Cell-derived Giant Membrane Vesicles Enclosing the Anthracycline Anti-Cancer Drug Doxorubicin and their Cytotoxicity

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Abstract: Although giant membrane vesicles prepared from paraformaldehyde-treated mammalian cells have been used to elucidate lipid and protein dynamics on the cell surface, there are few studies that have used them as a tool to deliver drugs to cells. Here we found that anti-cancer drug doxorubicin (Dox) was efficiently incorporated into and stably retained in giant membrane vesicles prepared from HeLa human cervical cancer cells. Intriguingly, coincubation of Dox-enclosed giant membrane vesicles with human pancreatic cancer-derived PK-59 and gastric cancer-derived KE-39 cells led to cell death. Microscopic observation of vesicle-docked cells revealed that docking of at least one to a few Dox-enclosed giant vesicles on the cellular cortex was sufficient to induce cell death, suggesting applicability of cell-derived giant membrane vesicles as an efficient drug delivery vector in cultured cell systems.

Keywords: Anti-cancer treatment, Extracellular vesicle, Doxorubicin, Drug delivery.

INTRODUCTION

Extracellular vesicles (EVs) are phospholipid bilayer membrane vesicles derived from a wide variety of cell types. EVs make up a heterogeneous population of particles that are involved in cell-to-cell communication in major patho/physiological processes and can be classified into multiple groups based on their generation pathways and size, including exosomes, microvesicles and apoptotic bodies [1, 2]. Small size EVs, such as exosomes and microvesicles, have been reported to possess a number of characteristics that qualify them as promising vehicles for drug delivery [3, 4]. On the other hand, application for drug delivery using large size EVs, such as apoptotic bodies, which are produced by cell fragmentation via apoptotic cell death, and giant plasma membrane vesicles, which are artificially generated in response to cellular stresses and/or chemical agents, have been rarely tested [5-7].

Giant membrane vesicles prepared by cellular treatment with paraformaldehyde (PFA) was first reported by Scott in 1976 [8] and they have been used for assessments of proteins and lipids on the plasma membrane to reveal mechanistic insights into the dynamic behavior of plasma membrane proteins and lipids [9-12]. Previously, we modified the original protocol to generate cell-derived giant membrane vesicles from PFA-treated HeLa human cervical cancer cells [13-15]. Although we demonstrated their ability to dock to the surface of exponentially growing HeLa cells without apparent cytotoxicity, there were few cells that showed transportability of the vesicle constituents, possibly because of the self-incompatibility of the membrane fusion event or an unknown cell surface mechanism that inhibited uptake of the extracellular vesicle components [15]. A search for cell types exhibiting better interaction with and uptake of cell-derived giant vesicles and a better understanding of the reason for variation in cell dependent intercellular vesicle transfer could improve applicability of cell-derived giant vesicles for drug delivery.

In this study, we assessed the interaction of HeLa cell-derived giant membrane vesicles with six kinds of human cell lines and three kinds of mouse cell lines. We found that, when the vesicles were applied to cells, several cell lines exhibited highly efficient uptake of the vesicle constituents. Using two kinds of human cancer cell lines, which exhibited efficient incorporation of the vesicle constituents, we showed that the cell-derived membrane vesicles, which enclosed anthracycline anti-cancer drug doxorubicin (Dox), exerted cytotoxicity after docking to the cellular cortex. Taken together, our findings suggest the capability of cell-derived giant membrane vesicles to carry and transport Dox to cultured cells, providing an additional membrane-encapsulated drug delivery material.

MATERIALS AND METHODS

Reagents and Stock Solutions

All reagents, enzymes, and stock solutions are listed in Supplementary Data.

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Cell Lines and Culture Conditions

Human cervical cancer cell line HeLa, human liver cancer-derived cell line Huh-7, human gastric cancer-derived cell line KE-39, human pancreatic cancer-derived cell line PK-59, human colon cancer-derived cell line colo320 and mouse ascites-derived macrophage-like cell line J774-1 were obtained from RIKEN cell bank (Tsukuba, Japan). Mouse fibroblast cell line C2C12 was kindly provided by Dr. Yuki Nakayama (Kumamoto University, Japan), mouse spermatogonia cell line GC-1 was kindly provided by Dr. Ko Eto (Kumamoto University, Japan) and human embryonic kidney cell line 293 was kindly provided by Dr. Terumasa Ikeda (Kumamoto University, Japan). HeLa cells, colo320 cells and GC-1 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) /Ham's F-12 nutrient mixture containing 5% fetal bovine serum (FBS) and 1 % penicilin-streptomycin solution (PS). Huh-7 cells were maintained in D-MEM (Low Glucose) containing 10 % FBS and 1 % PS. C2C12 cells and 293 cells were maintained in D-MEM (High Glucose) containing 10 % FBS and 1 % PS. KE-39 cells, PK-59 cells and J774-1 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-164) containing 10 % FBS and 1 % PS. Cells were cultured at 37 °C in a 5% CO2 incubator.

