* EVs can be blocked by antibodies to an EV recombinant tetraspanin (TSP), highlighting the potential usefulness of EV proteins in vaccines to prevent fluke infection and associated cholangiocarcinogenesis.

**MATERIALS AND METHODS**

**Ethics Statement**

Hamsters were maintained at Khon Kaen University (KKU), Thailand. The study was approved by the KKU Animal Ethics Committee (AEKKU 55/2554). Human bile specimens were collected as described elsewhere [8]. EVs were purified from ES products, using differential ultracentrifugation according to a modified protocol [21]. Briefly, parasite culture medium was centrifuged at 2000g for 30 minutes at 4°C to remove larger debris; supernatant was further centrifuged at 15,000g for 30 minutes at 4°C. ES supernatants were filtered using a 0.2-µm membrane (Schleicher and Schuell Bioscience) and ultracentrifuged at 110,000g for 2 hours at 4°C. The pellet was washed in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Roche) and ultracentrifuged for 1 hour. Crude pellet was resuspended in 200 µL of PBS/protease inhibitor cocktail and stored at 4°C.

**Preparation of EVs for Transmission Electron Microscopy**

A total of 5 µL of purified EVs was applied to a carbon-formvar coated grid and air dried. Grids were negatively stained in 2% uranyl acetate for 15 seconds. Stained grids were viewed in a JEM1011 transmission electron microscope (JEOL) equipped with a Morada side-mounted digital camera (Olympus).

**Fluorescence Labeling of EV Membranes**

EVs were labeled with Alexa Fluor 488 5-SDP Ester (AF488; Life Technologies) according to the manufacturer’s protocol. EV pellets were resuspended in 400 µL of PBS and mixed with 50 µg of AF488 for 1 hour at 4°C. The samples were collected by ultracentrifugation at 110,000g for 2 hours to remove excess dye, washed twice, and resuspended in 400 µL of PBS.

**Cell Cocultures and Fluorescence Microscopy**

To investigate internalization of EVs by mammalian cells, normal immortalized human cholangiocytes (H69) were grown to 80% confluence as described elsewhere [22]. Cells were starved of fetal calf serum (FCS) overnight, incubated with AF488-labeled EVs for 6 hours, and analyzed by fluorescence microscopy. Cell nuclei were stained with Hoechst dye and visualized using an AxioImager M2 ApoTome fluorescence microscope (Zeiss). Two technical and 2 biological replicates were performed, and at least 5 fields were analyzed in each replicate. Fluorescence was quantified using ImageJ.

**Ultraresolution Microscopy**

To convincingly demonstrate internalization of *O. viverrini* EVs by cholangiocytes, we used 3-dimensional structured illumination microscopy (3D-SIM). H69 cholangiocytes were grown to 50% confluence and cocultured with AF488-labeled EVs or an equivalent amount (based on fluorescence) of ES products (10 µg/mL) for up to 6 hours. Treated cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and stained with Alexa Fluor 568 Phalloidin (Life Technologies). Specimens were mounted in 5% N-propyl gallate (Sigma), and 3D-SIM was performed on isolated single cells using a DeltaVision OMX 3D-SIM system (Applied Precision, GE Healthcare). Solid-state lasers (405, 488, and 593 nm) provided wide-field illumination, and multiple-channel images were captured simultaneously by using 3 pco.edge scientific complementary metal-oxide-semiconductor cameras with a 512 × 512 pixel size. All data capture used a 60 × 1.4-numerical aperture oil objective and standard excitation (EX) and emission (EM) filter sets (405 nm EX/419–465 nm EM, 488 nm EX/500–550 nm EM, and 592.5 nm EX/608–648 nm EM). 3D-SIM images were sectioned using a 125 nm Z-step size. Raw 3-phase images were reconstructed as previously described [23]. Reconstructed
images were rendered in 3 dimensions, using IMARIS version 7. X (Bitplane Scientific).

