Development of a Membrane Fusible Drug Carrier from Erythrocytes by the Spontaneous Transfer of Viral Fusion Protein from Influenza Virus-Infected Cells

Kentaro Kogure, Otomo Okuda, Taku Itoh, Kyoko Hayashi, and Masaharu Ueno

Faculty of Pharmaceutical Sciences and Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitami, Toyama 930-01, Japan. Received November 8, 1996; accepted January 29, 1997

In order to develop a membrane fusible drug carrier from human erythrocytes, we attempted the reconstitution of influenza virus fusion protein hemagglutinin (HA) to an erythrocyte membrane. In this study, we succeeded in the preparation of HA-reconstituted erythrocytes (HA-erythrocytes) by the incubation of erythrocytes with influenza virus-infected CV-1 cells, and confirmed the ability of HA-erythrocytes to fuse with the cell membrane. Furthermore, by using an HA-reconstituted ghost (HA-ghost), which entrapped fluorescent-labeled ovalbumin, 25% of the protein was incorporated into cells through the fusion of the HA-ghost with the cell membrane.

Key words: fusion; drug carrier; protein transfer; erythrocyte; hemagglutinin

In recent years, application of the infectious mechanism of a virus, which can fuse with a lipid membrane, to carry drugs, enzymes or genes into target cells in vitro has been reported. The mechanism of infection of the influenza virus has been clearly identified, as follows. The influenza virus attaches to a receptor, sialic acid, on the cell surface, with hemagglutinin (HA), and is incorporated into the cell through endocytosis. Next, a change in the steric conformation of HA is caused by a decrease in the internal pH of the endosome. Then, as the virus becomes able to insert the hydrophobic part of HA into the endosomal membrane, the fusion of the virus with the endosomal membrane occurs, and the virus gene is released into the cytoplasm.

The mixture of liposomes and virus particles, or virosomes, which are viral fusion protein-reconstituted liposomes, have been attempted to be developed as a membrane fusible carrier of drugs, enzymes or genes by application of the virus-infection mechanism described above. However, there is a problem in those systems, i.e., it is difficult to entrap various substances, such as the enzymes, into those liposomes without a change in their structure and activity, because the detergents or organic solvents which cause their conformational alteration, are usually used at the step of liposome preparation. Therefore, we decided to apply the ghost of an erythrocyte instead of liposomes as membrane-fusible carriers, because it is possible to incorporate enzymes into ghosts by osmotic pressure. Previously, there have been several reports about the application of erythrocytes as membrane-fusible drug carriers. In those reports, the fusion of erythrocytes with the cell membrane and the release of drugs into cytoplasm occurred by the incubation of erythrocytes, which entrapped drugs, and membrane-fusible virus particles with the target cells. However, in the case of those systems, there was the possibility of infection of target cells with the membrane-fusible virus, despite treatment of the virus with something, such as ultraviolet rays, to inactivate its heredity. Therefore, we attempted to develop membrane-fusible erythrocytes, which have reconstituted viral fusion proteins on their surface, without the mediation of virus particles.

In recent years, it has been reported that various proteins of cell membranes are transferred spontaneously to artificial lipid vesicles or other cell membranes. Previously, we reported that the viral membrane protein HA was transferred from influenza virus-infected cells to dimyristoylphosphatidylcholine (DMPC) liposomes. Thus, from those reports and our previous study, since it was expected that a viral protein, such as the membrane-fusible protein HA, would be transferred to the erythrocyte membrane, in this study we attempted to develop a membrane-fusible drug carrier from the erythrocyte by the application of spontaneous protein transfer.

MATERIALS AND METHODS

Human blood was offered by an adult male volunteer. All reagents were of the highest grade commercially available.

To remove sialic acid from the erythrocyte surface for the efficient transfer of HA, 150 μl of 1.8 units/ml neuraminidase (NA) was added to 30 ml of 40% (v/v) erythrocyte suspension in an NA-treatment buffer (20 mM TES (N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid)–NaOH, 0.137 M NaCl, 2 mM CaCl₂ (pH 7.0)), and incubated at 37°C for 1 h.

