

The Influence of Lipid Rafts on Aging and Immunology

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Lipid rafts are operationally defined as cholesterol-rich membrane microdomains resistant to solubilization in nonionic detergents at low temperatures. Lipid rafts, which are quite different in lipid composition from the surrounding membranes, are of great importance to signal transduction, protein sorting and membrane transport. They have been implicated in a range of biosynthetic and endocytic processes and systems-signaling, molecular trafficking, diseases as well as being involved in the immune, vascular, digestive and reproductive systems. Dietary nutrients like fatty acids and vitamins of different types also play a critical and decisive role in the regulation of lipid rafts.

Table of Contents

Section One: Structures and properties of lipid rafts	1
Introduction	1
Characteristics of detergent-resistant membrane domains	2
Arguments concerning the existence of rafts in cell membranes	6
Relation between caveolae and lipid rafts	8
Section Two: Methods of studying lipid rafts	10
Introduction to different methods of investigating lipid rafts.....	10
Protocols.....	11
Results and discussion.....	14
Section Three: Lipid rafts in immune cells	16
Section Four: Dietary effects on lipid rafts.....	18
Fatty acids.....	18
Cholesterol.....	20
Vitamins.....	22
Section Five: Lipid raft-associated diseases.....	25
Insulin resistance and type 2 diabetes.....	25
Alzheimer’s disease.....	26
Age-Related Macular Degeneration.....	28
Section Six: Summary.....	32
Appendix.....	33
Bibliography.....	36
Vita.....	44

List of Figures

Figure 1.....	33
Figure 2.....	34
Figure 3.....	35

Section One: Structures and properties of lipid rafts

Introduction

Lipid rafts are microdomains with different lipid components than the adjacent membranes (1). They are liquid-ordered domains that are more tightly packed than the surrounding non-raft phase of the bilayer. The tighter packing results from a higher cholesterol content and a greater concentration of saturated hydrocarbon chains in raft sphingolipids and phospholipids, unlike the unsaturated fatty acids of phospholipids in the non-raft phase (2). The cholesterol-rich domains on cellular membranes are of great importance to signal transduction, protein sorting and membrane transport (3). Enriched with glycosphingolipids, sphingomyelin and cholesterol, lipid rafts are resistant to solubilization with non-ionic detergents because of the sphingolipid assemblies which are stabilized by addition of cholesterol. Therefore, we can take advantage of their insolubility in various concentrations of non-ionic detergents to isolate the components of lipid rafts.

A fundamental characteristic of lipid rafts is their ability to prevent undesirable protein-protein interactions, such as those that occur in disrupted signal transduction, because they can distinguish different proteins at the membranes (1). Specific protein-protein interactions such as the activation of signaling pathways involve a series of proteins which alter the sizes and compositions of lipid rafts when cells are subject to an intra- or extra-cellular signal. Such protein-protein interactions can be realized due to

another important property of lipid rafts that they can include or exclude proteins to variable extents.

Although lipid rafts are most abundant at the plasma membrane, they are also found in the biosynthetic and endocytic organelles. Cholesterol-sphingolipid rafts are first produced in the Golgi before moving towards the plasma membrane (4). Then they are endocytosed successively from cell surfaces (5). Rafts either recycle back from endosomes to the surface or return indirectly through recycling endosomes (6).

Characteristics of detergent-resistant membrane domains

The reason why non-ionic detergents can be applied in the extraction of lipid rafts is related with the chemical compositions and physical features of rafts. Lipid rafts are enriched with sphingolipid and cholesterol. They have very long saturated acyl chains and high acyl chain melting temperature, which makes it possible for raft domains to be resistant to detergents. Early research (7) showed that a sphingolipid and cholesterol-rich raft could be isolated by the property of its insolubility in non-ionic detergent of Triton X-100, which suggests that tight interactions between sphingolipid and cholesterol might help to form lipid microdomains because they are probably associated with any detergent-resistant proteins in cells. Sphingolipids are much more resistant to solubilization than phospholipids while cholesterol is also detergent-resistant in the microdomains where sphingolipids are located. In particular, insolubility might be due to glycosphingolipid lipid clusters. Later on, scientists found that glycosyl phosphatidylinositol (GPI) anchored proteins tend to gather in detergent-resistant membrane (DRM) domains, which provides supportive evidence for the idea that

membrane proteins might become detergent-insoluble when they interact with lipid rafts (8).

A few proteins bind to saturated-chain lipids, which are probably necessary to insert into tightly packed membrane domains. This fact provides a hypothesis that such acyl chains may play an important role in the targeting of these proteins. The proteins of this type first recognized are those anchored by GPI. It is necessary to have the GPI-anchor when these proteins bind to DRMs. Moreover, GPI-anchored proteins generally have saturated acyl chains, which proves that they are related to ordered domains. Particularly, they are likely to interact with lipids that can be detergent-insoluble and stabilize them in ordered microdomains.

Though early evidence (9) found that the insoluble material extracted from cells may be made up of a certain protein associated with some kind of lipids, yet detergent insolubility also exists even without any protein, which further indicates the existence of lipid-lipid interactions mentioned above. One research study shows that the high-melting temperature, saturated-chain phospholipid dipalmitoyl phosphatidylcholine is Triton insoluble in liposomes, while the low-melting temperature, unsaturated-chain phospholipid dioleoyl phosphatidylcholine is solubilized from the same liposomes (10). This result is probably associated with the insolubility by the tight packing ability and unusually high melting temperature of sphingolipids. This property of sphingolipids indicates that detergent-resistant phase enriched in tightly packed sphingolipids might be separated from a phase enriched in loosely packed phospholipids. One research paper reports a similar result showing that pure dipalmitoyl phosphatidylcholine is insoluble in Triton X-100 when it exists in the gel phase (11).