**Proteomic Analysis of EVs**

Sample preparation for proteomic analysis was performed as described previously [13]. Peptides were resuspended in 5% formic acid and analyzed by liquid chromatography tandem mass spectrometry (MS/MS). Ten microliters was injected onto a C18 trap column (Thermo Scientific Acclaim), flushed into an analytical column (Agilent), and eluted via a mobile phase gradient of 5%–80% solvent B over 120 minutes. The eluted material was directly applied to the nanospray source of a QSTAR Elite instrument (Applied Biosystems), and data analysis was conducted with Analyst 2.0 (Applied Biosystems).

**iTRAQ Analysis**

H69 cells were cocultured with the quantity of EVs that corresponded to 1.2 µg/mL of ES protein in PBS for 30 minutes, 3 hours, and 16 hours. Cells were washed using PBS containing protease inhibitor cocktail and lysed in 5 M urea, 2 M thiourea, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X–100, and 40 mM Tris (pH 7.4). Each sample was ground with a TissueLyser II (Qiagen) and centrifuged at 12 000 g for 20 minutes. The protein supernatant was precipitated with cold methanol and centrifuged at 8000 g. Air-dried pellets were redissolved in 0.5 M triethylammonium bicarbonate (TEAB)/0.05% SDS and then centrifuged at 12 000 g. Samples were resuspended in 0.5 M TEAB prior to reduction, alkylation, digestion, and iTRAQ labeling (AB Sciex) and then were analyzed on a 5600 TripleTOF mass spectrometer as described previously [24].

**Bioinformatic Analysis of Protein Expression Data**

For proteomic characterization of O. viverrini EV proteins, the O. viverrini genome database [25] was interrogated using MASCOT (Matrix-Science), allowing 2 missed cleavages and mass tolerance of 1.2 Da in MS mode and 0.8 Da on MS/MS product ions. Probability (P < .05) of false-discovery rate (FDR) was calculated for every search using the MASCOT decoy database facility. For iTRAQ, 2 biological and 2 technical replicates were conducted. Database searches were performed against NCBInr by using MASCOT, allowing 2 missed cleavages and a mass tolerance of 0.1 Da on MS and MS/MS modes. Results were validated using Scaffold v.4.2.1 (Proteome Software). Differentially expressed (dysregulated) proteins were determined using a Kruskal–Wallis test, and results are expressed in log2 ratios. Only proteins whose expression level underwent a significant (P < .05) log2 fold-change of >0.6 or ≤0.6 for upregulated and downregulated proteins, respectively, were further considered. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome functional enrichment analyses for dysregulated proteins were performed using KEGGMapper and DAVID [26]. Protein-protein interaction analysis was performed using String [27].

**Detection of EV Proteins in Bile of O. viverrini–Infected Humans and Hamsters**

Bile specimens were collected from O. viverrini–infected humans by aspiration of resected gallbladders and from experimentally infected and uninfected hamsters (controls) by puncture of the gallbladder from euthanized animals, and EVs were isolated as described above. Selected reaction monitoring (SRM) was performed on a 5600 TripleTOF to identify O. viverrini EV proteins in bile. All analyses were performed using SRM-initiated IDA experiments, in which a SRM survey scan triggered the acquisition of MS/MS spectra. Skyline 3.1 was used to generate SRM transitions and for data analysis.

**Cell Proliferation**

H69 cholangiocytes were cultured as described above until 80% confluent. Cells were starved of FCS overnight and cultured with the amount of EVs that corresponded to 1.2 µg/mL of ES protein over 72 hours at 37°C. Control cells were incubated with 1.2 µg/mL of ES products (not ultracentrifuged) or, for the negative control group, with 1.2 µg/mL bovine serum albumin (BSA). Cell proliferation was assessed by measuring cell index values in real time, using an xCELLigence SP system and E plates (Acea). Three biological replicates were analyzed, and 2-way analysis of variance (ANOVA) was performed.

**Blocking Internalization of EVs With A Recombinant O. viverrini TSP-1**

AF488-labeled EVs were incubated with mouse anti–O. viverrini TSP-1 serum [28] or normal mouse serum at different dilutions (1:2.5, 1:5, and 1:20) at room temperature for 1 hour. EV-antibody complexes were added to H69 cells and cocultured for 6 hours. Images were obtained using an ECLIPSE Ni-u confocal microscope (Nikon) at 60 times the original magnification. Thirty fields from 2 biological replicates were analyzed, and 1-way ANOVA was performed. Cell fluorescence was quantified using ImageJ.

