Oroxylin A promotes cancer cell-to-macrophage communication and improves immunotherapy in hepatocellular carcinoma model

Peiwen Wang[1], Xuefeng Zhang[1], Zhi Feng[1], Yufang Tang[1], Xiaolei Han[1], Tianxiao Mao[1], Sichan Li[1], Zhiyu Li[1], Qinglong Guo[1], and Xiaobo Zhang[1]

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Abstract

Emerging evidence suggest that oroxylin A, a natural flavonoid compound, induces apoptosis in HCC through multiple mechanisms. However, whether oroxylin A-induced apoptosis could exhibit modulatory effect on tumor microenvironment remains unclear. Here we investigate the effect of oroxylin A on communication between cancer cells and macrophages in vitro and mouse model. The data shows oroxylin A elicits apoptosis-related extracellular vesicles through caspase 3-mediated ROCK1 activation in HCC cells, which are able to regulate M1-like polarization of macrophage. Moreover, oroxylin A downregulates the population of M2-like macrophage and increase in T cells infiltration in tumor microenvironment, accompanied with suppression of HCC development and enhancement of immune checkpoint inhibitor treatment. Mechanistically, glycolysis proteins enriched in oroxylin A-elicited extracellular vesicles from HCC cells is transferred to macrophages where it contributes to ROS-dependent NLRP3 inflammasome activation, therefore promoting anti-tumor phenotype of macrophage. Taken together, this study highlights oroxylin A promotes metabolic shifts between tumor cells and macrophages, facilitating to inhibit tumor development and improve immunotherapy response in HCC model.

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Abbreviation

DEN, diethylnitrosamine

DMSO, dimethyl sulfoxide

DAPI, 4',6-diamidino-2-phenylindole

PBS, phosphate buffered saline

PMA, phorbol 12-myristate 13-acetate

2-DG, 2-Deoxy-D-glucose

NAC, N-Acetylcysteine

HCC, hepatocellular carcinoma

PARP1, poly(ADP-ribose) polymerase 1

ROCK1, Rho associated coiled-coil containing protein kinase 1

MYPT1, myosin phosphatase target subunit1

PD-1, programmed cell death 1

PD-L1, programmed cell death 1 ligand 1

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

PKM2, pyruvate kinase M1/2

LDHA, lactate dehydrogenase A

NLRP3, NLR family pyrin domain containing 3

ASC, apoptosis-associated speck-like protein containing a CARD

Abstract: Emerging evidence suggest that oroxylin A, a natural flavonoid compound, induces apoptosis in HCC through multiple mechanisms. However, whether oroxylin A-induced apoptosis could exhibit modulatory effect on tumor microenvironment remains unclear. Here we investigate the effect of oroxylin A on communication between cancer cells and macrophages *in vitro* and mouse model. The data shows oroxylin A elicits apoptosis-related extracellular vesicles through caspase 3-mediated ROCK1 activation in HCC cells, which are able to regulate M1-like polarization of macrophage. Moreover, oroxylin A downregulates the population of M2-like macrophage and increase in T cells infiltration in tumor microenvironment, accompanied with suppression of HCC development and enhancement of immune checkpoint inhibitor treatment. Mechanistically, glycolysis proteins enriched in oroxylin A-elicited extracellular vesicles from HCC cells is transferred to macrophages where it contributes to ROS-dependent NLRP3 inflammasome activation, therefore promoting anti-tumor phenotype of macrophage. Taken together, this study highlights oroxylin A promotes metabolic shifts between tumor cells and macrophages, facilitating to inhibit tumor development and improve immunotherapy response in HCC model.

Keywords: Apoptosis, Extracellular Vesicles, Oroxylin A, Macrophage, Glycolysis, HCC

Introduction

As the most common primary liver cancer, hepatocellular carcinoma (HCC) is among the leading causes of cancer-related mortality worldwide. Although the application of immune checkpoint inhibitor (ICI) therapy

targeting either the programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1) or cytotoxic T-lymphocyte (CTLA-4) pathways represents a major breakthrough for many types of cancers, the objective response rates for these agents as monotherapies for HCC are only ˜15–20%[1]. Tumor-associated macrophages (TAMs) are abundant in tumor microenvironment (TME) of HCC, which generally interacted with cancer cells or others to maintain immunosuppressive phenotype and correlates with a worse prognosis, therefore targeting TAMs could be a promising strategy to improve HCC therapy[2, 3].

The TAM compartment is highly dynamic and heterogeneous within tumors, among of which pro-inflammatory (so-called M1-like) populations exert tumoricidal functions and support the efficacy of various anticancer therapies, whereas the anti-inflammatory (so-called M2-like) TAM subsets promote tumor development and immune evasion[4]. The ratio of M1/M2 TAM has been closely linked with disease outcome and prognosis. NLRP3 interacts with ASC and caspase-1 to form the inflammasome complex and then induces caspase-1 activation and IL-1β secretion in response to stimulation, which are usually accompanied with macrophage M1 polarization[5]. On the other hand, the metabolic reprogramming also plays important roles in macrophage polarization process. M1-like macrophages are often associated with a high glycolytic metabolism and a robust ability to generate reactive oxygen species (ROS), and downregulation of oxidative phosphorylation (OXPHOS) and fatty acids oxidation (FAO), underlying their cytocidal functions[6]. Nevertheless, TAMs could be regulated in multiple ways in response to various TME perturbations, eventually influencing cancer progression and immunotherapy responses.