Preparation of Giant Membrane Vesicles

The detailed procedure has been described previously [14, 15]. Briefly, when HeLa human cervical cancer cells approached confluency (~1×10^7 cells/10-cm dish), 300 µl of 4% (v/v) PFA was added to 10 ml of culture medium. After gently mixing the medium and PFA, the cells were incubated for 90 min at room temperature. Cells were washed three times with 5 ml phosphate-buffered saline (PBS). After washing, 2 ml PBS was added to the culture dish and the cells were washed gently for 16 h at room temperature. The supernatant was transferred to an appropriate tube and stored at room temperature or 4°C.

Generation of Dox-enclosed Giant Membrane Vesicles

A total of 200 µl (~1×10^6/ml) giant membrane vesicles derived from HeLa cells was collected in a 1.5-ml tube, followed by mixing with doxorubicin (Dox) at 37°C for 6 h. The vesicles were washed with PBS by centrifugation at 2,500×*g* for 5 minutes. Washing was repeated three times to completely remove unincorporated Dox. Using the autofluorescence of Dox [16, 17] and references therein], fluorescent signals were analyzed by a ZOE Fluorescent Cell Imager (Bio-Rad) or XI71 microscope imaging system (Olympus) using ImageJ and cellSens standard software (Olympus). To estimate the Dox concentration in vesicles, 5 µl of 2 µM, 20 µM, 200 µM, and 2 mM Dox solutions was spotted on a glass plate. Fluorescence intensity in each spot was measured using ImageJ to create a standard curve that indicated the correlation between the Dox concentration and fluorescence intensity.

Treatment of Cultured Human Cancerous Cells with Giant Membrane Vesicles

To assess cellular uptake of constituents in vesicles, exponentially growing cells (~1×10^5 cells) were treated in a 3-cm culture dish with an appropriate number of giant membrane vesicles pretreated with either calcein-acetoxymethyl ester (calcein-AM) or Dox. Preparation of calcein-AM-stained vesicles has been described previously [15]. To count dead cells, floating cells, cells with abnormal morphology, and cells strongly stained with propidium iodide (20 µg/ml) were detected by a ZOE Fluorescent Cell Imager (Bio-Rad) or the XI71 microscope imaging system using ImageJ and cellSens standard software.

Statistical Analysis

Cells and vesicles were counted optically under a microscope using either bright field, the appropriate fluorescence field, or both. Unless stated otherwise, all data are presented as the mean ± standard deviation (SD). Within individual experiments, data points were based on a minimum of triplicate representative samples. Experiments were repeated at least three times. In some experiments, a t-test was performed to assume homoscedasticity.

RESULTS AND DISCUSSION

Interaction between HeLa cell-Derived Giant Membrane Vesicles and Various Cell Types

To elucidate whether the efficiency of cellular uptake of constituents in HeLa cell-derived giant membrane vesicles varied among cell types, we applied calcein-AM-labeled giant vesicles prepared from HeLa cells (Supplementary Figure **S1**) to nine kinds of cell lines. The cell lines used in the experiment were HeLa human cervical cancer cells, Huh-7 human liver cancer-derived cells, KE-39 human gastric cancer-derived cells, PK-59 human pancreatic cancer-derived cells, 293 human fetal kidney-derived cells, Colo320 human colon cancer-derived cells, C2C12 mouse fibroblasts, J774-1 mouse ascites-derived

