Induction of cell size vesicles from human lymphoma cell lines and their application to drug carriers

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Abstract Sodium butyrate (NaB) induced the membrane enclosed cell size vesicles from several IgM producing cell lines. We considered the application of the cell-derived vesicles (CDVs) to drug delivery system (DDS) using the lung cancer specific IgM producing AE6 cell line. Microscopic observation showed that the DiI fluorescence labeled AE6 vesicles were incorporated into the lung cancer cell line A549. The anticancer drug, actinomycin D (actD), contained in AE6 and Ramos vesicles decreased the A549 cell viability to 46 and 62% of control without actD, respectively. The cytotoxic effect in AE6 vesicles was superior to that in the Ramos vesicles that have the lung cancer non-specific IgM on their surfaces. However, the result of the Ramos vesicles suggests that the surface molecules other than IgM may interact with the A549 cells. In our method for vesicle production, more specific and abundant antibodies mounted vesicles can be generated by transfection of their genes into cells followed

by NaB treatment. These suggest that the CDVs may be useful for the development of a drug carrier for DDS.

Keywords Anticancer · Drug delivery system · Lymphoma · Sodium butyrate · Vesicle

Introduction

Proteoliposomes carrying membrane proteins on the surfaces were widely used for the characterization of membrane proteins (Rigaud and Levy 2003), the model of artificial cells (Noireaux et al. 2004), the construction of microreactor (Michel et al. 2004), and the establishment of drug delivery system (DDS; Park et al. 2004). They were prepared by the following process: the dissolution of lipids and membrane proteins, the formation of the lipid film by evaporation, the hydration of the lipid film with dispersion medium, and the capsulation of the lipid film. However, their membrane proteins cannot always keep native structure and activity. In particular, the three dimensional structure of multipass transmembrane proteins is not reconstructed. Among the proteoliposomes, the immunoliposomes carrying the antibodies on their surfaces can deliver a drug to the target via the antigen–antibody reaction, but N-hydroxysuccinimide used when the antibodies are linked to liposomes may decrease their functionality

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due to its linkage to the antigen binding sites. Thus, it is difficult to prepare the proteoliposomes carrying native membrane proteins.

We found the induction of cell size vesicles from an IgM producing human lymphoma cell line by the treatment of sodium butyrate (NaB) when we examined its cell cycle synchronization in G1 stage (Yamanaka et al. 2009). These cell-derived vesicles (CDVs) were different from physiologically secreted small ones in size and retained membrane proteins from original cells. In addition, their membrane is enclosed. It was confirmed by trypan blue staining (Yamanaka et al. 2009). CDVs were also stable at room temperature for 4 days and at 4 °C for 8 days. These suggest that CDVs are new findings and may be useful as alternatives of immunoliposomes or proteoliposomes. Then, we examined the vesicle induction in various human cell lines and considered the application of their CDVs to DDS.

Materials and methods

Cell lines and cell culture

The human B lymphoma lines, Ramos (RA1) (JCRB9119), Nawalwa (IFO50040), and PBCL-1, the human T lymphoma line MOLT-4 (JCRB9031), the human–human hybridoma lines, AE6, BD9, and HB4C5, the human monocyte lymphoma line, U937 (JCRB9021), the human myeloid leukemia line, K562 (JCRB0019) were used in this study. PBCL-1 cell line was established from human peripheral blood lymphocytes by treatment with phorbol 12-myristate 13-acetate in our laboratory, and expressed the B cell markers, CD19 and IgM, but not the T cell marker, CD3, and the NK cell marker, CD94. Three hybridoma lines were established as described previously (Kawahara et al. 1992; Murakami et al. 1985). Cells were maintained in ERDF medium (Kyokuto Pharmaceutical Industrial Co., Japan) containing 10% of fetal bovine serum (Thermo Fisher Science Inc., Japan), at 37 °C in humidified 5% CO₂/95% air.

Induction of CDVs by NaB

NaB (Sigma, USA) was dissolved in distilled water at a concentration of 10 M and stored at 4 °C. For each experiment, NaB stock solution was added to the

culture medium containing 4×10^5 cells at final concentration of 5 mM, and cells were cultured for 1 day. The induction of CDVs was confirmed by microscopic observation.

Incorporation of CDVs into lung cancer cells

According to manufacturer's instructions, AE6 cells were stained with the fluorescent dye DiI (Invitrogen, CA, USA) that can bind to cell membrane. The DiI labeled AE6 cells were immobilized to a culture dish by the adhesion protein, cell-Tak (Becton–Dickinson, USA), and cultured in the medium containing 5 mM NaB for 1 day. The induced CDVs were collected from supernatants by centrifugation at 3000×g for 5 min at room temperature. After washing with phosphate buffered saline (PBS), the DiI labeled CDVs were cultured with the human lung adenocarcinoma cell line A549 for 2 h. The binding and incorporation of the CDVs into A549 cells were analyzed using a fluorescence microscope.

Effect of CDVs containing anticancer drug on lung cancer cells

A549 cells (1.5×10^4 cells) were known to be almost dead by treatment with $1 \mu\text{g mL}^{-1}$ of actinomycin D (actD) for 24 h (Watanabe et al. 2002). Thus, CDVs were immersed in PBS containing $2 \mu\text{g mL}^{-1}$ of actD (Sigma, USA) for 30 min. The actD containing CDVs were thoroughly washed with PBS, collected by centrifugation at 3000×g for 5 min, and cultured with 5×10^4 cells of A549 cells for 1 day. As a control, the effect of $2 \mu\text{g mL}^{-1}$ of actD alone on A549 cells was examined. The viability of A549 lung cancer cells was examined by MTT assay because the CDVs did not show mitochondrial activity. The absorbance of the formazan formed by the living A549 cells was measured at 450 nm with 640 nm as reference.