As critical intercellular communicators, tumor-derived extracellular vesicles (EVs) have been recently documented to influence anti-tumor immune response[7, 8]. These mainly include exosome and microvesicles that vary in size, content and mechanism of formation. It has been reported that the exosomes derived from HCC cells promote macrophage activation and M2 polarization through transferring lncRNA or miRNA, facilitating to escape immune surveillance[9]. In addition, HCC-derived microvesicles leads to monocyte-to-macrophage differentiation and TME remodeling, thereby improving HCC progression[10]. However, the immunomodulatory effect of EVs from dying tumor cells upon drug treatment, broadly known as apoptotic cell-derived EVs (ApoEVs), including large membrane-bound vesicles like apoptotic bodies (ApoBDs) or smaller apoptotic microvesicles (ApoMVs), remains obscure.

Oroxylin A (OA, 5,7-dihydroxy-6-methoxyflavone), a natural flavonoid compound extracted from *Scutellaria radix* . Previous report has documented that OA treatment were able to induce apoptosis of HCC cells by activating caspase8 and mitochondrial pathway[11-13]. However, the dosage of OA used in previous study is very high compared with its plasm concentration in vivo after oral administration, implying that there could be other mechanisms involved in the inhibitory effect of OA on HCC in vivo. Whether OA-induced apoptosis could influence TME of HCC remines largely unexplored. In current study, we show that OA elicits HCC-derived ApoEVs depending on caspase 3-mediated ROCK1 cleavage, which exhibit capability of regulating macrophage polarization. The ApoEVs are enriched glycolysis-related proteins that promotes ROS-dependent NLRP3 inflammasome activation in macrophages. Moreover, OA downregulates M2-like macrophage accompanied with enhancement of T cell infiltration in tumor microenvironment, consistent with the observation of tumor suppression and sensitization of PD-1/PD-L1 blockage therapy. Taken together, our results reveal a novel mechanism besides OA induces apoptosis in HCC, which could serve as a potential strategy to improve clinical immunotherapy.

Materials and Methods

Chemicals and regents

Oroxylin A were provided by Dr. LI, Zhiyu (China Pharmaceutical University) with purification >99.6% analyzed by HPLC. DCFH-DA, DiI, Y27632, BMS-1, z-VAD-fmk, PMA and 2-DG were purchased from MCE (Shanghai, China). DMSO, DAPI, NAC were purchased from Sigma-Aldrich (Beijing, China). DEN was purchased from ThermoFisher Scientific (Bremen, Germany). CCl_4 was purchased from Nanjing Reagent (Nanjing, China). Cytochalasin B was purchased from Macklin (Shanghai, China). Proteinase K, Primary antibodies for β-Actin, GAPDH, Caspase3, cleaved caspase3, NLRP3, ASC and IL-1β were purchased from

ABclonal (Wuhan, China); Caspase1, cleaved PARP, Ki67, CD206, cleaved ROCK1 from Proteintech (Rosemont, IL, USA); F4/80, CD8-β and LDHA from Santa Cruz; ROCK1, RhoA from CellSignaling Technology (Danvers, MA, USA); CD11b-APC, F4/80-PE, CD206-FITC, MHCII-FITC, CD45-FITC, NK1.1-Cy5-PE, CD3-FITC, CD4-APC and CD8-APC form Elabscience (Wuhan, China). Alexa Fluro 488 or 546 conjugated secondary antibodies were obtained from ThermoFisher Scientific; HRP-conjugated secondary antibodies from ABclonal. ELISA kit for IL-1β detection was from Elabscience. Caspase 3/7 activity assay kit was from Promega (Madison, WI, USA).

Cell Culture and Treatment

HepG2, SMMC-7721, H22, THP-1 and HEK293T cells were obtained from National Collection of Authenticated Cell Cultures (Shanghai, China) and authenticated using STR (or SNP) profiling. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (for the HEK293T, HepG2) or RPMI-1640 medium (for the THP-1 cell line and SMMC-7721), supplemented with FBS (10% (v/v)) (HyClone), penicillin (100 IU) and streptomycin (100 mg/ml) (Solarbio Science & Technology Co., Ltd., Beijing, China). All these cells were maintained in the incubator at 37°C with 5% CO_2, and routinely tested and found to be negative for mycoplasma. HCC cells were treated with DMSO and OA (10μM) for 48h. For macrophage differentiation, THP-1 cells were treated with PMA (5ng/ml) for 48h to differentiate into macrophage. For co-culture model, THP-1 cells were seeded on 6-well plates and differentiated into macrophage, followed by HepG2 cell incubation on top chamber of Transwell insert with 0.4 μm pore (Corning, NY, USA). THP-1 derived macrophages were incubated with microparticles from HCC cells for 24h, followed by further analysis.

Plasmids and Generation of lentivirus system

To construct the pLifeAct-GFP, the annealing oligonucleotides for encoding LifeAct peptide reported previously[14] were ligated into pEGFP-N1 vector. To construct the plasmid expressing shRNA for target genes, the specific oligonucleotides were annealed and subcloned into pLKO.1 vector. Then the pLKO.1 shRNA plasmid was transfected along with lentiviral packaging plasmids into HEK293T cells to generate lentiviruses. The lentiviral supernatants were collected and used to infect cells with polybrene. Cells were selected with puromycin to obtain the one that target gene was stably silenced. All the oligonucleotides used in this study were synthesized by GENEWIZ (Suzhou, China) and the sequence was listed in Supplementary Table S1.