macrophage-like cells, and GC-1 mouse spermatogonia. Each cell line was treated with calcein-AM-labeled vesicles for 15 h and then the proportion of cells with accumulated calcein-AM fluorescent signals was measured (Figure **1**). We found that the distribution of fluorescent signals was significantly different depending on the cell line. J774-1, 293, PK-59, KE-39, and GC-1 cells showed intense intracellular fluorescence signals, whereas intracellular fluorescent signals were barely observed in HeLa, C2C12, Huh-7, and Colo-320 cells. HeLa, C2C12, Huh-7, and Colo-320 cells appeared to retain fluorescence-positive vesicles without any detectable damage to the vesicle surface. Although the mechanism(s) that facilitated or inhibited the transfer of vesicle constituents to cells was unclear, our findings indicated that the efficiency of cellular uptake of components in HeLa cell-derived giant membrane vesicles varied greatly depending on the cell type and that some human and mouse cell lines were competent to incorporate constituents in HeLa cell-derived giant membrane vesicles.

Generation of Dox-enclosed Giant Membrane Vesicles

During our analyses of HeLa cell-derived giant membrane vesicles, we found the anti-cancer drug doxorubicin (Dox) could be introduced into the vesicles. Because Dox emits autofluorescence [16, 17 and references therein], we monitored Dox incorporation in the vesicles under a fluorescence microscope (Figure **2A**). When HeLa cell-derived giant membrane vesicles were incubated at room temperature for 6 h in PBS with 20 nM, 200 nM, and 2 μM Dox, fluorescent signals in the vesicles were increased in a Dox concentration-dependent manner (Figure **2A** and **B**). It should be noted that, even after washing with an excess amount of drug-free PBS, followed by incubation in drug-free PBS for 24 h, there was no obvious reduction of fluorescent signals in the vesicles, indicating the potential of the vesicles to incorporate Dox from the extra-vesicle environment and stably retain the drug (Figure **2C**). By comparison with the standard curve of Dox fluorescence signals, we estimated that the vesicles could enclose up to 200 μM Dox when the vesicles were incubated in PBS with 2 μM Dox (Supplementary Figure **S2**). We assumed that such Dox enrichment in the vesicles against the concentration gradient might be explained by the formation of Dox-RNA/protein complexes in the vesicles, because HeLa cell-derived giant membrane vesicles contain RNAs and/or proteins [15, 18]. Although the exact Dox-binding partner(s) in the