Isolation of lipid rafts is accomplished, using non-ionic detergents, such as Triton X-100 and Brij (12). Since the upper limit of the solubility of Brij at 4°C is 0.5%, while rafts are usually prepared using Triton X-100 of 1% concentration, the two detergent concentrations are applied here. When either of the traditional detergents for isolation is applied, the cell membrane will dissolve but such a detergent cannot destroy the structures of the lipid rafts which, therefore, can be extracted. These detergent-resistant remnants of the lipid rafts have been referred to as DRMs, which are supposed to be subsets of the endogenous lipid rafts.

Non-ionic detergent is quite important for extraction of lipid raft fractions. The detergent gathers smaller lipid rafts into larger microdomains, which allows them to form bilayer vesicles (13). It shows that the function of such a detergent in the membrane is to motivate liquid-ordered microdomains to aggregate in number and size under certain conditions, usually at low temperatures. That is why the environmental condition of 4°C has been chosen for this experiment. A recent study (14) reports that under detergent-free circumstances, there is no obvious separation into high and low density fractions which come from membrane vesicles isolated from the cell. Moreover, only a light fraction is obtained, resembling lipid rafts. The two detergents mentioned above have different chemical structures, which result in different molecular properties of raft microdomains isolated by these two types of detergents. In particular, the orientation of the extracted rafts seems to be associated with the detergent used (15). It is reported that lipid rafts isolated using Brij tend to form low-density vesicles of right-side-out orientation while those extracted using Triton X-100 appear to form high-density vesicles of inside-out vesicles. These two detergents can thus be used to extract lipid rafts whose properties are

different in orientation, density and protein composition. Therefore, it makes sense to consider that the two detergents are capable of extracting different subsets of lipid rafts, indicating that they may be associated with the physicochemical characteristics of the isolated lipid rafts. It is likely that the structures of the detergents may determine to which extent the membranes could be inverted by adjusting the orientation and size of the gathered lipid rafts when the forming of vesicles is in process.

A recent study (16) that looks into the application of different detergents in preparing raft domains has found that these detergents are able to isolate lipid rafts due to the difference in their selectivity. For instance, Triton X-100 is a kind of highly-selective detergent, which is used to destroy protein-lipid interactions so as to extract rafts subsets that have low protein contents. In comparison, Tween 20, which is a typical low-selectivity detergent, is only able to extract raft subsets that have much higher protein contents. On the other hand, in spite of the fact that a highly-selective detergent is favored in terms of DRM fraction preparation, a weaker detergent, such as Brij, could also be a good candidate for raft isolation because it does not tend to disrupt the original protein-lipid interactions in the raft domains to such an extent as a highly-selective detergent. The application of a highly-selective detergent, like Triton X-100, appears to dissolve too many raft fractions, which might lead to the misunderstanding of the association between these fractions and the whole raft domains.

From the viewpoint of regarding lipid rafts as a heterogeneous collection of microdomains with different features in biophysics and biochemistry (15), we can also explain why the two detergents mentioned above are capable of extracting lipid rafts with different density or protein contents. As is known, lipid rafts are highly-dynamic

structures, which suggests that different fractions in them are exchanging from time to time. Therefore, Triton X-100 and Brij are likely to disrupt protein-lipid interactions to produce distinct fractions of raft domains.

Arguments concerning the existence of rafts in cell membranes

Since single rafts are too small to resolve, a key unanswered question is whether rafts exist in cell membranes. In other words, it is argued whether membranes have discrete microdomains in liquid-ordered phase or liquid-crystalline phase. A recent study shows that discrete liquid-crystalline phase microdomains could float in a liquid-ordered sea, which was found in sphingolipid-rich membranes (17). Meanwhile, DRMs are also found in cell membranes where lipid rafts exist in a detergent-soluble liquid-crystalline phase sea. In spite of the fact that liposomes are partially detergent-insoluble due to high levels of sphingolipid and cholesterol, the proposal that lipid rafts are present in cell membranes cannot be proved by partial detergent insolubility because membranes in a single uniform phase that has an intermediate property between the liquid-ordered phase and liquid-crystalline phase could also show the characteristic of partial insolubility. Therefore, lipid rafts might gather in a regulated way despite the fact that they do not exist constitutively.

When it comes to the interactions between proteins and lipids, there is a new perspective of the question of whether rafts exist. For instance, GPI-anchored proteins and glycosphingolipids have a high affinity for an ordered environment. Thus, once they exist in cell membranes, they tend to gather and form a raft microdomain, which can be used as a morphological marker. Many proteins and lipids have similar properties mentioned above, which contributes to the formation of visible lipid rafts. However,

these lipid rafts present in membranes are very difficult to observe because GPI-anchored proteins are located uniformly in cell membranes while glycosphingolipids do not appear in a larger cluster than a few molecules (18).

Fortunately, the clusters of certain proteins and lipids in plasma membranes have been found to support the proposal that lipid rafts really exist. A recent typical case is the IgE receptor in immune cells. Another example is some certain proteins which tend to gather in an ordered environment of DRMs, such as some GPI-anchored proteins and some transmembrane proteins. These proteins are capable of co-gathering when they are clustered independently, which suggests that they are present in rafts.

Although lipid rafts are considered to be dynamic structures that are estimated to be small when they are not activated, yet they may cluster together to generate much larger domains once receptor-ligand binding occurs. In other words, if the small raft components are crosslinked with antibodies or lectins, lipid fractions and raft protein will tend to aggregate while raft and non-raft components tend to be divided into micron-sized patches (19). The number of proteins in each raft depends on the packing density, which is less than 10 – 30 proteins. In spite of its small size, a lipid raft still contains a subset of all the raft proteins, which indicates that raft aggregation could activate raft signaling to a great extent. No sooner does ligand binding occur than receptors move into raft domains. As an example, scientists have observed the formation of an immunological synapse which is a specific link between T cells and antigen-presenting cells (20). But how these structures are associated with rafts has not been understood clearly though raft fractions play an important role in immunological synapses.