Isolation and Characterization of extracellular vesicles

The culture medium was used for isolation of EVs by sequential centrifugation as previously described[15]. Briefly, dead cells or cell debris were removed by centrifuging at 300 ×g for 10 minutes, and then the supernatant was centrifuged at 2000 ×g for 20 min to harvest ApoBDs, followed by centrifugation at 16,500 xg for 20 min to collect microvesicles (MVs). Next the supernatant was subsequently filtered by 0.2μm filters, and exosomes (EXOs) were then pelleted by ultracentrifugation at 120,000 ×g for 70 minutes. All centrifuge was performed at 4. The resulting EVs preparation was dissolved in either PBS or RIPA buffer (ThermoFisher Scientific), depending on the application, and either used immediately or stored at -80 degC. The ApoBDs was analyzed using a bead-enhanced quantitation method by flow cytometry (BD Biosciences) as described previously[16]. The number and size of MVs and EXOs were determined using MANTA ViewSizer 3000 (San Diego, CA, USA). For electron microscopy-based morphological analysis, MVs or EXOs samples were placed onto copper grids for 3˜5 min and then extra supernatants were removed, followed by staining using 3% phosphotungstic acid solution for 3˜5 min, and air-dried samples were photographed under JEOL-1230 transmission electron microscope (JEOL). The average apoptotic EVs yield obtained from HepG2 cells treated with OA was around 15 μg (determined by BCA) per ml of cell culture medium.

Quantitative Real-Time PCR

Cells were lysed using RNA-easy Isolation Reagent (Vazyme, Nanjing, China) according to the manufacture's protocol. The RNA concentration was measured by NanoDrop (ThermoFisher Scientific), and 1μg of total RNA was subjected to cDNA synthesis using HiScript III RT SuperMix (Vazyme). The Quantitative Real-Time PCR (qPCR) was performed by AceQ Universal SYBR qPCR Master Mix (Vazyme) using ABI-7500

system. Gene expression was calculated by $2^{-\Delta\Delta^{\wedge}\tau}$ method using GAPDH as a house-keeping gene. The primers are listed in Table S1.

RNA interference

Human THP-1 were seeded in 6-well plates (6×10^5cells/well). For gene silencing in each well, specific siRNA (50nM) and transfection agents Lipofectamine 2000 were mixed and then diluted with Opti-1640. After incubation for 20min at room temperature, the mixture were added into each well for 6h transfection, followed by differentiation using PMA for 48h. All siRNA was purchased from GenePharma Co., Ltd. (Suzhou, China), and the sequence information was listed in Table S2.

RNA sequencing

Total RNA was extracted from THP-1-derived macrophages that were treated with DMSO-EVs and OA-EVs. RNA sequencing was performed by Novogene Technology Inc. (Beijing, China). Basic analysis mainly included raw data filtering and quality control, reference genome comparison, alignment homogeneity analysis, saturation analysis, sample correlation analysis, expression analysis, and differential gene analysis. Enrichment analysis for signaling pathways was performed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Western Blot

Protein from cell culture supernatant was extracted using methanol-chloroform method. Cells or EVs were lysed into RIPA buffer supplemented with proteinase inhibitor cocktails (Roche) to obtain total protein. After determining protein concentration using BCA method, equal amount of protein was loaded to SDS-PAGE electrophoresis followed by transferring on the nitrocellulose membrane. After blocking with 3% BSA for 1 h, the membrane was incubated with primary antibody at 4 overnight. The next day, PBST washed it three times for 10 min each time, and the secondary antibody was incubated at room temperature for 1 h. After washing three times with PBST, the protein bands were viewed by ECL solution (Vazyme) using Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK).

LC-MS/MS (*Liquid chromatography with tandem mass spectrometry*)

Proteins of ApoBDs and ApoMVs were collected and digested using trypsin and purified, and then subjected to Easy-nLC 1200 (Thermo Scientific) with tandem a Q Exactive (Thermo Scientific) analysis. The data obtained were analyzed by ProteomeDiscover 2.1 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) and searched against the human Uniprot Reference Proteome with isoforms. The proteins with top 10% of the average label-free quantification (LFQ) intensity were subjected to pathway enrichment analysis using PANTHER classification system.

Animal Models

All mice were maintained on a 12h light/dark cycle with free access to food and water and the animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University. To establish the tumor microenvironment in vivo, mice aged 6-8w were injected with H22 cells ($5x10^5$ cells per mouse) intraperitoneally. After 3 days for injection, all mice were randomly divided into two groups and daily treated with scramble and OA (300mg/Kg) through oral administration for two weeks. The ascites was collected and the obtained cells represented the tumor microenvironment which were subjected to further analyzed by flow cytometry. To generate the subcutaneous HCC model in mice, 0.2 mL H22 cells ($5x10^6$ cells/mL) were injected into the right posterior flanks of 6-8w mice. Once the average of tumor volume is 50-100 mm^3, mice were given OA (300mg/Kg, i.g.) daily and BMS-1 (2mg/Kg, i.p.) every three day. Tumor volume was recorded continuously and mice were sacrificed after treatment for a week. For survival analysis, each mouse was subcutaneously inoculated with $1x10^5$ H22 cells, and the same treatment was performed for one month. To establish DEN/CCl_4-induced HCC model, mice were intraperitoneally injected once with DEN (25 mg/kg) on two-week and six-week old, respectively. And 10% CCl_4(0.5 mL/kg) were intraperitoneally

injected into mice at age of three weeks until the indicated time once a week. OA and BMS-1 were given as described above for two months.

