RESEARCH ARTICLE

Local oncolytic adenovirotherapy produces an abscopal effect via tumor-derived extracellular vesicles

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Short title: Abscopal effect by OBP-301 via EVs

Abbreviations

EVs, extracellular vesicles; ICI, immune checkpoint inhibitor; RFP, red fluorescent

protein; PARP, poly(ADP ribose) polymerase; DAPI, 4',6-diamidino-2-phenylindole;

PFU, plaque-forming unit; MOI, multiplicity of infection; qRT-PCR, quantitative

reverse transcription polymerase chain reaction.

SUMMARY (within 200 words)

Extracellular vesicles (EVs) play important roles in various intercellular communication processes. The abscopal effect is an interesting phenomenon in cancer treatment, in which immune activation is generally considered a main factor. We previously developed a telomerase-specific oncolytic adenovirus, Telomelysin (OBP-301), and occasionally observed therapeutic effects on distal tumors after local treatment in immunodeficient mice. Here, we hypothesized that EVs may be involved in the abscopal effect of OBP-301. EVs isolated from the supernatant of HCT116 human colon carcinoma cells treated with OBP-301 were confirmed to contain OBP-301, and showed cytotoxic activity (apoptosis and autophagy) similar to OBP-301. In bilateral subcutaneous HCT116 and CT26 tumor models, intratumoral administration of OBP-301 produced potent antitumor effects on tumors that were not directly treated with OBP-301, involving direct mediation by tumor-derived EVs containing OBP-301. This indicates that immune activation is not the main factor in this abscopal effect. Moreover, tumor-derived EVs exhibited high tumor tropism in orthotopic HCT116 rectal tumors, in which adenovirus E1A and adenovirus type 5 proteins were observed in metastatic liver tumors after localized rectal tumor treatment. In conclusion, local treatment with OBP-301 has the potential to produce abscopal effects via tumor-derived EVs.

INTRODUCTION

Recently, extracellular vesicles (EVs) have garnered high levels of scientific interest, as they are well known to play important roles in various intercellular communication processes such as immune responses, delivery systems and others.[1,2] EVs are recognized to carry cellular components such as proteins, messenger RNA (mRNA), microRNA (miRNA), DNA, lipids and transcriptional factors. In the field of cancer research, the involvement of cancer-derived EVs in mediating paracrine signaling in the tumor microenvironment, as well as cancer development and metastasis, is now recognized.[3] Furthermore, certain molecules key to their functionality have been identified.[4-6] However, determining the origins and functions of specific EV subsets is difficult or impossible.[7,8] EVs consisting of various subtypes (in terms of size, electrical potential, density, morphology, marker expression and dependency for a specific enzyme) are divided into several categories, including exosome (30 - 150 nm in diameter) derived from the endocytic pathway.[9-11] Key enzymes for the biogenesis of exosomes include NSMASE2 and RAB27A, while the secretion of exosomes is blocked by exosome inhibitors such as the selective inhibitor of a neutral sphingomyelinase GW4869.[6,10,12]

The abscopal effect was first reported in 1953 as a phenomenon involving tumor regression at a site distal to the primary site of radiotherapy,[13] and its mechanism is currently thought to involve activation of the immune system.[14,15] This phenomenon has attracted more attention since the advent of immunotherapy drugs such as immune checkpoint inhibitors (ICIs).[16,17] In addition to radiotherapy, other localized treatments such as oncolytic virotherapy have also been reported to induce the abscopal effect and produce potent antitumor effects on metastatic tumors, especially in combination with ICIs.[18-20]

We previously developed a telomerase-specific oncolytic adenovirus, Telomelysin (OBP-301), which is designed to replicate selectively in tumor cells and induce oncolytic cell death.[21] The safety of intratumoral administration of Telomelysin in humans was proven in a phase I clinical trial performed in the US.[22] Based on its safety profile and promising preclinical results of combination therapy with ionizing radiation, a phase I/II clinical trial to evaluate the safety and efficacy of this combination in patients with esophageal cancer is currently underway in Japan.[23,24]

In the process of developing OBP-301, we occasionally observed therapeutic effects on metastatic tumors after local administration of OBP-301 to primary tumors in immunodeficient mouse models[25] that could not be explained by induction of the abscopal effect via immune activation. This suggested that unknown non-immune mechanisms were responsible for the abscopal effect of oncolytic virotherapy. Interestingly, there is a previous study that reported that purified exosomes from hepatitis C virus-infected cells harbored viral protein and RNA,[26] and entry mechanisms of virus-containing EVs, such as clathrin-mediated endocytosis, and their intracellular dynamics have been also reported.[27,28] In the present study, we hypothesized that EVs may be involved in the abscopal mechanism and investigated whether tumor-derived EVs secreted after local treatment with OBP-301 could induce antitumor effects in tumors distal to the direct administration site as well as the primary tumors.

RESULTS

OBP-301 is present in Exo301

We investigated whether OBP-301 or OBP-301-related components, such as DNA, were present in Exo301 (EVs secreted from OBP-301-treated cells) by using cultured

HCT116 cells *in vitro*. Transmission electron microscopy (TEM) demonstrated that both normal exosomes (Exo) and Exo301 were spherical in shape. The Exo301 preparation appeared to contain both adenovirus-containing and free adenovirus particles (Figure 1A). Immunofluorescence staining showed that adenoviruses (green) were diffusely present throughout the cell, and that some adenovirus appeared to be co-localized (yellow staining) with EVs (red) in the area surrounding the cells (Figure 1B). Dynamic light scattering (DLS) revealed that the average size of Exo301 was 125 nm in diameter, which was larger than Exo, with an average size of 89 nm in diameter (Figure 1C).