Relation between caveolae and lipid rafts

In comparison with classical lipid rafts which are short of structural protein components, liquid-ordered domains may have a specific structural protein component, which tends to alter the features of lipid rafts in both appearance and function. In particular, as the structural component of caveolin-1 is inserted into a lipid raft, these microdomains invaginate and form caveolae. This new structure is flask-shaped with its location at or near the plasma membrane. A recent report (21) investigates vesicular trafficking events and signal transduction processes involving caveolins which function as scaffolding proteins to gather and organize particular lipid-modified signaling molecules within caveolae membrane. Another study (22) reports that the binding of caveolins with liquid-ordered domains appears to initiate the biogenesis of caveolae in the Golgi. It is proposed that lipid rafts can be considered to be precursors of caveolae that assist caveolins to participate in membranes.

Caveolins can generate a heterooligomeric complex in cells (21). As the functional assembly units of caveolae, these caveolin heterooligomers interact with cholesterol. Since the caveolin gene family is quite conserved from worms to humans, caveolins are very important in adjusting lipid raft function. Moreover, caveolins also modulate some signaling proteins, such as oncogenes. The interaction of caveolins with signaling proteins regulates their activation state, which suggests that adjusting the expression of caveolins could affect signaling pathways. It is known to all that caveolae are associated with cholesterol and that caveolin-1 tends to bind cholesterol. The loss of caveolae from the plasma membrane is attributed to the absence of cellular cholesterol. Therefore, caveolins also appear to play a significant part in the regulation and transport of

cholesterol levels in lipid rafts (23). In other words, signaling pathways may be affected by the regulation of caveolin expression.

In summary, classical lipid rafts contain glycosphingolipids, cholesterol, lipid-modified proteins with saturated acyl chains as well as transmembrane proteins. They are in liquid-ordered phase and are 50 nanometres in diameter. In contrast, the components of caveolae are mainly raft proteins, lipids and caveolins. They have morphological “cave-like” invaginations on the cell surface.

Section Two: Methods of studying lipid rafts

Introduction to different methods of investigating lipid rafts

The primary method of studying lipid rafts is based on the resistance of lipid rafts to extraction by Triton X-100 at 4°C (24). However, these DRM fractions are aggregates of raft domains, and thus, do not represent the native state of lipid rafts in cell membranes.

In addition, antibodies or lectins can be used to crosslink with raft components in living cells so that lipid rafts are grouped (25). As a result, these patches may be detected by standard light microscopy. Otherwise, single rafts are too small to resolve, which leads to the opinion that rafts are likely not to exist in cells.

The pilot application of single fluorescent molecule microscopy to living cells makes it possible to detect the membrane domains in a direct fashion (12). With a lipid probe of saturated acyl chains combined to these domains, the fluorescence microscopy can catch the images of single molecules through lipid probes in their motional path (single dye tracing, SDT). Since SDT renders it possible to track single lipid probe molecules in vivo and to follow their motion within a millisecond, such a novel method has been employed to search for lipid microdomains or rafts in the plasma membrane that is rich in certain probe molecules.

Another method of visualizing membrane lipid structure of living cells is the usage of two-photon microscopy, in which living cells are labeled with the fluorescent probe, Laurdan, which is an environmentally sensitive fluorescence probe. Due to any environmental stimulation, Laurdan will release red shifts at different wavelengths into

lipid rafts within the membranes in their natural state. A study compared the properties of microscopically visible domains with those of isolated DRMs, and thereby, providing strong support for the lipid raft hypothesis (3).

In fluorescence microscopy and in molecular biology, fluorescence resonance energy transfer (FRET) is a useful tool to find out how proteins interact with other molecules or how conformational changes occur in them. Therefore, FRET can be employed to detect whether two raft components are spatially close. For monitoring the complex formation between them, for example, one of them is modified with a donor and the other with an acceptor before these fluorophore-labeled molecules are blended. When they are separated, the donor excitation will lead to the donor emission which can be observed through fluorescence microscopy. In contrast, when the distance between the donor and the acceptor is less than 10 nm because of the interaction of the two components, the transfer of FRET from the donor and the acceptor will result in the apparent detection of the acceptor emission (26).

Protocols

1. Cell Lysis

- 1) Wash 300mg cells ($\sim 10^7$ cells) twice in PBS and add 2.7ml TNE to the pellet.
- 2) Disrupt cells with 15 strokes in a homogenator.
- 3) Transfer extracts to a micro centrifuge tube and spin at 15,000 rpm at 4°C; discard the pellet.
- 4) Recover the supernatant; spin the supernatant at 100,000x g for one hour and re-suspend the pellet in 450µl ice-cold 1xTNE-T (1xTNE containing 1% Triton X-100).

2. Sucrose Density Gradient Preparation

- 1) Prepare three 50 ml tubes labeled as follows: 13.75%, 22.5%, 31.25%.
- 2) Add 20 ml of 5% Sucrose and 20 ml of 40% Sucrose to the tube labeled 22.5%.
- 3) Add 10 ml of 5% Sucrose and 10 ml of 22.5% Sucrose to the tube labeled 13.75%.
- 4) Add 10 ml of 40% Sucrose and 10 ml of 22.5% Sucrose to the tube labeled 31.25%.
- 5) Add the following Sucrose solutions sequentially in Beckman SW55 Ti rotor tubes:
Use a large bore pipette tip (cut off approximately 2 mm from the tip of a 1 ml pipette tip) and "layer" the solutions very slowly into the tube.