The NA-treated erythrocytes were suspended into a 4-fold labeling buffer (0.1 mM fluorescein isothiocyanate (FITC) in phosphate-buffered saline (PBS) (pH 8.0)), and incubated at 4°C for 1 h. After the incubation, to terminate the labeling reaction, treated erythrocytes were resuspended into a 4-fold glycine buffer (0.3 M glycine, 91.3 mM NaCl, 1.79 mM KCl, 8.94 mM Na₂HPO₄, 0.98 mM KH₂PO₄ (pH 7.4)), and incubated at 4°C for 5 min. According to microscopic observation, the erythrocyte membrane and the hemoglobin contents were labeled.

Culture of CV-1 (monkey kidney) cells and subsequent infection with an influenza virus (NWS strain (H1N1)) were done as described previously. In addition, to ensure membrane fusion, the virus-infected CV-1 cells with the HA protein on the surface were treated with trypsin. Subunit HA1, which is the binding domain with sialic acid, was removed by this treatment. However, the fusibility of

* To whom correspondence should be addressed.

© 1997 Pharmaceutical Society of Japan

NII-Electronic Library Service
HA remained.

In order to transfer the viral fusion protein (HA) from the influenza virus-infected cells to erythrocytes, 600 μl of 6% (v/v) FITC-labeled erythrocyte (FITC-erythrocyte) suspension was added to a dish of the virus-infected cells, then incubated at 37°C for 1 h. After the incubation, the erythrocyte suspension was recovered and was centrifuged at 2000 × g for 5 min at 4°C.

To confirm the transfer of viral protein to erythrocytes, the HA-erythrocytes and other samples (10 μg/lane) were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel, then Western blot analysis was done with an anti-influenza virus rabbit serum.1,3,5

For induction of the fusion of HA-transferred FITC-erythrocytes (HA-FITC-erythrocytes) with cells, 400 μl of 25% (v/v) HA-FITC-erythrocyte suspension was incubated with virus-uninfected normal CV-1 cells at 4°C for 1 h. Then, 800 μl of warm fusion buffer (20 mM MES (2-(N-morpholino)ethanesulfonic acid)–NaOH, 0.137 M NaCl (pH 5.2), at 37°C) was added into the dish. After 5 min, the fusion buffer was discarded. Next, 800 μl of culture medium (at 37°C) was added into the dish and incubated at 37°C for 90 min for the induction of fusion. After the incubation, the cells were washed carefully several times with PBS to remove any unfused erythrocytes. The fusion of FITC-labeled erythrocytes with cells was observed as a fluorescent microscopic image using a BH-2 microscope (Olympus, Tokyo, Japan).

In order to prepare a model drug, 4 ml of 1 mM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was added to 16 ml of 0.2 mM ovalbumin in 50 mM phosphate buffer (pH 7.0), and the mixture was shaken at room temperature. After 1 h, 24 mg cystein was added into the mixture, and the shaking was continued for 30 min. Then, the mixture was dialyzed and freeze-dried.

To prepare the ghost, erythrocytes were suspended into 10-fold 5 mM sodium phosphate buffer (pH 7.4). Then, the suspension was centrifuged at 19000 × g for 20 min at 4°C, and the supernatant was removed. We repeated this procedure three times, and thus obtained the ghost of the erythrocytes. For entrapping NBD-labeled ovalbumin (NBD-Ov) into the ghost, a mixture of 35% (v/v) ghost suspension and NBD-Ov was dialyzed against 20 mM phosphate buffer (pH 7.4) for 30 min at 4°C, and additionally against PBS for 30 min at room temperature. After the dialysis, the recovered ghost suspension was centrifuged at 8000 × g for 20 min at 4°C.

