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Aralin, a type II ribosome-inactivating protein from *Aralia elata*, exhibits selective anticancer activity through the processed form of a 110-kDa high-density lipoprotein-binding protein: A promising anticancer drug

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ABSTRACT

Aralin from *Aralia elata* is a newly identified type II ribosome-inactivating protein, which preferentially induces apoptosis in cancer cells. In this study, we identified that the aralin receptor is a 110-kDa high-density lipoprotein-binding protein (HDLBP), which functions as a HDL receptor. The sensitivities of tumor cell lines to aralin were dependent on the expression levels of the 110-kDa HDLBP and its forced expression in aralin-resistant Huh7 cells conferred aralin sensitivity. HDLBP-knockdown HeLa cells showed a significant aralin resistance *in vitro* and *in vivo*. Conversely, ectopic expression of the 150-kDa HDLBP resulted in increased aralin sensitivity *in vivo*, accompanying enhanced expression of the 110-kDa HDLBP. Thus, these results showed that the110-kDa HDLBP in lipid rafts acted as an aralin receptor and that its expression levels determined aralin sensitivity, suggesting that aralin could be a promising anticancer drug for HDLBP-overexpressing tumors.

1. Introduction

Ribosome-inactivating proteins (RIPs), which are widely present in plants, inhibit protein synthesis through adenosine removal from eukaryotic ribosomal RNA as an RNA *N*-glycosidase. Type II RIPs are heterodimeric glycoproteins, comprising an A-chain, catalytic domain of RNA *N*-glycosidase, linked via a disulfide bond to B-chain, which mediates cell entry [1]. Type II RIPs enter the cell by binding to a cell surface receptor through the B-chain, cross the cell wall by endocytosis, and then reach the cytosol by retrograde transport through the Golgi apparatus to the endoplasmic reticulum (ER) [1].

Many laboratories are working with toxin conjugates and immunotoxins, some of which are being tested clinically on both solid tumors and hematological malignancies [2]. For type II RIP rViscumin, CD75s- and iso-CD75s-gangliosides, which are significantly expressed in pancreatic adenocarcinoma, act as rViscumin receptors. rViscumin provides an opportunity for treating pancreatic cancer [3,4].

Aralin is a type II RIP from *Aralia elata*, selectively induces apoptosis in various human tumor cells [5,6]. An analysis of the aralin receptor with rhodamine-conjugated aralin has shown that it is expressed around the cell surface of virus-transformed VA-13 cells. The binding of aralin is suppressed by Gal and not ricin [6]. Nonetheless, information on an aralin-specific receptor protein is still obscure.

High-density lipoprotein-binding protein (HDLBP) is a 150-kDa glycoprotein that is processed to a smaller form (105–110 kDa) and moved to the plasma membrane to act as an HDL receptor [7–9]. HDLBP expression is enhanced in cholesterol-overloaded cells [7,8,10], suggesting that this protein plays a role in HDL-mediated cholesterol removal. Thus, it appears that HDLBP expression and its processing pathway are controlled by physiological conditions [7,8,10].

In this study, we showed that the 110-kDa HDLBP in lipid rafts is an aralin receptor, suggesting that aralin is a promising anticancer drug for tumors in which HDLBP expression is deregulated.

Abbreviations: BSA, bovine serum albumin; ER, endoplasmic reticulum; Gal, galactose; HDLBP, high-density lipoprotein-binding protein; hHCC, human hepatocellular carcinoma; HRP, horseradish peroxidase; Lac, lactose; MβCD, methyl-β-cyclodextrin; RIP, ribosome inactivating protein.

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2. Materials and methods

2.1. Cell culture

Human normal fibroblast Hs68, cervical carcinoma HeLa, squamous cell carcinoma SCC-25, SV40-transformed simian kidney COS7, and hepatocellular carcinomas (hHCCs) Hep G2 and Huh7 cells were obtained from the Japanese Cancer Resource Bank (Tokyo, Japan).

2.2. Expression vectors

The 150-kDa human *HDLBP* cDNA (Open Biosystems, Huntsville, AL) was inserted into pcDNA3 vector (Invitrogen, Carlsbad, CA). The 110-kDa HDLBP (Asp475-Arg1328; Q00341) cDNA was amplified by PCR using the 150-kDa HDLBP cDNA as a template. The 3′- and 5′-primers used were as follows: 5′-primer, 5′-GCCAAGCTTGCC ACCATGAGTGAGAAGAGCAATTTGATCCGCATCG-3′; 3′-primer, 5′-CGTACCAACTTGGTCTCCCTATAG-3′. The amplified cDNA s were inserted into pcDNA3 (Invitrogen), pEGFP-N1 (Clontech, Palo Alto, CA) and PEGFP-N1 (Clontech) to obtain the 110-kDa HDLBP, EGFP-fused 110-kDa HDLBP at the N- and C-terminals, respectively. Using Lipofectamine PLUS (Invitrogen), HeLa/Huh7 cells were transfected with an empty vector and a *150-kDa HDLBP* expression vector and were designated HeLa/Huh7 VC and HBP1/2, respectively. Huh7 cells were also transfected with a *110-kDa HDLBP* vector and designated 110-kDa HBP2.