ExoCap® (EC) was used to extract exosomes from Exo301 (EC-Exo301) to avoid contamination (non-exosome substances and non-encapsulated adenovirus particles) associated with ultracentrifugation methods, and it was confirmed that EC preparations were not contaminated with non-encapsulated OBP-301 (EC-OBP-301) (Figure S1A). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that adenovirus E1A gene expression of EC-Exo301 was approximately 70% of Exo301 (Figure 1D). These findings suggest that OBP-301 or OBP-301-related components such as DNA and OBP-301-related proteins such as E1A (Figure S1B) were released from HCT116 cells, which are covered in EVs, after OBP-301 treatment.

Exo301 shows cytotoxic activity by mechanisms similar to OBP-301

We determined whether Exo301, which was thought to contain various components, such as viral particles, viral proteins, and viral DNAs (based on Figure 1), produced antitumor effects, following confirmation that OBP-301 produces dose-dependent cytotoxicity toward HCT116 cells (Figure S2A) and SW48 cells (Figure S2B). The titer of Exo301 isolated from HCT116 and SW48 cells was determined as 4.2×10^7 PFU and

1.3×10^8 PFU, respectively, indicating that Exo301 produced functional virus progenies in both cell types (Table 1). Cell viability assays showed that Exo301 produced potent cytotoxic effects on HCT116 and SW48 cells in a dose-dependent manner while Exo were ineffective (Figure 2A). Exo301 cytotoxicity was blocked by anti-CD63 antibody in a dose-dependent manner, reaching 60-70% inhibition on both cell lines at 1.0 μg/mL (Figure 2B) although anti-CD63 antibody itself did not significantly affect HCT116 cell growth (Figure S3). Similarly, EC-Exo301 produced a dose-dependent cytotoxic effect on HCT116 cells (Figure 2C). When Exo301 was treated with neutralizing antiserum to adenovirus type 5 (Ad5) to neutralize free OBP-301 or OBP-301 attached on the surface of EVs, cytotoxicity was inhibited approximately by half at 2.5 units of anti-Ad5 antiserum. However, cytotoxicity in the presence of 5 units was not different from baseline (0 units) values (Figure S4A), which may be due to cytotoxicity of the Ad5 neutralizing antiserum itself (Figure S4B).

Next, we compared the cytotoxic mechanisms of Exo301 and EC-Exo301 with OBP-301, which induces apoptosis and autophagy.[29] Western blot analysis demonstrated that treatment with both Exo301 and EC-Exo301 caused E1A induction, poly(ADP ribose) polymerase (PARP) cleavage, and p62 reduction (Figure 2D). These results indicate that Exo301 and EC-Exo301 produced similar effects on adenovirus replication, apoptosis, and autophagy. Determination of adenovirus replication efficacy after Exo301 or EC-Exo301 treatment of HCT116 cells (measured by qRT-PCR) indicated that adenovirus E1A gene expression after EC-Exo301 treatment increased at a constant rate until 72 h, while the replication rate after OBP-301 or Exo301 treatment slowed after 24 h (Figure 2E). This resulted in E1A gene expression after EC-Exo301 treatment being significantly higher than OBP-301 and Exo301 at 72 h (Figure 2E).

Western blot analysis showed that E1A protein expression after EC-Exo301 treatment of HCT116 cells continued until 72 h while E1A expression was decreased at 72 h compared to 24 h after OBP-301 or Exo301 treatment (Figure 2F); this observation is generally consistent with the PCR findings described above. These findings suggested that Exo301 possesses antitumor activity that is mechanistically similar to OBP-301. Moreover, 60-70% of this effect was observed following treatment with EC-Exo301, which contains only OBP-301-containing EVs or OBP-301-related components such as DNA.

Tumor-derived materials are delivered to distal non-tumor sites via tumor-derived EVs

Initial evaluation of the effect of the exosome secretion inhibitor GW4869 on cell growth and viral replication indicated that GW4869 produced cytotoxicity in a dose-dependent manner (Figure S5A), but did not cause apoptosis even at a high dose (Figure S5B) and did not affect viral replication (Figure S5C). We used an *in vitro* co-culture model to determine if RFP protein (representative tumor-derived material) expressed in HCT116-RFP cells seeded on the bottom chamber were delivered to HCT116 cells seeded on the upper chamber via EVs (Figure 3A). Western blot analysis of EVs isolated from the supernatant of HCT116-RFP cells (bottom chamber) treated with either OBP-301 or PBS showed that OBP-301 treatment increased RFP secretion compared to PBS (Figure 3B). Western blot analysis also showed that the exosome marker CD9 was increased by treatment with 100 multiplicity of infection (MOI) of OBP-301, which was dose-dependently inhibited by GW4869 (Figure 3C). Flow cytometry analysis of RFP uptake in HCT116 cells in the upper chamber showed that

RFP uptake was increased by treatment with 100 MOI of OBP-301 on the bottom

chamber, which was significantly inhibited by addition of 20 μM GW4869; however, no

significant difference was observed in the presence of 10 μM GW4869 (Figure 3D, 3E).

