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ACTIVITIES OF CELL FUSION AND LYSIS OF THE HYBRID TYPE OF CHEMICAL FUSOGENS (I) STRUCTURE AND FUNCTION OF THE PROMOTOR OF CELL FUSION

Kenji Honda[1] *, Yuki Maeda[1], Shigeru Sasakawa[1],
Hiroyuki Ohno[2] and Eishun Tsuchida[2]

1. Department of Research, Central Blood Center,
Japanese Red Cross, 4 Hiroo, Shibuya-ku, Tokyo 150, Japan
2. Department of Polymer Chemistry, Waseda University,
3 Ohkubo, Shinjuku-ku, Tokyo 160, Japan

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Summary: Monomeric lipids, for example, oleic acid, stearic acid, lauric acid, oleyl alcohol and p-isooctyl phenol, were introduced through ester or ether bonds to oligoethylene glycol with various molecular weights. The activities of hemolysis and fusion of these oligomeric lipids were examined using human erythrocytes in the presence or absence of high molecular weight of water-soluble polymers such as polyethylene glycol (Mw=6,000) or dextran (Mw=70,000). When the chain length of polyoxyethylene of the oligomeric lipids was less than 20, the activities of hemolysis and fusion were both enhanced. On the other hand, when the chain length was more than 20, the two activities were reduced to a different degree for each other.

INTRODUCTION

Recently, the technique of cell fusion is being extensively utilized in the fields of genetic engineering, cell technology, immunological technology, etc. (1-5). Especially, polyethylene glycol (PEG) is generally used as a fusogen, because (1) it can be utilized not only for animal cells but also for plant protoplasts, (2) the procedure is very simple, and (3) PEG, itself, is relatively stable and safe as materials compared with virus. The present authors have already found that as a result of the purification of commercial-grade PEG by reprecipitation, the fusogenicity of PEG almost completely disappeared, although its cell aggregability was not reduced at all (6). The chemicals removed in the purification were identified to be antioxidants like α-tocopherol or other phenolic derivatives, which are generally added to commercial-grade PEG,

* To whom correspondence should be addressed

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and it was ascertained that these fat-soluble additives promoted the cell fusion in the coexistence of PEG. It has, therefore, been concluded that commercial-grade PEG contains two components in regard to the activity of cell fusion: One is the high molecular weight of PEG and the other is the low molecular weight of lipids like α-tocopherol. The former induces only cell aggregation and the latter promotes membrane fusion. In this work, when the purified PEG-6,000 was used as the cell aggregating factor, water-soluble oligomeric lipids prepared by esterification or etherification of PEG of less than 1,000 of molecular weight with various fatty acids or alcohols were examined in regard to the potentials of the promotor of cell fusion.

MATERIALS AND METHODS

Heparinized human venous blood was collected from healthy donors. Plasma protains and buffy coats were removed by centrifugation (3,000 rpm, 5 min, at 5 °C) and erythrocytes were further washed with Eagle's basal salt solution (Eagle's M.E.M.(Nissui Seiyaku Co.,Ltd.,Nissui 2) 9.4 g/l + sodium cacodylate 1.6 g/l + glucose 1.0 g/l, pH 7.4). Washed erythrocytes were resuspended in Eagle's b.s.s., which contains 1.8 mM of calcium ions, at 1 % of hematocrit and 1 ml of this suspension was centrifuged (1,200 rpm, 5 min, at 5 °C). The supernatant was almost completely removed by aspiration and then 1 ml of 50 wt % of PEG solution of the same buffer was mixed with the precipitates. After this suspension was incubated at 37 °C for 1 min, 10 ml of Eagle's b.s.s., which prewarmed at 37 °C, was added to it and incubated at 37 °C for 5 min again. Then, fused cells were collected by centrifugation (1,200 rpm, 5 min, at 25 °C) and fixed with 1 % of glutaraldehyde for assay of fusion indices by optical microscopy. The fusion index was calculated from the ratio of fused cells to total cells in several fields of microphotographs with 500-600 cells counted. As for aggregated cells, all single cells making up the aggregated cells were counted. Lysed cells, that is, ghosts of erythrocytes were eliminated from the cell counting.

PEG-6,000, which purchased from Wako Junyaku Co.,Ltd., was purified as follows: 10 g of PEG dissolved in 100 ml of chloroform was poured into 2,000 ml of diethyl ether. The resulting white precipitates were dried in vacuo at room temperature over two days. The oligomeric PEGs which have various fatty acids or alcohols at the terminal OH groups were donated by Nippon Oils & Fats Co., Ltd. and used without further purification, as they used no additives such as antioxidants. The chemical compositions of the lipid-containing oligomeric PEGs were listed in Table I.