**IL-6 Detection**

H69 cholangiocytes were cultured as described above to monitor cell proliferation over 24 hours. Culture media were collected and centrifuged at 2000 g for 10 minutes to remove cell debris. IL-6 levels were quantified using an IL-6 enzyme-linked immunosorbent assay kit (R&D System) in accordance with the manufacturer’s instructions. Three biological replicates were analyzed, and statistical analysis was performed using 2-way ANOVA.

**RESULTS**

**Excretory/Secretory Products From O. viverrini Contain EV-Like Vesicles**

After filtration and ultracentrifugation of ES products from adult O. viverrini, we identified microvesicular structures by...
using negative staining in transmission electron microscopy (Figure 1). These spherical vesicles had an external diameter of 40–180 nm, resembling EVs recently reported from other trematodes [13, 14].

**Proteomic Analysis of *O. viverrini* EVs Reveals Conserved and Novel Proteins**

*O. viverrini* EVs were digested with trypsin and underwent LC-MS/MS. We identified 108 different proteins (Supplementary Table 1), 42 of which were homologous to mammalian EV proteins in Exocarta [29], including proteins that are diagnostic of mammalian exosomes, such as TSPs, HSP-70, and cytoskeletal, regulatory, and trafficking proteins. Gene ontology analysis of the proteins (Supplementary Figure 1) returned 212 biological process terms. Pfam analysis identified proteins with an EF hand domain as the most frequently represented (Supplementary Figure 1).

**O. viverrini** Exosome Proteins Are Identified in Bile Specimens From Infected Hosts

SRM analysis confirmed the presence of specific EV proteins in bile specimens obtained from *O. viverrini*-infected humans and hamsters. Seven of the top 15 proteins identified in the EV proteomic analysis (based on the number of peptides identified) were successfully quantified (Supplementary Figure 2).

**O. viverrini** EVs Are Internalized by Human Cholangiocytes

We monitored the uptake of AF488-labeled EVs by human cholangiocytes in vitro. Within 60 minutes, punctate fluorescent structures were detected, the number of which increased markedly over 6 hours (Figure 2A). Cell fluorescence of all fields from the 2 biological replicates revealed mean fluorescence coverage (±SD) of 62.44% ± 9.52% and 90.57% ± 4.58% at 0.5 hours and 6 hours, respectively. 3D-SIM confirmed the intracellular presence of AF488-labeled EVs within the cytoskeletal network of well-separated individual cells, as evidenced by their position between the basal and apical membranes outlined with phalloidin (Figure 2B, 2C, and Supplementary Movie). We attempted to colocalize EVs to defined organelles by using specific fluorescent markers but did not observe colocalization (not shown), suggestive of a cytoplasmic location.

**O. viverrini** EVs Promote Cell Proliferation and Stimulate Wound Healing and Tumorigenic Pathways in Cholangiocytes

H69 cholangiocytes were incubated for 72 hours with (1) crude ES products, (2) EVs purified from crude ES products, or (3) an
irrelevant protein control, BSA. We chose BSA as a control rather than EVs from another source because H69 cholangiocytes produce (and take up) their own exosomes in culture [30]. ES and EVs both induced significant ($P < .05$) cell proliferation as compared to BSA-treated control cells (Figure 3). A total of 705 proteins containing at least 2 peptides were identified and quantified with a 99.9% protein threshold and 1.2% FDR from 6863 unique peptides (peptide threshold, 95%; FDR, 0.82%) from H69 cholangiocytes that were cocultured with EVs (Supplementary Table 2); 396 proteins from cholangiocytes cocultured with EVs were differentially expressed ($P < .05$, by the Kruskal–Wallis test) and presented a fold-difference of $\geq 1.5$.