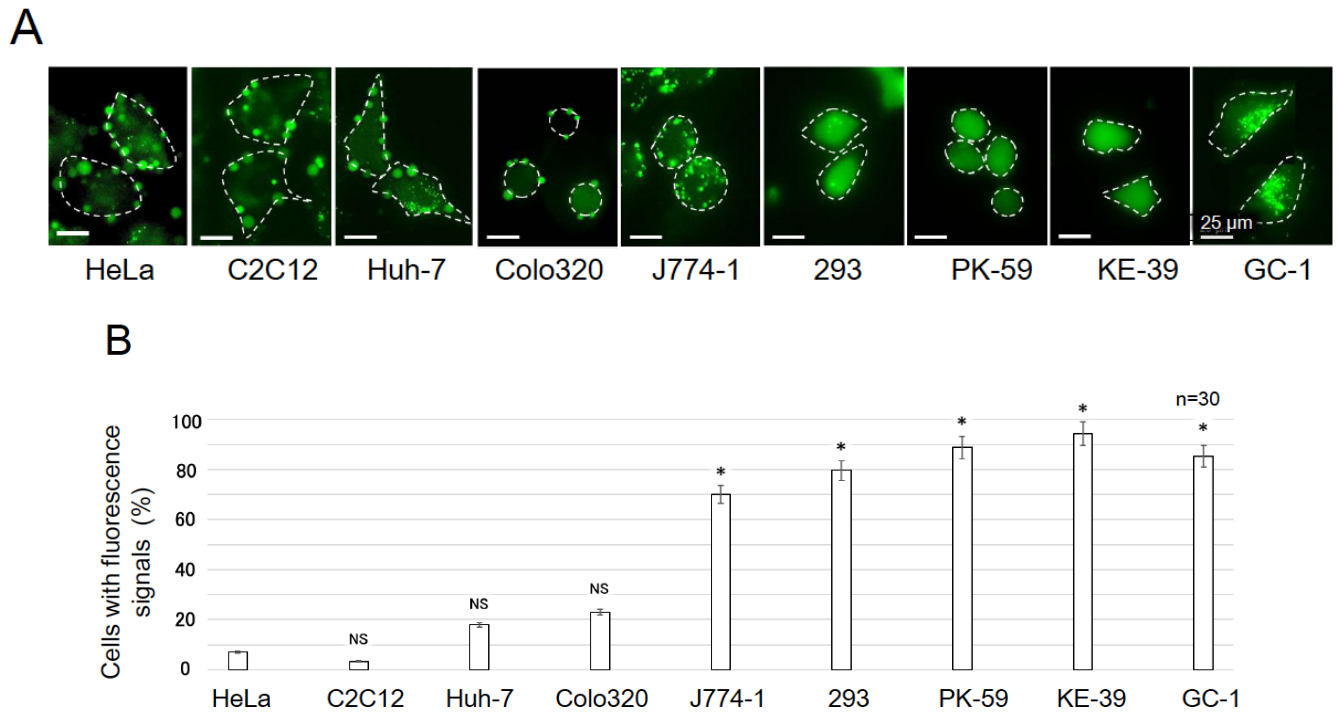

Figure 1: Cellular uptake of calcein-AM fluorescence signals from Hela cell-derived giant membrane vesicles. (**A**) Several cell lines were treated with calcein-AM-labeled giant membrane vesicles for 15 h, followed by microscopic observation. Representative microscopic images obtained under the appropriate fluorescence filter condition are shown. Bar = 25 μm. (**B**) Ratio of cells with fluorescence signals. n = 30. *p = 0.0003; ns, not statistically significant (t-test).