Immunofluorescence

For cell immunofluorescence, cells were fixed with 4% paraformaldehyde followed by washing with PBS. The samples were counterstained with DAPI, and then photographed under IX53 fluorescence microscope or IX81-FV1000 laser confocal microscope (Olympus). For tissue immunofluorescence, frozen sections were made by microtome (Leica CM3050S), and washed with PBS for 3 times for 5 min each time. The slides were blocked by 3% BSA at room temperature for 1h, followed by incubation with the appropriate primary antibodies overnight at 4 in a humidified chamber. The sections were then washed with PBS and incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. Staining with DAPI was performed before mounting and imaging. The images of tissue section were collected using the same measured parameters under Axio Scope A1 microscope (Zeiss).

Analysis of ROS production

THP-1 cells were plated and treated with PMA (5 ng/mL) for 48 h, followed by the addition of EVs for 6 h. Then cells were incubated with DCFH-DA solution with final concentration of 10 μM at 37 for 20 min. After washing with PBS three times, the photographs were taken under IX53 microscope (Olympus).

Flow cytometry

For the apoptosis analysis, HepG2 cells were treated as indicated, followed by staining with Annexin V-FITC/PI kit (KeyGEN) and flow cytometry analysis (BD Bioscience).

For the measurement of EVs uptake, the isolated EVs were resuspended in PBS, and stained with DiI dye (0.5 mM) at 4 for 20 min. After that, EVs were washed three times with PBS, and then added into THP-1-derived macrophage for indicated time before measurement using flow cytometry (BD Bioscience).

For the analysis of HepG2 cells in co-culture model, THP-1-derived macrophages were incubated with EVs for 24h. After that, the number of GFP labeled HepG2 cells were directly counted by flow cytometry analysis. For determination of Ki-67 expression, cells were permeabilized and incubated with anti-Ki67 antibody followed by Alexa Fluro 546- conjugated secondary antibodies before measurement by flow cytometry (BD Bioscience).

For flow cytometry-based analysis of cell phenotype in TME, the ascites from mice were harvested and the cell pellets were treated with ACK buffer (ThermoFisher Scientific) to lyse red blood cells. After that, the samples were incubated with anti-mCD16/32 (Elabscience) to block FcR, followed by staining for cell surface markers, as indicated before flow cytometry (BD Bioscience).

Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed with Prism software (GraphPad Prism 8.0 software). Survival curves were compared with the log-rank (Mantel-Cox) test. For comparisons of three or more groups, one-way analysis of variance (ANOVA) was performed with Tukey's multiple comparisons test. For comparisons of two groups, a two-tailed unpaired t test was performed. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and ns, no significance.

Results

OA elicits ApoEVs in HCC cells

To confirm the proapoptotic effect of OA at low concentration on HCC cells, we first treated HepG2 and SMMC-7721 cells using OA. As a result, OA treatment indeed resulted in apoptotic phenotype alteration in both HepG2 and SMMC-7721 cells as observed under microscopy (Fig. 1A). Moreover, OA treatment also lead to the increased cleavage of PARP and Caspase 3 in HCC cells, suggesting that apoptosis-associated

signaling pathways has been activated (Fig. 1B). Annexin V/PI staining assay showed the number of apoptotic HepG2 and SMMC-7721 cells was increased by about 10% and 3% in the presence of OA, respectively (Fig. 1C). In contrast, there was no significant apoptosis observed in L02 cells even treated with higher concentration of OA (Fig. S1A-D). Although the percentage of apoptosis is marginal, the production of ApoEVs including apoptotic bodies and microvesicles, but not exosomes, were largely elevated in the presence of OA as quantified by flow cytometry and nanoparticles tracking analysis (Fig. 1D-F). The typical appearances of EVs were visualized by transmission electron microscopy, and the diameters were over 200 nm (Fig. 1E and G). These data indicated that OA treatment induced the formation of ApoEVs (shorted as OA-EVs) in HCC cells.

Formation of OA-EVs requires ROCK1 activation

To elucidate the molecular mechanism by which OA elicited ApoEVs, the role of ROCK1 was investigated in formation of OA-EVs because caspase 3-mediated ROCK1 activation has been reported to regulate the formation of apoptotic bodies during apoptosis[17]. As expected, OA treatment resulted in the cleavage of ROCK1 in a dose- and time-dependent manner in HCC cells (Fig. 2A, Fig. S2A). Inhibition of caspase 3 using z-VAD-fmk largely attenuated the level of cleaved ROCK1 in the presence of OA (Fig. 2B), suggesting OA activated caspase-dependent ROCK1 cleavage. In addition, OA also increased the cleavage of MYPT1 (Fig. S2B), another caspase 3 target during apoptosis[18]. Consistently, ROCK1 signaling pathway was activated as characterized by the increased phosphorylation of cofilin and MLC2, as well as polymerization of actin due to OA treatment, and silencing ROCK1 disrupted the upregulation (Fig. 2C and S2B). Furthermore, generation of OA-EVs including ApoBDs and ApoMVs was dramatically blocked by both genetic and pharmacological inhibition of ROCK1 (Fig. 2D-G, Fig. S3A). The results indicate that ROCK1 activation is required for OA-elicited EVs in HCC cells. Interestingly, ROCK1 inhibition also diminished the anti-tumor effect of OA in a subcutaneous xenograft mouse model, although no difference was observed in cell proliferation *in vitro* (Fig. S3B, S4A-C), implying the roles of OA-EVs formation in tumor microenvironment*in vivo* .