These findings suggest that RFP proteins secreted from primary HCT116-RFP cells

were delivered to distal HCT116 cells via EVs, whose secretion was strongly increased

by OBP-301 treatment.

Localized treatment with OBP-301 produces abscopal effects via tumor-derived

EVs by a non-immune activation mechanism

We performed *in vivo* experiments to determine whether tumor-derived materials (OBP-

301 or OBP-related components) were delivered to distal tumors via EVs after local

treatment of primary tumors with OBP-301, and whether the EVs produced an abscopal

effect on the distal tumors. A bilateral subcutaneous tumor model using HCT116-RFP

and HCT116 was established in BALB/c nu/nu mice. The HCT116-RFP tumors

received three intratumoral treatments with OBP-301 or PBS, with or without

concurrent intraperitoneal administration of GW4869. In contrast, the HCT116 tumors

did not receive intratumoral treatments with either OBP-301 or PBS (Figure 4A). IHC

staining of HCT116 tumors demonstrated that RFP, E1A, and adenovirus type 5 (Ad5)

including capsid proteins were strongly expressed after OBP-301 treatment of the

HCT116-RFP tumors, effects that were blocked by GW4869 treatment (Figure 4B, 4C,

4D). In a bilateral HCT116 tumor model using BALB/c nu/nu mice, the antitumor

effects of three intratumoral administrations of OBP-301 or PBS, with or without

concurrent intraperitoneal GW4869 administration, were measured in the OBP-301-

treated tumor (treated tumor) and the tumors not directly treated with OBP-301

(untreated tumor) (Figure 4E). While OBP-301 treatment produced potent antitumor effects on treated tumors, which was not blocked by GW4869, OBP-301 treatment also produced strong antitumor effects on the untreated tumors, which was almost completely nullified by GW4869 (Figure 4F, Figure S6). Following confirmation of replication capability and dose-dependent cytotoxic activity of OBP-301 on CT26 cells *in vitro* (Figure S2C, S2D), the previous experiment was repeated in a CT26 bilateral tumor model using immunocompetent BALB/c mice (Figure 4G). Consistent with results in the HCT116 tumors, the antitumor effects of OBP-301 on untreated CT26 tumors were significantly blocked by GW4869, although not to the extent observed in the HCT116 tumors (Figure 4H). Furthermore, when the same experiment was conducted using immunodeficient NOD/SCID mice, which have a low natural killer (NK) cell activity (Figure 4I), the antitumor effects of OBP-301 on untreated tumors were also significantly inhibited by GW4869, which indicates that NK cell-mediated effects were not significant (Figure 4J). These findings suggest that OBP-301 or OBP-301-related components were delivered to distal tumors via tumor-derived EVs and produced an abscopal effect. Interestingly, the underlying mechanism of action does not appear to require systemic immune activation, which is currently believed to be the primary mechanism of the abscopal effect.

Tumor-derived EVs exhibit tumor tropism

The possibility of tumor-derived EVs exhibiting tumor tropism was investigated in *in vitro* and *in vivo* experiments. An *in vitro* triple co-culture model, in which HCT116-RFP cells were seeded on the bottom chamber and HCT116 and FEF3 cells were separately seeded on upper chambers was used to evaluate tumor tropism. Here, FEF

cells were used as non-transformed control cells, and RFP uptake in HCT116 or FEF3 cells after OBP-301 treatment of HCT116-RFP cells was evaluated (Figure 5A). Flow cytometry showed that accumulation of RFP protein secreted from HCT116-RFP cells after OBP-301 treatment was significantly greater in HCT116 cells than in FEF3 cells (Figure 5B). In contrast, a triple co-culture model, in which FEF3 cells stained with CellTracker Green (FEF3-Green) were seeded on the bottom chamber and treated with OBP-301, was used to show greater accumulation of green fluorescence secreted from FEF3-Green cells in FEF3 cells than in HCT116 cells (Figure S7). Moreover, addition of EVs isolated from the supernatant of OBP-301-treated HCT116 cells (HCT116 Exo) to a co-culture system of HCT116 and FEF3 cells resulted in co-localization of HCT116-derived EVs with HCT116 cells but not FEF3 cells (Figure 5C).

An orthotopic rectal tumor (HCT116-RFP) model with liver metastasis (HCT116-Luc) was used to evaluate tumor tropism of tumor-derived EVs *in vivo*. In this model, rectal HCT116-RFP tumors received intratumoral administration of OBP-301 or PBS, with and without concurrent intraperitoneal administration of GW4869, and primary rectal tumors, metastatic liver tumors and major organs were harvested to evaluate the biodistribution of RFP, E1A, and Ad5 (Figure 5D). IHC staining of RFP demonstrated that significant levels of RFP protein were detected in metastatic liver tumors after OBP-301 treatment, which was blocked by GW4869, whereas GW4869 did not affect RFP expression in the primary rectal tumors (Figure 5E, Figure S8A). In contrast, IHC analysis demonstrated that E1A and Ad5 proteins were significantly detected in both the primary rectal tumors and the metastatic liver tumors after OBP-301 treatment, which was blocked by GW4869 especially in the metastatic tumors (Figure 5F, 5G, Figure S8B, S8C). Uptake of RFP, E1A, and Ad5 in major organs was

relatively weak, surprisingly even in liver, compared to the primary tumor and metastatic tumors. These findings suggested that tumor-derived EVs exhibit strong tumor tropism, which can contribute to the abscopal effect of OBP-301.