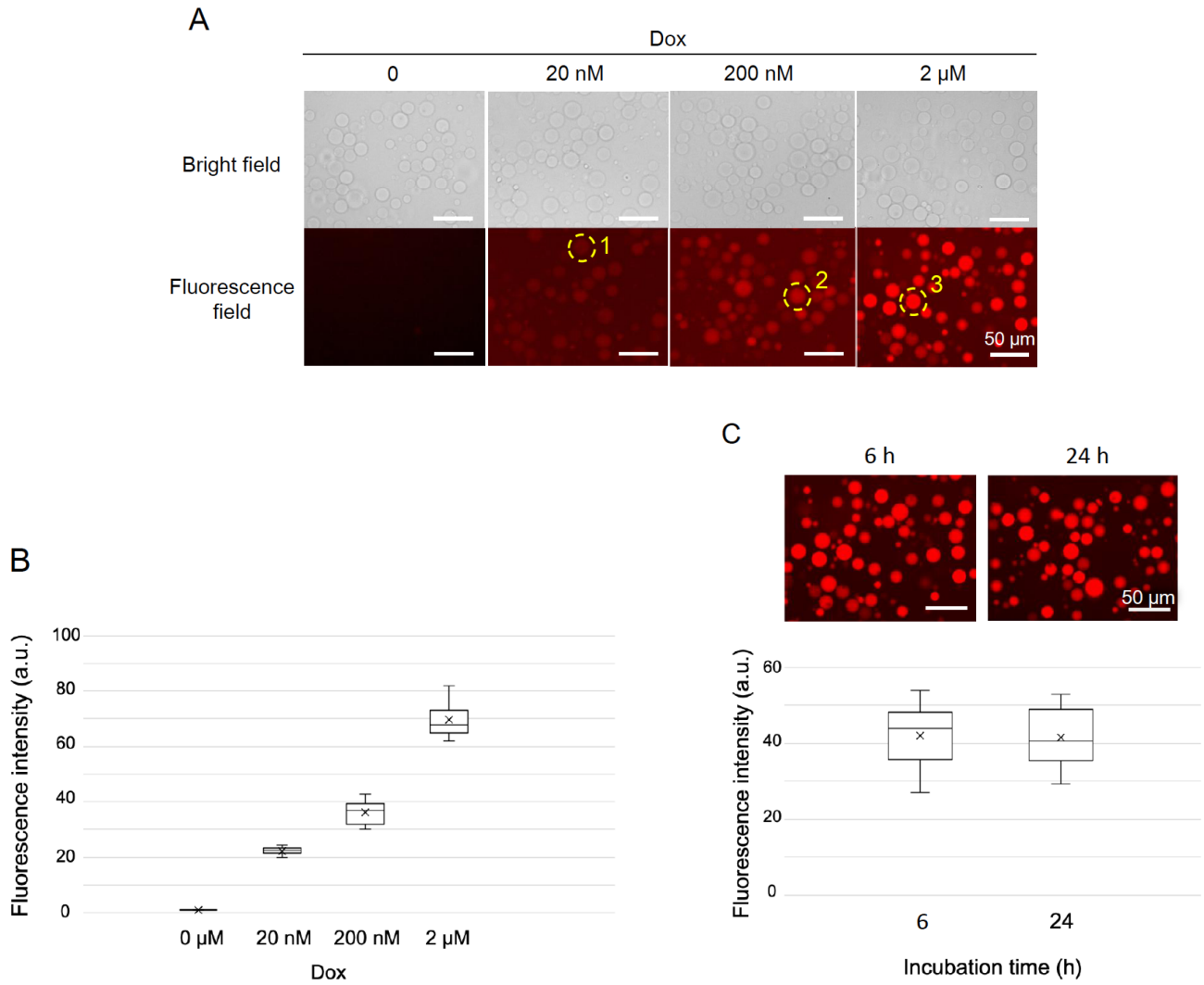

Figure 2: Concentration-dependent Dox incorporation in HeLa cell-derived giant membrane vesicles and estimation of the Dox concentration in vesicles. (**A**) HeLa cell-derived giant membrane vesicles were incubated with different concentration of Dox, followed by fluorescent microscopy. Circles 1, 2 and 3 with dashed lines indicate the vesicles subjected to estimation of Dox concentration in Supplementary Fig. S2. Bar = 50 μm. (**B**) Fluorescence intensities detected in the vesicles in A were measured and represented as arbitrary units (a.u.). (**C**) After incubation of the giant vesicles in PBS containing 2 μM Dox for 6 h, the vesicles were washed three times with excess amount of Dox-free PBS, followed by incubation for 24 h in Dox-free PBS (upper panel). Fluorescence intensities detected in the vesicles were represented as arbitrary units (a.u.) and shown in lower panel. Bar = 50 μm.

vesicles remain unclear, our data indicate that Dox can be introduced into and stably retained in HeLa cell-derived giant membrane vesicles.

Cytotoxicity of Dox-enclosed Giant Membrane Vesicles

To investigate the possibility that Dox-enclosed giant membrane vesicles can be used as carriers for drug delivery, we applied Dox-enclosed vesicles to human gastric cancer-derived KE-39 and human pancreatic cancer-derived PK-59 cell lines and determined whether they exerted cytotoxicity. At 24 h after addition of the Dox-enclosed vesicles, a large number of cells with abnormal morphology and propidium iodide (PI)-positive staining, which is indicative of cells undergoing cell death, were observed in both cell lines, in a Dox-concentration dependent manner, whereas addition of Dox-free control vesicles to cells resulted in no cytotoxicity (Figure **3A** and Supplementary Figure **S3**). Furthermore, microscopic observations of individual cells after vesicle docking to the cellular cortex revealed that that cell death was effectively induced in cells bound with at least one Dox-enclosed vesicle (Figure **3B** and Supplementary Figure **S4**, **S5A** and **B**). Although the mechanism(s) that facilitated the transfer of Dox in the vesicles to cells was unclear, our findings indicated that some human