sh*HDLBP1* and sh*HDLBP2* expression vectors that targeted the 1417–1437 and 3011–3031 regions, respectively, of *HDLBP* cDNA (NM_005336) were constructed using BLOCK-iT^{TM} Pol II miR RNAi Expression vector kits (Invitrogen) and the following pairs of top and bottom strands: sh*HDLBP1*, (top) 5′-TGCTGAGTCAACGCCT GACCTAACTTGTTTTGGCCATGACTGACAAGTTAGGAGGCGTTGACT-3′, (bottom) 5′-CCTGAGTCAACGCCTCCTAACTTGTCAGTCAGTGGCCAA AACAAGTTAGGTCAGGCGTTGACTC-3′; and sh*HDLBP*2, (top) 5′-TGC TGTCACGAATTTCACGGATCCGTGTTTTGGCCACTGACTGACACGGATC CGAAATTCGTGA-3′, (bottom) 5′-CCTGTCACGAATTTCGGATCCGTG TCAGTCAGTGGCCAAAACACGGATCCGTGAAATTCGTGAC-3′. HeLa cells were transfected with vacant vector and *shHDLBP1*/2 expression vectors by lipofection and were designated shC and sh1/2, respectively.

2.3. Preparation of cell membranes

Cells were suspended in Tris-buffered saline [(20 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing 0.5 mM of ethylenediaminetetraacetic acid, 1 mM of phenylmethanesulfonyl fluoride, protease inhibitor cocktail (Nacalai, Kyoto, Japan), 10 mM of NaF, and 1 mM of Na_3VO_4], homogenized with a Potter-Elvenjem homogenizer, and centrifuged at 800*g* for 10 min. The supernatants were centrifuged at 100,000*g* for 60 min, and the resulting pellets were solubilized in Tris-buffered saline containing 8 mM CHAPS by passing through a 22-gauge needle. The mixtures were centrifuged at 100,000*g* for 60 min, and the CHAPS-insoluble pellet was referred to lipid raft.

2.4. Detection of a cell surface receptor with aralin conjugated with Cy3, Cy5 or TAMRA

Aralin was purified from an *A. elata* young shoot [5] and labeled with Cy3/Cy5 (GE Healthcare Life Sciences, Uppsala, Sweden) or TAMRA (Molecular Probes, Eugene, OR). Cells attached to Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) were incubated with the labeled aralin, and examined under a fluorescence microscope.

2.5. Western blotting (WB)

Samples were subjected to 10% SDS–PAGE, transferred to a membrane and incubated with the following antibodies: anti-HDLBP (Abgent, San Diego, CA), anti-flotillin-1 (Santa Cruz Biotech., Santa Cruz, CA), anti-Na^+/K^+-ATPase (*ibid.*), anti-actin (*ibid.*), anti-Histone H1 (*ibid.*) or anti-GFP antibodies (Nacalai). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell signaling, Danvers, MA), HRP-conjugated anti-mouse IgG (Sigma–Aldrich, St. Louis, MO), or HRP-conjugated anti-goat IgG (Santa Cruz Biotech.). Signals were visualized with the ECL system (Amersham Biosciences, Buckinghamshire, UK).

2.6. Far Western blotting (FWB)

The blotted membranes were treated with 2 μg/ml of aralin and incubated with an anti-aralin antibody [5]. The membranes were visualized as described above.

2.7. Determination of the amino-acid sequence

The protein band corresponding to the 110-kDa band that was detected by FWB with the anti-aralin antibody was analyzed using lysyl endopeptidase and a nano-liquid chromatography–electrospray ionization mass spectrometry (LC/ESI/MS) system (Applied Biosystems, Carlsbad, CA). Peptide and protein identification were performed using Protein Pilot software, version 2.0 (Applied Biosystems). Each ESI/MS spectrum was searched for human species against the NCBI database.

2.8. Inhibition of tumorigenesis by aralin

The *A. elata* young shoots were homogenized in PBS (phosphate-buffered saline) and centrifuged at 8700*g* for 30 min at 4 °C. The resulting supernatant was used as the crude aralin extract. HeLa and its transfectants (1×10^6/200 μl PBS containing 50% Matrigel) were subcutaneously inoculated into the flanks of nude mice (BALB/cAJc1-nu/nu, Clea Japan, Tokyo, Japan). The crude extracts were orally injected into mice. Tumor volume was calculated as $0.52 \times (\text{short axis}^2 \times \text{long axis})$. Mouse care and handling conformed to the National Institutes of Health guidelines for animal research. The experimental protocols were approved by the Institutional Animal Care and Use Committee.

2.9. Statistics

The data are expressed as mean ± standard error (SE) of the indicated number of animals. Data comparisons were conducted with the Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Cell entry mechanism of aralin

HeLa cells were treated with 3.2 nM of Cy3-aralin at 4 °C for 15 min, Cy3-aralin, but not Cy3- bovine serum albumin (BSA), bound to the cell surface receptor. Binding was blocked by 10-fold excess unlabeled aralin, 5 mM Gal, lactose (Lac), and fucose but not mannose (Fig. 1A).