OA-EVs alters macrophage phenotype

Macrophage accounts for the largest fraction of the myeloid infiltrate in tumor microenvironment. We next ask whether OA-EVs could influence macrophage polarization. As shown in Fig. 3A, OA-EVs labeled with DiI-Red were incubated with THP-1-derived macrophages (Mφ) stably expressing GFP protein, the cellular internalization of OA-EVs by macrophages was enhanced with the culture time according to the flow cytometry analysis. Moreover, the uptake of OA-EVs led to the increased ROS production (Fig. 3B). The M1-like macrophages have been reported to exert tumor-eradicating effect in different types of cancer through producing factors such as Nitric Oxide (NO), ROS, tumor necrosis factor α (TNF-α), etc[6]. Indeed, the expression level of genes associated with M2-like polarization was significantly downregulated by OA-EVs in THP-1-derived macrophages co-cultured with HepG2 and mouse bone morrow-derived macrophage (BMDMs) polarized by interleukin 4 (*Il-4*) induction (Fig. 3C and D, Fig. S5A and B). Moreover, the numbers of HepG2 cells was decreased due to the co-culture with THP-1-derived macrophages treated by OA-EVs (Fig. 3E and Fig. S5C). Furthermore, HepG2 cells co-cultured with macrophages in the presence of OA-EVs also displayed low proliferation as evaluated by Ki67 expression (Fig. 3F). Taken together, these results demonstrate that OA-EVs can induce macrophage polarization towards to M1-like phenotypes which might contribute to tumor suppression.

OA-EVs activates inflammasome pathway in macrophage

To further explore the underlying mechanism of macrophage reprogramming induced by OA-EVs, RNA sequencing was performed to analyze gene expression. As a result, the expression level of genes associated with innate immune response and type I IFN response were upregulated in OA-EVs treated macrophage (Fig. 4A). NOD-like receptor signaling pathway which normally involved in inflammasome activation was enriched most by KEGG analysis (Fig. 4B). Moreover, the release of IL-1β, an indicator of inflammasome activation, was significantly increased (Fig. 4C and D). However, silencing of NLRP3 and ASC, two critical players in NOD-like receptor signaling pathway, diminished the secretion of IL-1β from macrophage in the presence

of OA-EVs (Fig. 4E and F). In addition, knockdown of ASC also affected the expression level of genes associated with M1 and M2 polarization of macrophage induced OA-EVs treatment (Fig. 4G). These data suggest that OA-EVs lead to NLRP3 inflammasome activation, and subsequently induce proinflammatory response in macrophage.

Glycolysis-associated protein from OA-EVs contributes to macrophage inflammasome activation

We next identified the components of OA-EVs responsible for activating inflammasome pathway in macrophage. Boiling and digestion with Proteinase K to denature proteins of EVs almost completely blocked secretion of IL-1β and caspase 1 activation, hallmarks for inflammasome activation, induced by OA-EVs in macrophage (Fig. S6A). Proteomic analysis and the PANTHER classification system showed that proteins associated with glycolysis were enriched in OA-EVs (Fig. 5A, Fig. S6B). Indeed, macrophages incubated with OA-EVs displayed increased glycolysis as evaluated the level of extracellular ATP and lactic acid (Fig. S6C). Treatment of macrophage with 2-DG, an inhibitor of glycolysis, exerted the same effect on macrophage incubated with OA-EVs as EVs with denatured protein did (Fig. 5B). Furthermore, enhanced glycolysis-associated cargo proteins seemed to increase ROS production which subsequently mediated inflammasome activation (Fig. 5C and D). OA-EVs derived from HepG2 cells with silenced PKM2 and LDHA gene encoding key glycolysis enzymes were not able to trigger inflammasome activation in macrophage (Fig. 5E). These data suggest glycolysis-associated proteins were sorted into OA-EVs and then transmitted to macrophage, where the cellular metabolic status was altered, resulting in the increased level of ROS and eventually leading to proinflammatory response.

OA remodels HCC immune microenvironment in vivo

Given the above results have shown OA-elicited ApoEVs are able to affect macrophage polarization *in vitro* , we next ask whether OA treatment could lead to HCC immune microenvironment reprogramming*in vivo* . Mouse H22 cells were injected intraperitoneally to generate syngeneic mouse model, and the cells obtained from ascites fluid of mice represent the tumor microenvironment and can be further analyzed using flow cytometry to help illustrate the mixed population of cells surrounding the tumor. As shown in Fig. 6A and B, OA treatment resulted in about 1.5-fold increase in MHC II expression (M1 marker) of $CD11b^+/F4/80^+$ macrophages, whereas reduced CD206 expression (M2 marker) by 17%, compared with control group. Meanwhile, the population of myeloid-derived suppressor cells (MDSCs) as characterized by $CD11b^+/Ly6G^+$ which have been associated with suppression of anti-tumor immune response was decreased in tumor microenvironment due to OA treatment (Fig. 6C). Furthermore, the populations of Natural Killer (NK) cells ($CD45^+NK1.1^+$), $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were concurrently upregulated in tumor microenvironment due to OA treatment (Fig. 6D-F). Collectively, the data suggest that OA treatment could promote M1-like macrophages in tumor microenvironment, as well as be beneficial for antitumor immune response.

