Lipid rafts in anticancer therapy: Theory and practice (Review)

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Abstract. To enlarge and stabilize transient raft patterns, proteins interact with membrane lipids. By lateral movements, these small lipid rafts (LRs) may coalesce into large platforms (raft clusters), allowing the alignment of transmembrane proteins that easily crosslink. The formation of raft clusters permits TNFR superfamily membrane receptors of the *L*o phase to subsequently cooperate with transducer and adaptor proteins to create receptosomes and/or signalosomes, even without external ligand binding. Chemical agents that disrupt actin and/or the microtubular network were also found to facilitate the death signal through the known death cascades. Hence, death machinery is triggered to initiate, transmit and execute the apoptosis (extrinsic and/or intrinsic) of tumor cells. Certain anticancer drugs such as edelfosine and aplidin convey these death signals, suggesting a disregard for the necessity of ligand-receptor communication. This also stresses the importance of existing LRs in tumor cells as a prerequisite for improving the strategies of anticancer therapies based on their physical and biochemical modifications. Tumor cells ignore the natural mechanisms of their elimination. As a result, the cytokines of the TNFR superfamily are unable to induce a physiological response guiding cells to programmed cell death. The clustering of LRs provides the opportunity to overcome the resistance of tumor cells to death ligand-induced apoptosis, thus offering a less harmful alternative, as well as a more efficient strategy, for eliminating tumor cells.

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1. Introduction

According to the fluid mosaic model proposed by Singer and Nicholson (1), the cell membrane is a phospholipid bilayer with peripheral, integral and transmembrane proteins. Over a decade ago, extensive research provided evidence that the plasma membrane is not entirely neutral liquid disordered (*L*d) fluid, but is rather compartmentalized into functional microdomains: cholesterol-rich liquid ordered (*L*o, with intermediate fluidity) microdomains, caveolae and clathrin-coated vesicles, as well as cholesterol-independent, non-raft domains (2). Thus, the plasma membrane consists of a mosaic of functional microdomains with complex organization, function and trafficking, indicating extensive influence on membrane proteins and their functions. Lipid rafts (LRs; plasma membrane islands with flotilline protein) and caveolae (plasma membrane invaginations formed from LRs by scaffolding caveolin proteins) are short-lived clusters that, depending on cell type, are roughly 50 nm in diameter (Fig. 1). Their existence in living cells has just recently been demonstrated using single-particle tracking (3) and fluorescence resonance energy transfer techniques (4). Caveolae and LRs were shown to represent membrane compartments enriched in a large number of signaling molecules whose structural integrity is essential to many signaling processes. However, in contrast to LRs, caveolae are not essential to signal transduction. In lymphocytes and neurons which lack caveolin, signal transduction occurs through rafts. According to theoretical models, LRs function separately by the altered partitioning of transmembrane proteins which, by oligomerization, achieve an active state or cluster, from individual rafts that differ in protein composition, to coalesce rafts into large platforms containing different crosslinkers (5). In both instances, extracellular signals are thought to increase the raft affinity of proteins. By this route, more proteins are driven into rafts, where they can be activated and recruit the other proteins necessary for signal transduction. After receptor crosslinking, their raft residency increases, indicating possible raft association (6). Alternatively, the clustering of LRs takes

