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News and Commentary

Role of membrane microdomain rafts in TNFR-mediated signal transduction

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The tumor necrosis factor receptor (TNFR) superfamily, with nearly 30 members identified so far in mammalian cells, regulates a variety of cellular responses depending on cell type and context, among which are (T cell) activation, proliferation, differentiation, survival and apoptotic and non-apoptotic cell death (reviewed, for example, in Locksley *et al.*[1]). These type I membrane proteins characterized by conserved extracellular cysteine-rich domains appear to transmit their signals via protein–protein interactions. Within the TNFR superfamily, death receptors such as Fas, TNFR1, DR3, DR4, DR5 and DR6 share a common intracellular protein-interaction domain, called the death domain (DD). Perhaps the best-characterized death pathway is the one triggered by the death receptor Fas, also called CD95 or APO-1, upon engagement by its ligand, Fas-L (for review see Krammer[2] and Mundle and Raza[3]). Fas then rapidly recruits to the membrane via a homologous DD interaction the adapter molecule FADD, which in turn recruits the initiator caspase-8 proenzyme via a homologous death effector domain (DED) interaction. The resulting death-inducing signal complex (DISC) leads to proteolytic autoactivation of caspase-8. Caspase-8 then activates other caspases, which are presumed to execute the apoptotic dismantling of the cell. Members of the TNFR superfamily that lack a DD (e.g. TNF-R2, CD27, CD30, CD40) are able to induce cell death via alternative mechanisms.[4] While the nature of molecular events taking place after DISC formation is now reasonably well documented and broadly accepted, little information is available on the initial events that are necessary to generate and regulate the very early formation of this protein complex. Indeed, many questions regarding this stage of death receptor mediated cell death remain open including the most fundamental: how is the signal initiated? Until recently, our thoughts on this matter completely failed to take into account the role of the plasma membrane itself, a structure that was often solely seen as a physical barrier between the external cell environment and the internal cell machinery. However, the cell membrane is composed of domains with diverse compositions and functions. Death receptor signaling has been studied in the context of plasma membrane heterogeneity. Thus, several groups have recently investigated the involvement of membrane 'rafts', sphingolipid–cholesterol membrane domains discovered in the membranes of all eukaryotic cells, in the initiation of TNFR signal transduction. Although sometimes contradictory and ambiguous, the results obtained so far do deserve discussion.

Rafts, membrane domains enriched in cholesterol and sphingolipids (sphingomyelins and glycosphingolipids), correspond to a particular phase of the lipid bilayer: the liquid-ordered (lo) phase that displays an intermediate fluidity between that of the liquid-disordered (ld) and gel phases.[5,6] The membrane structure is therefore currently believed to include lo phase rafts floating in a ld phase dominated by unsaturated phosphatidylcholine molecules in the exoplasmic leaflet. The differential behavior of the lo and ld phases in nonionic detergents provides the basis for an essential tool in raft investigation. Resistance to Triton X-100 at 4°C has been widely utilized as the basis for raft isolation.[5] Their existence on living cells had recently been unambiguously demonstrated using single-particle tracking[7] and fluorescence resonance energy transfer techniques.[8] Indeed, owing to their small size (<50 nm in diameter) and their dynamic behavior, rafts cannot be visualized via conventional imaging techniques. The resolution of conventional visible light microscopy prevents detection of rafts other than by their capacity to coalesce, for example by lateral crosslinking of known raft molecules, leading to patches.

Over the last few years, the role of microdomain rafts has been well studied in signal transduction generated by multi-chain immune recognition receptors (MIRRs), such as TCR, BCR and FcεRI. Their role in pathways mediated especially by four members of the TNFR superfamily (Fas, TNFR1, CD40 and $p75^{NTR}$) has been investigated only more recently.

Using a biochemical approach, it has been shown that a considerable fraction of Fas is constitutively partitioned into sphingolipid- and cholesterol-rich membrane rafts in primary cells such as thymocytes.[9] Similarly, a constitutive association of CD40 with rafts in dendritic cells,[10] and of TNFR1 in U937 and Hela cells[11,12] has been demonstrated. $P75^{NTR}$, one of the NGF receptors, has been shown to be specifically enriched in caveolae, a specialized membrane microdomain.[13,14] Using immunofluorescence techniques, others reported that Fas colocalization with raft-associated proteins in Jurkat cells is not constitutive but rather follows its engagement by the ligand.[15,16] In the same way, a partial relocalization of TNFR1 from detergent-soluble to detergent-insoluble fractions was observed in human skin fibroblasts.[17]

These apparent discrepancies in the constitutive or post-ligand localization of these two receptors in rafts probably reflect the use of diverse cell types, but also of diverse raft isolation techniques. Indeed, it is now well known that

nonionic detergents such as Triton X-100 are able to substantially solubilize the lo phase, therefore making possible the loss of components weakly associated with the rafts.[18] This could be the case for Fas prior to ligation. In addition, the phase behavior of the membrane is, in all likelihood, strongly altered at 4°C. Drevot *et al.*[19] recently reported a new method using polyoxyethylene ether Brij 98, which allows both better discrimination of ordered phases and isolation of rafts at physiological temperature. Using this new technique, it has been shown that Fas is strongly associated with lipid rafts prior to ligation at a level that is not changed after Fas engagement.[9] However, Fas ligation might trigger compositional changes in lipid rafts (see below).

