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Induction of neuron-like tubes and liposome networks by cooperative effect of gangliosides and phospholipids

Kazunari Akiyoshi[a,b,*], Ayako Itaya[c], Shin-ichiro M. Nomura[a,d], Naoaki Ono[e], Kenichi Yoshikawa[f]

[a] *Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan*
[b] *PRESTO, Japan Science and Technology Corporation, JST, Japan*
[c] *Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida-Hommachi, Sakyo-ku, Kyoto 606-8501, Japan*
[d] *Research Fellow of the Japan Society for the Promotion of Science, Japan*
[e] *ATR, 2-2-2 Hikaridai, Seika-cho, Soraku-gun, Kyoto 619-0288, Japan*
[f] *Department of Physics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecyo, Sakyo-ku, Kyoto 606-8502, Japan*

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Abstract **Although there is a rather large abundance of gangliosides in neurons, their functional role is still unclear. We focused on a physicochemical role of gangliosides in the formation of tubular structures, such as axons or dendrites in neurons. When a ganglioside, GM3, was added to cell-size liposomes that consisted of dioleoylphosphatidyl-choline, tubular structures were induced and liposome networks connected by the tubes were observed by differential interference microscopy and fluorescence microscopy. The potential for various gangliosides to induce tubes was dependent on the structures of their hydrophilic head group. With a large excess of gangliosides, the tubes are destabilized and small fragments, or micelles, are generated. The phenomenon was suggested by physical model calculation. Gangliosides may play a role as building material in neural unique tubular structures.**

Key words: Ganglioside; Neuron; Giant liposome; Nanotube; Network structure; Microscopic observation

1. Introduction

Gangliosides play a role in various cellular functions, especially in the neural system, such as the differentiation of neurons and the formation of axons, dendrites and synapses [1–3]. Recently, it has been found that gangliosides form microdomains on the outermost surface of the cell membrane [4]. Such microdomains play important roles in signal transduction, and as a result induce the formation of dendrites [5]. Gangliosides accumulate at high levels in brain tissue (more than 10 mol% of total lipids). However, the reason for such a high accumulation of gangliosides in the brain is not yet clear. The brain consists of various neurons with quite unique tubular structures. The structures of ganglioside oligosaccharides in the cell membrane of neurons change drastically during their differentiation [6]. Therefore, gangliosides are believed to be related to the specific morphogenesis of neurons. Compared to various physiological studies, there have been few physicochemical studies on the effects of gangliosides on morphological changes in lipid bilayer membranes [7,8]. We investigated the spontaneous morphological changes in giant cell-size liposomes in the presence of gangliosides as a model cell membrane.

2. Materials and methods

2.1. Lipids

Dioleoylphosphatidyl-choline (DOPC) was obtained from Fluka. Monosialoganglioside-GM1 (bovine brain) was obtained from Avanti Polar-Lipids. Monosialoganglioside-GM2 (bovine brain), monosialoganglioside-GM3 (bovine brain), disialoganglioside-GD1a (bovine brain), disialoganglioside-GD1b (bovine brain), disialoganglioside-GD3 (bovine brain), trisialoganglioside-GT1b (bovine brain), tetrasialoganglioside-GQ1b (bovine brain), asialo-ganglioside-GM1 (bovine brain), and *N*-oleoyl-D-sphingomyelin (bovine brain) (SM) were obtained from Sigma Chemical Co. Lactosyl ceramides (bovine, butter milk) (Lac-Cer) were obtained from Funakoshi Co. GM3 labelled with NBD (nitrobenzo-2-oxa-1,3-diazole) (GM3-NBD) was obtained from Peptide Institute, Inc. 2-[4-(2-Hydroxy-ethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2′,7′-{[bis(carboxymethyl)amino] methyl}fluorescein (calcein) were obtained from Dojin Chemical Co. $MgCl_2{\cdot}6H_2O$ and $CoCl_2{\cdot}6H_2O$ were obtained from Wako Chemical Co. Deionized water (Mill-Q system; Millipore) was used in all experiments.