To elucidate whether aralin cell entry was mediated by the receptor, SCC-25 cells were treated with TAMRA-aralin for up to 120 min at 4 °C. After 15 min, TAMRA-aralin was observed on the cell surface, and after 120 min, aralin on the cell surface was

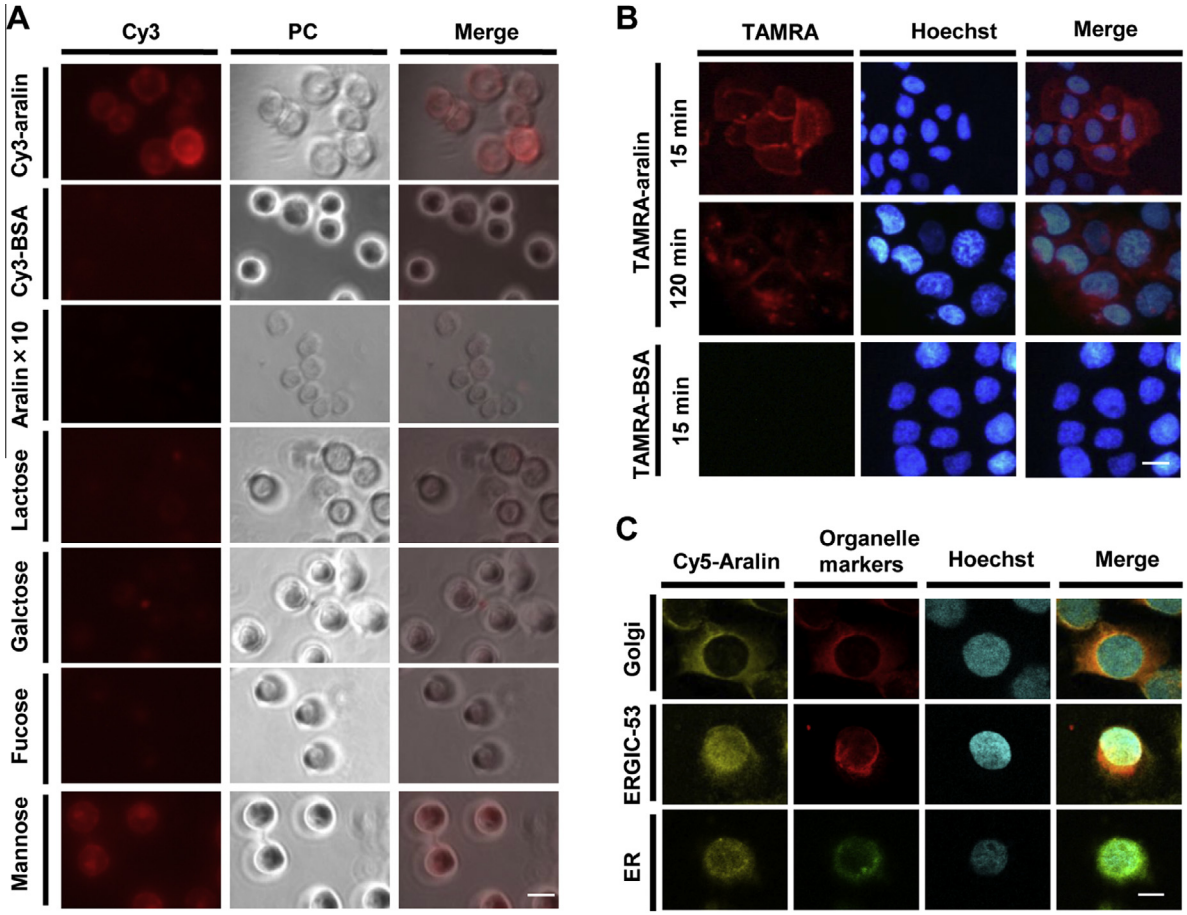

Fig. 1. Implication of cell surface receptors in aralin cell entry. (A) HeLa cells were treated with 3.2 nM Cy3-aralin for 15 min at 4 °C with or without 10-fold excess unlabeled aralin, 5 mM lactose, galactose, fucose, or mannose and examined under a fluorescent microscope. Cy3 and phase-contrast (PC) merged images are also shown. Cy3-bovine serum albumin was used as a negative control. (B) SCC-25 cells were treated with 3.2 nM of TAMRA-aralin at 4 °C up to 120 min. The nuclei were stained with Hoechst 33258. (C) Intracellular localization of aralin. The Golgi apparatus, the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC)-53 and ER in SCC-25 cells treated with Cy5-conjugated aralins for 45 min at 4 °C were visualized with BODIPY TR ceramide, anti-ERGIC-53/p58-Cy3™ and pEF/myc/ER/GFP, respectively. Bars indicate 10 μm.

reduced with diversion into the cytoplasm (Fig. 1B). To analyze the intracellular localization of aralin, the Golgi apparatus, the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC)-53 and ER were visualized using BODIPY TR ceramide (Molecular Probes), anti-ERGIC-53/p58-Cy3™ (Sigma–Aldrich) and the pEF/myc/ER/GFP expression vector (Invitrogen), respectively, together with Cy5-aralin (Fig. 1C). These results indicated that aralin was localized in the Golgi apparatus, ERGIC-53 and ER. The order of amount of Cy5-aralin was the Golgi apparatus > ERGIC-53 > ER, suggesting that aralin was incorporated into cells via cell surface receptor mediated endocytosis and diffused into the cytosol as did other type II RIPs [1].

3.2. Processed form of the 110-kDa HDLBP acted as an aralin receptor

The cytoplasmic and membrane fractions of HeLa cells were analyzed by FWB with the anti-aralin antibody. Aralin bound primarily to the 110-kDa band, and the binding was diminished in the presence of 5 mM of Lac (Fig. 2A). To identify the 110-kDa band, LC/ESI/MS analysis was performed. The obtained peptide sequences predicted that the 110-kDa band was a HDLBP.