PD-1/ PD-L1 Inhibitor enhances the antitumor activity of OA on HCC model in vivo

It has been believed that IFN-γ inducible PD-L1 expression dependent on activation of STAT3 of tumor cells inhibit the anti-tumor effect of T cells[19]. As showed in Fig. 4A, OA-EVs induced upregulation of various genes associated with IFN signaling pathway. In addition, the mRNA expression level of PD-L1 was increased in macrophages co-incubated with OA-EVs compared with DMSO-EVs (data not shown). The results prompted us to further investigate whether blockage of PD-L1/PD-1 signaling could enhance the antitumor activity of OA on HCC. As a result, the combination of OA and BMS-1 (an inhibitor of PD-1/PD-L1) significantly suppressed tumor growth compared with control or single agent treatment in subcutaneous HCC mice model (Fig. 7A). Furthermore, blocking PD-1/PD-L1 signaling pathway markedly prolonged survival of mice bearing tumor together with OA administration (Fig. 7B). Moreover, the combination treatment significantly reduced the numbers of tumor load of liver in DEN/CCl4-induced HCC model (Fig. 7C). Further analysis showed that OA treatment lead to decrease in population of M2-like macrophages ($CD206^+$ $F4/80^+$), and increase in infiltrated $CD8^+$ T cells of mice liver tumors (Fig. 7D and E), which might contribute to the synergistic effect with blockage of PD-1/PD-L1 signaling. Taken together, our

results suggest that OA-induced ApoEVs from HCC cells trigger TAM reprogramming, facilitating to tumor suppression, and inhibition of PD-1/PD-L1 pathway could further boost the suppressive effect of OA on HCC progression (Fig. 7F).

Discussion

As a major flavonoid component derived from traditional Chinese herb-Huangqin, OA has been well documented to suppress tumor growth in different cancer models[20]. Although different mechanisms of action have been associated with the effect of OA on HCC progression in previous studies from our group and others[13, 21], it is still less known how OA influences tumor microenvironment. The complex interaction between immune cell and cancer cell in tumor microenvironment has been well-known to play key roles in cancer development, and then targeting immunosuppressive microenvironment has made great breakthrough in HCC therapy clinically[22-24]. In current study, we found that OA elicited apoptosis-associated EVs in HCC, which can alter macrophage polarization into M1-like phenotype via enhancing glycolysis and subsequent ROS production. The data from mouse models also confirmed the reduced M2-like TAMs in tumor microenvironment and enhanced antitumor immune response by OA treatment. Our results suggest that OA could be able to suppress tumor growth through regulating tumor immune microenvironment.

OA activated proapoptotic pathways in HCC cells by multiple mechanisms [25, 26]. The results from current study showed that caspase 3, an executor of apoptosis, has been activated in both HepG2 and SMMC-7721 cells by OA, which is in line with previous observation. During cell apoptosis, caspase 3-dependent cleavage of ROCK1 removes an auto-inhibitory region yielding a constitutively active kinase fragment. This results in phosphorylation of downstream targets that promote contractile force generation leading to cell shrinkage, nuclear disintegration and membrane blebbing, which ultimately generated ApoBDs that are typically 1–5 μm in diameter[17, 27, 28]. Consistently, OA treatment also induced caspase-mediated ROCK1 activation in HCC cells. Indeed, genetic and pharmacological inhibition of ROCK1 markedly diminished the formation of ApoBDs and ApoMVs (<1μm in diameter) by OA, both of which were generated by the outward budding and fission of the plasma membrane[29]. Although several kinases have been thought to regulate apoptotic membrane blebbing, recent report by utilizing CRISPR/Cas9 gene editing approaches found that ROCK1 is a key regulator for ApoBDs formation in apoptotic cells[17]. Our results demonstrated that OA elicited ApoEVs from HCC cells in a ROCK1-dependent way was consistent with previous findings.

It is known that loss of ROCK1 activity could disturb the clearance and removal of apoptotic cells by phagocytes, therefore regulating tissue homeostasis. Julian *et al.* showed that mice with mutant ROCK1 being resistant to caspase cleavage (ROCK1nc) developed fewer tumor after exposure to DEN treatment compared with wild-type mice (ROCK1wt), which might be attributed to greater neutrophil infiltration and then acute sterile inflammation[30]. Interestingly, we found silencing ROCK1 did not affect the antiproliferative effect of OA on HCC cells*in vitro* , however, Fasudil (a marketed inhibitor of ROCK1) attenuated the anticancer effect of OA *in vivo* . It is possible that infiltrated neutrophils resulted from ROCK1 blockage is beneficial for suppressing tumor initiation, but promoting progression at late stage, due to the dual functions of neutrophil including protumor and antitumor effect on cancer development[31]. Given that ROCK1 is also involved in other cellular processes such as cell contraction, migration, adhesion, survival and proliferation[32-34], it remains to be investigated the roles played by ROCK1 activation by OA in tumor or immune cell themselves or the interaction networks of microenvironment.

ApoEVs could modulate immune response based on “find-me” or “eat-me” signal in surface has been well documented[35]. Although ApoEVs formation was generally believed to ensure the immunological silent characteristic of apoptosis by limiting the release of intracellular contents before secondary necrosis, there are evidence showing that ApoEVs-derived damage-associated molecular pattern proteins (DMAPs) can initiate inflammatory response [27, 36]. EVs from cancer cells treated with radiation also influenced macrophage polarization in tumor microenvironment, contributing to radiation-induced bystander effect (RIBE) [37]. In present study, OA treatment remodeled tumor immune microenvironment, which might be resulted from OA-EVs activated NLRP3 inflammasome pathway in macrophage. Recent pan-cancer analysis showed that NLRP3 inflammasome was closely associated with cancer immunotherapy response[38]. Indeed, we observed

that OA enhanced tumor suppression with PD-1/PD-L1 inhibitor together. Nevertheless, chemotherapy-elicited EVs from breast cancer cells has been reported to exert enhanced pro-metastatic activity through recruiting monocytes into lung tissue[39]. The difference between OA-EVs and other EVs elicited by traditional chemotherapy needs to be closely clarified to rule out the effect of OA-EVs on pro-metastatic niches in distant organs.