2.2. Preparation of giant liposomes (GLs) and tubes

GLs were prepared by the method reported previously [9]. GLs of neutral phospholipids such as DOPC spontaneously form in the presence of appropriate concentration of magnesium ions (10 mM) without the use of an organic solvent. It is known that charged lipids or a mixture of neutral phopholipids with charged lipids form unilamellar giant vesicles under natural swelling [9]. Magnesium ions have a high affinity for the negatively charged phosphate group in neutral phospholipids. By the addition of magnesium ions, neutral phospholipids are, therefore, expected to behave as positively charged lipids, inducing the spontaneous formation of GLs. DOPC was dissolved and stored at 10 mM in chloroform:methanol (1:1, v/v). SM and sphingoglycolipids were dissolved and stored at 1 mM in chloroform:methanol (1:1, v/v). Suspensions of sphingoglycolipids, DOPC and DOPC/sphingoglycolipids (various molar fractions of gangliosides) were prepared as follows: 0.1 μmol (total lipid concentration) of dry lipid film was prepared by evaporating a chloroform:methanol (1:1, v/v) solution containing various lipids in a 10-mm test tube under a nitrogen stream, and then stored for at least 4 h under aspiration. The resulting lipid film was swollen with 100 μl of HEPES buffer (10 mM, pH 7.0, 10 mM $MgCl_2$), where the final concentration of lipid was 1 mM. For

*Corresponding author. Fax: (81)-3-5280-8027.
E-mail address: akiyoshi.org@tmd.ac.jp (K. Akiyoshi).

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fluorescence imaging of liposomes and tubes, thin films of DOPC/GM3 containing GM3-NBD were prepared by adding GM3-NBD to the starting lipid mixture at 2.0% by mol.

2.3. Microscopic observation

A cover glass was fixed at the center of a slide glass by using double-sided adhesive tape (the thickness is about less than 0.5 mm). DOPC liposomes were injected from one side of the space between the slide glass and the cover glass. GM3 suspensions containing GM3-NBD were injected from the other side. The two solutions were mixed gently at the center of the slide glass. A differential interference microscope and a fluorescence microscope (E600, Nikon, Japan) with a 100-W high-pressure Hg lamp as a light source were used to observe the liposomes and tubes. The images were captured with a digital camera (C4743-95, Hamamatsu Photonics, Japan) and recorded and analyzed with an image processor (Aqua Cosmos, Hamamatsu Photonics, Japan). In the fluorescence quenching experiment, a DOPC lipid film was swollen with 100 μl of HEPES buffer (10 mM, pH 7.0, 10 mM $MgCl_2$) containing calcein (0.1% by mol). These DOPC liposomes and GM3 were mixed on the slide glass, and then $CoCl_2$ solution for quenching was injected. In observing suspensions after the hydration of thin films of various mixtures of DOPC/gangliosides, the experiments were carried out several times for each sample and the average ratios of liposomes and tubes were estimated for 15–20 areas in the slide glass. The numbers of liposomes and tubes were counted in each area. The average percentages of tubes were evaluated as following four regions: +++, 50%<; ++, 30–50%; +, 10–30%; −, <10%.

2.4. Electron microscopic observation

Electron microscopy was carried out with negative staining using a JEM-100SX (JEOL) operated at an acceleration voltage of 80 kV. 5 μl of the sample solution and 5.0 μl of an aqueous solution of phosphotungstate as a stain (1.0% by weight, pH 7.0) were dropped on a Collodion-coated 200 Cu mesh grid (Nisshin EM Co., Ltd., Japan). After 4 min, the excess liquid was absorbed at the periphery of the grid using filter paper. This grid was then stored for at least 1 h under aspiration.

