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EDITORIAL

Multifaceted nature of membrane microdomains in colorectal cancer

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Abstract

Membrane microdomains or lipid rafts are known to be highly dynamic and to act as selective signal transduction mediators that facilitate interactions between the cell's external and internal environments. Lipid rafts play an important mediating role in the biology of cancer: they have been found in almost all existing experimental cancer models, including colorectal cancer (CRC), and play key regulatory roles in cell migration, metastasis, cell survival and tumor progression. This paper explores the current state of knowledge in this field by highlighting some of the pioneering and recent lipid raft studies performed on different CRC cell lines and human tissue samples. From this literature review, it becomes clear that membrane microdomains appear to be implicated in all key intracellular signaling pathways for lipid metabolism, drug resistance, cell adhesion, cell death, cell proliferation and many other processes in CRC. All signal transduction pathways seem to originate directly from those peculiar lipid islands, thereby orchestrating the colon cancer cells' state and fate. As confirmed by recent animal and preclinical studies in different CRC models, continuing to unravel the structure and function of lipid rafts - including their associated complex signaling pathways - will likely bring us one step closer to better monitoring and treating of colon cancer patients.

Key words: Actin; Caveolae; Cytoskeleton; Combined imaging; Detergent-resistant membranes; Drug targeting; Electron microscopy; Lipid domains; Membrane rafts; Prognosis; Staging; Tomography; Lipid-mediated therapy

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INTRODUCTION

In recent years, high-speed multidimensional live-cell confocal laser imaging[1], often combined with combinatorial labeling approaches[2] and/or subsequent correlative electron microscopy studies[3], has allowed high-throughput studies that add missing pieces of temporal and spatial resolution and detail to the cell membrane puzzle. At the nanometer length scale, semi-automated transmission electron tomography (TET) has enabled generation of accurate three-dimensional models of the fine architecture of the cell membrane and its associated proteins[4,5]. Meanwhile, advances in the high-throughput analyses of chemical and molecular biology have allowed researchers to catalogue the lipidomics of the cell with hitherto unseen sensitivity[6,7], complementing recent computer simulations of lipid membranes[8,9]. Over the last decade, this

progress in analytical chemistry and in rapid microscopy-based imaging has underpinned the exponential growth in our understanding of the dynamic and multifaceted role of the cell membrane in various physiological processes.

The cell membrane is unquestionably one of the most studied subcellular components in the modern era of cell biology[10-12]. This is not simply because it is an attractive target for drug designers[13], but also because it acts as a selective protector of the interior of the cell[14]. Key regulatory roles for the cell membrane have been demonstrated in cell homeostasis and differentiation, cell death and cell survival pathways, inter- and intracellular signaling, cell development and movement, and trans- and intercellular transport mechanisms[15,16]. Of special interest are unique lipid domains within the cell membrane that have been shown to be involved, directly or indirectly, in such lipid-mediated cell regulation. These small domains, also known as lipid rafts, were initially described by Palade through the use of electron microscopy[17] and later carefully identified by Simons *et al*[18] through a combined biochemistry and imaging approach. These rafts lie as discrete patches, known as detergent-resistant membrane structures, in the plasma membrane of cells and are rich in sphingolipids and cholesterol[18,19]. The fatty acid chains of lipids within these rafts tend to be tightly packed, creating ordered lipid domains that float in a sea of poorly-ordered lipids within the membrane[20]. Lipid rafts are highly dynamic and temperature sensitive, are able to form large clusters and to interact with the cell's internal molecular and structural compartments[21,22]. From recent studies, it is becoming ever more evident that rafts act as highly dynamic and selective guardians between the cell's external and internal worlds, which makes researchers view them as important structural and molecular targets for altering cell function and behavior (for a review[23]).

With regard to the biology of cancer, it has been demonstrated that lipid rafts play important mediating roles in cell migration, metastasis, cell survival and tumor progression[24,25]. The literature of the past five years contains over one thousand original research papers that have studied the role of these peculiar lipid islets in cancer. This illustrates their importance and their perceived potential in future cancer cures and/or as markers for tumor staging and, hence, diagnosis and prognosis. This literature also shows the presence of lipid rafts in almost all existing experimental cancer models, *in vitro* and *in vivo*. The presence and role of rafts have also been highlighted within relevant human and clinical settings[26,27], including in colorectal cancer (CRC)[28,29]. Lipid rafts in CRC cells were observed initially in 1998 by Orlandi and Fishman[30] and studied extensively by many others subsequently (see next section). So far, however, no dedicated paper has addressed "raft biology and pathobiology" in CRC. Here, therefore, we carefully review the current state of knowledge by highlighting some of the pioneering and recent raft studies carried out on different CRC cells and tissue samples.

