Disruption of Endolysosomal RAB5/7 Efficiently Eliminates Colorectal Cancer Stem Cells

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Abstract

Given that cancer stem cells (CSC) play a key role in drug resistance and relapse, targeting CSCs remains promising in cancer therapy. Here we show that RAB5/7, which are involved in the endolysosomal pathway, play key roles in the maintenance of CSC survival via regulation of the mitophagic pathway. Inhibition of RAB5/7 efficiently eliminated colorectal CSCs and disrupted cancer foci. In addition, we identified mefloquine hydrochloride, an antimalarial drug, as a novel RAB5/7 inhibitor and promising colorectal CSC-targeting drug. Endolysosomal RAB5/7 and LAMP1/2 mediated parkin-dependent mitochondrial clearance and modulated mitophagy through lysosomal dynamics. In a patient-derived xenograft (PDX) model of colon cancer, treatment with mefloquine resulted in suppression of mitophagic PINK1/PARKIN and increased mitochondrial disorder and mitochondria-induced apoptosis without apparent side effects. These results suggest that the combination of mefloquine with chemotherapeutic agents in the PDX model potentially disrupts the hierarchy of colorectal cancer cells and identify endolysosomal RAB5/7 and LAMP1/2 as promising therapeutic targets in CSCs.

Significance: These findings show that endosomal/lysosomal RAB5 and RAB7, which regulate mitophagy, are essential for the survival of colon cancer stem cells.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/79/7/1426/F1.large.jpg

Introduction

Colorectal cancer morbidity and mortality have been increasing year after year (1). Cancer stem cells (CSC) construct the cancer cell hierarchy in tumor tissue (2, 3) and play a critical role in the formation of cancer diversity and acquisition of resistance to therapy (4–6). Thus, CSCs have gained attention as an attractive target for the novel cancer therapy (7). Stem cells and CSCs possess characteristic metabolic mechanisms for the maintenance of stemness properties and self-protection from cytotoxic substances, including oxidant stress, radiation, and chemotherapeutic agents (6, 9). Upregulation of reactive oxygen species (ROS) metabolism is one of the representative systems that characterize stem cell and CSC properties (7). By screening cell surface antigens for lower ROS-expressing cells, we previously identified CD107a [lysosomal-associated membrane protein 1 (Lamp1)] as a marker of lower ROS-expressing cells with immature cell properties (10). LAMP1 is the main constituent protein of the endosome/lysosome pathway. The lysosome pathway fuses to the autophagy pathway and constitutes the autolysosome (11). Although autophagy is known to play key roles in the retention of stemness involving self-renewal, cell dormancy, and metabolism (12), little is known about the role of the endosome/lysosome in CSCs. In this study, we aimed to clarify the role of the endosome/lysosome pathway in the maintenance of colorectal CSCs and to identify key regulators of the endosome/lysosome pathway that potentially become a target for the development of curative colorectal cancer treatment based on the CSC concept.
Materials and Methods

Cell preparation and chemical regents

Human colorectal cancer cell lines HT29, DLD-1, HCT116, SW480, and Caco2 were cultured in DMEM supplemented with 10% FBS (GE Healthcare) and penicillin/streptomycin (Invitrogen). Primary colorectal cancer samples were obtained from Osaka University, Japan, after receiving informed consent from the patients and approval from the Research Ethics Board of Osaka University (Osaka, Japan). Cells and mice were treated with 5-Fluorouracil (5-FU; Wako), oxaliplatin (L-OHP; Wako), SN-38 (Wako), chloroquine (Thermo Fisher Scientific), hydroxychloroquine (Sigma-Aldrich), quinine (Sigma-Aldrich), and melfloquine (Sigma-Aldrich).

Flow cytometric analysis

Flow cytometry was performed with the BD FACS Aria Ilu (BD Biosciences). Doublet cells were eliminated with FSC-A/FSC-H and SSC-A/SSC-H. Dead cells were eliminated with 7-AAD (BD Pharmingen). A negative control (isotype controls; BD Biosciences), LC3B imaging vector (Thermo Fisher Scientific), Lyso-tracker (Thermo Fisher Scientific), CD133 (Miltenyi Biotec, DEU), and CD44v9 (Clone RV3, Cosmo Bio Co) were used for cell labeling.

IHC

Subcutaneous tumors were enucleated and embedded in paraffin. After antigen retrieval, treatment, and blockade of endogenous peroxidase activity, the 4-μm-thick sections were stained with antibodies. List of antibodies used for IHC analysis and Western blotting were supplied in the Supplementary files. Terminal dUTP nick-end labeling (TUNEL) assays were carried out using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International).