DISCUSSION

The abscopal effect is widely believed to be caused by systemic activation of a cancer immune response, although the precise mechanisms remain unknown. As with radiotherapy, oncolytic virotherapy has been recently recognized as a promising treatment strategy that induces an abscopal effect in addition to its strong local effects.[30] We recently reported that our telomerase-specific oncolytic adenovirus produced an abscopal effect on murine cancer cells in *in vivo* experiments using immunocompetent mice, and it synergistically enhanced antitumor effects with ICIs.[20] We characterized the immune modulation produced by the oncolytic adenovirus as a mechanism for the abscopal effect, and observed significant recruitment of CD8-positive T lymphocytes in distal tumors, as well as the primary tumors to which the oncolytic adenovirus had been directly administered.[20] Notably, we observed that this effect was occasionally observed in *in vivo* experiments using immunodeficient mice, which prompted us to examine additional mechanisms for the abscopal effect.

While EVs have recently become a highly topical research field, we are not aware of any previous reports describing the involvement of EVs in the biology of adenoviruses, especially in the process of spreading viral progeny from infected cells to surrounding cells or tissues. Although we hypothesized that OBP-301 may be delivered to distal sites via EVs, one of the biggest challenges in this study was to conclusively demonstrate that OBP-301 was present within tumor-derived EVs secreted after OBP-

301 treatment. This is because EVs and adenovirus particles are very similar in size and are very difficult to separate with the commonly used ultracentrifugation method. Thus, EVs prepared by ultracentrifugation are expected to contain free OBP-301 particles that are present in the culture medium, which was shown in TEM images (Figure 1A). Because of this, there was a concern that the cytotoxic effects of Exo301 would be unrelated to the EVs and attributable to free OBP-301. We addressed this concern in the following two ways. We used the magnetic-bead-based ExoCap® system to extract purified EVs, as well as an antibody toward the exosome surface marker CD63 to physically block cellular uptake of EVs.[31] A cell viability assay showed that ExoCap®-purified Exo301 (EC-Exo301) produced a potent cytotoxic effect against HCT116 cells (Figure 2C). Moreover, anti-CD63 blocked 60-70% of the cytotoxicity of Exo301 (Figure 2B), which was consistent with PCR data that demonstrated adenovirus E1A copy number of EC-Exo301 was approximately 70% of Exo301 (Figure 1D). Western blot analysis was used to examine the cytotoxic mechanisms and demonstrated that both Exo301 and EC-Exo301 showed induction of autophagy and apoptosis in HCT116 cells, which was mechanistically similar to OBP-301 (Figure 2D, 2F). This suggests that Exo301 contained viable OBP-301 or DNA producing viable OBP-301. Although the particle size of Exo301 was slightly larger than normal Exo, this is considered reasonable if EVs contained an adenovirus particle of similar size to that of EVs (Figure 1C). Based on these findings, we were confident that tumor-derived EVs contained OBP-301 or OBP-301-related components and were responsible for the observed cytotoxic activity. However, EVs production by cells is unstable and varies depending on cell types, culture conditions, hypoxia, and treatment, which makes EVs-related research challenging.[32]

While the *in vivo* evidence that local treatment with OBP-301 produced potent antitumor activity on distal tumors via EVs as well as on the primary tumor was very definitive, another intriguing observation in this study was the tumor tropism of tumor-derived EVs. There appears to be no further reports regarding tumor tropism of tumor-derived EVs, with respect to key tropism-mediating molecules, beyond a 2015 report describing a possible mechanism for metastatic organotropism via tumor exosome integrins.[6] Although we have not been able to identify a mechanism in this study, we confirmed the phenomenon of tumor-derived EVs having tumor tropism, as has been previously described.[33-35] Most notably, EVs secreted from primary rectal tumors showed surprisingly high tumor tropism in an orthotopic rectal tumor model with liver metastasis. Here, tumor tropism was confirmed by the high uptake of tumor-derived RFP and adenovirus E1A in metastatic liver tumors after OBP-301 treatment of the primary rectal tumor. In comparison, other major organs (liver and spleen) exhibited little to no uptake of RFP, E1A and Ad5 (Figure 5E, 5F, 5G). Typically, when oncolytic adenoviruses such as OBP-301 are systemically administered *in vivo* they are non-specifically trapped in the reticuloendothelial system of the liver and spleen.[36,37] Thus, the results of the present study suggest that tumor-derived EVs imparted a masking property upon OBP-301. This further suggests that tumor-derived EVs offer great promise as a drug carrier for cancer therapeutics, which is expected to become an intriguing topic in the field of drug delivery systems.

In the present study, we have revealed a novel aspect of EVs, namely that tumor-derived EVs secreted following OBP-301 treatment can play an important role in the abscopal effect of OBP-301, which appears to be mediated through the direct delivery of OBP-301 to the metastatic site, rather than by indirectly activating a systemic

immune response. Although a lot of work is needed to clarify the detailed mechanisms, the observed abscopal effect represents a therapeutic mechanism that can be widely applied to other oncolytic viruses.[38] Therefore, this study represents an important step toward initiating subsequent breakthroughs in the development of cancer therapeutics.