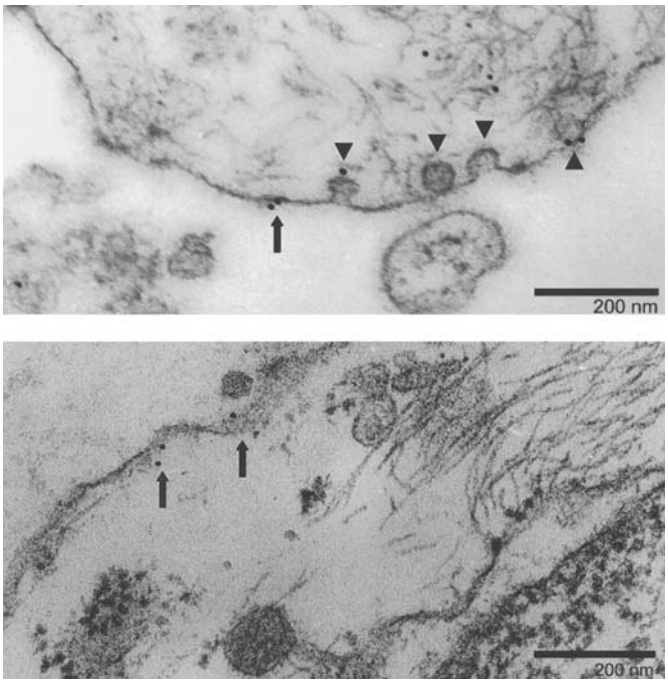

Figure 1. Ultrastructure of the plasma membrane in HaCaT keratinocytes (JEOL 1200 EX electron microscope). Top panel: post-embedded immunogold labeling. Caveolin 2 was identified with immunogold 18-nm diameter particles. Bottom panel: Flotillin 2 was identified with immunogold 18-nm diameter particles. Lipid rafts are indicated by arrows; caveolae by arrow heads.

place without ligand binding, allowing membrane receptors to position themselves in configurations that trigger the construction of signalosomes that, in turn, initiate the transmission and propagation of signals. The abovementioned models are not mutually exclusive. Next, the endocytosis of LRs and caveolae follows the clathrin-independent process (7). They are endocytosed to early endosomes in order to directly recycle to the cell membrane or revisit the Golgi apparatus through recycling endosomes (8). More recent data, addressing how receptors aggregate and initiate the raft clustering process to activate cellular signaling, will be discussed below.

LRs are enriched in cholesterol and sphingolipids that are packed together to form a highly ordered structure. In detail, phospholipids with saturated hydrocarbon chains pack tightly with cholesterol but, nevertheless, remain mobile in the plane of the membrane which predominantly remains disordered and fluidal. By this route, the inner (cytoplasmic, cytofacial) leaflet of LRs is formed by proteins tethered with glycophosphatidylinositol anchors, and is somehow attached to the outer (exoplasmic, exofacial) leaflet with proteins anchored by the saturated palmitoyl or myristoyl groups. In addition, cholesterol-binding proteins are preferentially targeted to the LRs. The amino acid composition of transmembrane proteins is essential to their hydrophobicity and anchorage in the LRs. Moreover, LRs float and are believed to be a major location of the plasma membrane proteins indispensable to the maintenance of cell and tissue viability. As previously shown, some of these proteins have a high affinity to rafts and play a significant role in signal transduction (9-16). In the LRs, double-acylated and palmitoylated proteins in particular contribute to these processes (17).

2. Lipid raft properties and their respective functions

LRs can change their size and composition in response to intracellular and extracellular stimuli. The identification of these intrinsic/extrinsic factors and their function remains elusive. However, signal transduction has been shown to be initiated by the clustering of transmembrane proteins. For instance, oligomerized proteins have a higher affinity for LRs than the monomers from which they are formed. Thus, the clustering of LRs may facilitate the activation of signal transduction pathways, allowing for the induction of the enzymes involved in signal cascades or the assembly of active receptors with adaptor proteins (2). Furthermore, the spatial separation of raft proteins involved in signal transduction affords protection from non-raft components (enzymes, inhibitory adaptor proteins) that would otherwise inhibit intracellular signaling. Given their small size and the limited representation of available membrane-bound receptor proteins, the importance of rafts in signal transduction must have to do with their dynamics (i.e. the ability to cluster and recruit non-raft proteins). This feature has led to the use of chemical crosslinking in order to identify rafts and their constituents. Approximately 10-30 proteins have been found in individual rafts by this approach (18). In addition, raft proteins are sometimes transiently connected to the cytoskeleton or to cellular lectins, enabling rafts to cluster in a novel manner. The observation that protein raft affinity increases with protein acylation by saturated fatty acids (SFAs) leads to the assumption that polyunsaturated fatty acids (PUFAs) might weaken the associations of proteins with LRs. Indeed, PUFAs replace SFAs in acylated proteins, causing them to dissociate from rafts (19).