Colorectal cancer cell xenograft and PDX mouse models

All studies were conducted in accordance with recognized ethical guidelines (Declaration of Helsinki, Japanese Ethical Guidelines for Human Genome/Gene Analysis Research, and Ethical Guidelines for Medical and Health Research Involving Human Subjects in Osaka University). We obtained informed written consent from all patients. All animal experiments were conducted according to the institutional ethical guidelines for animal experimentation (Osaka, Japan). A 1.0 × 10^5 of HCT116 cells in 100 mL of 1:1 v/v DMEM/Matrigel (BD Biosciences) were injected subcutaneously into the flanks of NOD/SCID mice (CLEA Japan). As a preclinical model, surgically resected samples were cut into pieces (3–4 mm) and transplanted subcutaneously into NOD/SCID mice under approval of Institutional review Board from Osaka University Hospital (25–062–011). For therapeutic effects assessment, when the tumor reached approximately 10 mm³, mice were randomly assigned to one of six groups administered Control (vehicle plus PBS), L-OHP, SN-38, chloroquine diphosphate (CQ), or melfloquine hydrochloride (MQ) alone, or a combination (L-OHP or SN-38 plus CQ or MQ). Vehicle solution and CQ contained 5% DMSO (Wako). MQ was formulated in 5% DMSO. CQ and MQ were dosed at 25 mg/kg once every 3 days by oral gavage. L-OHP contained 5% DMSO and was administered intraperitoneally weekly at 20 mg/kg/day. Tumor volume (width² × length)/2 was measured once every 3 days until 21 or 30 days from the start of therapy. All control mice received an equal volume of DMSO.

Microarray analysis

Total RNA was extracted using the miRNeasy Mini Kit (QIA-GEN). The array platform was the SurePrint G3 Human Gene Expression Microarray 8 × 60K Ver.3.0 (Agilent). Microarray data were analyzed using GeneSpring software (version 14.9; Agilent) and QIAGEN's Ingenuity Pathway Analysis software (QIAGEN). The data were normalized by the percentile shift to the 75th percentile and the threshold raw signal set to 1.0. The data were filtered using the fold change cut-off of 2.0 and P ≤ 0.05. The scores associated with each form of analysis were calculated using the logarithm of the P value (Fisher exact test). P < 0.05 was set as significant.

Statistical analysis

Data are shown as the mean ± SD of the indicated number of experiments. In xenograft mouse models, data are mean ± SEM. Two-sided t tests were used. Two-sided P values <0.05 were considered significant. These analyses were carried out using JMP version 13.0 (SAS Institute).

Full methods are described in the Supplementary Materials and Methods.