950 μ l of 40% Sucrose

950 μ l of 31.25% Sucrose

950 μ l of 22.5% Sucrose

950 μ l of 13.75% Sucrose

950 μ l of 5% Sucrose

- 6) Gently keep the tube of Sucrose solution layers at 4°C for at least 12 to 16 hr (i.e., overnight). A linear gradient will form.

3. Isolation of lipid rafts from total cell lysates

- 1) Layer the crude cellular extract on top of the Sucrose gradient solution.
- 2) Insert the tube into a pre-chilled SW55 Ti rotor at 4°C. Balance the rotor with another tube of equal mass and centrifuge the Sucrose gradient at 4°C for 4 hr at 50,000 rpm (approximately 237,000x g).
- 3) After centrifugation, prepare sixteen microcentrifuge tubes on ice. Remove the centrifuge tube from the rotor and place on ice.

4) Carefully transfer 300 μ l of fractions from the top of the gradient to the microcentrifuge tubes with large-bore pipette tips.

5) Assay the fractions by SDS-PAGE; run Western immunoblot analysis.

4. Hints

1) Every solution must be ice-cold and procedures are performed on ice.

2) If it is difficult to get the pellet in TNE-T into solution, it is appropriate to sonicate it for a few seconds.

3) When recovering the supernatant, make sure that the pellet is intact.

4) In terms of preparing different concentrations of sucrose, the buffer will be the same composition as the extract to be fractionated by centrifugation. The presence of sucrose or glycerol in the starting extract will also affect the migration of proteins.

5) When layering the solution very slowly into the tube, it is useful to prop the centrifuge tube in a bucket of ice and tilt the tube to approximately 30° to assist in the gentle layering of each Sucrose solution on top of the previous solution. If the layering is not gentle enough, the fractionation will be smeary. While layering, notice the barely visible layers of solution.

6) Be careful not to overload the gradient with too much protein when layering the crude cellular extract on top of the Sucrose gradient solution. This requires empirical determination for the protein of interest and its migration in Sucrose.

Results and discussion

Lipid rafts are isolated using extraction with cold 1% Triton X-100, followed by sucrose density sedimentation. A raft marker called the GPI-anchored GM1 ganglioside is used in Western immunoblot analysis in order to locate the raft-containing fractions.

Figure 1 shows the results from the experiment where 5 μ l antibodies are added into the fractions with the incubation time of 24 hours. Roughly speaking, the blot has a high background, and thus, no specific proteins could be observed. In the control experiment (Figure 2), 2.5 μ l antibodies are added into the fractions with the incubation time of 12 hours. Rafts are visible between the 5% and the 30% interface. In particular, from Lane 15 and 16, specific proteins could be observed, which indicates the extraction of lipid rafts. Moreover, the whole proteins from cell lysis are also used to run Western immunoblot analysis for further references (Figure 3).

In terms of the high background in Figure 1, there might be a couple of reasons. First of all, blocking of non-specific binding might be insufficient. In this case, the blocking incubation period could be increased and another blocking agent may be chosen as a better candidate. Secondly, cross-reactivity between blocking agents and antibodies will lead to an overall membrane staining. Under most circumstances, the addition of a mild detergent to the incubation and washing buffers will eliminate such a problem. Thirdly, the concentration of antibody is probably too high or the incubation time might be too long. The higher the antibody concentration and the longer the incubation time is, the greater the likelihood for non-specific staining is. Fourthly, membranes tend to dry out during the incubation process. Finally, washing of unbound antibodies may be insufficient. So the number of washes may be increased.

In Figure 2, similar problems occur with regard to high backgrounds, which could be solved according to the discussion above. Besides, a smile effect of the bands is observed. This is probably due to fast or hot migration, which could be altered by changing the pH or slowing down the migration or running the gel on ice. Furthermore, uneven white spots exist on the blot, which is attributed to air bubbles trapped against the membrane during transfer or antibodies which are not evenly spread on the membrane. In order to avoid uneven white spots, bubbles must be removed when the gel for transfer is prepared.

Section Three: Lipid rafts in immune cells

The lipid rafts at the cell surface may contribute to signal transduction. In a lipid raft, single receptors may be activated by ligand binding and then cluster together on concentration platforms on which non-raft enzymes such as membrane phosphatases will not be able to exert a negative influence on the signaling complex (25). By grouping together, individual rafts are able to connect different proteins and form a signaling complex. A typical example is the combination of doubly acylated non-receptor tyrosine kinases and G proteins from separate rafts.

In the models for signal initiation in lipid rafts, signaling occurs in either single rafts or clustered rafts. In single rafts, ligand binding crosslink with receptors in living cells so that these receptors are ready for further activation and signaling. In clustered rafts, raft coalescence may come into being when crosslinking proteins in different rafts are combined together through activated receptors. Both of these models may function at the same time to lead to an even larger network of interactions among different proteins that will be involved in the whole signaling process. As mentioned above, lipid rafts have a liquid-ordered phase that prevents signaling complexes from such negative factors as non-raft enzymes in the surrounding liquid-disordered phase. After that, the complete signaling pathway is activated by the concentration of a series of clustered rafts which can include or exclude proteins according to the initial stimulations.

Researchers have recognized the presence and importance of lipid rafts in cellular signaling by comparing the initial signaling processes within lipid rafts and the situations

with the disruption of lipid rafts (25). If the cholesterol from the membrane is removed from lipid rafts, there will be negative changes in cellular function due to the disruption of the lipid rafts.

The B cell receptor (BCR) tends to move into a lipid raft domain when B cells come up with an antigen. Then it produces a signal to make the cell transform into plasma cells which can release antibodies accordingly. However, when lipid rafts are deprived of cholesterol in B lymphocytes, the BCRs tend to lose their ability to produce the signal so that antibodies are formed.