3. Results

3.1. Formation of liposome networks

GLs at the micron scale have attracted growing interest as cell models [10–14]. GLs are useful for following morphological changes with time without perturbation by microscopy. When DOPC is swollen in the presence of magnesium ion, GLs on the order of several tens or hundreds of micrometers in size are obtained (Fig. 1A) [9]. We investigated the morphological changes in DOPC-GL upon the addition of gangliosides. A thin film of the ganglioside GM3 and GM3 labelled by NBD (2 mol%) was swollen with HEPES buffer (10 mM, pH 7.0, containing 10 mM $MgCl_2$). GM3 formed small aggregates smaller than 1 μm (Fig. 1B). Suspensions of both GL and GM3 were mixed on a slide glass with a cover glass, and the mixture was observed by differential interference microscopy and fluorescence microscopy. Interestingly, liposome networks formed immediately after the two suspensions were mixed (Fig. 1C,D). To elucidate the structure of these networks, GL was prepared in the presence of a water-soluble fluorescent probe, calcein, and then mixed with the GM3 suspension. To this suspension was added the fluorescence quencher Co(II) ion, which does not penetrate a bilayer membrane. The calcein in the bulk phase of the network was quenched by Co(II) ion. Fig. 1E shows that the inside of the tubular structures contains the fluorescent probe, which prevents penetration by Co(II) ion. This indicates that the liposomes were connected by tubular structures with an internal water phase. The size of tubes in the network was less than 1 μm judging from Fig. 1D.

3.2. Morphological change of GLs in the presence of gangliosides

To obtain further information on the effect of gangliosides in the induction of morphological changes in liposomes, we performed hydration experiments with thin films of various ganglioside/DOPC mixtures. For example, when a thin film containing 2 mol% of GM1 was hydrated in HEPES buffer (10 mM, pH 7.0, containing 10 mM $MgCl_2$), tubular structures as well as liposomes were observed. In the presence of 10 mol% of GM1, well-extended straight-chain tubular structures (1–10 μm wide) were mainly formed. Furthermore, in the presence of more than 40 mol% of GM1, the tubular structures disappeared and changed to small liposomes (smaller than 1 μm) (Fig. 2, the rightmost picture). Similar morphological changes that depended on the structures of gangliosides occurred with other mono-sialo gangliosides such as GM2, GM3 and also poly-sialo gangliosides such as GD1a, GD1b, GD3, GT1b and GQ1b (Table 1); tubular structures are induced at low levels and small vesicles are formed with an excess of ganglioside. The optimal content for tube formation depends on the structure of the gangliosides. For example, more than 10 mol% of GM3 is needed to induce a tubular structure, while only 5 mol% is needed for GM1 or GM2. On the other hand, the tubes were still seen in the presence of 70

Table 1
Tubular formation in the presence of various gangliosides

mol %	GM1	GM2	GM3	GD1a	GD1b	GD3	GT1b	GQ1b	Lac-Cer	Sphingo
2	−	−	nd	−	−	−	−	nd	nd	nd
5	++	++	−	++	+	−	++	−	+	+
10	+++	++	++	+++	++	++	++	++	−	−
20	+++	+++	+++	+++	+	++	++	++	−	−
30	++	++	++	+	+	+	−	++	−	−
40	−	++	++	−	−	−	−	−	−	−
50	−	+	+	nd	nd	nd	nd	nd	nd	nd
60	−	−	++	nd	nd	nd	nd	nd	nd	nd
70	nd	nd	+	nd	nd	nd	nd	nd	nd	nd
80	nd	nd	−	nd	nd	nd	nd	nd	nd	nd
90	nd	nd	−	nd	nd	nd	nd	nd	nd	nd

+++, 50%<; ++, 30–50%; +10–30%; −, <10 and nd, not determined. Percentages stand for the ratio of the tubes in the microscopic images. The data were obtained by the microscopic observation of suspensions after the hydration of thin films of ganglioside/DOPC mixtures in HEPES buffer (10 mM, pH 7.0, 10 mM $MgCl_2$).