Results

Characterization of LC3B/LysoTracker-labeled cells

Autophagy and lysosome activity in colon cancer cells were assessed by the LC3B BacMam imaging system and LysoTracker, respectively. Colorectal cancer cell lines HCT116, HT29, Caco2, and DLD1 were used for subsequent assays (Fig. 1A). Flow cytometric analysis revealed that both the LC3B and Lyso-Tracker-labeled cells were localized mainly in the CD44v9 and CD133 colon CSC—expressing fraction (Fig. 1A). To confirm acidification of lysosome, we used acridine orange and obtained compatible results with LysoTracker (Supplementary Fig. S1A). Proliferation of LC3B<sup>high</sup>/CD44v9<sup>−</sup> cells and LysoTracker<sup>high</sup>/CD44v9<sup>−</sup> cells was significantly slower than LC3B<sup>low</sup>/CD44v9<sup>−</sup> cells and LysoTracker<sup>low</sup>/CD44v9<sup>−</sup> cells, respectively (P < 0.05; Fig. 1B). Cell-cycle analysis revealed an enriched G<sub>0</sub>–G<sub>1</sub> cell fraction in both the LC3B<sup>high</sup>/CD44v9<sup>+</sup> and LysoTracker<sup>high</sup>/CD44v9<sup>+</sup> cell fractions, 54.7% and 58.3%, respectively (Fig. 1C). Sphere formation activity, a common characteristic of stem cells, in 200 LC3B<sup>high</sup>/CD44v9<sup>−</sup> and LysoTracker<sup>high</sup>/CD44v9<sup>−</sup> cells was significantly higher than in the same number of LC3B<sup>low</sup>/CD44v9<sup>−</sup> and LysoTracker<sup>low</sup>/CD44v9<sup>−</sup> cells (P < 0.05). Single-cell sphere formation activity was significantly higher in LysoTracker<sup>high</sup>/CD44v9<sup>−</sup> cells than LC3B<sup>high</sup>/CD44v9<sup>−</sup> cells (P < 0.05) and was not dependent on CD44v9 expression status (Fig. 1D). Chemosensitivity assay of trans-L-diaminocyclohexane oxalatoplatinum (L-OHP), a key chemotherapeutic agent for colorectal cancer treatment, revealed a higher chemoresistance ability of LC3B<sup>low</sup>/CD44v9<sup>−</sup> cells and LysoTracker<sup>low</sup>/CD44v9<sup>−</sup> cells compared with LC3B<sup>low</sup> or LysoTracker<sup>low</sup> cells (P < 0.05; Fig. 1E). Immunofluorescence microscopy demonstrated increasing numbers of LC3B and LysoTracker-labeled cells with increasing L-OHP concentration (Fig. 1F).
Cell populations in which the autophagy and lysosomal pathways were activated and had the properties of CSCs. **A,** Colon CSC fractions (CD44v9⁺ or CD133⁺) of HCT116 and HT29 were classified into two subpopulations based on autophagy or lysosome activity. **B,** Proliferation assay of LC3B<sup>high</sup> or LysoTracker<sup>high</sup>/CD44v9⁺ (red lines) and LC3B<sup>low</sup> or LysoTracker<sup>low</sup>/CD44v9⁺ (blue lines) HCT116 cells. Data represent mean ± SD (n = 6) of independent experiments of fractions differentially sorted by flow cytometry. *P* < 0.05. **C,** Cell-cycle assay of LC3B<sup>high</sup> or LysoTracker<sup>high</sup>/CD44v9⁺ cells and LC3B<sup>low</sup> or LysoTracker<sup>low</sup>/CD44v9⁺ cells. **D,** Sphere formation assays compared LC3B<sup>high</sup> or LysoTracker<sup>high</sup>/CD44v9⁺ cells with LC3B<sup>low</sup> or LysoTracker<sup>low</sup>/CD44v9⁺ cells. Top, 200 cells seeded into each well. Bottom, a single cell seeded into each well. Scale bars, 100 μm. Data represent mean ± SD (n = 5). *P* < 0.05. **E,** Chemosensitivity assays of LC3B<sup>high</sup> or LysoTracker<sup>high</sup>/CD44v9⁺ cells (red lines) and LC3B<sup>low</sup> or LysoTracker<sup>low</sup>/CD44v9⁺ cells (blue lines). Chemosensitivity was indicated by viability after treatment with L-OHP for 72 hours. Data represent mean ± SD (n = 6). *P* < 0.05. **F,** Immunofluorescence staining using HCT116 cells treated with L-OHP. Scale bars, 20 μm.

Figure 1.
Autolysosome-activated cells are tumor-initiating and long-term dye-retaining cells

To assess and compare the tumorigenic activity of LC3B and LysoTracker-labeled cells, limiting dilution assays and serial transplantation assays were performed with HCT116 cells. Each 1.0 × 10^2 cells from the LC3Bhigh/CD44V9+ and LysoTrackerhigh/CD44V9+ fractions formed subcutaneous tumors, but CD44V9+ cells not labeled with LC3B and LysoTracker did not form tumors (Table 1). Serial transplantation assays, which more strictly reveal tumorigenic and self-renewal activity, revealed that only LysoTrackerhigh cells form tumors, but not LysoTrackerlow cells existing in the CD44V9+ expressing cell fraction. LC3B labeling was not strict for the isolation of tumorigenic cells; 50% of LC3Blow/CD44V9+ cells formed tumors in NOD/SCID mice (Table 1). Because LC3B and LysoTracker labeling status closely correlated with cell-cycle status, long-term dye-retaining ability was assessed by intraperitoneal BrdUrd injection. Long-term dye-retaining ability also revealed cell dormancy or slow growth as unique characteristics of stem cells and CSCs. After 7 weeks of BrdUrd injection, BrdUrd retention was assessed on tumors derived from LC3Blow/CD44V9+, LC3Bhigh/CD44V9+, LysoTrackerlow/CD44V9+, and LysoTrackerhigh/CD44V9+ cells. BrdUrd retention was observed in tumors derived from LC3Bhigh/CD44V9+ and LysoTrackerhigh/CD44V9+ cells and typically existed at the edge of tumor foci (Supplementary Fig. S1B).

