Cryoprotective Effect of Gelatin and Albumin on Recombinant Human Tumor Necrosis Factor Liposome

Kiyotada YASUI,* Hiroshi FUJIOKA, and Yasuhiko NAKAMURA

Department of Formulation Research, Dainippon Pharmaceutical Co., Ltd., 5–51, Ebie 1-Chome, Fukushima-ku, Osaka 564, Japan. Received April 13, 1993

A search for a cryoprotective agent of a liposome containing recombinant human tumor necrosis factor (rHuTNF) was undertaken. Various additives were added and liposomes were freeze-thawed. As a result of these studies, albumin and gelatin were found to suppress the leakage of rHuTNF from liposome. This cryoprotective effect was only observed when the additives were added to the inside of the liposomal membrane; their addition to the outside of the membrane stimulated the leakage. This characteristic was clearly different from well-known cryoprotective agents, such as saccharides which have the cryoprotective effect on both sides of the liposomal membrane. Although this cryoprotective effect was also observed for recombinant human interleukin-1α (rHuIL-1α), it was not observed for fluorescein sodium. These effects of gelatin and albumin were restricted to macromolecular proteins such as rHuTNF and rHuIL-1α, but both substances were very effective cryoprotectants for a liposome containing protein.

Keywords liposome; freeze-thawing; cryoprotectant; gelatin; albumin; rHuTNF

Progress in gene technology has given us various proteins such as recombinant human tumor necrosis factor (rHuTNF), interleukins and interferons. These proteins have strong antitumor and other activities, but they also have various toxicities which restrict their practical use. Liposome are a delivery vehicle which can increase the activities or decrease the side effect of a pharmaceutical.

The antitumor effects of interleukin-2 and other cytokines were reportedly increased by the use of liposomes.[1-4]

Liposomes are not stable in long term storage without being frozen or freeze-dried, but there are two major problems with freeze-thawing and freeze-drying of liposomes: one is a change in size and the other is leakage of the contents due to freezing injury. Size change was reported to be completely suppressed by saccharide addition.[5,6] In the current study, no size change was found in saccharide added samples. Although there are many reports on liposomal lyophilization, complete cryoprotection for content leakage has not yet been achieved. Leakage was also suppressed by saccharide addition to some extent,[7] but the cryoprotective effect was incomplete. Glycerin, dimethyl sulfoxide (DMSO) and betain also suppressed leakage,[8-10] but, again, the effect was incomplete.

In this study, a search for a liposomal cryoprotectant was undertaken under the following conditions. The first requirement was strong cryoprotective effects without use of special conditions, such as immersion in liquid nitrogen. The second was safety and the absence of biological activity, and the third was stability in long-term storage.

Materials and Methods

Materials Egg phosphatidyl choline (EggPC, NC-10S) was purchased from Nihon Yushi Co., Ltd. Injectable hydrolysis gelatin (M.W. 7000) was purchased from Nippi Co., Ltd. rHuTNF and human interleukin-1α (rHuIL-1α) were produced in our laboratories. Fluorescein sodium was purchased from Wako Pure Chemical Co., Ltd.

Preparation of Liposomes EggPC (1 g) was dissolved in 10 ml of chloroform and this solution was evaporated until a film was formed on the bottom of the flask. Drug solution (1 ml), 188 mg/ml of sodium uranine, 2.4×10^6 JRU/ml of rHuTNF, 8.51 mg/ml of rHuIL-1α and 5 mM phosphate buffered saline (pH 7.0, 9 ml) were added to the flask and then intra-membrane additives were mixed with this solution and the flask was shaken with glass beads 1 mm in size. These multi lamellar vesicle (MLV) liposomes containing various intra-membrane additives were passed through a polycarbonate filter (0.2 μm) to control liposomal size and were separated from free drug by gel permeation chromatography (Sepharose 4B).

Extra-membrane additives were mixed with the MLV liposomes.

These liposomes had 0.22—0.24 μm diameter as determined by a Coulter nanosizer (Coulter Co., Ltd.) in Table I, and were MLV when observed by electron microscope, data was not shown.

Freeze-Thawing Procedure Samples were frozen in a freezer at −70 °C for 1 h and then thawed at room temperature until completely thawed.

Determination of rHuTNF and rHuIL-1α and Fluorescein Sodium rHuTNF and rHuIL-1α were determined by enzyme immunoassay (EIA) as reported by Sunahara *et al.*[11]

Free rHuTNF and rHuIL-1α were determined without treatments, and total rHuTNF and rHuIL-1α contents were determined after triton X-100 addition because of solubilization of liposomal membrane. rHuTNF latency was calculated by the following equation.

$$(1 - \text{free drug/total drug}) \times 100 \quad (1)$$

The concentration of fluorescein sodium was determined by fluorescein spectrophotometry (emission 510 nm, excitation 495 nm). Fluorescein sodium has the nature of self-quenching in the liposomal membrane, so free fluorescein sodium concentration was determined without treatment, and total fluorescein sodium content was determined after triton X-100 addition. Fluorescein sodium latency was calculated by Eq. 1.