A recent model describes the initial steps that lead to phosphorylation of T cell receptors (TCRs) in lipid rafts. Within T cells in a resting status, the TCRs seem not to incorporate with lipid rafts very frequently. However, antigen presentation by antigen-presenting cells (APCs) helps to associate the TCRs with lipid rafts. Similar to what happens to the BCRs, the TCRs will no longer release signals due to antigen attachment when rafts are extracted from T lymphocytes.

Furthermore, lipid rafts seem to be involved in another important signaling system of the EGF-receptor family, EGFR (Epidermal Growth Factor Receptor). A study suggests that the EGFR is more likely to accept its donor, together with cholesterol when it is tested in artificial membranes. That is why high-affinity EGFR exists in the domains with adequate cholesterol (27). In conclusion, lipid rafts have been implicated in a huge range of other processes and systems-signaling, molecular trafficking, diseases such as HIV and malaria as well as being involved in the immune, vascular, digestive and reproductive systems.

Section Four: Dietary effects on lipid rafts

Fatty acids

Dietary n-3 polyunsaturated fatty acids (PUFAs) are beneficial in preventing various diseases, such as autoimmune and inflammatory diseases, coronary heart disease, diabetes, and cancer. PUFAs may change T cell functional responses and the effects of PUFAs on T cells might be due to lipid raft disruption.

A large number of studies have investigated the role of long-chain (n-3) PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which could be associated with the inhibition of tumor growth (28-30). Generally speaking, the mechanism for the growth-inhibitory effects of PUFAs on cancer cells is associated with their interactions in membrane phospholipids of tumor cells. Particularly, the PUFAs change the composition of plasma membranes where signaling pathways occur so that they may help to inhibit the growth of cancer cells.

In a recent study (31), cells were prepared in a medium with long-chain PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Originally, the DRMs had a higher concentration of saturated fatty acids and a lower concentration of PUFAs, EPA and DHA than membrane phospholipids. After the incubation with EPA and DHA, the composition of DRMs was changed in such a way that there was less sphingomyelin and cholesterol and more phosphatidylcholine. Such a change in the composition of lipids may be attributed to the movement of lipids between DRMs and any other membranes.

Alternatively, it may also be due to the hydrolysis process of lipids in DRMs. In the paper

mentioned above, it reported that neutral sphingomyelinase activity was enhanced by EPA and DHA. Meanwhile, more ceramides were generated within the DRMs of the cells treated with EPA and DHA. Since sphingomyelin hydrolysis generated ceramide that took part in the process of apoptosis, the change in lipid composition which induced the increase of neutral sphingomyelinase activity might be the reason why PUFAs could exert inhibitory effects on the growth of cancer cells. Therefore, long-chain PUFAs may alter the types of lipids in raft microdomains as well as the composition of fatty acids, which would further change raft functions. However, it is controversial that the function of ceramide is always linked to its potency to induce cell death, because the dynamic balance between cellular levels of ceramide and other sphingolipids can regulate opposing signaling pathways. For example, low concentrations of ceramide could promote T-cell activation through the up-regulation of expression of TCRs, while higher concentrations of ceramide might induce cell death so as to terminate T-cell responses.

On the other hand, the concentration of epidermal growth factor (EGF) receptors in DRMs decreased after the treatment of EPA and DHA, while the number of phosphorylated EGF receptors was increased by PUFAs. In fact, the phosphorylation process of EGF receptor occurred after they migrated out of rafts. In addition, loss of cholesterol resulted in depletion of EGF receptor from lipid rafts and promoted its binding to EGF and phosphorylation, which produced further proapoptotic signals. Finally, the processes mentioned above led to cell apoptosis, reducing tumor cells.

Not only does dietary fatty acid composition tend to alter lipid compositions and structures within DRMs, but also is associated with some transcription factors which can regulate expression of caveolin, such as peroxisome proliferator-activated receptor- γ and

sterol regulatory element binding protein (SREBP) (32). Peroxisome proliferator-activated receptor- γ is likely to increase expression of caveolin while SREBP may manipulate cell membrane composition and regulate lipid synthesis (33). One of the best examples involved in intracellular signaling is sterol carrier protein (SCP)-2, which has very high affinity for binding and can shuttle many types of lipids in a very selective manner. SCP-2 is also known to be capable of distributing signaling lipids between intracellular sites and lipid rafts (34). These recent studies propose that SCP-2 plays a key role in cholesterol and phospholipid transfer as well as in manipulating a number of transcription-related lipid signaling pathways in raft microdomains of cell membranes.

Furthermore, PUFAs can help to ameliorate aging-related disease, such as brain ischemia and Alzheimer's disease. For instance, DHA, which is enriched in neuronal membranes, participates in membrane activation and translocation processes by esterifying with phosphatidylserine at raft microdomains. In this way, DHA could provide a binding site for transcriptional factors so as to down-regulate apoptotic caspases (35). That is why DHA are able to promote the growth of neurons and the development of brains. This result also supports the clinical finding that neurological deficits probably result from the deficiency of n-3 fatty acids.

Cholesterol

Located among the hydrocarbon chains of sphingolipids, cholesterol serves as a dynamic link that clusters lipid raft microdomains (36). Cholesterol is known to have higher affinity to raft sphingolipids than to unsaturated phospholipids, serving as a spacer between the raft phase and the nonraft phase. Experimental results also show that despite

the insolubility property of raft-linked lipids and proteins, raft proteins tend to become detergent-soluble when cholesterol is isolated by methyl- β -cyclodextrin or saponin (37). Therefore, loss of raft cholesterol could make most proteins separated from lipid rafts, and subsequently, these proteins lose their normal functions.