On the other hand, LRs are vulnerable to factors affecting the composition of the cell membrane. Either poorer representation of cholesterol or a higher contribution of gangliosides (glycosphingolipids) in the membrane phospholipid bilayer releases proteins from LRs. This aspect of LRs has led to the wide use of inhibitors of cholesterol synthesis, factors that deplete cholesterol, exogenous gangliosides, or PUFAs to disarray LRs in numerous experimental approaches. Similarly, cholesterol sequestration by antibiotics or pore-forming agents disrupts rafts, bringing an end to their functionality. Recent developments in molecular biology techniques that knockout or knockdown certain genes have provided evidence supporting the notion that some proteins are preferentially-located in LRs. The dissociation of these proteins from rafts by dominant-negative mutants or gene silencing can be rescued by the addition of exogenous cholesterol. The unique structural characteristic of LRs has prompted several groups to examine their physiological relevance in detail, as well as the implications of the finding that some substances facilitate death signal transduction (20,21).

3. Signal transduction in lipid rafts

The arrangement of raft clusters allows interactions among adaptors, scaffolds and anchoring proteins to accurately manage the space and time formation of signaling complexes. Correspondingly, clustered rafts lead to the amplification of the signal through the concentration of signaling molecules

assembled with oligomerized receptors, and by the respective multiplication of second messengers. Likewise, selective protein affinities to rafts exclude certain negative modulators of signal transduction. It has also been proposed that raft clusters interact with cytoskeleton and second messengers at the cytoplasmic leaflet of rafts.

Although microdomains are believed to regulate signaling, it should be stressed that both the facilitation and retardation of signal transduction may occur at these sites. Over a dozen hormones, cytokines, growth factors and macromolecules have been reported to utilize LRs as a platform for interaction with cognate receptors or as a basis for intracellular trafficking (2). Irrespective of the abundant representation of EGFR in caveolae and LRs, EGFR signaling is enhanced by cholesterol depletion (22,23). Similarly, downstream to EGFR, the Ras/Raf/Mek/MAPK cascade is distinctly active in cholesterol-deprived cells (24). It is known that, under these circumstances, cholesterol-independent cell surface EGFR overwhelms LR signaling (11,25). Thus, it seems plausible to situate EGFR signaling predominantly in non-cholesterol-dependent microdomains. Another example of repressed signal transduction comes from the caveolin-1-dependent inhibition of eNOS signaling in endothelial cells (26). In caveolin-1 knockout mice, eNOS activity is higher because the enzyme is liberated and binds calcium-calmodulin, a positive regulator of eNOS (27).

However, the bulk of the evidence indicates that LRs segregate signal transduction molecules in the absence of the ligand while facilitating signaling in its presence. LRs provide an environment in which spatially-segregated signaling modules can be assembled into active complexes (2). In recent years, a great deal of attention has been paid to the elucidation of the details of these molecular events. LRs play a significant role in a variety of well-described ligand-receptor interactions, but their range is beyond the scope of this review.

Our research is focused on human colon cancer and the 'immune escape' of colon adenocarcinoma cells. These cells are immune to death signals and counterattack when targeted by non-specific mechanisms of the whole-body defense system. We aim to outline the role of LRs in the aforementioned phenomenon. The central position of this study is envisaged by death ligands of the TNF-α superfamily (TNF-α, FasL, TRAIL), which regularly eradicate undesirable and harmful cells.