Melfoxiquine hydrochloride inhibits lysosomal LAMP1/LAMP2 expression and disrupts CD133/CD44V9 colon CSCs

Some antimalarial drugs potentially inhibit autophagy. The autophagy and lysosome pathways are not completely independent and fuse downstream to form the autolysosome pathway. Although no drug is currently applied clinically as a lysosome inhibitor and whether antimalarial drugs have lysosome-inhibitory actions is unclear, we hypothesized that some antimalarial drugs possess strong lysosome-inhibitory effects. We screened clinically approved antimalarial agents quinine hydrochloride hydrate (QN), CQ, hydroxychloroquine sulfate (HQ), and MQ to investigate whether autophagic activity or lysosomal chloride hydrate (QN), CQ, hydroxychloroquine sulfate (HQ), or MQ with or without 5-FU, L-OHP, or SN-38 cotreatment. We used 5-FU, L-OHP, and SN38 (an active metabolite of irinotecan) because they are key drugs in colorectal cancer treatment. A combination effect was not observed with 5-FU–based combinations, but the combination of L-OHP with CQ or MQ resulted in significant suppression of cell viability in both K-ras wild-type (Caco2 and HT29) and mutant (HCT116 and SW480) cell lines. With the SN38–based combination, a combination effect was observed only with MQ cotreatment (Fig. 2D). A drug combination effect was confirmed with isobologram analysis (13, 14). CQ, HQ, and MQ exhibited antagonistic effects on the individual dose of 5-FU. Although CQ demonstrated a moderate synergistic effect on L-OHP, it had an additive effect on SN38. HQ demonstrated an additive effect on L-OHP and SN38. Interestingly, only MQ had a strong synergistic effect that depends on the individual dose of L-OHP and SN-38 (Fig. 2E). We performed isobologram analysis of multiring combination. Similar to the results of the isobologram analysis using a single agent, only MQ showed a synergistic effect with L-OHP and SN-38 under the treatment of 5-FU (Supplementary Fig. S1C). Flow cytometric analysis was performed to assess the effects of each chemical agent on stem cell fractions. The CD44V9+/CD133+ CSC population was increased from 54.6% in control to 84.1% with L-OHP treatment and 80.1% with SN38 treatment. The CD44V9+/CD133+ CSC population decreased to 37.5% with CQ treatment, 39.3% with HQ treatment, and 9.4% with MQ treatment. The CD44V9+/CD133+ CSC population decreased to 0.8% and 0.1% with combined treatment of MQ with L-OHP or SN-38, respectively, but was not decreased by the combination of CQ or HQ with L-OHP or SN-38 (Fig. 2F), and we confirmed that MQ treatment induce intracellular ROS (Supplementary Fig. S1D).

Melfoxiquine suppresses CD44V9 and LAMP1/2 expression in the PDX model

To elucidate the mechanism of the antitumor effect of MQ, we established a xenograft mouse model in which two colon cancer patient-derived tissues were implanted subcutaneously. These models were treated with CQ, MQ, or MQ with L-OHP (Fig. 3A). IHC staining of CD44V9, LC3B, LAMP1, and LAMP2 was performed using enucleated tumor specimens to confirm the in vitro results. Decreased expression of CD44V9, LAMP1, and LAMP2 was observed with MQ treatment (Fig. 3B) (Supplementary Fig. S2A).

Melfoxiquine inhibits lysosomal activity by targeting RAB5 and RAB7

Microarray analysis was performed to identify the gene targeted by MQ. Cluster analysis with whole genome probes