Scientists have found that lipid rafts are first assembled in the Golgi complex (36). Particularly, cholesterol is synthesized in the endoplasmic reticulum (ER). Its synthesis process is very similar to that of ceramide, which is known as the hydrophobic backbone of sphingolipids. In the Golgi complex, most of the sphingolipid head groups are linked with ceramide. A recent study has found that the content of cholesterol and sphingolipids increases from the ER to the plasma membrane because lipid rafts are not allowed to go back and forth between the ER and the Golgi complex (38). That is why lipid rafts can only travel from the Golgi complex to the plasma membrane, where they aggregate and take part in many important signaling pathways. The supply of lipid rafts to cell membranes is strictly manipulated by cholesterol and sphingolipid concentrations within the Golgi complex.

On the other hand, it is important to know that cholesterol is harmful, to some extent. Therefore, its concentrations in cells should be tightly limited and regulated in several ways. For example, a complicated system of transcriptional regulation is set up to monitor the biosynthesis and cellular uptake of cholesterol. In addition, cholesterol may be deposited into fat droplets in an esterified form. If these regulation processes are disrupted for some reason, a lot of serious aging diseases related with lipid metabolism will come into being, such as Alzheimer's disease. Broadly speaking, the mechanism of such diseases involves dysregulation of cholesterol metabolism and accumulation of

cholesterol in metabolic overload, which is associated with high levels of triglyceride and low levels of high-density lipoprotein in different aging-related diseases. High-fat diets including saturated fat and red meat contain large amounts of cholesterol, which exerts negative influence on such diseases as type 2 diabetes, obesity and vascular diseases. Moreover, the dynamics and composition of lipid membranes are highly sensitive to any disorders in cholesterol metabolism. One example is that people are at higher risk for developing Alzheimer's disease when the plasma cholesterol levels in their bodies are found to increase. Recent researches show that removal of cholesterol from cells helps to inhibit the synthesis process of a certain transmembrane protein called APP (39). It is also observed that the patients who are treated chronically with cholesterol synthesis inhibitors are likely to have far less risk for developing Alzheimer's disease. The mechanism involved is discussed in detail in the following section.

Vitamins

An increased concentration of end products of lipid peroxidation is the evidence most frequently quoted for the involvement of free radicals in human disease (40). Lipid peroxidation is initiated by the attack on a fatty acid or fatty acyl side chain of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. The occurrence of lipid peroxidation in biological membranes causes impairment of membrane functioning, changes in fluidity, inactivation of membrane-bound receptors and enzymes, and increased nonspecific permeability to ions. For example, deformation of red blood cells after exposure to peroxides causes them to become leaky to potassium ions. It is also noticed that antioxidant nutrients are

able to interact with lipid membrane microdomains and down-regulate signaling pathways involving inflammatory responses and atherosclerosis so as to protect vascular endothelium from environment toxicity.

Evidence predominantly from in vitro studies suggests that antioxidant vitamins can prevent or mitigate lipid peroxidation (41). Vitamin C protects bio-membranes against peroxidative damage in the aqueous phase. Vitamin E is considered to be the predominant lipid-soluble, chain-breaking micronutrient antioxidant. Some studies suggest that vitamin C may enhance the effects of vitamin E by reducing tocopheroxyl radicals, which means that vitamin C and vitamin E have synergistic effects.

Besides, a significant inverse linear correlation between malondialdehyde levels and natural antioxidant levels was recorded (42). Lipid peroxidation products are significantly increased in groups of subjects with deficient levels of vitamin C, vitamin E and both vitamins, if compared to group with normal vitamin levels. The results show that the deficiency in two key antioxidants for lipid peroxidation inhibition means the insufficient defense against free radicals and the increased lipid peroxidation.

Furthermore, scientists have investigated Carboxyethyl-hydroxychromans (CEHC), hydrosoluble vitamin E metabolites, which are excreted through the renal filter (43). Because of the highest levels of CEHC in hemodialysis (HD) patients, it makes sense to look into CEHC accumulation which may affect patients' plasma antioxidant status. It is obvious that in these patients there is a significant impairment of the non-enzymatic antioxidant network. In addition, increased antioxidant consumption by sustained oxidative stress has been proposed to occur in these patients. With the dialysis-dependent

leakage of antioxidants, this could lead to a higher susceptibility to oxidative damage of target biomolecules.

Section Five: Lipid raft-associated diseases

Insulin resistance and type 2 diabetes

In contrast to type 1 diabetes that lack sufficient insulin in bodies, type 2 diabetes are associated with decreased ability of cells or tissues to react to normal levels of insulin, which is called insulin resistance (44). Scientists have proposed that lipid rafts might serve as a critical platform where insulin signaling is compartmentalized to function in adipocytes (45). As a family of sialic acid-containing glycosphingolipids, gangliosides tend to play a structurally and functionally important role in raft microdomains. One recent paper reports that the most abundant ganglioside is called GM3, which is over-expressed when the level of TNF- α increases in adipocytes (46).

TNF- α is known to induce dedifferentiation of adipocytes and reduce gene expression on components of insulin receptor signal transduction pathway, such as GLUT4 genes and IRS-1 (47). Particularly, TNF- α triggers a moderate decrease of insulin-induced phosphorylation of insulin receptors, leading to a more prominent inhibition of insulin-stimulated phosphorylation of IRS-1 without any impacts on the expression of either insulin receptors or IRS-1. Since many studies have proved that TNF- α is a critical component involved in insulin resistance (48, 49), it is intriguing to investigate the relationship between TNF- α and GM3 so as to demonstrate the mechanism of insulin resistance in type 2 diabetes.