RESULTS AND DISCUSSION

Fig.1 shows the correlations of the degree of hemolysis to the chain length of polyoxyethylene and the concentration of (a) oleic acid-containing PEG and (b) oleyl alcohol-containing PEG. These results demonstrate the

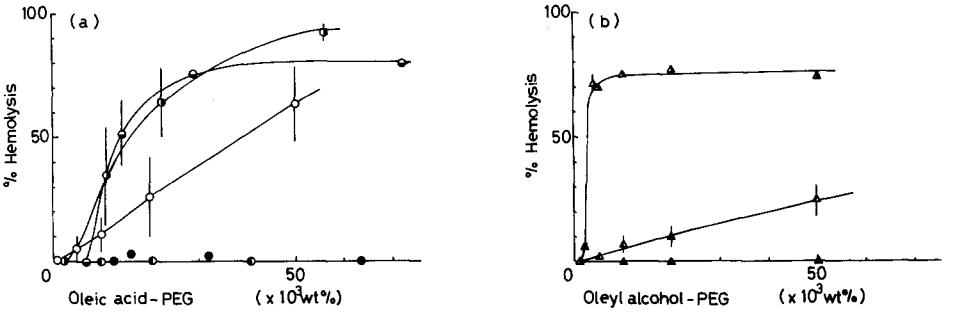

Fig.1 Dependencies of the chain length of (a) oleic acid-PEG conjugates and (b) oleyl alcohol-PEG conjugates on the hemolytic activity to human erythrocytes in the absence of purified PEG-6,000. Cell density 10^7 cells/ml, in Eagle's b.s.s. containing 1.8 mM Ca^{2+}, pH 7.4, 37 °C, 6 min. % hemolysis in the ordinate was determined from the absorbance at 578 nm of supernatant of cell suspension after incubation with oligo-PEG, where the absorbance after complete hemolysis with distilled water was made 100 %. (a) -◑- n=9, -◒- n=14, -◐- n=23, -●- n=40, -○- oleic acid (b) -▲- n=10, -▲- n=20, -▲- n=50.

following characteristics of these oligomeric lipids on the hemolytic activity: (1) a strong dependency of the chain length of polyoxyethylene, (2) a significant enhancement of the hemolytic activity of the lipids due to the introduction to oligomeric PEG, and (3) an obvious effect of the flexibility of the lipid moiety. When the chain length of polyoxyethylene exceeded a certain value, the hemolytic activity was completely reduced. For example, oleic acid-

Table I

Chemical composition of fatty acid-PEG and fatty alcohol-PEG conjugates

Components (%)	Oleic acid-PEG(n)			Lauric acid-PEG	Oleyl alcohol-PEG
	n=9	n=14	n=23	(n=9)	(n=10)
free lipid	1.2	1.5	1.1	3.1	0.5
free PEG	3.7	1.5	1.6	14.0	0.3
lipid-PEG conjugate	95.1	97.0	97.3	82.9	99.2

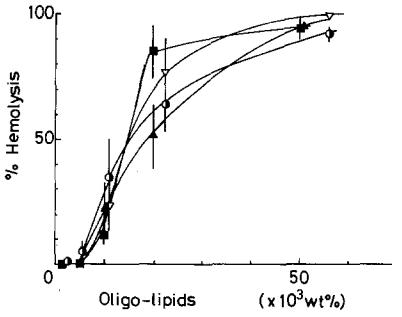

Fig.2 Effect of terminal hydrophobic groups (R) of fatty acid-PEG(n=9) conjugates on the hemolytic activity to human erythrocytes in the absence of purified PEG-6,000. The conditions were the same as those of Fig.1.
-▲- $CH_3(CH_2)_{10}$-COOH, -◑- $CH_3(CH_2)_7CH{=}CH(CH_2)_7$-COOH,
-▽- $CH_3(CH_2)_{16}$-COOH, -■- C_8H_{17}--OH.

containing PEG with 23 of the chain length was not hemolytic at all. On the other hand, oleyl alcohol-containing PEG with 20 of the chain length had still a hemolytic activity. Generally, if the chain length was less than 20, the hemolytic activity of the oligomeric lipids was higher than that of original ones. This is because the lipids became water-soluble due to the binding to PEG so that they could more easily interact with plasma membranes, especially with the phospholipid bilayers. Moreover, the hemolytic activity of ether type of the oligomeric lipids was higher than that of ester type of the derivatives. It may come from the fact that ether bond is generally more flexible than ester bond. In addition, there appeared a critical concentration of the oligomeric lipids at which the hemolysis was suddenly initiated. It was nearly equal to the critical micelle concentration of those surfactants (7-9). Therefore, it is suggested that the formation of regularly ordered structure in solution of the lipids like micelle is necessary for the initiation of hemolysis.