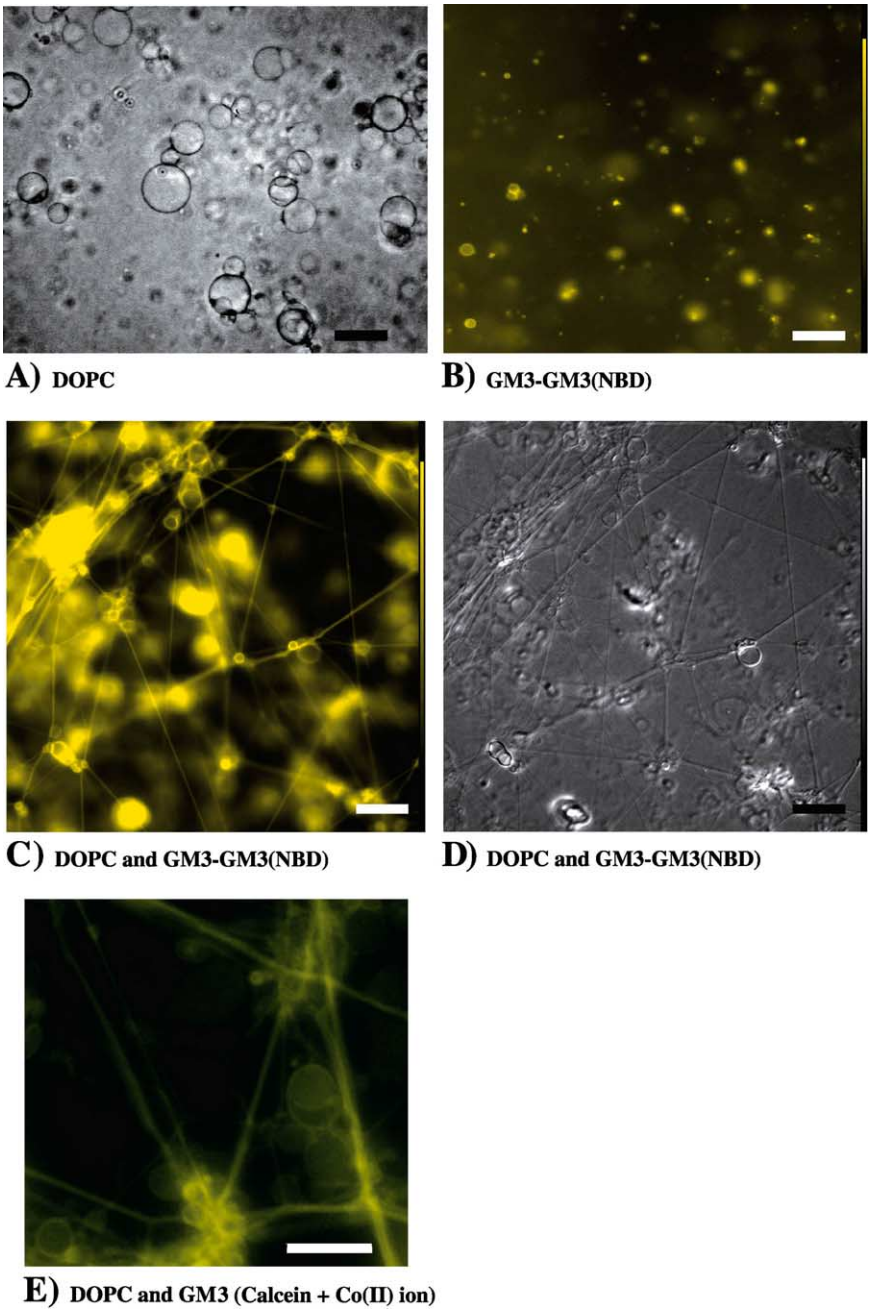

Fig. 1. Optical microscopic observations of a DOPC giant liposome (A), GM3-GM3-NBD (B), a mixture of DOPC liposome and GM3-GM3-NBD in HEPES buffer (10 mM, pH 7.0, 10 mM $MgCl_2$ (C, D), and a mixture of DOPC liposome and GM3 in the presence of calcein (0.05 mM) and $CoCl_2$ (0.025 mM) in HEPES buffer (10 mM, pH 7.0, 10 mM $MgCl_2$) (E). Bar = 20 μm. A and D: differential interference microscopic images, B, C and E; fluorescence microscopic images.