The 110-kDa HDLBP was detected in the cytoplasm and CHAPS-insoluble membrane (lipid raft) fractions (Fig. 2B). In addition, FWB with the anti-aralin antibody showed that aralin bound to the 110-kDa HDLBP in a lipid raft. HeLa cells were treated with methyl-β-cyclodextrin (MβCD), which disintegrates membrane lipid rafts by depleting cholesterol [11]. The membrane cholesterol levels detected by filipin were dose-dependently reduced by MβCD (Fig. 2C). Lipid rafts were then prepared from the HeLa cells and determined the amount of the 110-kDa HDLBP by WB. The 110-kDa HDLBP levels in lipid rafts were IC_{50} reduced by MβCD, whereas the levels in the CHAPS-soluble membrane fractions were negligible (Fig. 2D). Thus, the 110-kDa HDLBP existed in membrane lipid rafts.

To test whether the expression level of the 110-kDa HDLBP determined aralin sensitivity, we analyzed the expression levels of the 110-kDa HDLBP in human normal Hs68, HeLa, Hep G2, and Huh7 cells with Cy3-aralin. The cell surface of HeLa cells was intensely labeled with Cy3-aralin, whereas binding was hardly detected in Huh7 and Hs68 cells (Fig. 2F). The expression levels of the 110-kDa HDLBP in lipid rafts in these cells were analyzed by FWB. Similar to the Cy3-aralin-labeling experiments, the order of expression of the 110-kDa HDLBP bound to aralin was HeLa > Hep G2 > Huh7 = Hs68 (Fig. 2G). IC_{50} values of HeLa, Hep G2, Huh7, and Hs68 were estimated to be 19.3, 36.4, >1000, and 902.5 ng/ml, respectively (Fig. 2H and I).

3.3. Gain-of- and loss-of-functional analyses of HDLBP for aralin sensitivity in vitro

To further elucidate the function of the 110-kDa HDLBP in selective cytotoxicity of aralin, the 150-kDa HDLBP-overexpressing