Table 1. Limiting dilution and serial transplantation assay of HCT116 cells

<table>
<thead>
<tr>
<th>Fraction/cell number</th>
<th>Limiting dilution</th>
<th>Serial transplantation</th>
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<tr>
<td></td>
<td>1.0 × 10^2</td>
<td>5.0 × 10^2</td>
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<td></td>
<td>1.0 × 10^3</td>
<td>5.0 × 10^3</td>
</tr>
<tr>
<td>LC3B High/CD44V9+</td>
<td>3/4</td>
<td>3/4</td>
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<tr>
<td>LC3B Low/CD44V9−</td>
<td>0/4</td>
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<tr>
<td>LysoTracker High/CD44V9+</td>
<td>4/4</td>
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<td>LysoTracker Low/CD44V9−</td>
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Figure 2.
Meltoquine efficiently inhibited the lysosomal pathway and eliminated CSCs compared with chloroquine and hydroxychloroquine. A, Cells were treated with the indicated concentrations of QN, CQ, HQ, and MQ for 48 hours and cell viability determined by CCK-8. B, Flow cytometry showing the expression of LC3B, LysoTracker, and Lamp in HCT116 cells after treatment with CQ, HQ, and MQ. The proportion of each antigen-expressing cell type was calculated by comparison to a negative control (isotype controls). C, Western blot analysis of mTOR, Lamp1, Lamp2, and LC3 in HCT116 cells. Cell lysates were prepared every 48 hours after treatment with L-OHP, CQ, and MQ (all doses are IC50). D, HCT-116, SW480, Caco2, and HT29 were treated with 43 mmol/L CQ, 47 mmol/L HQ, 8 mmol/L MQ, and/or 4.0 μmol/L oxaliplatin (L-OHP) for 48 hours (all doses are IC50). Cell viability was assessed by CCK-8. Data represent mean ± SD (n = 6). * P < 0.05. E, Assessment of combined drug effects with isobologram analysis. Isobologram analyses were used to determine whether the interactions between 5-FU or L-OHP or SN-38 and CQ, HQ, and MQ were additive, synergistic, or antagonistic. F, Flow cytometry showing expression of CD44v9 and CD133 in HCT116 cells by treatment with CQ, HQ, MQ, and L-OHP or SN-38.
clearly divided each condition in PDX tumors into four clusters of MQ, MQ with L-OHP, control, and CQ (Fig. 4A). Gene clusters were also divided into four clusters: C1, C2, C3, and C4. We focused on the C3 cluster, which contained 1,106 gene probes and indicated genes specifically downregulated by MQ treatment. Analyzing the C3 cluster using the Ingenuity Pathway Analysis (IPA), we identified the predominant canonical pathways. The top nine molecular and cellular functions and canonical pathways are listed in Fig. 4B. First, we focused on the phagosome maturation pathway associated with lysosome maturation. Mapping the C3 cluster genes in the phagosome maturation pathway revealed broad disruption of the lysosomal maturation process (Fig. 4C), indicating that MQ broadly inhibited lysosome formation in the early and late stages. Western blot showed that expression of RAB5 and RAB7, regulators of early and late lysosome biogenesis (15–17), was specifically inhibited by MQ treatment (Fig. 4D). Analysis of protein structures and MQ docking simulations revealed the existence of MQ-binding sites in RAB5 and RAB7 (Fig. 4E). Transmission electron microscopy revealed accumulation of ballooning lysosome, a feature of lysosomal dysfunction (12), was significantly increased by MQ treatment (Fig. 4F). These results indicate that mefloquine inhibited lysosomal activity by targeting RAB5 and RAB7.
MQ inhibited lysosomal activity by targeting Rab5 and Rab7. A, Hierarchical clustering under microarray analysis for PDX mouse model tumors under each treatment using GeneSprings software (version 14.9). The microarray was classified into four clusters. In the group treated with mefloquine, the genes with significantly decreased expression were clustered. B, The top nine molecular and cellular functions and canonical pathways are shown. Threshold criteria considered for the analysis are \(-\log P > 3.0\) and \(P < 0.05\). C, IPA canonical pathway modeling of the phagosome maturation pathway. Genes with relatively lower expression in MQ vs. control are depicted in green. D, Western blot analysis of RAB5 and RAB7 in HCT116 cells. E, Molecular docking simulations of mefloquine and target protein (RAB7 or RAB5). The free energy of the binding of mefloquine and RAB5 was \(-39.55\) kJ/mol, and mefloquine and RAB7 \(-40.33\) kJ/mol. F, HCT116 cells treated as control, CQ, or MQ were fixed and imaged by transmission electron microscopy. Representative images taken at \(x30,000\) magnification are shown. Lysosome sizes were recorded as the mean diameters of the lysosomes in 10 fields. Statistical analyses were performed using Student t tests (*) \(P < 0.05\) Scale bars, 0.2 μm.