Results and discussion

Induction of CDVs from cell lines

The CDV induction by the treatment of NaB was examined in various human lymphoma and hybridoma lines. NaB induced the vesicles from Ramos,

Table 1 Induction of CDVs in various human cell lines

Cell line	Cell type	Ig product	Induction rate (%)
Ramos (RA1)	B lymphoma	IgM	70
Namalwa	B lymphoma	IgM	60
PBCL-1	B lymphoma	IgM	60
AE6	B cell hybridoma	IgM	40
HB4C5	B cell hybridoma	IgM	40
BD9	B cell hybridoma	IgG	0
MOLT-4	T lymphoma	Non	0
U937	Monocyte lymphoma	Non	0
K562	Leukemia	Non	0

Namalwa, PBCL-1, AE6 and HB4C5, but not from BD9, MOLT-4, U937, and K562 (Table 1). The induced all cell lines were IgM producers. Among the cell lines tested, Ramos the best induction rate of approximately 70% of total cell number (Fig. 1a). The induced CDVs were 4–6 μm thus of similar diameter as that of the cells (Fig. 1b). However, all cells were dead after NaB treatment for 1 day. The CDVs formation started from 8 h after treatment (Fig. 1c). NaB is known as a histone deacetylase inhibitor that can suppress cell proliferation by G1

arrest and induce apoptosis (Vidali et al. 1978; Schwartz et al. 1998), but the molecular mechanism concerning selective cell vesicle induction was not understood.

In CHO cells Jiang and Sharfstein (2008), and other research groups reported that NaB enhanced the expression of foreign genes including monoclonal antibody, but induced apoptosis. There are no reports on vesicle induction. Therefore, our approach for CDV induction may not be applicable to CHO cells.

Incorporation of CDVs into lung cancer cells

The specific antigen of IgM produced by Ramos cells was not identified. Shoji et al. (1994) reported that the human–human hybridoma line AE6 produced a lung cancer specific monoclonal antibodies. In addition, its IgM productivity could be increased by retinoids (Inoue et al. 1999). Thus, AE6 cell line was used to examine the ability of the CDVs to bind to the target cells. Microscopic observation showed that the DiI fluorescence labeled AE6 vesicles were incorporated into A549 lung cancer cells (Fig. 2). The number of the incorporated CDVs was not determined because they appeared to be degraded

Fig. 1 Induction of cell size vesicles from Ramos cells by the treatment of NaB. **a** Before and **b** after centrifugation, and **c** time course. The transparent spheres are vesicles. All magnifications are the same

Fig. 2 Microscopic observation of the incorporation of the AE6 vesicles into A549 cells. *White color* in (b) indicates the DiI fluorescence labeled AE6 vesicles in the A549 cells. **a** Bright, and **b** fluorescent fields

within the A549 cells. It is known that the membrane of CDVs was enclosed (Yamanaka et al. 2009). These results suggest that the specific IgM mounted CDVs may be applicable as a drug carrier directed against the target cells.

Effect of CDVs containing anticancer drug on lung cancer cells

The cytotoxic effects of the actD containing AE6 vesicles on A549 lung cancer cells were examined. As control for antibody reactivity, IgM mounted Ramos vesicles, non-specific for lung cancer cells, were used. The actD containing AE6 and Ramos vesicles decreased the A549 cell viability to 46 and 62% of that without actD, respectively (Fig. 3). The IgM abundance in AE6 vesicles was 2 times lower than that in Ramos vesicles (data not shown). This

Fig. 3 Cytotoxic effects of the actD containing CDVs on A549 lung cancer cells. Each column shows the average values and *error bars* represent the corresponding standard deviations ($n = 3$)

implies that the cytotoxic effect of AE6 vesicles was superior to that of Ramos vesicles. In addition, the result of the Ramos vesicles suggests that the surface molecules other than IgM may interact with the A549 cells. Although the reactivity of the CDVs with normal cells has not been examined yet, in the case of reactivity, the use of more specific anticancer drug may be required.

Buttgereit et al. (2000) reported efficient gene transfer into lymphoma cells. Actually, we have succeeded in the expression of membrane proteins such as fructose transporter on the surface of the human myeloma cell line SC-01MFP (Tsukamoto et al. 2009). In our method for vesicle production, membrane proteins, from the original cells were retained on the vesicle surfaces (Yamanaka et al. 2009). Therefore, more specific and abundant antibodies mounted CDVs can be generated by transfection of their genes into cells followed by NaB treatment.

Recently, polyethylene glycol modified liposomes have been reported (Immordino et al. 2006). These liposomes are known as stealth liposomes and can extend blood circulation time by avoiding the immune system in the body. This modification technique may lead to the improvement of antigenicity and non-specific reactivity of CDVs.

Conclusions

We reported a simple method for the production of cell size vesicles from lymphoma and hybridoma cell lines by the treatment with NaB. The lung cancer specific IgM and anticancer drug carrying CDVs had the advantage of decreasing the viability of lung cancer cells. The induced CDVs can be designed after transfection of the vesicle producer cells with desired genes. Therefore, more effective drug carrier may be developed by improving the interaction of the vesicles with their target.

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