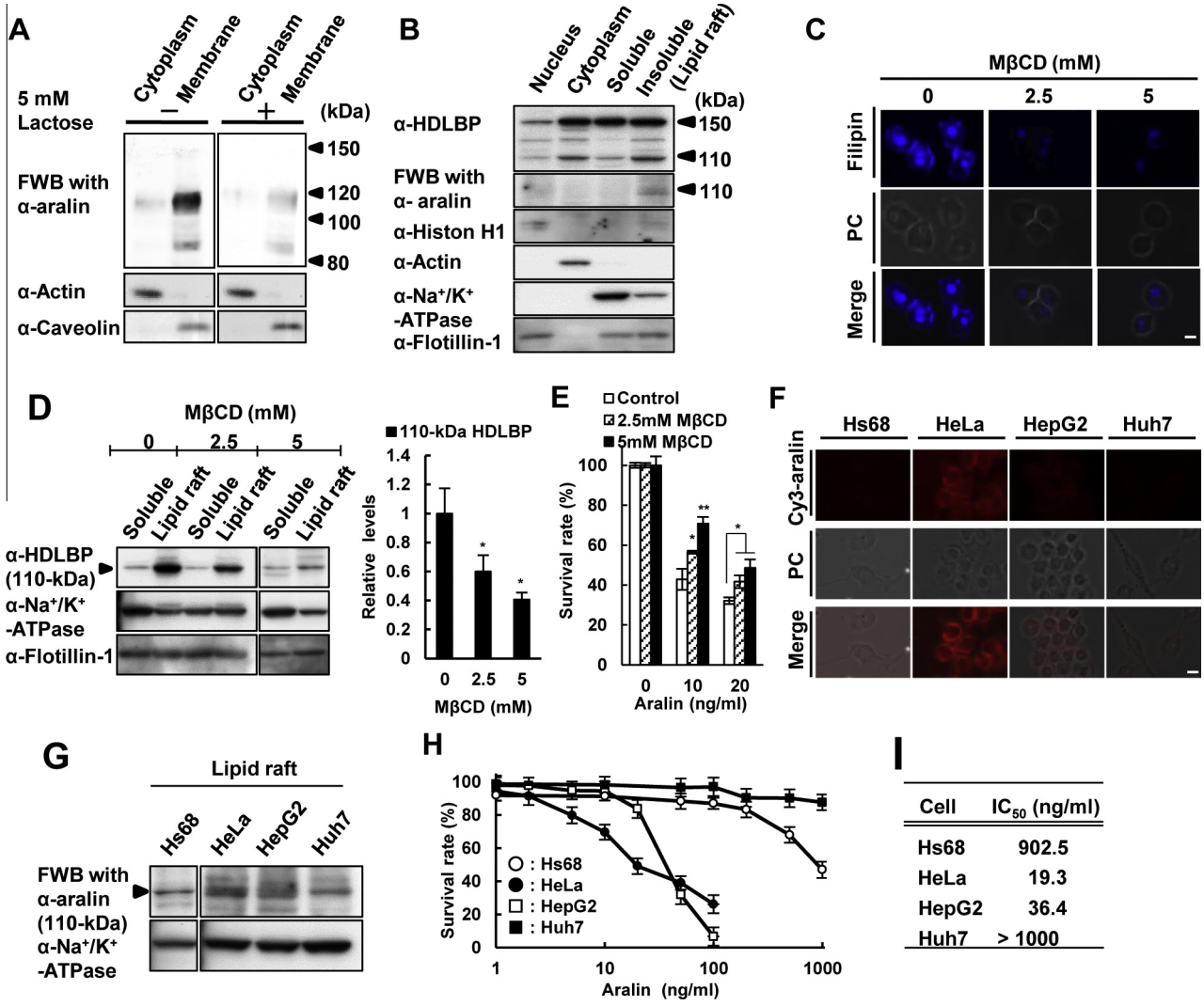

Fig. 2. Aralin specifically bound to the 110-kDa HDLBP in lipid rafts. (A) The cytosolic and membrane fractions from HeLa cells were analyzed by FWB with an anti-aralin antibody with or without 5 mM of lactose. The amino-acid sequence of the 110-kDa band was determined by nano-liquid chromatography-electrospray ionization mass spectrometry. (B) Intracellular localization of the 110-kDa HDLBP. The nuclear, cytoplasmic, and CHAPS-soluble/insoluble (lipid raft) membrane fractions were analyzed with WB and FWB. The expression levels of actin, histone H1, Na^+/K^+-ATPase, and flotillin-1 were analyzed by WB with the corresponding antibodies as markers of subcellular fractions and loading controls. (C and D) The 110-kDa HDLBP existed in lipid rafts. HeLa cells were treated with 2.5 and 5 mM methyl-β-cyclodextrin (MβCD) for 1 h at 37 °C to destruct the lipid raft. (C) The cells were then fixed with 1% glutaraldehyde and treated with 50 μg/ml filipin to visualize membrane cholesterols. (D) The CHAPS-soluble membrane/lipid raft fractions from HeLa cells treated with MβCD were analyzed with WB and quantified. *Significant difference from the untreated control ($P < 0.02$), $n = 3$. (E) Effect of MβCD on aralin sensitivity. Cells were treated with 2.5 and 5 mM MβCD and then additionally treated with aralin. Significant difference from the untreated control (*$P < 0.03$, **$P < 0.005$), $n = 3$. (F and G) Expression levels of the 110-kDa HDLBP lipid rafts determined aralin sensitivity. (F) Detection of the aralin cell surface receptor in Hs68, HeLa, Hep G2, and Huh7 cells with Cy3-aralin. (G) The expression levels of the aralin receptor in lipid rafts in these cells were determined by FWB. (H and I) Cytotoxic effects of aralin on cancer cells. Cells were treated with aralin (H) and estimated IC_{50} values (I). Bars indicate 10 μm.

HeLa subcell lines HBP1, HBP2, and vacant vector-introduced VC cells were established. Although the expression levels of the 150-kDa HDLBP in HBP1/2 cells were increased to approximately 1.4-fold of VC cells, the binding levels of Cy3-aralin on these cells were similar (Fig. 3A). Expectedly, the expression levels of the 110-kDa HDLBP in lipid rafts of HBP1/2 cells detected by FWB were unchanged (Fig. 3B). Aralin sensitivities of these transfectants were also similar (Fig. 3C), showing that forced expression of the 150-kDa HDLBP did not directly augment the 110-kDa HDLBP.

Conversely, in sh1 and sh2 cells, the binding levels of aralin with the cell surface were significantly reduced compared with that in shC cells (Fig. 3D). The expression levels of the 110-kDa HDLBP in lipid rafts of sh1 and sh2 cells detected by WB and FWB were reduced to approximately 40% and 20% of shC cells, respectively (Fig. 3E). As a result of the reduction of the 110-kDa HDLBP, the sensitivities of sh1 and sh2 cells against aralin were lowered to factors of 2.6 and 6.2, respectively, compared with that of shC cells (Fig. 3F)

Using aralin-resistant Huh7 cells, we analyzed the effects of ectopic expressions of the 150-kDa and 110-kDa HDLBP on the aralin sensitivity. The 150-kDa HDLBP vector-transfected Huh7 subcell lines HBP1/2 cells were established. Cy3-aralin binding levels and aralin sensitivities of these cells were similar to VC cells, even though the expression levels of the 150-kDa HDLBP HBP1/2 cells were 1.6-fold higher than that of VC cells (Fig. 3G–I).

Processing mechanism of the 150-kDa HDLBP to the smaller 110-kDa form is not yet known. To clarify which site is removed, *EGFP-tagged 150-kDa HDLBP* at the N- terminal (EGFP-HDLBP) and the C-terminal (HDLBP-EGFP) vectors were introduced into COS7 cells and cell lysates were analyzed by WB with anti-GFP