Figure 4.
Meltoquine demonstrates promising antitumor effects on colon cancer cells

To confirm the antitumorigenic effect of MQ and validate the clinical application of MQ, we established three xenograft mouse models in which HCT-116 cells or two colon cancer patient-derived tissues were implanted subcutaneously. The PDX mice were treated with L-OHP, CQ, MQ, or a combination of L-OHP with CQ or MQ (Fig. 5A). Compared with the vehicle control, MQ reduced tumor growth in all three models. The inhibitory effect of MQ on tumor growth was almost the same as L-OHP alone. In any mouse model, the combination of MQ with L-OHP drastically reduced tumor volume compared with either agent alone (Fig. 5B and C). TUNEL staining showed induced apoptosis in the tumor treated with MQ and L-OHP (Fig. 5D). In addition, IHC analysis showed no reduction of CD44v9, LAMP1, or LAMP2 expression after treatment with L-OHP, CQ, or CQ and L-OHP (Fig. 3B). Treatment with MQ and the combination of MQ and L-OHP was well tolerated and no weight loss was observed (Fig. 5B). No effects on appearance or behavior were confirmed, and there were no obvious changes in the major organs macroscopically or microscopically (Fig. 5D). We confirmed the expression of LAMP1, LAMP2, LC3, RAB5, and RAB7 in healthy normal tissues by IHC staining. In contrast to the tumor foci, by the treatment of MQ, expression of LAMP1, LAMP2, RAB5, and RAB7 did not decrease in the healthy normal tissues (Supplementary Fig. S2B). We assessed the blood test of mice model and confirmed that MQ did not induced apparent side effects (Supplementary Table S1A).

We also confirmed an antitumor effect of the combination of MQ with SN-38 in vitro because SN-38 had a synergistic effect with MQ, as well as L-OHP, in vitro (Fig. 5E). In any mice, the combination of MQ and SN-38 had a strong antitumor effect as in mice treated with MQ and L-OHP (Fig. 5F). To confirm that tumors are eradicated after cessation of treatment, when tumors were grown over 200 mm³, these mice were treated by above treatments for 30 days. Each tumor was observed without treatment by each reagent until day 45. Only the mice treated by MQ+SN-38 revealed no apparent signs of tumor regrowth. In addition, when the tumors were sampled from primary mice at just after 30 days of each treatment and then serially transplanted into secondary mice, no tumor reformation was observed in the MQ+SN-38 group alone (Supplementary Fig. S3A; Supplementary Table S1B). We developed an orthotopic transplant mouse model and peritoneal metastasis mouse model. In both mouse models, MQ with SN-38 showed significant reduction of tumor compared with other treatment (Supplementary Fig. S3B and S3C).

Downregulated expression of RAB5 and RAB7 was also confirmed by IHC staining of the PDX models shown in Fig. 5A. Expression of RAB5 and RAB7 was suppressed by treatment with MQ, but not by other agents (Fig. 6A).

Meltoquine impaired mitochondrial function and inhibited mitophagy

To examine additional effects of MQ, we focused on pathways associated with mitochondria because oxidative phosphorylation and mitochondrial dysfunction were the top two pathways in IPA analysis (Fig. 4B). IHC analysis and Western blotting were performed to determine mitochondrial status and metabolism. IHC analysis revealed suppression of PINK1 and Parkin, major factors involved in mitophagy (18) only in mice treated with MQ (Fig. 6A). Western blotting also revealed suppression of TOMM20 expression in addition to PINK1 and Parkin (Fig. 6B). TOMM20 is a constituent of the mitochondrial outer membrane and responsible for the recognition and translocation of cytosolically synthesized mitochondrial preproteins. Mapping of C3 cluster genes in the mitochondrial dysfunction pathway revealed broad disruption of components of the inner mitochondrial membrane, including complex I, III, IV, and V, and inhibition of downstream signals involving Parkin and PINK1, suggesting that MQ treatment induced mitochondrial disruption (Supplementary Fig. S4A). Transmission electron microscopy also supported mitochondrial degradation induced by MQ treatment, ballooning of the mitochondria, and disappearance of the cristae (Fig. 6C). IHC staining showed high expression of BAX, cytochrome C (CYC), and cleaved caspase-3 in mice treated with MQ, suggesting that MQ treatment induced mitochondrial apoptosis (Fig. 6D). We confirmed the dynamics of mitophagy, RAB5, and RAB7 in CSCs, finding that all of these were enhanced in colon CSCs (Fig. 6E). These results suggest that mitophagy was increased and abnormal mitochondria removed in CSCs. To elucidate whether RAB5 or RAB7 is involved in CSC maintenance, we knocked down the expression of the RAB5 or RAB7 gene in HCT116 and HT29 colorectal cancer cells and performed in vitro functional assays. Western blotting confirmed RAB5/7 knockdown by siRNA for RAB5/7, and revealed the suppression of LAMP1, PINK1, and PARKIN by treatment with RAB5/7 siRNA (Fig. 6F). In HCT116 cells, the CD44v9+/CD133+ CSC population was decreased from 62.5% to 15.3% or 26.5% with RAB5 siRNA, and to 30.0% or 30.9% with RAB7 siRNA. In HT29 cells, the CD44v9+/CD133+ CSC population was decreased from 66.6% to 21.7% or 38.3% with RAB5 siRNA, and to 40.9% or 41.5% with RAB7 siRNA (Fig. 6G). We performed double knockdown of both RAB5 and RAB7 and confirmed the decreased number of CD44v9+/CD133+ cells by flow cytometric analysis. Western blot analysis also indicated that PINK1, Parkin, and LAMP1 expression decreased as a result of double knockdown of RAB5/7. Furthermore, sphere formation assay revealed that double knockdown of RAB5/7 strongly inhibited the sphere-forming ability similar to MQ treatment (Supplementary Fig. S4B–S4D).