OA-EVs seems to transmit glycolysis-associated proteins into macrophage, instead of DMAPs as reported previously. The increasing evidence demonstrate that metabolic reprogramming of cancer and immune cells is closely associated with anti-tumor immune response[40]. The oncologic mutation of cancer cells which lead to aerobic glycolysis allows cancer cells to compete for essential nutrients or reduce the metabolic fitness of tumor-infiltrating immune cells, therefore displaying immune-suppressive effect[41, 42]. On the other hand, the metabolic status of innate and adaptive immune cells shifts from mitochondrial oxidative phosphorylation (OXPHOS) to glycolysis in the activated state, thereby being beneficial for engulfing or killing cancer cells[42-44]. Previous report demonstrates that HCC-derived ectosomes enriches PKM2 which mediates macrophage differentiation and tumor microenvironment remodeling[10]. However, whether the anti-cancer compound could affect the metabolic interaction of cancer cells and immune cells is still obscure. OA has been shown to suppress glucose metabolism in HCC cells[45]. We showed in this study glycolysis-associated proteins were mainly sorted into OA-EVs, which were transferred into macrophages where they lead to ROS-dependent M1-like polarization. Interestingly, integrin signaling pathway was also enriched among of proteins from OA-EVs, which might facilitate to the binding of EVs with receipt cells. However, the mode of OA-EVs interaction with macrophage surface and the mechanisms that mediate transfer of cargo proteins into macrophage are still unraveled in current study.

Taken together, OA treatment elicited a large number of ApoEVs in HCC cell, which was able to induce proinflammatory phenotype of macrophages, probably contributing to tumor microenvironment remodeling and consequent antitumor immune response in mouse HCC model. Although the emerging reports have showed that natural compounds including flavonoids could remodel tumor-immunosuppressive microenvironment through multiple mechanisms[46], our results elucidate potential actions of OA on tumor microenvironment by influencing the metabolic crosstalk of cancer cells and immune cells.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

XZ and QG conceived and designed the experiments; PW, XZ, ZF, YT, XH and TM performed the experiments; PW, XZ, ZF, SL and YT analyzed the data; XZ, ZL and QG contributed reagents/materials/analysis tools; XZ wrote the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

All the animal experiments were performed in accordance with the guidelines of the Provision and General Recommendation of Chinese Experimental Animals in China, and were approved by the Ethics Committee of China Pharmaceutical University.

Consent for publication

The authors approved for the manuscript being published.

Competing interests

The authors declare that they have no competing interests.

Reference

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Figure Legends

Fig. 1 OA elicits apoptosis-related extracellular vesicles in HCC cells. (A) Images for HepG2 and SMMC-7721 cells treated with or without OA. Apoptotic cells were indicated with arrow (scale bar: 100μm). (B) After treatment of HepG2 and SMMC-7721 cells with OA as indicated for 48h, the cell lysates were subjected to immunoblot analysis for the expression of cleaved PARP, cleaved caspase 3 and total caspase 3 in, using β-actin as loading control. (C) HepG2 and SMMC-7721 cells were treated with OA as indicated for 48h, followed by flow cytometry analysis after staining with Annexin V-FITC/PI kit. Representative photos (left) and quantified data (right) were shown. (D) Flow cytometry analysis of apoptosis-related extracellular vesicles derived from HepG2 and SMMC-7721 cells was performed. The representative photos (left) and quantified data (right) were shown. (E-F) Nanoparticle tracking analysis of microvesicles (E) and exosomes (F) from HepG2 cells was carried out. The representative photos (left) and quantified data (right) were shown. (G) Electron microscope analysis of extracellular vesicles from HepG2 treated with OA was performed and the representative photos were shown (scale bar: 0.5μm for upper, 200nm for lower).

Fig. 2 Generation of OA-EVs requires caspase-dependent ROCK1 activation. (A) Cells were treated as indicated, and the lysates were subjected to immunoblot analysis for the expression of total ROCK1, cleaved ROCK1 (c-ROCK1) and RhoA using β-actin as loading control. (B) Cells were treated with DMSO and OA (10μM) with or without pan-caspase inhibitor (z-VAD-fmk, 10μM) for 48h, followed by analysis of caspase 3/7 activity and the expression of cleaved PARP (c-PARP), cleaved ROCK1 (c-ROCK1), total ROCK1 and β-actin in HepG2 and SMMC-7721 cells. (C) SMMC-7721 cells were transiently transfected with pLifeAct-GFP plasmid and treated with DMSO and OA (10μM) for 48h. After that, immunofluorescence was performed for analysis of GFP labeled LifeAct peptide expression, which indicated F-actin formation (scale bar: 5μm). (D) HepG2 cells with or without ROCK1 knockdown (ROCK KD) were treated with DMSO and OA (10μM) for 48h, followed by collection of ApoBDs and flow cytometry analysis for quantification. Upper panel: representative photos; Lower panel: quantified data. (E) HepG2 cells were treated with DMSO and OA (10μM) with or without ROCK1 inhibitor (Y-27623, 10μM) for 48h, followed by flow cytometry analysis same as (D). (F and G) HepG2 cells were treated as described above. After collection of ApoEVs, nanoparticle tracking analysis was performed. The representative images (left) and quantified data (right) were shown.