More importantly and beyond the localization of these receptors to rafts, the biological importance of rafts in TNFR signaling has been documented at the molecular level: CD40 triggering leads to membrane-raft-restricted recruitment of TRAF-2 and -3 and to activation of the tyrosine kinase Lyn.[10] Similarly, FADD and caspase-8 absent from rafts in non-stimulated thymocytes are immediately recruited to these membrane compartments upon Fas crosslinking.[9] As the membrane targeting of both FADD and caspase-8 is, at least in mouse thymocytes and embryonic fibroblasts, both necessary and sufficient to initiate Fas-induced cell death signaling,[20–22] these results suggest that rafts represent the membrane site from which, upon engagement by its ligand, Fas initiates its signaling cascade.[9] These observations were further corroborated by the fact that decrease of cellular free cholesterol leading to raft domain disruption significantly blocked both DISC formation and Fas-mediated cell death in mouse thymocytes and L1210-Fas T cells. Such a treatment, however, had been reported to have no effect on Fas clustering and Fas-L-induced apoptosis in Jurkat and SKW 6.4 cells.[23] It would seem important in these studies to quantify raft depletion after treatment with these drugs to be able to draw firm conclusions as to membrane-raft-dependent modulation of signaling events. For instance, the efficiency of raft depletion is cell-type-dependent: according to the amount of membrane cholesterol these drugs may only inefficiently extract cholesterol from the inner leaflet of the plasma membrane.[24] On the other hand, divergent findings may only suggest that sequences necessary for the spatial localization of TNFR to different cell compartments may vary from one cell type to another. In the specific case of Fas, these observations should be considered in the context of the proposed two pathway model of cell death signaling:[25] a difference between type 1 and 2 cells might be found at the plasma membrane, more precisely at the level of raft involvement.

Why and how DISC formation is preferentially formed within rafts is still unclear. Until recently, it was thought that each trimer of FasL molecules interacts with three Fas molecules, resulting in the formation of a trimeric receptor complex. However, recent findings by the groups of M Lenardo[26] and G Ruberti[27] redefine this proposed model of Fas assembly. They show not only that Fas can assemble into oligomers (probably trimers) even in the absence of ligand, but also that this preassembly is a prerequisite for the binding of FasL to its receptor Fas. Ligand engagement may then induce either a conformational rearrangement of the individual receptor chains or alternatively a superclustering into large complexes.[28,29] Rafts might then be considered as higher order structures allowing bridges between multiple trimeric receptors and thus optimizing the biological response. This specific lipid environment might in addition foster signaling. For example, in the absence of ligand, DD-containing receptors could be maintained in an inactive state, through association with negative regulatory molecules, preventing spontaneous aggregation of the receptors (as SODD for TNFR1[30]). Following FasL binding, such DISC formation inhibitors could then be excluded from lipid rafts. On the other hand, molecules required for signaling might be concentrated in lipid rafts. This model proposes that the relocalization of cytosolic adaptor molecules to lipid rafts induces signaling by allowing them to meet their targets, because of the highly increased probability to meet their receptor binding partners. In this context, the data reported by Cottin *et al.*[12] are of particular interest: using a mutagenic approach, they show that the DD of TNFR1 is necessary for the localization of this receptor to the rafts, therefore suggesting a new role for the DD.

Coalescence in membrane rafts following TNFR engagement might provide a membrane compartimentalization which stabilizes receptor clusters and thereby favors adaptor protein recruitment. In this respect, the reports by Grassme *et al.*[15] and Cremesti *et al.*[16] describing a ceramide-dependent formation of Fas clusters upon ligation might be highly significant. The number and/or the stability of initial DISCs formed after engagement of constitutively raft-associated Fas receptors might be rather limited. Activation of caspase-8 would then lead through sphingomyelinase activity to ceramide production, which in turn would induce coalescence of elementary rafts and possibly also reorganization of raft membrane domains. This notion is supported by the results from Xu *et al.*[31] showing that ceramide in model membrane systems promotes the formation of the Lo phase. This would strongly amplify Fas signaling by further recruitment of FADD and caspase-8, as well as stabilizing DISC formation. Thus, Fas signaling initiation could be viewed as a self-strengthening multistep process (Figure 1). In this case, the role of ceramide would become essential when (initial) DISC formation is below a certain threshold, which could be found in various (patho)physiological conditions.