Discussion

In hematopoietic stem cells, acute myelogenous leukemia cells, and progenitor cells, the autophagy pathway is closely related to the maintenance of stem cells (12, 19, 20). In solid cancer, there have been several reports suggesting that the autophagy pathway is also involved in CSCs (21–23). However, a few studies have suggested a relationship between the lysosome and CSCs. This is the first study to reveal critical roles of phagosome/lysosome maturation factors in CSCs and cancer cell maintenance.

There have been many reports that inhibition of autophagy improves the anticancer drug resistance of cancer cells (24, 25). CQ and HQ are applied clinically in among other cancers (26, 27). In this study, we clarified that targeting lysosomes had a strong effect on cancer cells, and that inhibiting lysosomes is important for targeting CSCs. Meltoquine, an
MQ had antitumor effects, and combined treatment of MQ with anticancer drugs drastically abrogated the tumorigenic activity of cancer cells in PDX mouse models. A, In the HCT116 model and two PDX models, each animal was treated with CQ (25 mg/kg; \(n = 4\)), MQ (25 mg/kg; \(n = 4\)), L-OHP (5 mg/kg; \(n = 4\)), or combinations (CQ + L-OHP or MQ + L-OHP; \(n = 4\)) via injection. B and F, Tumor volume and body weight were measured every 3 days until day 21. C, Tumors from treated mice on day 21. D, Top, analysis of apoptosis (blue, DAPI staining of nuclei; cyan, TUNEL-positive staining) in PDX-derived tissues from animals treated with each drug. Bottom, hematoxylin and eosin–stained sections of mouse liver, kidney, and heart from one representative mouse in each treatment group. Scale bars, 20 \(\mu m\). E, In the HCT116 model and PDX model, each animal was treated with CQ (25 mg/kg; \(n = 4\)), MQ (25 mg/kg; \(n = 4\)), SN-38 (20 mg/kg; \(n = 4\)), or combinations (CQ + SN-38 or MQ + SN-38; \(n = 4\)) via injection.
Figure 6.
MQ disrupted mitochondrial function and inhibited mitophagy. A, IHC analysis of RAB5, RAB7, PINK1, and Parkin in tumors from PDX models on day 21 after therapy. Scale bars, 100 μm. B, Western blot analysis of PINK1, Parkin, and TOMM20 in HCT116 cells. C, Representative images taken at ×10,000 or ×30,000 magnification in transmission electron microscopy. The cells treated with MQ exhibited mitochondrial swelling and dissolved cristae compared with the control cells. White scale bars, 0.2 μm; black scale bars, 500 nm. D, IHC analysis of TOMM20, Bax, CYC, and cleaved caspase-3 in tumors from PDX models on day 21 after therapy. Scale bars, 100 μm. E, Western blot analysis of PINK1, Parkin, RAB5, RAB7, and TOMM20 in CSC fractions of HCT116 or HT29 cells. F, Western blot analysis of RAB5, RAB7, PINK1, PARKIN, and Lamp1 in HCT116 and HT29 cells transfected by negative control or RAB5 or RAB7 siRNA. G, Flow cytometric analysis was performed to assess the effects of each RAB5 or RAB7 siRNA on stem cell fractions. The CD44 v9<sup>+</sup>/CD133<sup>+</sup> CSC populations of siRNA RAB5 or RAB7 were decreased compared with negative controls.
antimalarial drug that characteristically inhibits lysosomes, efficiently eliminated CSCs and had a synergistic effect with anticancer drugs used for colon cancer. In the PDX models, meloquine alone had nearly the same effect as the anticancer agent and a stronger antitumor effect than CQ in combination with an anticancer drug. We also clarified the pharmacologic mechanisms and target molecules (RAB5 and RAB7) of meloquine using microarray analysis of the PDX treatment model. RAB proteins are members of the RAS GTPase superfamily and key regulators of membrane trafficking and fusion events (28). RAB5/RAB7 is involved in early and late lysosome formation (15, 16). Early endosomes mature into late endosomes before fusing with lysosomes, and this maturation involves a switch from RAB5 to RAB7 (17).