MEMBRANE MICRODOMAINS IN COLORECTAL CANCER CELLS

Researchers have shown, for example, that lipid rafts in CRC cells act as go-betweens for cell death-mediated signaling[31,32], as portals for bioactive compounds[33], and as congregation regions for adhesion proteins and major histocompatibility complex class 1 (MHC-1) molecules[34]. However, despite the abundance of literature available on the biology and pathobiology of lipid rafts in cancer[35], only a few dedicated papers presently address the importance of these membrane domains in the process of CRC. While lipid rafts have been thoroughly described in other malignant tumor models such as cancers of the breast, lung and prostate, this does not imply that we should overlook their important role in the onset and development of CRC (see below). Unraveling their structure and function - including their associated complex signaling pathways - could eventually form the basis for future therapeutic interventions.

Labeling and morphometric imaging approaches

Although lipid rafts have been studied extensively through dedicated labeling and microscopy techniques[36], relatively little is known about their fine structure in CRC cells or tissue at the nanometer scale[37]. The majority of CRC membrane raft studies use immunofluorescence microscopy, often in conjunction with protein blotting and/or flow cytometry. Microscopic identification of lipid rafts in CRC cells is frequently performed by direct labeling against specific molecular components of membrane rafts[32,38]; the classic example is the use of fluorescently-labeled cholera toxin against ganglioside II3-N-acetylneuraminosyl-gangliotetraosylceramide (GM1)[39-41]. Indirect identification approaches are frequently used as well, such as staining against molecular targets or proteins that are supposed to associate with CRC lipid microdomains[42]. However, combinatorial protein blotting studies on isolated raft protein fractions must be an essential part of this approach to unambiguously identify the association of raft-specific proteins with lipid rafts. The different caveolin (Cav) isoforms - predominantly Cav-1 and Cav-2 - are also popular protein targets for raft detection[43,44], but should be used with caution because the degree of Cav expression in cancer cells depends on the differentiation status of the cells and the cancer model studied[35].

Depending on the imaging approach applied, lipid rafts have been reported to range from between 30 and 100 nanometers up to almost one micrometer in size[20,45,46]. We have applied correlative fluorescence and electron microscopy (CFEM) to confirm that the same holds true for CRC cells[37]. CFEM analysis of Caco-2 cells labeled for GM1 allowed the direct observation of fluorescently-labeled lipid rafts by light-optical microscopy and by electron microscopy (Figure 1A). We observed that the smaller lipid rafts could be easily resolved under electron microscopy, but could not be clearly seen by confocal imaging. In some