Last but not least, studies of the structural organization of rafts and their involvement in the physiopathology of TNFR signaling should provide valuable information on antitumor strategies. Indeed, dysregulated signaling events in raft membrane microdomains leading to oncogenic transformation have been reported recently. For instance, B-cell lineage non-Hodgkin's lymphomas (NHLB) are neoplastic B cells that show dysregulated B lymphocyte growth characteristics. Pham *et al.*[32] have recently shown that unlike normal B cells, aggressive NHL-B cells display constitutive expression of nuclear NF-κB by maintaining an assembled, scaffold-like signaling platform, called a signalosome, within the lipid raft microdomain. The CD40 signalosome appears to be initiated through autochtonous production and cognate binding of CD40L to CD40 on the lymphoma cell. Therefore, disruption of the signalosome/raft signaling by anti-CD40 or anti-CD40L

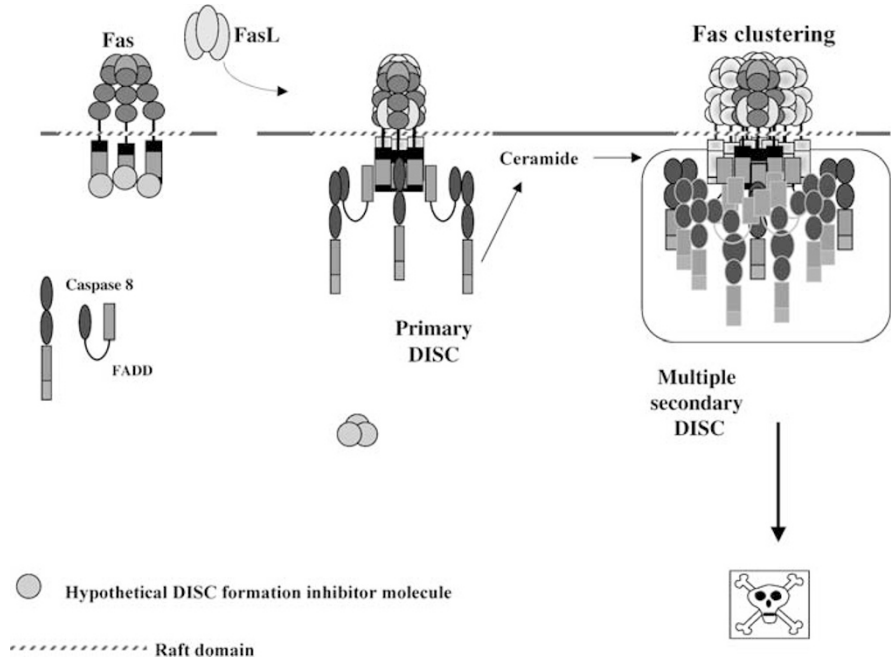

Figure 1 Fas/CD95 signaling initiation: a hypothetical self-strengthening multistep process. DD-containing receptors such as Fas are localized in rafts and maintained there in an inactive state, through association with potential negative regulatory molecules, preventing spontaneous aggregation of the receptors. Following FasL binding, such DISC formation inhibitors could then be excluded from lipid rafts, and molecules required for signaling such as FADD and caspase-8 are recruited to lipid rafts. The number and/or the stability of initial DISCs formed within rafts after ligation of Fas receptors might be in a first step rather limited. Activation of caspase-8 would then lead to ceramide production by acid sphingomyelinase activity, which induces in turn coalescence of elementary rafts and possibly also reorganization of raft membrane domains. This would strongly amplify Fas signaling by further recruitment of FADD and caspase-8, as well as stabilizing DISC formation

antibodies downregulates constitutive expression of NF-κB in NHL-B promoting both lymphoma cell growth inhibition and cell death induction. It can also be envisioned that exaggeration of raft-dependent signaling could benefit tumor elimination. Since the death receptor Fas activates apoptosis signaling in rafts in both normal and transformed cells,[9,15,16] cytotoxic drugs can be used in chemotherapy via enhancement of raft-dependent killing of tumor cells. Such a notion has found experimental support in a recent study conducted by Gajate and Mollinedo on the antitumor ether lipid ET-18-OCH(3), which has been reported to promote apoptosis in tumor cells through intracellular activation of Fas/CD95.[33] These authors show that the effect of ET-18-OCH(3) relies on its ability to induce cocapping of Fas-containing raft membrane domains. Such a cocapping presumably amplifies raft-dependent Fas signaling, possibly through structural reorganization of raft membrane microdomains. Thus, rafts could represent a potential target for therapeutic intervention.

The recent studies mentioned above have already changed the way scientists in the field view the coordination between initial steps associated with this family of receptors and cell death signaling. While we do not know exactly yet the answer to the question of how the signal is intiated, these studies have emphasized some raft-related parameters. However, there is a need to investigate further the dynamics of membrane rafts during TNFR signal transduction. Especially, approaches integrating imaging and biochemical methodologies should help further explain raft behavior and role in TNFR-mediated signaling.

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