In contrast to the tumor foci, by the treatment of MQ, expression of LAMP1, LAMP2, RAB5, and RAB7 did not decrease in the healthy normal tissues (Supplementary Fig. S2B). We hypothesize that healthy lysosomes may be transformed into abnormal lysosomes in cancer, and meloquine seems to target this abnormal lysosome; thus, side effects of meloquine may be reduced in normal cells. However, further study is necessary to clarify the correlation between abnormal lysosomes and cancer. In addition, in this study, we could not show whether the MQ effect is totally dependent on Rab5/Rab7. Because the effect on sphere formation of either MQ treatment or Rab5/7 double knockdown was potent, and it was difficult to see any additional effects of MQ for Rab5/Rab7 double knockout status.

Recent investigations have demonstrated that RAB5 mediates parkin-dependent mitochondrial clearance (29) and RAB7, which regulates lysosomal transport and fusion, provides a mechanism by which mitochondria modulate lysosomal dynamics (30). In this study, meloquine targeting RAB5 and RAB7 disrupted mitochondrial function and inhibited mitophagy (31), eliminating damaged mitochondria. Whether abnormal mitochondria are increased in cancer is not clear, but mitochondrial DNA copy number is increased in several cancers, including colon cancer (32). Considering the effect of meloquine, which effectively eliminates CSCs, the mechanism of eliminating damaged mitochondria seems to be important for CSCs. There are no reports on the relationship between CSCs and mitophagy. However, Vazquez-Martin and colleagues (33) clearly indicated that mitophagy is necessary for efficient nuclear reprogramming of somatic cells into induced pluripotent stem cells (iPSC). We confirmed that mitophagy was elevated in the CSC population. However, to confirm the correlation with mitophagy, damaged mitochondria and CSCs require further investigation.

Meloquine has already been clinically applied as an antimalarial drug and has fewer side effects than CQ and HQ when applied clinically to cancer (26, 27, 34). Meloquine has a stronger antitumor effect than oxaliplatin and irinotecan, key drugs for the treatment of colon cancer, without depending on a K-ras mutation and with fewer serious side effects than chemotherapeutic agents. Drug repositioning, which aims to expand the indication of drugs, has rapidly progressed in recent years worldwide (35). Drug repositioning saves money and time compared with developing new medicines (36). The benefits of applying meloquine to the treatment of colorectal cancer are extremely high from the viewpoint of ensuring patient quality of life and reducing medical costs. Oxaliplatin is highly effective, but once it fails, readministration becomes extremely difficult (37). In addition, there are no markers predicting side effects (37). Thus, irinotecan should be used instead of oxaliplatin as a concomitant medicine in clinical research following the salvage line. Irinotecan has diarrhea as a serious side effect, but the high-risk group can be excluded by measuring UGT1A1 (38). Other side effects are fewer with irinotecan than oxaliplatin and has the merit of easy reintroduction (38). This study indicates that irinotecan had a synergistic effect with meloquine in vitro and in vivo. We are planning phase I and phase II trials for advanced/recurrent colorectal cancer.

We expect that meloquine may induce depletion of CSCs and, due to a synergistic effect, demolish the cancer hierarchy, including cancer precursor cells, when given in combination with cytotoxic anticancer drugs. A single CSC-targeting therapy may not be sufficient to eradicate cancer, because potential treatment evasion by non-CSC plasticity, in which non-CSCs acquire stem cell properties, should be considered (39, 40).

In conclusion, our study shows that lysosomal activity is more closely related to the CSC properties of colon cancer cells than autophagic activity. The combination of meloquine and oxaliplatin or irinotecan efficiently disrupts the tumorigenic activity of colon CSCs. Accordingly, we suggest that meloquine is a promising candidate for colon CSC–targeting therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Disruption of Endolysosomal RAB5/7 Efficiently Eliminates Colorectal Cancer Stem Cells

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