The degree of incorporation of NBD-Ov entrapped in the HA-ghost into the cells by the fusion was measured according to the fluorescence intensity of the labeling reagent. The procedure of inducing fusion was the same as that involved in the fusion of FITC-erythrocytes with cells, described above. Thus, the fluorescence intensities of ghost suspensions, which were recovered during the process of induction of fusion, and of cells treated for fusion, were measured at 520 nm excited at 360 nm in a Shimadzu fluorophotometer RF-5000 (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

In order to transfer the viral protein from the influenza virus-infected cells, which were treated with trypsin to remove the sialic acid receptor subunit HA1, to erythrocytes, the receiver was incubated with the donor cells at 37°C for 1 h. After the incubation, the recovered erythrocyte suspension was subjected to Western blotting (Fig. 1) to confirm the transfer of viral protein. As shown in Fig. 1, the positive band (near a molecular weight of 33000) against anti-virus serum was observed in the erythrocytes (lane 1) incubated with influenza virus-infected cells, and also in the virus-infected cells (lane 3), and was considered to be an HA2 subunit. Previously, we obtained a similar result when the receiver of the protein transfer was a liposome.1,3 Thus, it was confirmed that the viral protein of influenza virus-infected cells can also transfer to the erythrocyte membrane, as well as to liposomes. We referred to those erythrocytes, which were reconstituted with the viral protein HA, as HA-erythrocytes.

Next, to confirm the fusion of HA-erythrocytes with target normal cells, we used FITC-labeled erythrocytes (FITC-erythrocytes) for distinguishing them from the target cells (Fig. 2). Figure 2 shows the microscopical images after the treatment of HA-transferred FITC-erythrocytes (HA-FITC-erythrocytes) with target normal cells by an acidic buffer for 5 min. In the case of the FITC-erythrocytes, which were not preincubated with virus-infected cells, there was no morphological alteration of the erythrocytes after the treatment with the acidic fusion buffer with normal cells (data not shown). On the other hand, as shown in Fig. 2, many HA-FITC-erythrocytes attached densely on the cell surface, like corn. The shape of the erythrocytes was altered variously, and the outline of the labeled erythrocytes extended significantly onto the target cell surface (Fig. 2B). Although treatment with an acidic buffer (pH 5.2) was not done, a similar phenomenon of HA-erythrocytes was observed (data not shown). However, the degree of change with acidic treatment was higher than that without the treatment. From these results, we considered that HA-erythrocytes can fuse with normal target cells, especially in the case of pre-treatment with an acidic buffer.

![Fig. 1. Western Blotting with an Anti-influenza Virus Serum of 1) HA-Erythrocytes, 2) Non-treated Erythrocytes, 3) Influenza Virus-Infected CV-1 Cells, and 4) Uninfected CV-1 Cells.](image-url)

Positions of the molecular weight marker are each indicated beside. Experimental conditions are described in Materials and Methods.
acids buffer.

Next, we examined the efficiency of incorporation of the drugs from a membrane fusible erythrocyte ghost into target cells. To estimate the incorporation, we used NBD-labeled ovalbumin (molecular weight about 45000) as a model drug, and referred to this labeled protein as NBD-Ov. The NBD-Ov was entrapped into erythrocyte ghosts (NBD-Ov-ghosts), and the NBD-Ov-ghosts were incubated with influenza virus-infected cells in order to transfer HA protein to that carrier membrane (HA-NBD-Ov-ghosts). Then, after HA-NBD-Ov-ghosts were treated with normal cells by an acidic fusion buffer (pH 5.2), the fluorescence intensity of NBD-Ov, which was incorporated into the target cells through membrane fusion of the ghosts, was measured. As shown in Fig. 3, the percentage of incorporation of NBD-Ov into target cells from HA-NBD-Ov-ghosts was more than 30%, but that value from normal ghosts, which were not pre-treated with virus-infected cells, was about 5%. Therefore, the difference between both values corresponded to the amount of incorporated NBD-Ov into the target cells via fusion by the transferred HA protein on the ghost membrane surface.

In this study, we succeeded in developing a membrane fusible drug carrier from human erythrocytes due to the application of spontaneous protein transfer. Previously, there were several reports that drug- or gene-loaded liposomes or erythrocytes were fused with cells via a membrane fusible virus, such as influenza or sendai viruses. However, if those viruses still have infectious ability, that procedure poses a danger to target cells, especially in the postulated case of incorporation of these carriers into the body in the future. However, our present method is very simple and safe. Therefore, we considered that our new carrier-ghosts may become a useful tool for therapy or cellular engineering.

REFERENCES