One pioneer approach is to test obese-diabetic mice in which the GM3 synthase mRNA levels in adipose tissues are significantly higher than in controls (50). In the

experiment, an inhibitor of glucosylceramide synthase called D-PDMP was used so as to clarify whether the elevated level of GM3 contributed to the inhibition of insulin-dependent insulin receptor internalization as well as the removal of insulin receptors from lipid raft membranes treated with TNF- α . As was expected, the dissociation of insulin receptors from raft microdomains was partially blocked while the internalization of insulin receptors was effectively suppressed after GM3 depletion by D-PDMP. When treated with TNF- α , the level of GM3 increased by nearly 100% within DRMs in contrast to the results in normal adipocytes. Additionally, a recent study reports that mice in short of GM3 synthase tend to exhibit enhanced insulin signaling (51). These findings are consistent with the results from another research paper which examines the effect of TNF- α on the function and composition of DRMs and concludes that the loss of insulin receptors from raft domain membranes is probably attributed to elevated level of GM3. Subsequently, it induces the inhibition of insulin's metabolic signaling (50). In other words, GM3 depletion may become a potential target for mitigating the TNF- α -induced inhibition of insulin receptor accumulation in lipid raft microdomains when it comes to the future treatment of insulin metabolic signaling defect in insulin resistance.

Alzheimer's disease

Lipid rafts are involved in Alzheimer's disease, known for regulating protein processing and signaling (52). Formation of senile plaques containing the amyloid- β -peptide is a hallmark of this disease. Amyloid- β -peptide is derived by proteolytic cleavage from the amyloid precursor protein called APP by β -secretase and γ -secretase. It has been shown that lipid membrane microdomains as well as caveolae provide a critical

platform where APP is processed to be different kinds of β -amyloid peptides (53). Although the mechanism of Alzheimer's disease is not well-known yet, researchers suggest that it is a key point to investigate in depth how APP processing is regulated within lipid raft microdomains. It is found that β -amyloid peptide production is somehow dependent on the integrity of lipid raft microdomains while lower levels of cholesterol help decrease β -amyloid peptide production (53).

When it comes to processing of APP to form its intracellular domain, a lipid membrane microdomain associated protein called Flotillin-1 plays an important role as an APP intracellular domain interacting protein (54). With the help of Flotillin-1, APP may be guided towards lipid raft microdomains. Therefore, Flotillin-1 is regarded as a participant in the processing and localization of APP. Another study also supports this proposal, reporting that there is a positive correlation between the concentration of Flotillin-1 and the progression of Alzheimer's disease (55).

In addition to Flotillin-1, cholesterol also takes an active part in regulating different cleavage processes of APP. Apolipoprotein E is known as a major apolipoprotein in the brain, serving as a carrier to deliver cholesterol within central nervous system. It exerts significant influence on compositions of lipid microdomain and properties of enzymes, transport proteins and receptors that mediate β -amyloid peptide production and degradation (56). Besides, there is a positive correlation between the concentration of β -amyloid peptide in the brains of Alzheimer's disease patients and levels of total low-density lipoprotein and cholesterol in serum. One clinical study has found that the increase in cholesterol level during mid-life is likely to elevate the risk of developing Alzheimer's disease (57). Furthermore, removal of cholesterol is shown to inhibit the

formation of β -amyloid peptides in neurons by shifting the partitioning of APP from lipid rafts to surrounding lipid bilayer. Since β -amyloid peptides are derived from APP cleaved by a certain enzyme called β -secretase, the existence of APP and β -secretase together is very likely to increase the production of β -amyloid peptides. However, considering that cell surface rafts are very small and contain only a subset of proteins, APP is less likely to meet with β -secretase in the same rafts. Therefore, the formation of β -amyloid peptides is supposed to be limited on cell membranes. Conversely, the production of β -amyloid peptides is strongly stimulated after antibody cross-linking which is attributed to clustering of a large number of surface rafts where APP and β -secretase co-exist.

Even after processing of APP, certain interactions between lipid rafts and β -amyloid peptides are likely to have impacts on the development of Alzheimer's disease. A number of recent studies have shown such an important role of lipid rafts in β -amyloid peptides production. For example, raft binding may result in a conformational change that triggers amyloid plaque formation (58). Another result shows that the ganglioside GM1, known as one raft component, tends to bind β -amyloid peptides and probably alter their conformation, too.

Age-Related Macular Degeneration

Age-Related Macular Degeneration (AMD) is the major cause of vision loss in elderly people. The percentage of older people is increasing rapidly among the whole population, especially in developed regions and countries, while the people at 65 and

older tend to suffer much more from this disease. Therefore, AMD is becoming an important clinical problem and an attractive research topic.

Oxidative stress is believed to be one of the mechanisms behind the development of AMD. It is a biological process where cellular damage is caused by reactive oxygen intermediates (ROI), which is related with many diseases, especially age-related disorders. ROIs include free radicals, singlet oxygen, and hydrogen peroxide, which are usually the byproducts of oxygen metabolism. Generally speaking, cumulative oxidative damage is responsible for aging and paid great attention to in the pathogenesis of AMD.

The retina is particularly susceptible to oxidative stress because of a large amount of polyunsaturated fatty acids present within lipid raft domains (59). A characteristic of retinal lipids is that the photoreceptor membranes of both rods and cones contain a lipid bilayer that have very high proportion of long-chain PUFAs found in all phospholipid classes. These PUFAs are quite susceptible to oxidation since they have a number of double bonds. Therefore, the retina is correspondingly susceptible to lipid peroxidation which may lead to loss of membrane function and structural integrity (60). There is also evidence that the ability to present an anti-oxidant defense at the macula diminishes with increasing age.

In order to reduce oxidative damage, many researchers have investigated the effects of nutritional antioxidant supplements on the onset and natural course of AMD (61). Although the relationships between dietary and serum levels of antioxidants and AMD are less clear yet, people believe that such nutrients as vitamins, PUFA, lutein, zeaxanthin and zinc may help lower the risk of AMD or slow down the progression of dry macular degeneration.