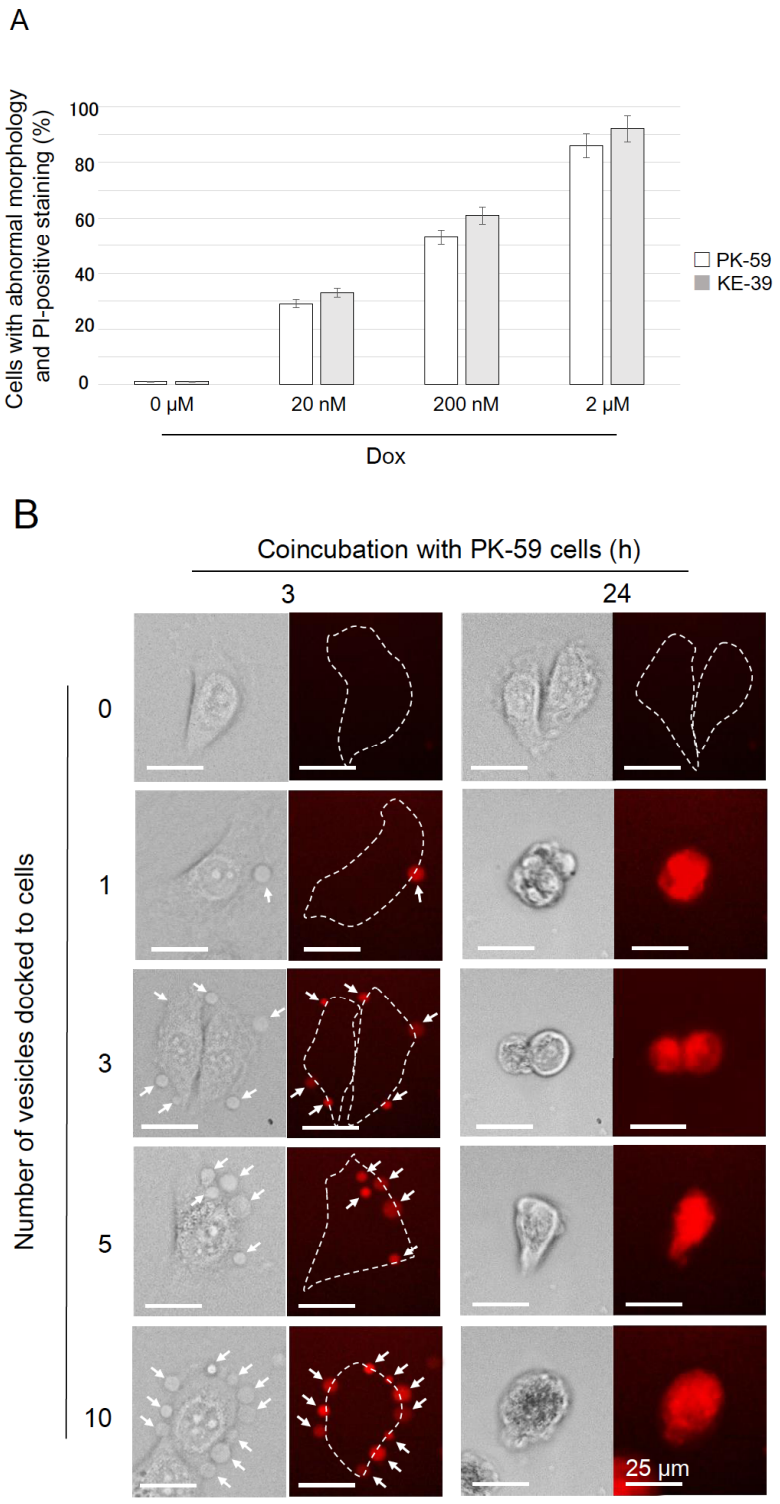

Figure 3: Cytotoxic effect of Dox-enclosed vesicles on cultured human cancer cells. (**A**) HeLa cell-derived giant membrane vesicles were incubated in PBS with 0 nM, 20 nM, 200 nM, and 2 μM Dox and then applied to exponentially growing PK-59 and KE-39 cells. After 24-h co-incubation, cells with abnormal morphology were detected and the ratio of such cells was calculated as dead cells. (**B**) Microscopic observation of PK-59 cells at a fixed time point after addition of Dox-enclosed vesicles. Cells docked with no (zero), one, three, five, and 10 vesicles are shown. Images were obtained at the indicated time under the bright field (left side) and red fluorescence filter (right side) to visualize autofluorescent signals from Dox. Vesicles docked to the cellular cortex are indicated by arrows. White-dashed lines indicate the cellular cortex of the observed cells. Bar = 25 μm.

cancer cell lines were competent to incorporate Dox residing in HeLa cell-derived giant membrane vesicles and suggested cytotoxicity of the Dox-enclosed giant, membrane vesicles.

CONCLUSION

The application of HeLa cell-derived giant membrane vesicles to multiple cultured human and mouse cell lines showed that the constituents in cell-derived vesicles were incorporated differentially depending on the cell line. Notably, we found the capability of HeLa cell-derived giant membrane vesicles to enclose Dox and suggested their applicability as a drug delivery vector. Our data not only expand the applications of cell-derived giant membrane vesicles, especially in cell culture and cell engineering systems, but also provide useful information for researchers who are interested in developing membrane-encapsulated drug delivery systems in pharmacological and medical research fields [19-22].

ABBREVIATIONS

Dox, doxorubicin; **PFA**, paraformaldehyde; **PI**, propidium iodide; **PBS**, phosphate buffered saline.

SUPPLEMENTARY DATA

Supplementary Data are available at *Online*.

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CONFLICT OF INTEREST

None declared.

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