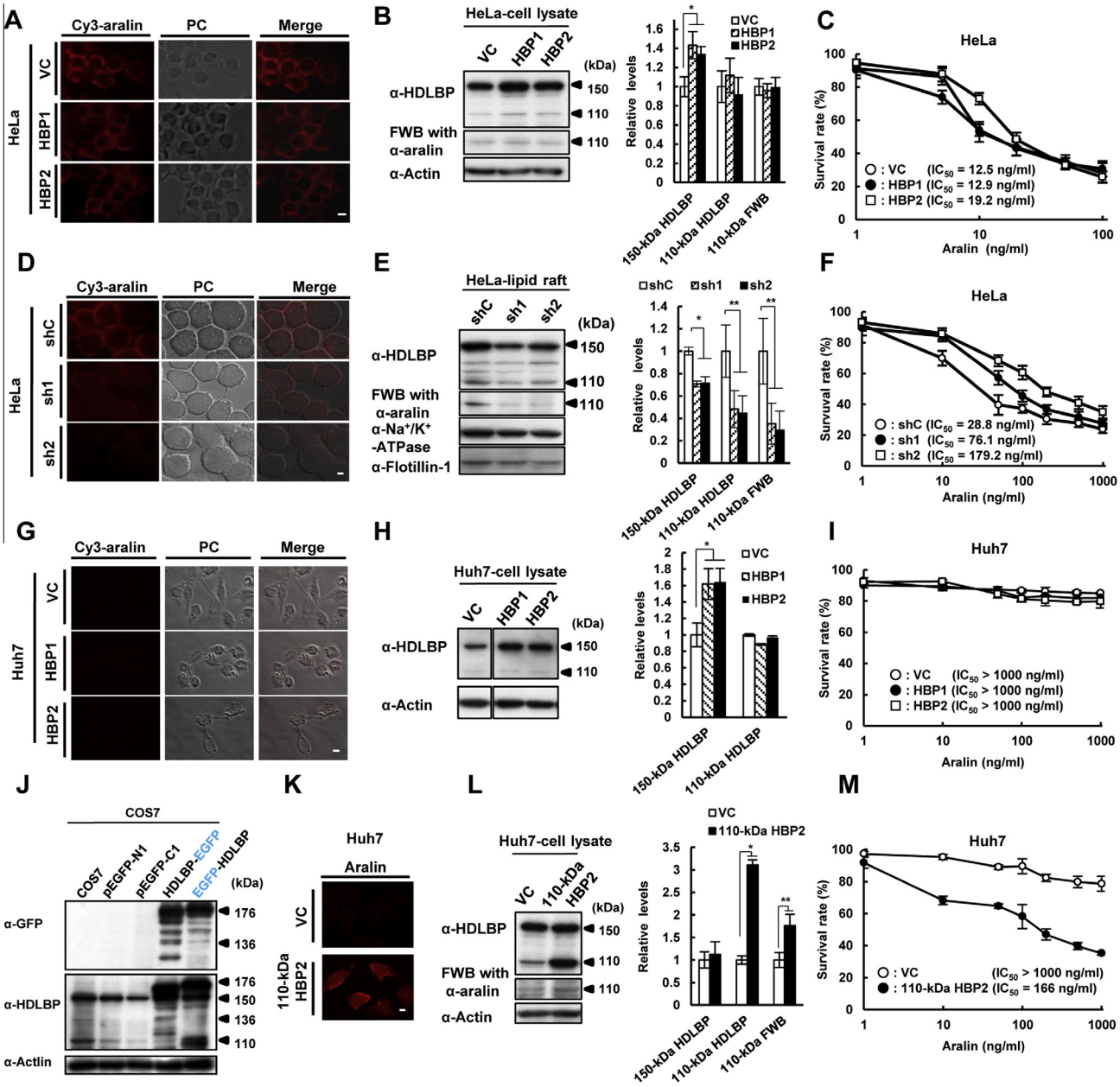

Fig. 3. Gain-of- and loss-of-functional analyses of HDLBP for aralin sensitivity *in vitro*. (A–C) Gain-of-functional analysis of HDLBP in HeLa cells. (A) Analysis of the aralin cell surface receptor. The aralin cell surface receptors in the 150-kDa HDLBP-overexpressing HeLa-derived HBP1/2 and VC cells were visualized with Cy3-aralin for 15 min at 4 °C. (B) The expression levels of HDLBP in HBP1/2 cells. The cell lysates of VC and HBP1/2 cells were analyzed by WB and FWB, and quantified. *Significant difference from VC cells ($P < 0.03$), $n = 3$. (C) No inhibitory effects of aralin on the 150-kDa HDLBP-overexpressing cells *in vitro*. VC and HBP1/2 cells were treated with aralin for 36 h. Their IC_{50} values are indicated in parentheses. (D–F) Loss-of-functional analysis of HDLBP. (D) Decrease of the cell surface receptor by HDLBP downregulation. shC and sh1/2 cells were incubated with Cy3-aralin. (E) The membrane lipid rafts were analyzed with WB and FWB and quantified. Significant difference from shC cells (*$P < 0.001$, **$P < 0.05$), $n = 3$. (F) shC and sh1/2 cells were treated with aralin, and their IC_{50} values were estimated. (G–I) Effects of the ectopic expression of the 150-kDa HDLBP on the aralin sensitivity of Huh7 cells. (G) Analysis of aralin receptor on the cell surface of the 150-kDa HDLBP-overexpressing Huh7 transfectants HBP1/2 cells was performed with Cy3-aralin. (H) Expression levels of the 150-kDa HDLBP in Huh7 transfectants were analyzed by WB and quantified. *Significant difference from VC cells ($P < 0.05$), $n = 3$. (I) Forced expression of the 150-kDa HDLBP in Huh7 cells had no effect on the aralin sensitivity. (J) The N-terminal region of the 150-kDa HDLBP was removed during processing steps. The 150-kDa HDLBP tagging with EGFP at the N-terminal (EGFP-HDLBP) and at the C-terminal (HDLBP-EGFP) were expressed in COS7 cells and analyzed by WB with anti-GFP and anti-HDLBP antibodies. The EGFP-fused 150-kDa and 110-kDa HDLBPs were expressed as 176 and 136-kDa proteins, respectively. pEGFP-N1/C1 were used as vector controls. (K–M) Ectopic expression of the 110-kDa HDLBP (Asp475-Arg1328) in Huh7 cells conferred aralin sensitivity. (K) The 110-kDa HDLBP-overexpressing Huh7 transfectant 110-kDa HBP2 and VC cells were analyzed with Cy3-aralin and the merged images are shown. (L) Analysis of expression levels of the 110-kDa HDLBP in VC and 110-kDa HBP2 cells. Cell lysates were analyzed with WB and quantified. *Significant difference from VC cells (*$P < 0.001$, **$P < 0.02$), $n = 3$. (M) Aralin sensitivity was acquired by ectopic expression of the 110-kDa HDLBP. Cells were treated with aralin and estimated IC_{50} values are indicated in parentheses. Bars indicate 10 μm.

and anti-HDLBP antibodies. Both fused proteins were expressed as 176-kDa proteins. The processed form of the 136-kDa EGFP-tagged 110-kDa HDLBP was substantially observed, when HDLBP-EGFP was expressed (Fig. 3J). These data suggested that the N-terminal region was removed from the 150-kDa HDLBP during the processing steps.