MATERIALS AND METHODS

Reagents and oncolytic adenovirus

A PKH26 Red Fluorescent Cell Linker Mini Kit (cat. MINI26-1KT; Sigma-Aldrich, St. Louis, MO, USA) was used to stain EVs. CellTracker™ Green CMFDA (cat. C7025; Invitrogen, Waltham, MA, USA) and CellTracker™ Blue CMAC (cat. C2110; Invitrogen) were used to stain HCT116 and FEF3 cells. ExoCap™ Streptavidin CD9/CD63/CD81 Set (cat. MEX-SA123; MBL International, Woburn, MA, USA) were used to extract pure EVs. GW4869 (Sigma-Aldrich) was used to inhibit exosome secretion, which has been utilized in both in vitro and in vivo studies.[39,40] Adeno-X™ Rapid Titer Kit (cat. 632250; Clontech Laboratories, Inc., CA, USA) was used for titer determination. Telomelysin (OBP-301) is a telomerase-specific oncolytic adenovirus in which the hTERT promoter drives the E1A and E1B genes linked with an internal ribosome entry site.[21] OBP-301 is designed to replicate in tumors, many of which have high telomerase activity, and lead to oncolytic cell death; however, OBP-301 does not replicate in normal tissues.

Cell lines and culture

The human colon carcinoma cell lines HCT116 and SW48, HCT116 expressing red fluorescent protein (HCT116-RFP), HCT116 expressing firefly luciferase (HCT116-

Luc), the murine colon carcinoma cell line CT26, and normal human fibroblast cells (FEF3) were used in this study. HCT116 and CT26 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), HCT116-RFP and SW48 was obtained from AntiCancer, Inc. (San Diego, CA, USA), HCT116-Luc was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Japan), SW48 was purchased from Horizon Discovery (UK), and FEF3 was obtained from the Wister Institute (Philadelphia, PA, USA). HCT116 and HCT116-Luc were cultured in McCoy's 5A medium, HCT116-RFP and SW48 were cultured in RPMI1640 medium, and CT26 and FEF3 were cultured in DMEM medium. All culture media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (100 U/mL).

Isolation and characterization of EVs

Normal EVs were isolated by ultracentrifugation of supernatants collected after cell culture in FBS-free medium for 48 h (Exo). EVs were also isolated by the same process following OBP-301 treatment for 24 h (Exo301) of HCT116 cells at 35 MOI and SW48 cells at 5 MOI. Briefly, the ultracentrifugation method involved centrifuging the collected supernatants at 2,000 × g for 10 min to remove cells and debris, followed by another centrifugation at 100,000 × g for 70 min at 4 °C after filtration through a 0.22 μm filter. The pellets were then rinsed with phosphate buffered saline (PBS), ultra-centrifuged again with the same conditions, and dispersed in PBS.

Protein concentrations were measured using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). Exosome sizes were measured using a dynamic light scattering method with a Zetasizer

Nano ZSP (Malvern Instruments, Malvern, UK). Exosome morphology and structure were visualized using a transmission electron microscope (TEM) (H-7560, HITACHI, Hitachi, Japan).

Cell viability assay

HCT116 and SW48 cells (3.0×10^3 cells/well in a 96-well plate) were treated with various concentrations of Exo, Exo301, or OBP-301 (n=5). Cell viability was determined 3 days after treatment using a Cell Proliferation Kit II (XTT) (Roche, Basel, Switzerland) according to the manufacturer's protocol. The inhibition assay involved adding anti-CD63 antibody (1:1000, cat. 10628D, Invitrogen) to the culture medium to inhibit exosome function. The percentage of viable cells relative to untreated cells (0 μg/mL) was plotted. All error bars indicate 95% confidence intervals.

Western blot analysis

Proteins (20 μg) extracted from cells or EVs were electrophoresed on 10% SDS-polyacrylamide gels and transferred to Hybond-polyvinylidene difluoride membranes (GE Healthcare UK Ltd., Amersham, UK). The membranes were incubated overnight at 4°C with primary antibodies against E1A (1:500, cat. 554155; BD Pharmingen, San Jose, CA, USA), PARP (1:1000, cat. 9542; Cell Signaling Technology, Danvers, MA, USA), p62 (1:1000, cat. 5114; Cell Signaling Technology), β-actin (1:5000, cat. A-5441; Sigma-Aldrich), CD9 (1:1000, cat. 10626D; Invitrogen), and RFP (1:1000, cat. R10367; Invitrogen), followed by incubation with secondary antibodies for 1 h at room temperature. The Amersham ECL chemiluminescence system (GE Healthcare UK Ltd.) was used to detect the peroxidase activity of the bound antibody.

Immunofluorescence staining

HCT116 cells treated with 50 MOI of OBP-301 or PBS were incubated with anti-CD9

antibody (1:500, cat. 10626D; Invitrogen) overnight at 4°C after blocking with

Blocking-One (Nacalai Tesque, Kyoto), followed by incubation with FITC-conjugated

anti-adenovirus antibody (Hexon) (1:200, cat. AB1056F; CHEMICON, Temecula, CA,

USA) or anti-mouse IgG Alexa Fluor 647 secondary antibody (1:1000, cat. A21237;

Invitrogen). DAPI (4',6-diamidino-2-phenylindole) (1:1000, cat. D1306; Invitrogen)

was used for DNA staining. Colocalization of CD9 and adenovirus was observed using

a LMS780 laser scanning confocal microscope (Carl Zeiss, Tokyo, Japan).