TABLE I. Characteristics of Liposomes Containing rHuTNF, rHuIL-1α and Fluorescein Sodium

	Content	Entrap ratio (%)	Diameter (nm)
rHuTNF	1.8×10^6 (JRU/ml)	98.2	223±45
rHuIL-1α	0.24 (mg/ml)	99.1	232±65
Fluorescein sodium	8.5 (mg/ml)	97.4	218±32

Results and Discussion

Cryoprotective agents are generally added to a substance to reduce the amount of free water. Although there is free water on both sides of liposomal membrane, cryoprotective agents generally are added only to the outside and there were few reports about their addition to the inside. Representative cryoprotective agents sucrose and glycerin were therefore added to the inside of liposomal membrane and rHuTNF latency was determined after freeze-thawing. Gelatin, albumin and polyethylene glycol (PEG) were also

added to absorb the free water. Concentrations of gelatin and albumin in the range of 0.2 to 5% did not produce any cryoprotective effect. As shown in Fig. 1, all additives except for sucrose exhibited the higher latency than non additive control.

In general, saccharides are strong cryoprotective agents for liposomes, but they had little effect when added to the inside of the liposomal membrane. In contrast, this study showed that albumin and gelatin have a strong cryoprotective effect. In MLV liposomes, there are several lipid bilayers on the inside of the aqueous layer. The distance between membrane layers is about 12—15 nm[12] and the molecular size of albumin is 3.8×15 nm.[13] Further, it was reported that liposomal membranes interact with a hydrophobic region of albumin.[14] Albumin has the potential for the lipid–protein–lipid bridging among the membranes of MLV liposome. Gelatin also contains hydrophobic amino acids such as tryptophan, and there is the potential for protein–lipid interaction. But the molecular weight of this gelatin is about 7000 and its molecular size is much smaller than albumin, thus it is too small to interact with lipid–protein–lipid.

To determine the cryoprotective effect of gelatin, it was added to the inside of the liposomal membrane, trehalose was added to the outside of the membrane and liposomal latency was determined after freeze-thawing. As shown in Fig. 2, trehalose dose-dependently suppressed the rHuTNF leakage from liposome whether or not it contained gelatin.

Crowe *et al.* reported that trehalose has a good cryoprotective effect on liposome containing Na and inulin when added to both sides of the membrane,[5] and Miyajima *et al.* also reported that mannitol and D-glucose suppressed the leakage of calscein when added to both sides of liposomal membrane.[8]

In this study, trehalose didn't exhibit a cryoprotective effect on rHuTNF liposome when it was added to the inside of liposomal membrane. It was reported, however, that the cryoprotective effect of trehalose depends on the trehalose/lipid ratio, so further studies on this are needed. This suggested the unique nature of liposome containing protein and also suggested there may be a different mechanism of cryoprotective effect between trehalose and gelatin.

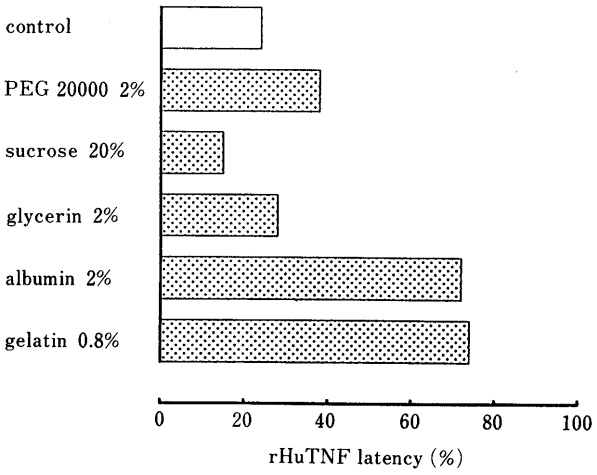

Fig. 1. rHuTNF Latency of Liposomes Containing Various Additives (Dotted Column) or Not (Open Column) in the Inside of the Liposomal Membrane after Freeze Thawing

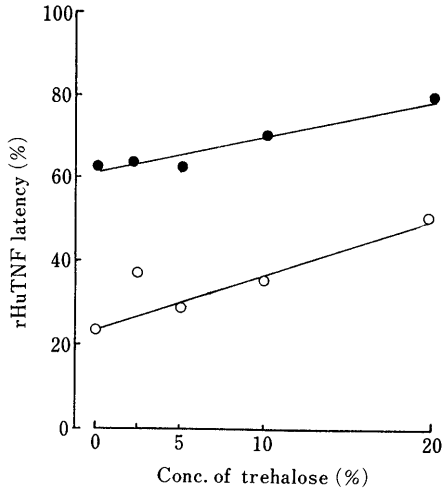

Fig. 2. Effect of Trehalose Concentration in the Outside of Membrane on rHuTNF Latency of Liposomes Containing Gelatin (●) or Not (○) after Freeze Thawing