Then we constructed the 110-kDa HDLBP (Asp475-Arg1328) expression vector and introduced into aralin-resistant Huh7 cells. Huh7-derived 110-kDa HBP2 cells possessed a functional aralin receptor rather than VC cells (Fig. 3K). The expression level of the 110-kDa HDLBP in this transfectant determined by WB and FWB were significantly higher those in VC cells (Fig. 3L). As expected,

the 110-kDa HBP2 transfectant obtained aralin sensitivity and its IC_{50} value was estimated to be 166 ng/ml (Fig. 3M).

3.4. Orally administrated aralin inhibited tumorigenesis through HDLBP

To assess the anticancer activity of aralin *in vivo*, HeLa cells were subcutaneously injected into the flanks of nude mice. After 24 h, the crude extract of *A. elata* containing 2 μg aralin (equivalent to 100 μg/kg)/150 μl PBS/animal was orally administered thrice per week. After 3 weeks, the tumor volumes were measured. Orally administered aralin significantly suppressed HeLa cell tumorigenesis as it did *in vitro* (Fig. 4A and B). Using this system, we analyzed the inhibitory effects of aralin on the tumorigenesis of HeLa transfectants. When the inoculated cells developed palpable tumors, 1, 2, and 3 μg of aralin were administered in sequence for 19, 7, and 7 days, respectively. After 70 days, the volumes of VC-derived tumors treated with aralin were reduced to 60% of the PBS-injected controls, whereas the volumes of HBP1-originated tumors treated with aralin were diminished to 43% of the controls (Fig. 4C a/b, D a/b and Ea). The expression levels of the 110-kDa HDLBP in each of two tumors developed by VC and HBP1 cells were analyzed by WB. These results implied that, compared with those of VC-originated tumors, in HBP1-derived tumors, the 110-kDa HDLBP is more actively processed (Fig. 4F).

Conversely, sh2-originated tumors were highly resistant to aralin, as they were *in vitro*, whereas the inhibition rate of shC cells was estimated to be 65% of the PBS controls (Fig. 4C c/d and D c/