Flow cytometry

To evaluate cellular uptake of EVs, a non-contact coculture system was used, in which

HCT116-RFP cells were seeded in the lower chamber, and HCT116 and/or FEF3 cells

were individually seeded in the upper chambers. HCT116-RFP cells were treated with

OBP-301 or PBS, and the culture medium was changed to FBS-free medium 24 h after

treatment. HCT116 cells and/or FEF3 cells in the upper chambers were subjected to

flow cytometry (BD FACSLyric, BD Biosciences, San Jose, CA, USA) 48 h after the

medium change.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis
Total RNA was extracted from cells using the miRNeasy Mini Kit (Qiagen, Valencia,

CA, USA) after cell lysis, and from EVs using the Total Exosome RNA & Protein

Isolation Kit (Invitrogen). Reverse transcription reactions were performed at 22°C for

10 min, 42°C for 15 min, 95°C for 5 min, and 4°C for 5 min. PCR amplification was conducted with 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s. Data analysis was performed using a StepOnePlus^{TM} real-time PCR system (Applied Biosystems, Waltham, MA, USA). The relative gene expression was determined using the ΔΔCT method. Primers and probes were predesigned by the manufacturer (Applied Biosystems). TaqMan E1A probe: 5'-CTGTGTCTAGAGAATGC-MGB-3', TaqMan E1A primers: E1A-F 5'-CCTGAGACGCCCGACATC-3', E1A-R 5'-GGACCGGAGTCACAGCTATCC-3'.

In vivo experiments

To evaluate the delivery of tumor-derived RFP via EVs, a bilateral subcutaneous tumor model was established by implanting 2×10^6 HCT116-RFP cells in one flank and 2×10^6 HCT116 cells in the other flank of 5-week-old female immunodeficient BALB/c nude mice. When HCT116-RFP tumors reached ≈ 200 mm^3 in volume, HCT116-RFP tumors were treated with intratumoral administration (i.t.) of PBS, intraperitoneal administration (i.p.) of GW4869 (2.5 µg/g), OBP-301 (i.t., 1×10^8 plaque-forming unit [PFU]), or OBP-301 (i.t., 1×10^8 PFU) + GW4869 (2.5 µg/g) (i.p. 6 h before OBP-301 treatment) three times every two days while HCT116 tumors were not treated directly with OBP-301. Three days after the last treatment, the mice were sacrificed and all tumors were harvested and fixed in formalin (n=8).

To evaluate the antitumor effects of OBP-301, a bilateral subcutaneous tumor model was established by implanting 2×10^6 HCT116 cells in both flanks of 5-week-old female immunodeficient BALB/c nude mice (n=9-10), or implanting 1×10^6 CT26 cells in both flanks of 5-week-old female immunocompetent BALB/c mice (n=9-10) or

NOD/SCID mice (n=4). When the larger of the two tumors reached 50-100 mm^3, the larger tumors were treated with PBS (i.t.), GW4869 (i.p., 2.5 µg/g), OBP-301 (i.t., 1 × 10^8 PFU), and OBP-301 (i.t., 1 × 10^8 PFU) + GW4869 (2.5 µg/g) (i.p. 6 h before OBP-301 treatment) three times every two days. The smaller tumors were not treated directly with OBP-301, and tumors were monitored until 28 days after the initiation of treatment or tumors exceeded 4,000 mm^3. The perpendicular diameter of each tumor was measured twice a week until 28 days after the initiation of treatment, and tumor volume was calculated using the following formula: tumor volume (mm^3) = $a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant used to calculate the volume of an ellipsoid.

For the orthotopic rectal tumor model with liver metastasis, 2×10^6 HCT116-RFP cells were implanted into the rectum and 4×10^6 HCT116-Luc cells were injected into the superior mesenteric vein of 5-week-old female BALB/c nude mice. When the luminescence intensity after luciferin injection of the upper abdominal region, measured using an IVIS Imaging System (Xenogen, Alameda, CA, USA), reached 1.0 E+04, the HCT116-RFP rectal tumors were treated with PBS (i.t.), GW4869 (i.p., 2.5 µg/g), OBP-301 (i.t., 1 × 10^8 PFU), and OBP-301 (i.t., 1 × 10^8 PFU) + GW4869 (2.5 µg/g) (i.p. 6 h before OBP-301 treatment) three times every two days. Three days after the last treatment, the mice were sacrificed, and all main organs (primary rectal tumor, metastatic liver tumor, brain, heart, lung, liver, kidney, and spleen) were harvested and subjected to immunohistochemical (IHC) staining to determine the biodistribution of tumor-derived RFP, adenoviral E1A and Ad5 proteins (n=4-5).

Mice were maintained in specific pathogen-free conditions in the animal laboratory of Okayama University. All animal protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue samples cut at 2 μm were deparaffinized and soaked in 0.3% H_2O_2 for 10 min at room temperature to extinguish endogenous peroxidase activity. After antigen retrieval by heating in 10 mM sodium citrate (pH 6.0) buffer solution or 10% EDTA using a microwave for 14 min, the samples were incubated with primary antibodies against RFP (1:1000, cat. R10367; Invitrogen), E1A (1:200, cat. bs-7697R; Bioss Inc., Woburn, MA, USA) and adenovirus type 5 (Ad5) (1:200, cat. ab6982; abcam, Cambridge, UK) overnight at 4°C, and then with peroxidase-linked secondary antibody for 30 min at room temperature. After washing, the samples were stained with 3,3'-diaminobenzidine (Dako, Santa Clara, CA, USA) for visualization, and counterstained with Meyer's hematoxylin.