4. TNFR superfamily and lipid rafts

The TNFR superfamily, including functional receptors to TNF-α, FasL and TRAIL (TNF-α-related apoptosis-inducing ligand), have been shown to be associated with LRs (28,29). Among the 30 identified members, two types of TNFR receptors were distinguished: those composed of death domain (DD) proteins (TNF-R1, Fas, DR3, DR4, DR5, and DR6) and those that lack DD (TNF-R2, CD27, CD30, and CD40) (30). It has been shown that a considerable fraction of Fas, CD40, and TNF-R1 is constitutively partitioned into rafts. As a result, in certain cell types rafts of the *L*o phase enriched with TNFR float in the *L*d phase dominated by unsaturated phosphatidylcholine molecules at the exofacial leaflet. Nonetheless, the co-localization of TNFR with raft-associated proteins is also inducible. In certain cell types, ligand (TNF-α) binding receptors are complexed and translocate to the LRs. Additionally, LRs seem to be essential to the TNF-α-mediated activation of RhoA, but dispensable in the activation of the NF-κB and MAPK pathways in smooth muscle cells (31).

In general, depending on cell type, TNF-R1 receptor mediates two opposite responses, one leading to cell death and another to improved cell viability. Until now, it was assumed that these opposite responses occurred in sequence, with the survival signal (complex I) transpiring first to herald the subsequent death signal (complex II) (32). However, according to Doan *et al* (33), there is spatial separation between the signals mediated by TNF-R1 and the non-raft (survival) and LR (death) signals.

One common building block, essential to signal transmission from TNF-R1, is the DD, created by the homotrimerization of receptors, followed by TNFR-associated death domain (TRADD) protein assembly. The death signal of extrinsic apoptosis requires the association of TRADD with the Fas-associated death domain (FADD) protein, followed by procaspase-8 assembly to form death-inducing signaling complex (DISC), with subsequent activation via a homologous death effector domain interaction (34). The biological importance of rafts in TNFR signaling has been demonstrated on triggered CD40 (35) for the first time. After activation, TRAF-2 and -3 adaptor proteins were intracellularly recruited to rafts (36). Furthermore, raft depletion of cholesterol blocked both DISC formation and death ligand-induced cell death. Protein-protein interactions have been studied in detail (e.g. signalosomes, receptosomes), whereas the initial events of ligand engagement to LRs and the origin of DISC formation remain obscure. It is widely acknowledged that, unless death ligands are not trimers, no molecular interaction occurs with their cognate receptors. The theory that the prerequisite for sensing the extracellular signal is the attachment of the ligand trimer to its oligomerized TNFR superfamily receptor has been questioned in view of recent data suggesting that at least some of the TNFR superfamily receptors assemble without ligand action (37,38). In contrast, recent observations point to the preassembly of receptors as a requirement for ligand binding.

What, then, is the role of the ligand? Most likely, the ligand functions to change the conformation of the receptor protein, so that the latter can attract specific protein(s) or so that receptors cluster into higher order complexes with bridges between trimeric receptors setting aside multiple secondary DISC (39). This option might, at least in part, explain the facilitated signal transduction and amplification of the death signal at LRs. Actually, the lipid composition of the raft seems to foster the signal, because cytosolic adaptor molecules gather at these sites, allowing a higher incidence of binding to the receptors and to other partners required for signal transduction. Alternatively, prior to ligand action, TNF-R1 receptors remain inactive as spontaneous aggregation is inhibited by suppressors of the death domain. The proposal that DD residues of TNF-R1 encourage localization at LRs (40) is uncertain in the face of the differential regulation of TNF-R1 signaling, regardless of similar representation at the lipid bilayer (*L*o vs. *L*d) (33).

Presumably, there is also a role for ceramides, which have been shown to endorse the arrangement of the *L*o lipid phase (41). The conversion of sphingomyelin into ceramide can play a membrane structural role, with consequences for membrane

microdomain function, membrane vesiculation, fusion/fission and vesicular trafficking (42). In addition, the autolytic cleavage of the procaspase-8 dimer after TNF-R1 receptor activation causes caspase-8-dependent activation of sphingomyelinase. As a result, ceramides are generated by sphingomyelinase, which is activated by caspase-8. Finally, ceramides gain access to the cell membrane, so that the recruitment of FADD and caspase-8 is exaggerated and the process of signal initiation and transmission is apparently strengthened.