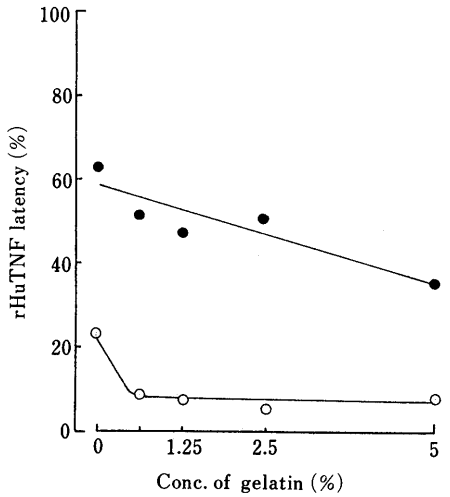

Fig. 3. Effect of Gelatin Concentration in the Outside of the Membrane on rHuTNF Latency of Liposomes Containing Gelatin (●) or Not (○) after Freeze Thawing

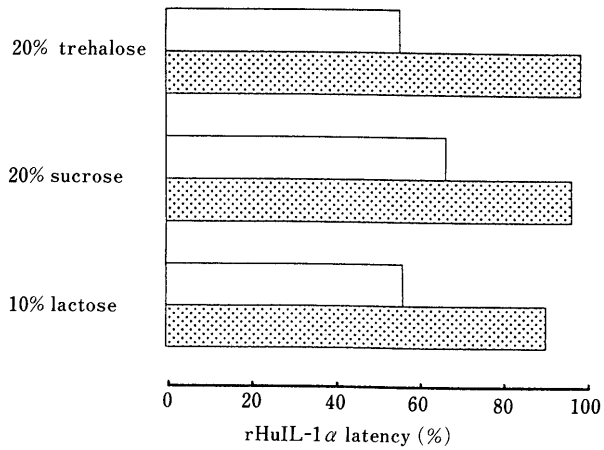

Fig. 4. rHuIL-1α Latency of Liposomes Containing 0.8% of Gelatin (Dotted Column) or Not (Open Column) in the Case of Saccharide Additions to the Outside of the Liposomal Membrane after Freeze Thawing

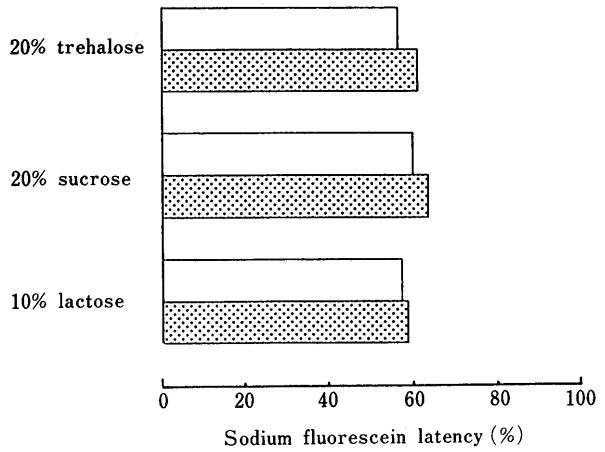

Fig. 5. Fluorescein Sodium Latency of Liposomes Containing 0.8% of Gelatin (Dotted Column) or Not (Open Column) in the Case of Saccharide Additions to the Outside of the Liposomal Membrane after Freeze Thawing

Gelatin was added to the outside of the membrane and rHuTNF latency was determined after freeze-thawing, as shown in Fig. 3, this addition caused an increase in rHuTNF leakage from the liposomes, even though gelatin was also present in the inside of the membrane.

It is therefore clear that gelatin only exerts the cryoprotective effect when added to the inside of the membrane. This property is quite different from the well-known liposomal cryoprotectants such as saccharides and DMSO.

The reason for the increase of rHuTNF leakage from the liposome when gelatin was added to the outside of the membrane is not still clear. But dextran T 100 is reportedly a destabilizer in spite of a large quantity of unfreezable water.[8]

We were then interested in the different drugs from rHuTNF. We used rHuIL-1α as a protein and sodium fluorescein as a low molecular compound.

Although gelatin suppressed the rHuIL-1α leakage from liposome, it didn't suppress fluorescein sodium leakage, as shown in Figs. 4 and 5.

The cryoprotective effect of gelatin and albumin was restricted to the macromolecules, rHuTNF and rHuIL-1α and was not observed in small molecules such as fluorescein sodium.

Gelatin is representative substance which has the property of gelation at low temperature. So, gelation may contribute to increasing the liposomal stabilization on freezing.

But its molecular weight is 7000 and this gelatin does not have the property of gelation. Albumin also has a cryoprotective effect and also lacks the property of gelation. Gelation therefore is not related to the cryoprotective effect.

The mechanisms of freezing injury to living cells have long been studied and accumulated evidence suggests there are several mechanisms of freezing injury. (1) The formation of intracellular ice found by microscopic observation.[15] (2) Osmotic dehydration of cells, resulting from the formation of ice outside, but not inside the cells, as shown by the volume shrinkage of cells.[16] (3) The integrity of the lipid membrane may suffer on freeze-thawing, if a phase transition from a liquid like (or liquid crystal) to a crystalline state occurs. This process has been studied in model systems.[17]

The cryoprotective mechanism of gelatin is not yet clear, but proteins were reported to prevent liposomal damage during freezing,[18] and it is suggested that this lipid–protein interaction played an important role in cryoprotection.

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