Statistical analysis

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is modified version of R commander that was designed to add frequently used biostatistical functions. The Pearson Chi-square test or the Fisher exact test was used for categorical variables, and the Mann-Whitney *U* test was used for continuous variables. A probability (*P*) value less than 0.05 was considered statistically significant.

AUTHOR CONTRIBUTIONS

Y.K. and S. Kuroda designed the experiments and wrote the manuscript. Y.K., N.K., K.K., T.T., M.H., C.Y., R.S., and Y.H. conducted the experiments. S. Kuroda, S. Kikuchi, M.N., S. Kagawa, H.T., and T.F. assisted in interpretation of the results. Y.U. provided reagents and all authors reviewed the manuscript.

CONFLICT OF INTERESTS

Y.U. is President and CEO of Oncolys BioPharma, Inc., the manufacturer of OBP-301 (Telomelysin). H.T. and T.F. are consultants of Oncolys BioPharma, Inc. The other authors have no competing interests to disclose.

KEY WORDS

Extracellular vesicles, Exosome, Abscopal effect, Oncolytic adenovirus, Local treatment, Systemic delivery, Drug delivery system.

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Table 1. Titer of Exo301

Cell line from which Exo301 was isolated	Titer (PFU)
HCT116	4.2×10^7
SW48	1.3×10^8

PFU, plaque forming unit.

FIGURE LEGENDS

Figure 1. Characterization of EVs

(A) Representative TEM images of EVs isolated from HCT116 cells (Exo) and HCT116 cells treated with OBP-301 (Exo301). Scale bar, 100 nm. (B) HCT116 cells treated with PBS or OBP-301 (50 MOI) were subjected to immunofluorescence staining for CD9 (exosome marker, red), Hexon (adenovirus marker, green) and DAPI (nuclear marker, blue) 24 h after treatment. The right bottom image is a magnified view of the boxed region in the merged image. Scale bars, 20 μm (left upper) and 2 μm (right bottom). (C) Particle size of Exo and Exo301 were measured by DLS. (D) DNAs extracted from EC, Exo301, and EC-Exo301 were subjected to qRT-PCR for the adenovirus E1A gene (n=3). E1A copy numbers are expressed as fold change relative to Exo301. * $p<0.001$.

Figure 2. *In vitro* cytotoxic activity of Exo301

(A) Viability of HCT116 and SW48 cells 3 days after treatment with Exo or Exo301 at the indicated concentrations were assessed using the XTT assay (n=5). (B) Anti-CD63 antibody or IgG (for control) was added to the culture medium at the indicated concentrations together with Exo301 to inhibit exosome function, and the viability of HCT116 and SW48 cells was subsequently assessed using the XTT assay 3 days after treatment (n=5). (C) Viability of HCT116 cells treated with EC or EC-Exo301 was assessed using the XTT assay 3 days after treatment (n=5). (D) HCT116 cells were harvested 48 h after treatment with an IC_{25} dose of OBP-301, Exo301 or EC-Exo301, or a dose of EC that was equivalent to that used in the EC-Exo301 isolation process. Whole cell HCT116 lysates were then subjected to western blot analysis for PARP, p62, E1A and β-actin. (E) HCT116 cells were treated with OBP-301, Exo301, or EC-Exo301

for 2 h and were harvested at the indicated time points after removing the treatments.

The extracted DNA was subjected to qRT-PCR analysis of adenovirus E1A gene levels

(n=3). E1A copy numbers are described as fold change relative to time = 0 h. * $p<0.005$,

** $p<0.0001$. (F) HCT116 cells treated with an IC$_{25}$ dose of OBP-301, Exo301, or EC-

Exo301 were harvested at the indicated post-treatment time points. Whole cell HCT116

lysates were subjected to western blot analysis for PARP, p62, E1A and β-actin.

Figure 3. *In vitro* model of delivery of tumor-derived substances to distal tumor sites via tumor-derived EVs

(A) Experimental design for the co-culture model. Briefly, HCT116-RFP cells and

HCT116 cells were seeded in the lower and upper chambers, respectively. HCT116-RFP

cells were treated with PBS or OBP-301 (100 MOI) and the culture medium was

changed to FBS-free medium 24 h after treatment. Cells were incubated for another 48

h and then harvested for analysis. GW4869 was added to the culture medium at 6 h

prior to PBS or OBP-301 treatment to block exosome secretion. (B) EVs isolated from

culture medium in the bottom chamber after PBS or OBP-301 treatment of HCT116-

RFP cells were subjected to western blot analysis for RFP, E1A, and CD9. (C) EVs

isolated after GW4869 treatment at the indicated doses in addition to OBP-301

treatment were subjected to western blot analysis for CD9. Relative intensities to non-

treatment condition (measured by Image J) are provided beneath the blot. (D, E)

HCT116 cells in the upper chamber after treatment of HCT116-RFP cells in the bottom

chamber with OBP-301 and GW4869 were subjected to flow cytometry to measure RFP

uptake (n=3). Representative data for each treatment are shown in (D), and statistical

assessments of RFP amounts in each treatment are shown in (E). * $p<0.01$, ** $p<0.001$.