Fig. 3 OA-EVs promotes M1 like polarization of macrophage. (A) HepG2 cells were treated with OA (10μM) for 48h. After collection of OA-EVs, they were labeled with DiI-Red, and then incubated with THP-1-derived macrophage (Mφ) expressing GFP for indicated time. Immunofluorescence (left) and flow cytometry analysis (right) of the uptake of OA-EVs was performed and the representative photos were shown (scale bar: 200μm). (B) OA-EVs were collected and added into THP-1-derived macrophage for 24h. After washing with PBS, macrophages were labeled DCFH-DA, a ROS detecting probe, and then counterstained with DAPI, followed by photographing using fluorescence inverted microscope in different field of culture dish. The representative images (left) were shown (scale bar: 200μm) and the intensity of fluorescence (right) were quantified by Image J software on three different culture fields. (C) The co-culture model of THP-1-derived macrophages (Mφ) with HepG2 cells were established as indicated, and the OA-EVs were directly added into macrophages. (D) qPCR analysis of expression level of indicated genes in co-cultured THP-1-derived macrophages (Mφ) with DMSO-EVs and OA-EVs, respectively. (E and F) Flow cytometry analysis of the number (E) and Ki67 expression (F) of the co-cultured HepG2 cells when DMSO-EVs and OA-EVs were incubated with macrophages for 24h, respectively.

Fig. 4 OA-EVs activates inflammasome pathway in macrophage. (A) Heat map of differentially expressed genes associated with immune response in THP-1 derived macrophages treated by DMSO-EVs and OA-EVs. (B) KEGG enrichment analysis of differential gene expression in THP-1 derived macrophages treated with DMSO-EVs and OA-EVs. (C) Immunoblot analysis of IL-1β and caspase-1 (p20) in culture supernatants (SN), pro-IL-1β and pro-caspase-1 in lysates (Input) of THP-1 derived macrophages treated with DMSO-EVs and OA-EVs (2× represents double EVs). (D) ELISA analysis of IL-1β in culture supernatants of THP-1 derived macrophages treated with DMSO-EVs and OA-EVs. (E) Immunoblot analysis of IL-1β and caspase-1 (p20) in culture supernatants (SN), pro-IL-1β and pro-caspase-1 in lysates (Input) of THP-1 derived macrophages with OA-EVs after silencing NLRP3 and ASC, respectively. (F) ELISA analysis of

IL-1β in culture supernatants of cells treated as described in (E). (G) Realtime qPCR analysis of NOS2, CD206 and ASC gene expression in THP-1 derived macrophages with DMSO-EVs and OA-EVs following silencing of ASC gene expression.

Fig. 5 OA-EVs enriched glycolysis-associated proteins contribute to inflammasome activation in macrophage. (A) The proteome analysis of OA-EVs was performed by mass spectrometry (n=3). Top: list of the 50 most abundant proteins identified from OA-EVs. Bottom: the enrichment analysis of signaling pathways. (B) Immunoblot analysis of THP-1 derived macrophages IL-1β and caspase-1 (p20) in culture supernatants (SN), and pro-IL-1β and pro-caspase-1 in cell lysates (Input) of THP-1 derived macrophages incubated with EVs in the absence or presence of 2-DG. (C) Immunofluorescence analysis of intracellular ROS (green) in THP-1 derived macrophages in the presence of EVs with or without 2-DG. Top: representative images (scale bar: 100μm); Bottom: quantification data. (D) THP-1 derived macrophages were treated with EVs in the absence or presence of NAC, followed by western blot analysis of the supernatants (SN) and lysates (Input) for the expression of IL-1β and caspase-1 (p20) and their precursor forms. (E) PKM2 and LDHA genes were silenced in HepG2 cells, followed by EVs collection after control or OA treatment. Then EVs were added into THP-1 derived macrophages for 24h, and the supernatant (SN) and lysates (Input) were subjected to immunoblot analysis for the expression of IL-1β and caspase-1 (p20) and their precursor forms.

Fig. 6 OA treatment alters immune microenvironment of HCC*in vivo*. Cells were from the ascites were pretreated as described in "Materials and Methods". For M1-like macrophages (A), cells were incubated with three fluorescence conjugated antibodies including F4/80-PE, CD11b-APC and MHC II-FITC. Both F4/80 and CD11b positive cells was gated, in which MHC II expression was compared by flow cytometry analysis. For M2-like macrophages(B), the MHC II antibody was replaced with CD206-FITC, and the analysis was performed as (A). (C-F) Cells were incubated with two fluorescence conjugated antibodies as follows: Ly6G-PE and CD11b-APC for MDSCs (C); NK1.1-Cy5-PE and CD45-FITC for NK cells (D); CD4-APC and CD3-FITC for $CD4^+$ T cells (E) and CD8-APC and CD3-FITC for $CD8^+$ T cells (F), followed by flow cytometry for analysis and the positive cell population was gated.

Fig. 7 PD-1/PD-L1 inhibitor enhances the antitumor effect of OA in mouse HCC model. (A) Representative image and growth of subcutaneous H22 HCC tumor. (B) Survival curve of mouse bearing subcutaneous H22 HCC tumor. (C) Experimental scheme, liver images and tumor number of DEN/CCl_4 HCC model. (D) Immunofluorescence analysis of F4/80 (red) and CD206 (green) in tumor tissue. Left: representative images (scale bar: 50μm); Right: counting number of double-positive cells of three different fields. (E) Immunofluorescence analysis of CD8 alpha (red). Left: representative photographs (scale bar: 50μm); Right: the number of positive cells of three different fields. (F) A schematic diagram depicting the potential mechanism of action of OA in HCC.