**Figure 2.** Internalization of *Opisthorchis viverrini* secreted extracellular vesicles (EVs) by human cholangiocytes. A, Fluorescence images of Alexa Fluor 488–labeled EVs (green) internalized by H69 cholangiocytes. Cholangiocytes internalized the EVs within 1 hour of coculture, and maximum internalization was observed by 6 hours. Control cells were incubated with phosphate-buffered saline. Hoechst dye (blue) was used to label cell nuclei. B, Three-dimensional structured illumination microscopy (3D-SIM) fluorescence image of the edge of a well-separated individual cholangiocyte after 6 hours incubation with Alexa Fluor 488–stained EVs (green). Lateral ($xy$) overview of cell showing EVs present within the cytoskeletal actin network (red) of the cell. C, Rendered axial ($xz$) view of the inset in panel A revealed EVs between the apical and basal surfaces of the cell that were stained by phalloidin (red).
of activated PAK-2p34 by proteasome, and Cdh1-mediated degradation of Skp2 pathways were differentially regulated in cholangiocytes cocultured with EVs (Figure 4C). Expression of 17 proteins associated with apoptosis was regulated by EVs, and protein-protein interactions revealed that the majority of dysregulated cholangiocyte proteins formed a cluster belonging to the proteasome complex (Supplementary Figure 4).

Uptake of EVs and Proinflammatory Cytokine Production by Cholangiocytes Can Be Blocked With Antibodies to an O. viverrini EV TSP

Because of the roles of TSPs in the formation of EVs by mammalian cells and their subsequent uptake by recipient cells [31] and the recent identification of TSP-1 on the surface of O. viverrini [28], we explored the role of O. viverrini TSP-1 in the uptake of EVs by cholangiocytes. Antiserum to recombinant O. viverrini TSP-1 blocked the uptake of EVs by cholangiocytes as compared to normal mouse serum (P range, <.0001 to <.001; Figure 5). ES and EVs both stimulated the secretion of significantly more IL-6 from cholangiocytes than did control cells incubated with medium alone (P range, <.001 to <.01; Figure 6), and IL-6 secretion was significantly reduced when EV internalization was blocked by antiserum to O. viverrini TSP-1 (P < .05; Figure 6).

DISCUSSION

While the mechanisms that drive chronic liver fluke infection toward cancer are multifactorial, a pivotal role for fluke ES products in promoting a tumorigenic phenotype has been demonstrated [7]. The intracellular localization of O. viverrini ES proteins in cholangiocytes of infected hosts has prompted speculation on the mechanisms underlying this process of parasite protein internalization and its potential pathologic and carcinogenic consequences [6, 7]. Herein we show that O. viverrini secretes EVs that are internalized by human cholangiocytes in vitro and trigger a cascade of inflammatory and protumorigenic changes within the cell, thereby providing a plausible mechanism by which ES proteins are taken up by biliary cells of infected hosts and contribute to the development of CCA in infected individuals.

Investigations of the interactions between EVs derived from single-celled parasites and the cells that they encounter in an infected host have shed light on a novel means of host-parasite cellular communication [11, 12]. Protozoan parasite EVs are also capable of modulating proinflammatory immune responses and inducing production of regulatory cytokines in recipient cells [19]. EV production by helminth parasites, however, has received considerably less attention. A seminal study recently showed that the parasitic nematode Heligmosomoides polygyrus secretes exosomes that contain microRNAs that suppress type 2 innate responses [15]. However, little is known about the