Investigations have shown that diet poor in vitamin A, C and E and low plasma levels of antioxidants tend to lead to the development of AMD, which indicates that this supplementation enhances antioxidant defense and might prevent or retard AMD (62). As is mentioned above, these anti-oxidant vitamins are able to react directly and non-enzymatically with ROIs, generating harmless products and terminating free radical chain reactions. Also, the retina contains high levels of these vitamins in rod outer segments and RPE, and the concentrations within these tissues are very sensitive to dietary intake of them (63). Therefore, the consequences are consistent with previous studies which have shown that photochemical retinal injury is due to oxidative stress and that the antioxidant vitamins A, C, and E protect against this type of injury.

What is more, many scientists have been focusing on a supplementation of long-chain PUFA of the n-3 family, which is known as DHA, as it addresses primary prevention of AMD (60). A supplementation of n-3 PUFAs could be proposed to certain subjects at risk for AMD for primary prevention.

Furthermore, high plasma levels of lutein and zeaxanthin are associated with reduced risk of neovascular AMD (64). It is known that lutein and zeaxanthin are two major components in macular pigments which serve to filter blue light, and thus, protect against this possible source of photooxidative damage to the neurosensory retina. Lutein and zeaxanthin accumulate in the eye lens and macular region of the retina and their concentrations in the macula are greater than those found in plasma and other tissues. Because of their very high absorptivity, lutein and zeaxanthin in the inner retina form a very efficient filter for blue-light that reaches the back of the eye. This reduction in blue-light intensity could significantly reduce the oxidative stress on the retina, and therefore,

contribute to the reduction in risk of AMD, which is shown in many recent researches (64, 65). That is why higher dietary intake of lutein and/or zeaxanthin is associated with decreased likelihood of having large or extensive intermediate drusen and neovascular AMD.

In addition, zinc is the most abundant trace element in the human eye. It acts as a cofactor for CuZn-SOD and is involved in the regulation of catalase activity. Therefore, zinc is a key component in antioxidant defenses (66). Also, it induces the synthesis of metallothionein, which functions in clearing hydroxyl radicals, and stabilizes membrane lipids against oxidation.

Section Six: Summary

Recent studies show that lipid rafts can be isolated from cells with non-ionic detergents as extraction agents. It indicates that such sphingolipid and cholesterol-rich membranes appear to exist and are involved in signal transduction through GPI-anchored proteins as well as high-affinity receptors. Lipid rafts also play a critical role in trafficking and sorting through endocytic and secretory pathways, which is involved in diverse immune processes related with aging. Rafts have a close relationship with diabetes, different types of cancers, Alzheimer's disease, and many other aging-related diseases. Therefore, the properties of rafts can be used in treatment or prevention of any of these diseases in future. In addition, the investigation of dietary nutrients, such as fatty acids and vitamins, tend to become an important topic because of their participation in the regulation of lipid rafts.

Appendix



Figure 1 Blots with 5 μ l antibodies added and the incubation time of 24 hours

Lane

18 17 16 15 14 13 12 11 10



Figure 2 Blots with 2.5 μ l antibodies added and the incubation time of 12 hours

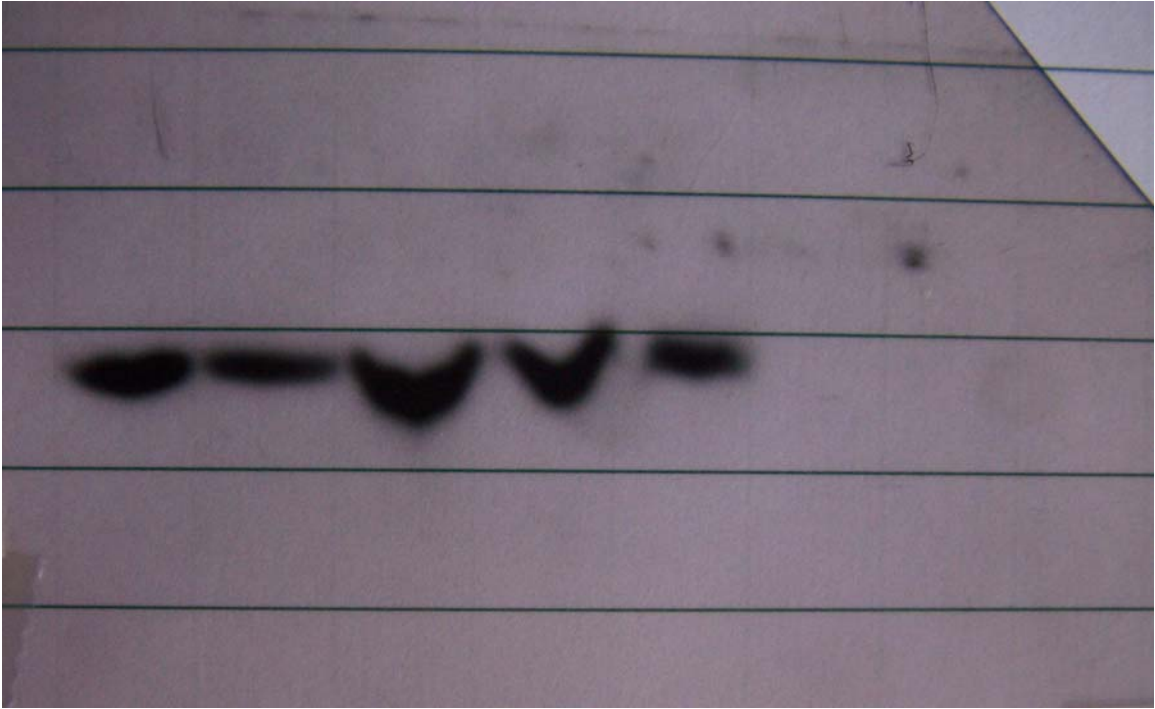


Figure 3 Blots of the whole proteins from cell lysis

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