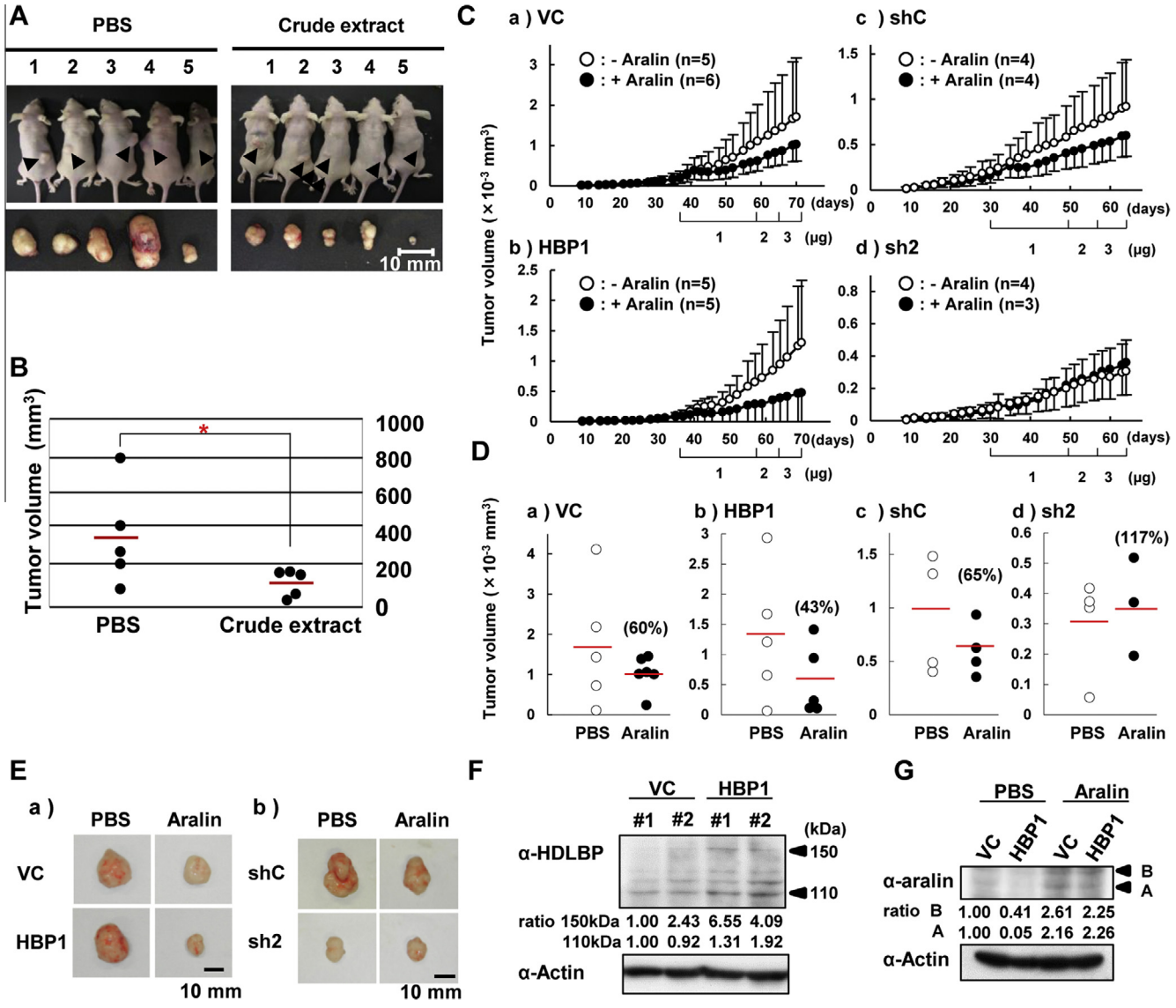

Fig. 4. Orally administered aralin inhibited tumor growth *in vivo* through high-density lipoprotein-binding protein (HDLBP). (A) HeLa cells (1×10^6 cells) were subcutaneously injected into 6-week-old nude mice flanks. After 24 h, the crude extract of *A. elata* containing 2 μg (equivalent to 100 μg/kg) aralin was orally administered thrice/week/animal through a stomach tube. After 3 weeks, the tumors were photographed. (B) The tumor volumes with or without aralin were quantified. *Significant difference from the PBS-treated control ($P < 0.05$), $n = 5$. (C–E) HDLBP expression levels affected aralin sensitivity *in vivo*. (C) When HeLa transfectants were developed to approximately 150–200 mm^3, the crude extracts containing 1, 2, and 3 μg of aralin/day/animal were orally administered to mice for 19, 7 and 7 days, respectively. (a) VC, (b) HBP1, (c) shC, and (d) sh2 cells-transplanted mice were treated with or without the crude extract. (D) (a) VC, (b) HBP1, (c) shC, and (d) sh2-derived tumor volumes with or without aralin were quantified. (E) Photographs of typical tumors by HeLa transfectants. At the final treatment, VC- and HBP1-derived tumors (a) and shC- and sh2-derived tumors (b) were photographed. (F) Enforced expression of the 150-kDa HDLBP in HeLa cells enhanced aralin sensitivity *in vivo*. Each of two tumors developed by VC and HBP1 cells were lysed and analyzed by WB. (G) Orally administered aralin was distributed to tumors. After 24 h of administration with PBS or 3 μg of aralin, VC- and HBP1-originated tumors were removed and analyzed by WB with anti-aralin antibody. A and B indicate the A-chain and B-chain of aralin, respectively.

d). Furthermore, the tumors formed by sh2 cells were significantly smaller than those of other transfectants (Fig. 4C d and E b).

To elucidate whether orally administered aralin was distributed to tumors, VC and HBP1-derived tumors were removed after the final treatment with 3 μg aralin and analyzed by WB with the anti-aralin antibody. The A-chain and B-chain of aralin were substantially detected (Fig. 4G), indicating that orally administered aralin was distributed to tumors *in vivo*.

4. Discussion

In this study, gain-of- and loss-of-functional analyses of HDLBP *in vitro* and *in vivo* showed that the 110-kDa HDLBP of HDL receptor in lipid rafts acted as an aralin receptor. This is the first demonstration that the 110-kDa HDLBP mediates RIP cell entry through endocytosis.

In this study, we observed that forced expression of the 150-kDa *HDLBP* RNA in HeLa cells did not affect the expression levels of the 110-kDa HDLBP or its sensitivity toward aralin *in vitro*, whereas the tumorigenesis of HBP1 cells was more efficiently suppressed compared with that of VC cells. In contrast, sh1/2 cells, which expressed reduced levels of the 110-kDa HDLBP in lipid rafts, were highly resistant to aralin *in vitro*. The tumorigenesis of sh2 cells was resistant. Furthermore, tumors formed by sh2 cells without aralin were significantly smaller than those formed by the vacant vector-introduced shC cells, showing that the 110-kDa HDLBP was essential for tumor growth. The processing pathway of HDLBP to functional HDLBP may be regulated by physiological conditions *in vivo* [7,8,10,11]. Therefore, the difference in the aralin sensitivities of HBP1 cells between *in vitro* and *in vivo* may be caused by differences in processing control.

Based on these data, we assume that the reduction of cytoplasmic cholesterol by forced expression of HDLBP triggers the excessive incorporation of cholesterol through the CLA-1 scavenger receptor in tumor cells, by which the abnormal growth and survival of tumor cells is maintained [12]. Alternatively, it is possible that protein kinase C is activated downstream of ApoA-I, which is a component of the HDL/HDLBP signaling pathway, resulting in rapid cell division [9].

The expression level of the 110-kDa HDLBP in HeLa cells were higher than those in Hep G2, Huh7, and normal Hs68 cells with a high aralin sensitivity, whereas the expression level of the 110-kDa HDLBP in Huh7 and Hs68 cells were extremely low with a high aralin resistance. Forced expression of the 110-kDa HDLBP (Asp475-Arg1328) in aralin-resistant Huh7 cells conferred aralin sensitivity, suggesting that, in the processing steps, the N-terminal region of the 150-kDa HDLBP is removed by endoprotease Asp-N. Thus, it was demonstrated that the specific target of aralin was the 110-kDa HDLBP, whose expression in lipid raft determined aralin sensitivity.

The young shoots of *A. elata* (the Japanese Angelia tree, taranoki in Japanese) are a popular food, particularly in the spring season. The root bark is a widely used folk medicine with tonic, anti-arthritic, and anti-diabetic effects. Taken together, our present data suggested that aralin could be a promising anticancer and cancer prevention drug for tumors in which HDLBP expression is deregulated.

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