mol% of GM3, while they disappeared in the presence of more than 40 mol% of GM1 or more than 60 mol% of GM2. As the size of the oligosaccharide moiety of gangliosides increases, tubes are induced at a lower ganglioside concentration, while tubes change into small liposomes at lower concentrations. Once the tubular structures were formed, they were quite stable after 24 h at room temperature or even after heating at 45°C for 1 h. Lactosyl-cerebroside and SM are not as effective for the induction of tubular structures (Table 1). In the presence of other kinds of phospholipids, such as phosphatidic acid, phosphatidylserine, and phosphatidylethanolamine, large liposomes formed, but not tubular structures.

Interesting morphological changes were also observed by electron microscopy of GM1 and GD1a systems, which mainly exist in the brain, as shown in Fig. 3. Dipolar liposomes, in which two nanotubules (25 nm wide) extended from a liposome, were observed in the GD1a system. Unipolar liposomes connected to nanotubes (60 nm wide) were formed in the GM1 system. The results of electron microscopy and optical microscopy indicate that various radii of tubes such as

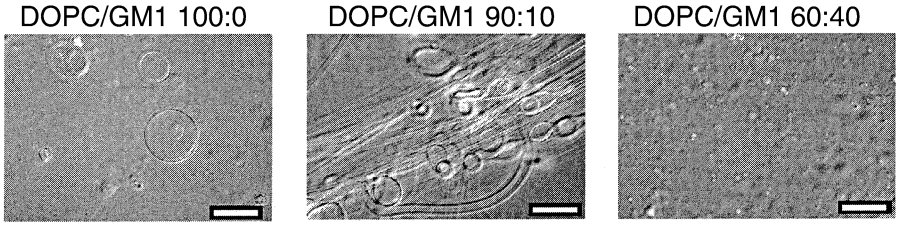

Fig. 2. Microscopic images of ganglioside (GM1)/DOPC suspensions. The tubule formed and fragmented with higher concentration of the ganglioside in DOPC (see Table 1). Bar = 20 μm.

both nano- and micron-size tubes are induced in the presence of gangliosides.

4. Discussion

Lipid tubes and nanotubes are subjects of growing interest in nanotechnology and biotechnology [15–17]. However, there have been few reports on liposome networks, and these were constructed by micromanipulation using a micropipette [18] and by piercing liposomes with a carbon nanotube [19]. To the best of our knowledge, this is the first report of self-organizing three-dimensional liposome networks in situ. Many liposomes can be connected spontaneously at once.

What factors determine the morphological changes in the presence of gangliosides? The formation of tubes in one-component systems has been explained by a mechanism involving helical ribbons based on the chiral packing of chiral lipids [15,17,19–21]. However, in multi-component and dynamic systems such as the mixtures of two lipids described here, other factors should be considered in the formation of tubes. Tubes are characterized by the existence of an orthogonal anisotropic plane in a bilayer structure. A tube cross-section is similar to the structure of a vesicle, with a high curvature of the outer surface. On the other hand, the other tube section follows a tighter curve. Tubes may be intermediate structures in the morphological change from GLs to small liposomes or small aggregates. In general, the morphology of the self-assembly of amphiphilic molecules, especially in the fluid state, can be discussed in terms of a packing parameter, $V/a_o l_c$, where a_o is the optimal surface area, V is the volume of the hydrocarbon, and l_c is the critical chain length [22]. The molecular structures of phospholipids with double acyl chains are well suited for the formation of bilayers with a tight mean curvature, such as GLs. DOPC sometimes shows polymorphism which includes a tubular structure in the hydration of a thin film of DOPC [23,24]. In our experiment, DOPC mainly formed GLs under controlled conditions in the presence of Mg^{2+}. On the other hand, gangliosides are cone structures with small packing parameters (0.4–0.5) (Fig. 4A). Therefore, most gangliosides except GM3 tend to form small micelles [25,26]. The anisotropic structures of tubes should be stabilized by the cooperative effect of gangliosides and phospholipids, which have different packing parameters. Among gangliosides systems examined, the tubes were still seen even in the presence of 70 mol% of GM3. The ability of GM3 for perturbation or destabilization of GL is weak because GM3 has a higher packing parameter (0.5 <) than other gangliosides and it forms vesicles. This is a plausible explanation for the higher stabilization of tubes in the presence of the higher concentration of GM3.

