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Note

The delivery of protein into living cells by use of membrane fusible erythrocyte ghosts

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Abstract

We examined the effects of pretreatment of monkey kidney normal cells (CV-1) and human cervical tumor cells (HeLa) with membrane fusible erythrocyte ghosts (MFEG) encapsulating superoxide dismutase (SOD-MFEG) on adriamycin (ADM) cytotoxicity. The decrease in CV-1 cells with ADM was inhibited markedly, and the initiation of decrease in HeLa cells was delayed for 2 days by the pretreatment with SOD-MFEG, indicating that the delivered SOD held the activity in the cells.

Keywords: Membrane fusion; Erythrocyte; Intermembrane protein transfer; Protein delivery

Recently, we developed the membrane fusible erythrocyte ghosts (MFEG), as a tool for delivery of protein into living cells, by reconstitution of the membrane fusible protein hemagglutinine (HA) of influenza virus on erythrocyte membranes (Kogure et al., 1997). The HA protein was reconstituted by intermembrane protein transfer (Cook et al., 1980) from virus-infected cell to erythrocyte ghost. However, it is not yet confirmed morphologically that protein is delivered certainly into the cells by MFEG, and not examined that the protein holds the activity even after the delivery. In this study, to confirm this, we examined the effects of pretreatment of living monkey kidney normal cells (CV-1) and human cervical tumor cells (HeLa) with MFEG encapsulating superoxide dismutase (SOD) on adriamycin (ADM) cytotoxicity.

The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The infection of CV-1 cells with influenza virus (NWS strain (H1N1)) were performed as described previously (Kogure et al., 1997). Proce-

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dures for preparation of erythrocyte ghost (EG) and labeling of EG with fluorescein isothiocyanate (FITC) were also described in previous report (Kogure et al., 1997). To prepare Rhodamine-labeled ovalbumin (R-Ov), 1 mM rhodamine-B-isothiocyanate was added 0.25 mM ovalbumin solution (1:1), and the mixture was shaken at room temperature for 1 h. Then, glycine (a final concentration was 5 mM) was added, and the mixture was shaken for 30 min followed by dialysis and freeze-dry. Encapsulation of R-Ov or SOD into EG and transfer of HA from cell to EG were performed as described previously (Kogure et al., 1997). For fusion of MFEG with the cells, 400 μM of 25 vol.% MFEG suspension was incubated with the cells (1.0×10^6 cells per 35 mm dish) at 4°C for 1 h. The membrane fusion was induced as described previously (Kogure et al., 1997).

To confirm morphologically the delivery of protein encapsulated in MFEG into cells by membrane fusion, we observed the normal CV-1 cells after incubation with FITC-labeled MFEG encapsulating R-Ov using a fluorescent microscope OLYMPUS BX50-34-FLA-1 (Fig. 1). To observe the both FITC (ex, 495 nm; em, 520) and rhodamine-B-isothiocyanate (ex, 554; em, 578) at the same time, wave lengths, for suitable excitation, and emission were achieved by combination of several filters. The empty FITC labeled-MFEG fused with the CV-1 cell membrane was recognized as bright lumps of the cells (stars in Fig. 1B), i.e. the shape of MFEG was remained still after fusion treatment. Although number of MFEG was a few for clear observation (Fig. 1), in fact MFEG attached densely on the cell surface as reported previously (Kogure et al., 1997). In the case of MFEG encapsulating R-Ov, around of the lump (stars in Fig. 1C) was highly whitish in color (arrows in Fig. 1C). This whitish region should be the diffused R-Ov in cytoplasm, indicating that R-Ov was present at the inside of the cells.

ADM has been suggested to become reduced semiquinone radicals in cytoplasm (Keizer et al., 1990). And then, by donation of an electron from the reduced ADM, oxygen is reduced to O_2^-. As O_2^- and derived reactive oxygen species (ROS) were considered to cause cytotoxic events such as