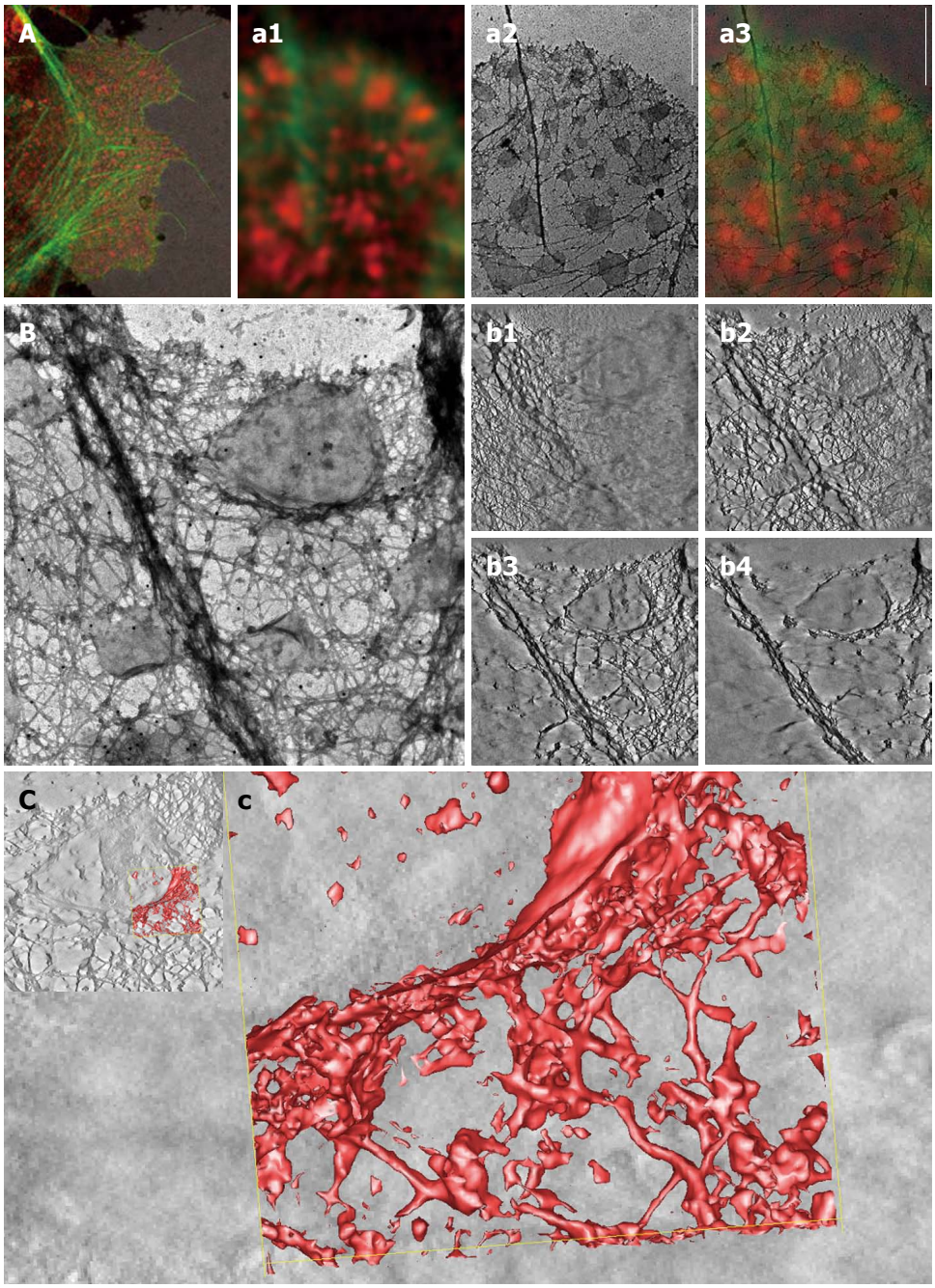

Figure 1 High-resolution imaging of membrane domains in human Caco-2 colorectal cancer cells. These data come from high-resolution correlative fluorescence and transmission electron microscopy (CFEM) studies on whole mounts. Human colorectal cancer (CRC) cells (Caco-2) were cultured on formvar-coated nickel grids and then treated with Triton X-100 in cytoskeleton stabilization buffer, leaving detergent-resistant membranes behind[37]. These membrane fractions were labeled with the membrane raft marker GM1 CTxB-594 (red) and the actin cytoskeleton was stained with phalloidin-488 (green)[48]. A: Low-magnification overview of the peripheral cytoskeleton reveals actin-rich lamellipodia and associated filopodial extensions. Note the numerous GM1-positive lipid rafts interspersed throughout the extracted cytoplasm. The corresponding high-magnification CFEM analysis (a1-a3) of one of these lamellipodium-filopodial regions reveals the complex architectural nature of the leading edge, showing an intricate cytoskeleton-rich matrix and the close structural relationship with the lipid rafts (a1). Subsequent TEM analysis of exactly the same region not only allowed us to determine the exact size and shape of lipid rafts, but also provided an accurate idea of whether the rafts were located on top or beneath the lamellipodium: i.e. apical or basal (a2). The corresponding merged information (a3) clearly shows the additional value of applying different imaging techniques on the same cell: the electron-microscope data reveals small detergent-resistant membrane islands that could not be resolved by advanced confocal microscopy. Scale bar, 2 μm; For the electron tomographic analysis of a detergent-resistant membrane island and the surrounding cytoskeletal matrix (show in B and C), whole mounts of Caco-2 cells were prepared as outlined under A. Then we performed transmission electron tomography imaging at 1.5° incremental steps under dual-axis tilting (B) and then carried out subsequent segmenting (b1-b4) of the XYZ-tilting series. The single-image slices obtained *via* the IMOD tomography software show the sample at different heights from bottom to top (b1-b4) spanning a height of about 300 nm; C: An area of the entire tomogram was next selected (c) and a three-dimensional model generated of the membrane raft-cytoskeleton interface (c), showing the close interaction of fine cytoskeletal fibers at the rim of the detergent-resistant membrane.