What role do LRs play in 'immune escape'? We and others have observed that transformed cells resist TNF-α-induced apoptosis throughout the impaired formation of death signalosome and/or the stimulation of NF-κB-dependent cell viability (43,44). Seemingly, LRs must be scrutinized to ascertain whether raft restructuring affects TNFR superfamily death signals. For instance, in ROS-induced cell death (H_2O_2), LRs play a significant role in allowing the assembly of TNF-α receptor-associated factor 2 (TRAF2) and TNF-α receptor-interacting protein (RIP). Notably this process occurs without the participation of TNFR1. Subsequently, c-Jun NH_2-terminal kinase is activated as a critical downstream target of RIP and TRAF2 (45). This was first suggested by evidence that non-Hodgin's lymphoma neoplastic cells are featured by the constitutive activity of NF-κB, resulting from the presence of survival signalosomes at the LRs (46). The disruption of CD40 signaling, through the use of specific antibodies that target ligand binding, impaired viability and evoked lymphoma cell death.

Recently, it was shown that TRAF2 and TRAF1 cooperate in CD40 signaling in response to signals from the TNFR superfamily (36). Accordingly, these proteins represent possible candidates for the disarray of the CD40 signal.

5. Lipid rafts in cell transformation

As mentioned above, another elusive function of LRs is their involvement in cell transformation. The NF-κB transcription factor plays a crucial role in this process, as a prolonged pro-inflammatory response by subsequent expression of pro-inflammatory genes causes cells to be predisposed to tumorigenesis. To block the nuclear signal from NF-κB, certain factors (penetratin peptide) go through the cell membrane to interfere with IκB degradation (47). Inhibited degradation of the latter is essential to NF-κB inactivation. Similarly, efforts aimed to repress the survival signal from death receptors in the rafts might be regarded as a possible tool that protects against tumor development. In colon cancer, both the futile attacks of the immune system and the enhanced viability of cancer cells permit tumor development. Resistance to apoptosis is linked to tumorigenesis as it allows the survival of cells with genomic lesions and promotes cell resistance to immune-based destruction (48).

Apparently, a prominent role in these processes is played by IGF-I, which sends contradictory signals that interfere with the cell death induced by different ligands of the TNF superfamily. LRs segregate proapoptotic from antiapoptotic signaling in colon carcinoma cells, illuminating the behind the scenes process of 'immune escape' (49). APC mutation is the initial step in colon tumorigenesis and leads to the constitutive activation of ß-catenin, a cytoskeleton protein that, under these circumstances, is no longer sequestered and acts as a transcription factor that stimulates anti-apoptosis genes. Caveolin-1, a scaffold protein that bends the plasma membrane to form caveolae, suppresses expression of apoptosis protein survivin inhibitor via a mechanism involving diminished ß-catenin-Tcf/Lef-dependent transcription (50).

It is very likely that the dysfunction of the caveolae microdomains fills one gap in the understanding of colon cancer development. Failures in apoptosis are a major hallmark of cancer cells. Thus, disturbances in intracellular redistribution and mitochondrial targeting of proapoptotic factors may shed more light on tumor development. In response to TNF-α, the ganglioside (GD3) is trafficked to the mitochondria to promote apoptosis. This process is mimicked by acidic sphingomyelinase, confirming the contribution of LRs to TNF-α-mediated cell death (51).