Fig.2 shows the correlation of the degree of hemolysis to the chemical structure of the terminal hydrophobic groups using the ester type of the oligo-

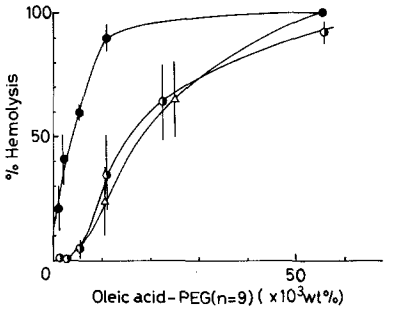

Fig.3 Effect of high polymers added to oligo-PEG on the hemolytic activity of oleic acid-PEG(n=9) conjugate. -◑- no high polymers added, -●- purified PEG-6,000 added, 50 wt %, -△- dextran (Mw=70,000) added, 5 wt %.

meric lipids. There appeared no significant difference in the degree of hemolysis among lauric acid-, stearic acid- and oleic acid-containing PEGs. In addition, the hemolytic activity of p-isooctyl phenol-containing PEG was the same as those. The capabilities of hemolysis of these oligomeric lipids were greatly enhanced in the presence of high molecular weight of PEG-6,000 as shown in Fig.3. This is because erythrocytes are swollen to spherocytes in 50 wt % of PEG-6,000 solution so that the plasma membranes became unstable. On the other hand, the similar effect was not observed in the presence of dextran (Mw=70,000, 5 wt %). Dextran is known to protect plasma membranes (10). Thus, the high molecular weight of polymers added to the oligomeric lipids had an important influence on hemolysis. There are two different behaviors of the high polymers used as a cell aggregating factor. High molecular weight of PEG more strongly interacts with plasma membranes, especially with membrane-bound proteins, than dextran so that it destabilizes the membranes. However, PEG is known to be superior to dextran in regard to the capability of cell aggregation (11).

Fig.4 shows the correlations of the degree of cell fusion to the chain length and the concentration of (a) oleic acid-containing PEG and (b) oleyl alcohol-containing PEG. There appeared no difference in the fusogenicity in

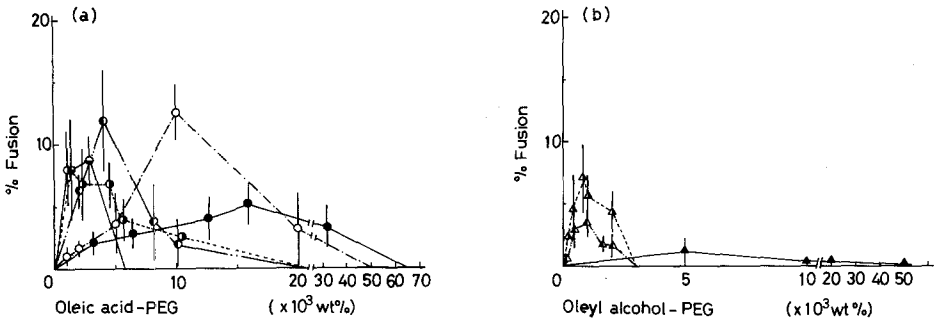

Fig.4 Dependencies of the chain length of (a) oleic acid-PEG conjugates and (b) oleyl alcohol-PEG conjugates on the fusion activity to human erythrocytes in the presence of 50 wt % of purified PEG-6,000. (a) -◑- n=9, -◒- n=14, -◐- n=23, -●- n=40, -○- oleic acid (b) -◭- n=10, -◭- n=20, -▲- n=50.

the range of the chain length of 10-20. However, the fusogenicity was greatly reduced above 40 of the chain length. It was a general tendency that the degree of cell fusion was reduced and the concentration of oleyl groups at which the maximum degree of cell fusion appeared became higher as the chain length increased. The order of fusogenicities of hydrophobic groups bound to the terminal OH groups of PEG was as follows: oleic acid $>$ oleyl alcohol $\simeq$ lauric acid $\simeq$ stearic acid $\gg$ p-isooctyl phenol. Of the conventional fusogens previously reported, hemolysis was found to increase with increasing activity of cell fusion. On the other hand, the fusion activity of the oligomeric lipids reported in this paper could be regulated by the chain length of polyoxyethylene and the concentration of the polymers applied and, furthermore, their fusion activity was not necessarily in parallel with the hemolytic activity. This suggests that the suppresion of lysis and the promotion of cell fusion could be achieved at the same time by controlling the chain length of polyoxyethylyne, the hydrophobicity of the introduced lipid moieties and the concentration of the polymers applied.

As the result that the fusion activities of the new type of polymeric fusogens were examined using various kinds of promotors of cell fusion, the hybrid type of fusogen consisting of purified PEG-6,000 and oleic acid-containing PEG-1,000 was found to be more fusogenic 1.5-2.0 fold than commercial PEG-6,000. In further developments, by selecting the combination of the promotor and the aggregator of cell fusion, more fusogenic but less lytic polymers could be prepared.

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