At a molecular level, another important factor for the induction of tubes may be the branched structures of the ganglioside oligosaccharides. Gangliosides have anisotropic branched structures that can stabilize one large curvature (oligosaccharide chain) and another curvature (branched sialic acid), for two orthogonal planes (Fig. 4A). Thus, gangliosides seem to have characteristics favorable for the stabilization of tubular structures.

We present a simple model to explain the transition from giant vesicles to tubes. In the spontaneous curvature model of Helfrich, the bending energy per unit area of a membrane is given by, $2\kappa(H-\chi_0)^2$ where κ is the bending modulus, χ_0 denotes the spontaneous curvature, and H gives the mean curvature of the membrane [27]. Under reasonable approximation, we consider that the bending energy is the most dominant factor to interpret the stability of tube [22]. We assume that the curvature of the tubular membrane is determined by the difference in the lipid composition between the inner and outer layers. The spontaneous curvature of the binary membrane can be approximated by the linear average, $\chi_0(\rho) = \rho\chi_0^G + (1-\rho)\chi_0^D$, where χ_{0G} and χ_{0D} denote the spontaneous curvature of GM and DOPC, respectively, and ρ denotes the molar fraction of GM. Fig. 4A illustrates the structures of DOPC and a series of mono-sialo gangliosides (GM). Con-

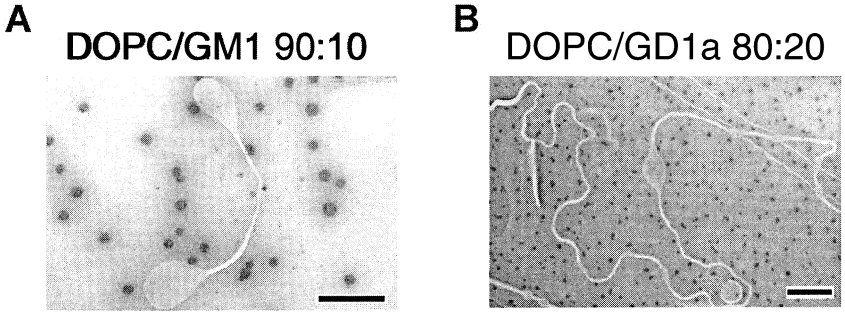

Fig. 3. Electron micrographs of tubular structures of A: GM1/DOPC (bar = 1 μm) and B: GD1a/DOPC (bar = 0.5 μm) in HEPES buffer (10 mV, pH 7.0, 10 mM $MgCl_2$).