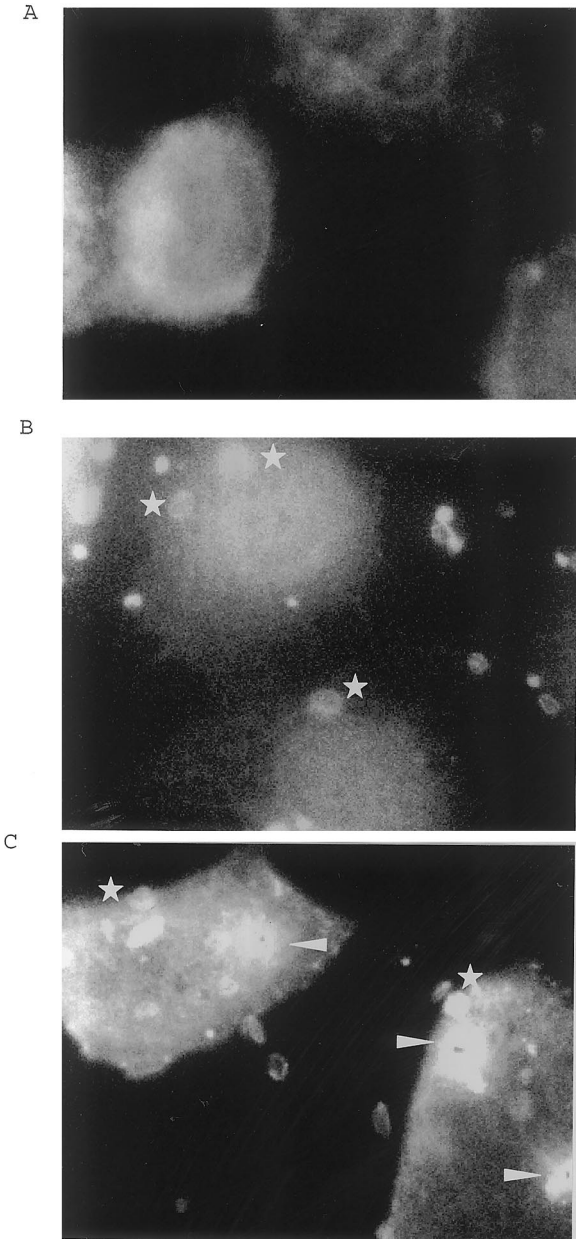

Fig. 1. A fluorescent microscopic image of (A) nontreated CV-1 cells and those of membrane fusion of FITC-labeled MFEG, which were (B) empty and (C) encapsulating R-Ov, with CV-1 cells (× 1000). Membrane-fused MFEG and released R-Ov in the cytoplams were indicated by stars and arrows, respectively.

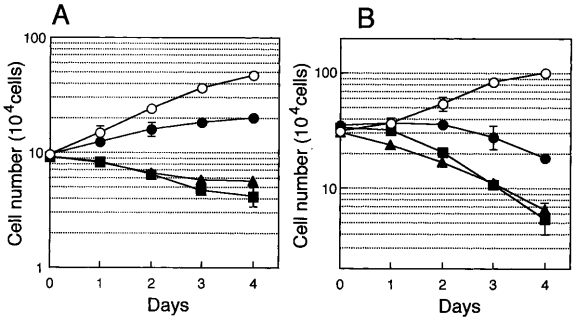

Fig. 2. Effect of pretreatment of (A) CV-1 cells and (B) HeLa cells with MFEG encapsulating SOD. Control (open circle) and MFEG (closed square) show changes of number of cells, which were fused with empty MFEG, treated without or with ADM, respectively. MFEG plus SOD (closed triangle) and SOD-MFEG (closed circle) indicate cells fused with empty MFEG along with SOD added exogenously and with SOD-encapsulated MFEG, respectively.

lipid peroxidation, ROS were suggested to participate into the ADM toxicity (Keizer et al., 1990). If SOD delivered into the cells holds the activity, ADM toxicity will be inhibited by scavenging of O_2^- with the SOD. Next, to clarify whether the delivered enzymes can function in the cells, we examined the effects of pretreatment with MFEG encapsulating SOD (SOD-MFEG) on ADM cytotoxicity against CV-1 cells and HeLa cells. ADM was added to the cells (1.0×10^6 cells per 35 mm dish), and incubated at 37°C. Then, the medium was exchanged for fresh medium, the cells were cultured for further 3 days. Treatment with ADM, in the absence of SOD, decreased the numbers of both CV-1 cells and HeLa cells (Fig. 2). As, neither DNA fragmentation nor nuclear condensation was observed (data not shown), both cell types had undergone necrotic, and not apoptic cell death. However, the conditions of ADM treatment necessary for induction of the death of both cell lines were different, i.e. the ADM concentration and the periods for treatment of CV-1 cells and HeLa cells were 30 μg/ml for 24 h and 1 μg/ml for 5 h, respectively. It was noted previously that the activities of antioxidant enzymes such as catalase and SOD in various tumor cells of mouse, rat and human origin were lower than those in normal cells (Sun, 1990), indicating that tumor cells are more susceptible for ROS than their normal counterparts. For this, the susceptibilities of HeLa cells to ADM was significantly different from that of CV-1 cells. Next, we studied the effects of the pretreatment of both cell lines with SOD-MFEG on ADM toxicity. The activity of SOD (about 6700 units per mg) encapsulated in MFEG was kept even after encapsulation. The cells were fused with 400 μl of MFEG encapsulating 25 μg/ml SOD. Growth of the cells was not affected by the treatment with empty MFEG (data not shown). Since the percentage of protein incorporation through fusion of MFEG was about 25% (Kogure et al., 1997), the amount of SOD delivered would be estimated as about 2.5 pg per cell. The pretreatment with SOD-MFEG caused significant recovery of the decrease in CV-1 cell number by ADM treatment by about 40% (Fig. 2A). That is, the delivered SOD inhibited the ADM toxicity. On the other hand, HeLa cell number was not restored, but initiation of the decrease in HeLa number was delayed for 2 days by the pretreatment with SOD-MFEG (Fig. 2B). Since delivered SOD inhibited the ADM toxicity, the hypothesis that ROS especially O_2^- is the cause of ADM cytotoxicity (Keizer et al., 1990) was confirmed. As described above, since the defense system against ROS in HeLa cells is assumed to be lower than CV-1 cells, the protective effect of SOD-MFEG in HeLa cells would be lower than that in CV-1 cells. However, the exogenous addition of SOD (a final concentration was 10 μg/ml) simultaneously with fusion of empty MFEG did not affect the decrease in cell number of either line by ADM. Thus, it was clarified that SOD was delivered certainly into the cells, and did function as the active enzyme. Furthermore, as the protective effect of SOD-MFEG was recognized in not only CV-1 cells, but also HeLa cells, it was appeared the availability of MFEG does not depend on the kind of cells. The life time of delivered SOD would be at least 2 days, because in HeLa cells the decrease in cell number was prolonged for 2 days by treatment with SOD-MFEG. That is, degradation of the delivered SOD at the inside of cell should spend at least 2 days. To prevent the degradation system, we are planing to deliver protease inhibitors into cytoplasm together with

SOD using MFEG. This project is now in progress.

Consequently, we confirmed the protein delivery using MFEG by the morphological observation and the inhibition of ADM toxicity by treatment with SOD-MFEG, and clarified that delivered enzyme held the activity.

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