6. Lipid rafts as targets in anticancer therapy

Over a decade ago, it was postulated that drug-induced apoptosis in cancer chemotherapy resulted from the activation of cell death signaling (52). This theory led to extensive research envisioning the contribution of different death signaling pathways to anticancer drug approaches. It was assumed that, if death receptors activate apoptotic signaling in rafts, tumor-bearing subjects would benefit from drugs which enhance the raft-dependent killing of tumor cells. Not surprisingly, a plethora of evidence supported this suggestion. Initially, Gajate and Mollinedo (53) noticed that antitumor ether 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phospho-choline (ET-18-OCH(3), edelfosine) amplified raft-dependent Fas signaling, probably through the structural reorganization of raft membrane microdomains by the intracellular activation of Fas/CD95. It became evident that LRs represent a potential target for therapeutic intervention. The same group, led by Mollinedo, reported that the molecular mechanism of edelfosine was reliant on Fas ligand action (54). Notably, drug sensitivity was dependent on drug uptake and Fas expression, regardless of the presence of other death receptors, such as TNF-R1 or DR5, in the target cells. Fas and DISC were also found to be constitutively overexpressed in LRs, and FasL was recruited into LRs for maximum Fas receptor contact and cell death-inducing potency (55). Finally, it appeared that, during Fas-mediated cell death, caspase-3 was a component of the Fas DISC in LRs as caspase-3, caspase-8 and the FADD were recruited to LRs following agonist anti-Fas treatment (56).

Confoundingly, FasL up-regulation and even FasL itself was not essential to Fas-dependent cell death (57). These authors have shown that another anticancer drug, aplidin, is incorporated into membrane rafts and that actin-linking proteins were conveyed into Fas-enriched rafts. FADD, procaspase-8, procaspase-10, c-Jun NH_2 kinase and Bid were concomitantly translocated to LRs following Fas/FasL interaction in a FasL-independent way (58). By this route, certain anticancer drugs were demonstrated to act through the raft-dependent process.

TRAIL has been considered a promising candidate in anticancer therapy for a long time, as this death ligand selectively targets transformed, but not normal, cells (59). Recent reports indicate that this view is not as optimistic

when LRs are taken into consideration. It appears that decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) fail to induce apoptosis after TRAIL ligation, as they lack and have a truncated cytoplasmic death domain, respectively. Moreover, the two act dissimilarly; DcR1 prevents the assembly of DISC by tritating TRAIL within LRs, whereas DcR2 is co-recruited with functional receptor DR5 within the DISC, where it inhibits initiator caspase activation (60). Consequently, rafts might play a dual role in extrinsic and intrinsic apoptotic pathways; one encouraging and the other impeding these events.

Cellular motility is another important attribute of metastasizing tumor cells. Therefore, several cytoskeletal disrupting agents are used in anticancer therapy. A considerable body of evidence indicates that LRs and integrin signaling are tightly coupled in order to target Rac and Rho GTPases to the plasma membrane (61,62). Inhibitors of microtubules and actin filaments can enhance signaling by G protein-coupled receptors (GPCR) with the subsequent activation of adenylyl cyclase (AC) and increased cAMP synthesis. Recent findings indicate that, in T-lymphoma cells, the depolymerization of microtubules and actin filaments restricts cAMP formation by regulating the localization and interaction of GPCR-Gs-AC in LRs (63). Again, LRs became a point of interest in improving the efficacy of tumor cell eradication.

7. Conclusions and perspectives

The inhibition of tumor cell transformation and cancer cell elimination is the most convenient approach to the prevention of proliferative diseases. Extensive research points to LRs being a platform which transduces signals that evoke cell death. There is extensive evidence that the infiltration of the plasma membrane with cholesterol and sphingolipids is associated with the activation of death-inducing signaling pathways. In addition, it is important to emphasize that the TNFR superfamily is unable to cope with the 'immune escape' of tumor cells, unless death signals can reach their targets and execute apoptosis. The surpassing of plasma membrane proteins in extrinsic apoptosis evoked by TNFR superfamily ligands indicates that this specific formula of anticancer drugs is able to affect crosslinking of transmembrane proteins in order to facilitate the transmission of the death signal in tumor cells. This observation has profound consequences, since molecular mechanisms of tumor cell elimination do not differ from regular cells in terms of quality, but rather in terms of quantity. Fortunately, there are several redundant pathways that could be activated to overcome 'immune escape'. LRs seem to represent a common platform in the initiation of these events because they spatially and temporally coordinate either type of death pathway. Therefore, it is critically important to obtain detailed knowledge of the molecular mechanisms that govern the activation of death cascades originated from LRs when comtemplating the comprehensive and efficient improvement of anticancer therapy.

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