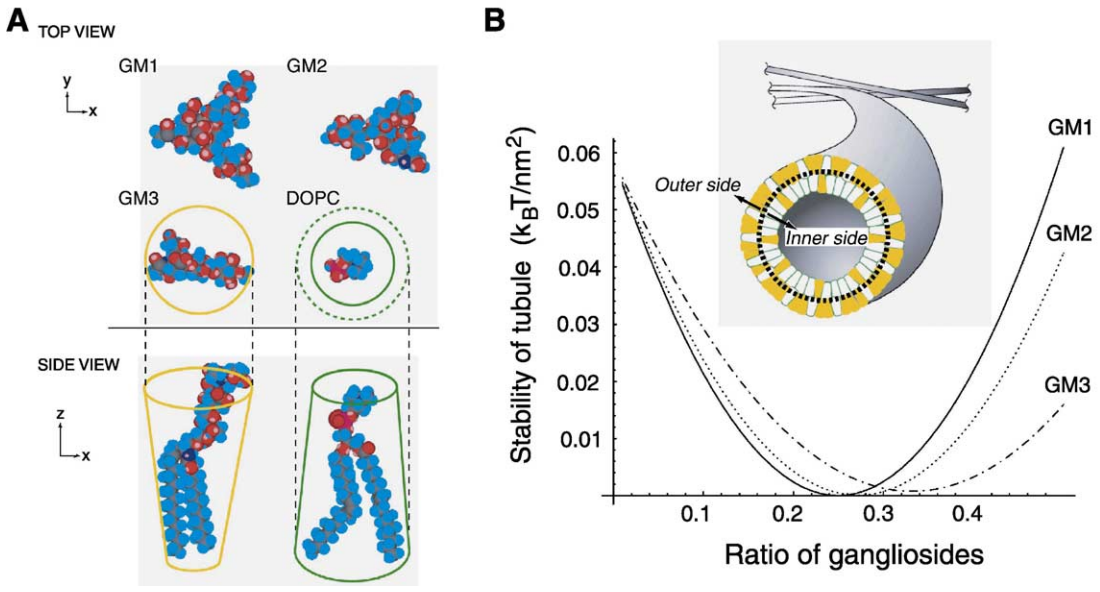

Fig. 4. A: Schematic representation of 3-D models of a series of gangliosides (GM) and a lipid (DOPC). B: Stability of tubule represented with bending energy as a function of the total ratio of gangliosides for GM1, GM2 and GM3. $\chi_0^{GM2} = 1/6.60$ nm^{-1}. $\chi_0^{GM3} = 1/8.88$ nm^{-1} [25,26].

sider a tube for which the radius of the middle surface (i.e. between the inner and outer layers) is R, and the thickness of the membrane is $2dr$. Since the length of the tube is much longer than its radius, the ends of the tube can be neglected. The average bending energy of the tubular membrane can be approximated as

$$u = 2\kappa\left(\frac{R-dr}{2R}\left(-\frac{1}{2(R-dr)}-\chi_0(\beta\rho)\right)^2 + \frac{R+dr}{2R}\left(\frac{1}{2(R+dr)}-\chi_0(\rho)\right)^2\right) \quad (1)$$

where ρ denotes the ratio of GM in the outer layer, and β represents the one-sidedness of the ratio of GM. The bending modulus κ is considered to be independent of the composition. The bending energy of a membrane, which forms a tube with the most stable radius, depends on the total ratio of GM (ρ) and the ratio of GM in the outer layer (β). The difference in the lipid composition between the inner and outer layers is essential for the transition from vesicle to tube. Fig. 4B shows the stability of tube represented with bending energy at the most stable radius of the tube as a function of the total ratio ρ for different species of gangliosides (GM1, GM2, GM3) when β is fixed at 0.5. The spontaneous curvatures are estimated from their surface areas. It is clear that they can form tubular membranes most easily at an appropriate ratio of GM and DOPC. The minimum value shifts to the right when the surface area of the head group of GM becomes smaller. This explains the observations shown in Table 1 well.

Gangliosides stimulate the formation of dendrites and the development of axons in various neuron cultures [28]. Furthermore, gangliosides improve learning and memory in the aged rat [6]. Many factors such as membrane proteins and membrane bound proteins should be considered in the formation of unique cell morphology in neurons. Microtubules are an important determinant of cell architecture. One hypothesis is that actin polymerization effectively pushes the leading edge of the axon [29]. Our results show that gangliosides assist in the formation and stabilization of unique tubular structures by physicochemical mechanisms. In molecular evolution, gangliosides may have been the lipids that led to the unique morphology of nerve cells.

5. Conclusion

This paper describes formation of various size tubes in the mixture of gangliosides with phospholipids such as DOPC and also formation of novel liposome networks by addition of ganglioside to GLs. These results suggest that gangliosides assist in the formation and stabilization of unique tubular structures, like those present in neurons, by physicochemical mechanisms. This may be one of the possible reasons for a high accumulation of gangliosides in the neuron. Gangliosides have suitable chemical structures for stabilization of a tubular structure in the appropriate mixture with phospholipids. The mechanism is supported by physical model.

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