Figure 4. Abscopal effects of OBP-301 mediated by tumor-derived EVs *in vivo*

(A) Experimental design for (B), (C), and (D). In brief, in a bilateral subcutaneous

tumor model with HCT116-RFP and HCT116 using BALB/c nu/nu mice, HCT116-RFP

tumors were treated with intratumoral administration of PBS or OBP-301 (1×10^8 PFU)

and/or intraperitoneal administration of GW4869 (2.5 µg/g) three times every two days,

and harvested 7 days after treatment initiation (n=8). (B) Representative images of IHC

staining for RFP in HCT116 tumors in each treatment are shown on the left, and

statistical assessment of RFP staining intensity (measured by Image J) is shown on the

right. Scale bar, 200 µm. * $p<0.05$. (C) Representative images of IHC staining for

adenovirus E1A in HCT116 tumors in each treatment are shown on the left, and

statistical assessment of E1A staining intensity (measured by Image J) is shown on the

right. Scale bar, 200 µm. * $p<0.05$, ** $p<0.01$, *** $p<0.005$. (E) Experimental design

for (F). Briefly, in a HCT116 bilateral subcutaneous tumor model using BALB/c nu/nu

mice, larger HCT116 tumors were treated with intratumoral administration of PBS or

OBP-301 (1×10^8 PFU) and/or intraperitoneal administration of GW4869 (2.5 µg/g)

three times every two days, and both tumors were monitored until 28 days after

treatment initiation to evaluate antitumor effects of the treatments (n=9-10). (F)

Volumes of both HCT116 tumors were monitored twice a week until 28 days after

treatment initiation. "Treated tumor" indicates the tumor directly treated with OBP-301,

and "Untreated tumor" indicates the tumor not directly treated with OBP-301. * $p<0.01$,

** $p<0.005$, *** $p<0.001$. (G) Experimental design for (H). Briefly, in a CT26 bilateral

subcutaneous tumor model using BALB/c mice, larger CT26 tumors were treated with

intratumoral administration of PBS or OBP-301 (1×10^8 PFU) and/or intraperitoneal

administration of GW4869 (2.5 µg/g) three times every two days, and both tumors were monitored until 18 days after treatment initiation to evaluate antitumor effects of the treatments (n=9-10). (H) Volumes of both CT26 tumors were monitored twice a week until 18 days after treatment initiation. "Treated tumor" indicates the tumor directly treated with OBP-301, and "Untreated tumor" indicates the tumor not directly treated with OBP-301. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (I) Experimental design for (J). Briefly, in a CT26 bilateral subcutaneous tumor model using NOD/SCID mice, larger CT26 tumors were treated with intratumoral administration of PBS or OBP-301 (1×10^8 PFU) and/or intraperitoneal administration of GW4869 (2.5 µg/g) three times every two days, and both tumors were monitored until 11 days after treatment initiation to evaluate antitumor effects of the treatments (n=4). (J) Volumes of both CT26 tumors were monitored twice a week until 11 days after treatment initiation. "Treated tumor" indicates the tumor directly treated with OBP-301, and "Untreated tumor" indicates the tumor not directly treated with OBP-301. * $p<0.05$, ** $p<0.01$.

Figure 5. Tumor tropism of tumor-derived EVs

(A) Experimental design of the triple co-culture model used for (B). Briefly, HCT116-RFP cells were seeded in the bottom chamber, and HCT116 cells and FEF3 cells were individually seeded in the upper chambers. HCT116-RFP cells were treated with OBP-301 (100 MOI) and the culture medium was changed to FBS-free medium 24 h after treatment initiation. Cells were incubated for another 48 h and then harvested for analysis. (B) HCT116 cells and FEF3 cells in the upper chamber were subjected to flow cytometry for RFP uptake (n=3). Representative data for each cell line are shown on the left, and statistical assessments of RFP amounts in each cell line are shown on the right.

* *p*<0.05. (C) EVs isolated from culture medium of OBP-301-treated HCT116 cells (HCT116 Exo) were added to a co-culture system of HCT116 cells and FEF3 cells. The cells were observed by fluorescence microscopy after 48-h incubation. HCT116 cells, FEF3 cells, and HCT116 Exo were stained green with CellTracker Green, blue with CellTracker Blue, and red with PKH26, respectively. Scale bar, 100 nm. (D) Experimental design for (E), (F), and (G). Briefly, in an orthotopic model of rectal tumors (HCT116-RFP) with liver metastasis (HCT116-Luc) using BALB/c nu/nu mice, HCT116-RFP tumors were treated with intratumoral administration of PBS or OBP-301 (1×10^8 PFU) and/or intraperitoneal administration of GW4869 (2.5 μg/g) three times every two days, and primary rectal tumors and metastatic liver tumors along with major organs (brain, heart, lung, liver, kidney and spleen) were harvested 7 days after treatment initiation (n=4-5). (E) Representative images of IHC staining for RFP in the primary rectal tumor and metastatic liver tumor in each treatment are shown on the left and statistical assessment of RFP staining intensity (measured by Image J) in primary rectal tumors, metastatic liver tumors and major organs are shown on the right. Scale bar, 200 μm. * *p*<0.05. (F) Representative images of IHC staining for E1A in primary rectal tumors and metastatic liver tumors in each treatment are shown on the left and statistical assessment of E1A staining intensity (measured by Image J) in primary rectal tumors, metastatic liver tumors and major organs are shown on the right. Scale bar, 200 μm. *** *p*<0.001. (G) Representative images of IHC staining for Ad5 in primary rectal tumors and metastatic liver tumors in each treatment are shown on the left and statistical assessment of E1A staining intensity (measured by Image J) in primary rectal tumors, metastatic liver tumors and major organs are shown on the right. Scale bar, 200 μm. * *p*<0.05. ** *p*<0.005.