Figure 3. Opisthorchis viverrini extracellular vesicles (EVs) drive proliferation of human cholangiocytes. O. viverrini EVs (open triangles) and excretory/secretory (ES) products (closed circles) promoted the proliferation of human cholangiocytes. Asterisks represent the time point from which cell growth remained significantly different between test and control groups after each treatment. *P < .05. Abbreviation: BSA, bovine serum albumin.
Figure 4. Comparison of proteins that were significantly regulated and biological pathways regulated in cholangiocytes cocultured with Opisthorchis viverrini extracellular vesicles (EVs). A, Heat map of the proteins from human cholangiocytes with a significant fold-regulation after the Kruskal–Wallis test and that underwent a 2-fold change ($\log_2 = 1.5$) at at least one of the time points assessed after incubation with EVs from O. viverrini. B, Top 25 Kyoto Encyclopedia of Genes and Genomes pathways regulated after incubation with EVs. C, Reactome pathways regulated after incubation with EVs. Pathways that are involved in wound healing and cancer are indicated by red font. Abbreviations: HIV, human immunodeficiency virus; HTLV-1, human T-lymphotropic virus type 1; mRNA, messenger RNA; UTR, untranslated region.
production and particularly the biological effects of EVs from parasitic flatworms (platyhelminths). EVs were reported in the ES products from the flatworms Fasciola hepatica and Echinostoma caproni [13] and, more recently, Dicrocoelium dendriticum [14], and while the authors showed binding of fluke EVs to cell lines, neither the functional implications nor in vivo evidence of EV uptake by tissues of infected hosts were addressed. We show here for the first time that helminth EVs are unequivocally internalized by host cells and that major EV proteins can be detected in bile specimens from naturally infected humans and experimentally infected hamsters. From proteomic analyses, we identified 108 proteins from O. viverrini EVs, and only a handful (13) had been previously identified in a proteomic analysis of the ES products of the fluke [32].

Cholangiocytes line the bile ducts and provide the first line of defense against pathogens in the biliary system. We show here that O. viverrini EVs drive production of IL-6 from recipient human cholangiocytes, implicating these EVs in the hepatic disease process. IL-6 has been associated with chronic periductal fibrosis and CCA in patients with opisthorchiasis [33], and IL-6 has been implicated in the maintenance of a chronic inflammatory milieu that could lead to tumorigenesis [34]. O. viverrini EVs were also capable of driving proliferation of cholangiocytes, a phenomenon that has been convincingly
documented in the hamster infection model [35]. This relentless cell proliferation, coupled with other carcinogenic insults such as chronic immunopathology and elevated intake of dietary nitrosamines [2], culminates in the establishment of an environment that is conducive to malignant changes.

Uptake of EVs by cholangiocytes resulted in dysregulated expression of proteins with documented roles in wound healing and cancer. Indeed, wound repair has long been implicated in tumorigenesis, and there are striking histological and molecular similarities between tumor stroma and wounded tissues [36]. Nineteen KEGG pathways related to cancer and wound healing processes. The proteasome plays a critical role in cell-cell junction assembly and organization, and they are upregulated during cell proliferation and wound healing [38].

Other important cholangiocyte proteins underwent dysregulated expression after coculture with EVs, notably proteins involved in the proteasome complex. Most of the proteins involved in this complex were upregulated in cholangiocytes exposed to EVs and are known to interact with other proteins involved in cancer and wound healing processes. The proteasome complex regulates directly or indirectly many important cellular processes and has been suggested as a therapeutic target for cancer [39, 40].

EV uptake by dendritic cells can be blocked with antibodies to EV TSPs [41]. TSPs are thought to be involved in the regulation of protein assembly and microRNA recruitment in mammalian exosomes [31] and influence cell selectivity [42]. Our findings highlight an important role for TSP-1 in *O. viverrini* EV uptake by cholangiocytes. Although TSPs are found on the surface membranes of all exosomes, their discovery here is particularly pertinent owing to their abundance on the surface membranes of parasitic helminths [43] and their importance in the development of the *O. viverrini* tegument [28]. TSPs are efficacious helminth vaccine antigens [44–46], and our findings here suggest that the mechanism of vaccine efficacy is linked to interruption of parasite EV uptake by recipient host cells in vivo and subsequent disruption of key physiological (and pathological) processes.

Our study describes for the first time the release of EVs in the secreted products of the carcinogenic liver fluke, *O. viverrini*, and highlights the role of fluke EVs in promoting an inflammatory yet simultaneously modulatory (wound healing) environment that ultimately facilitates the development of biliary cancer. Our findings do, however, offer hope for the eventual control of this debilitating neglected tropical disease by highlighting a key physiological process that can be potentially interrupted via subunit vaccines targeting key EV surface molecules.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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