cases, no fluorescent label could be detected at all, supporting earlier observations regarding the heterogeneous size and composition of lipid rafts[46,47]. The samples prepared for CFEM investigation were also readily used for subsequent TET[48], which generated a stack of virtual XYZ sections through the structure of interest (Figure 1B). When

combined with computer modeling, we obtained a three-dimensional view of the structure at a typical resolution of approximately 5-8 nm (Figure 1C). From these tomographic data, it became apparent that a web of fine cytoskeletal fibers, derived from the surrounding cytoskeletal matrix, accumulates at the circumference of rafts. These cytoskeleton-raft interactions probably indicate that membrane rafts require an intact cytoskeleton lattice for proper functioning of raft-associated subcellular processes[49].

Raft-mediated cell death

There has been an extraordinary increase in research activity aimed at understanding the mechanisms and processes that underlie cell death, which occurs in different forms. The best-studied cell death mechanisms are apoptosis and necrosis (for a recent review[50]). Whether by apoptosis or necrosis, cell death is initiated, triggered and regulated by a cascade of signaling pathways that involve different molecules. As we continue to unravel the pathways of cell death, novel findings emerge concerning the complex role that the cell membrane - and in particular rafts and their associated molecules - play in the tightly coordinated process of programmed cell death and cell survival[25]. A few reports unambiguously illustrate that rafts are key signal transducers when it comes to death of CRC cells. Remacle-Bonnet *et al*[51] showed that lipid rafts segregate pro- from anti-apoptotic insulin-like growth factor-I (IGF-I) receptor signaling in various human colon adenocarcinoma cell lines (HT29-D4, HRT-18, HCT-116, HCT-15, COLO-205, SW480, SW620, HCT-8/E11 and HCT-8/E11R1) when exposed to tumor necrosis factor-α (TNF-α). However, the pro-apoptotic effect of IGF-1 was not observed in all CRC cell lines tested, and SW480, SW620, HRT-18 and COLO-205 cells seem to be exceptions. The authors found that the paradoxical pro-apoptotic action of IGF-I is conveyed *via* the phosphoinositide 3-kinase (PI3K)/Akt pathway and that integrity of lipid rafts is necessary for proper anti-apoptotic cell signaling (Figure 2). In contrast, the activation of the Erk 1/2 and p38 MAPK pathways that transmit the IGF-I anti-apoptotic signaling is independent of lipid rafts.

These unexpected findings, obtained by incubating the different cell lines with the cholesterol-depleting agent methyl-β-cyclodextrin (Me-β-CD), showed the complicated functions that lipid rafts can display, depending on the molecules they are exposed to in the tumor microenvironment. Here, lipid rafts acted to precisely regulate whether CRC cells would survive or die. This might also partially explain the different and conflicting data reported in CRC cell death studies.

Another groundbreaking finding comes from a careful comparative *in vitro* and *in vivo* study in which sugar-cholestanol derivates provoked cell death in COLO-201, HT29 and Colon-26 cells, including Balb/c mice that contain Colon-26 tumors[38]. In this study, Hahismoto *et al* showed that chemically-synthesized sugar-cholestanols, with mono-, di- and tri-saccharides attached to cholestanol, are transported into the cell's interior *via* membrane microdomains; however, cholestanol without sugar moieties was not taken up. Biochemical analysis revealed that all N-acetyl-D-glucosamine-based sugar-cholestanols accumulate quite rapidly within the mitochondria of CRC cells, gradually increasing the release of cytochrome C from these organelles within the cytoplasm. Subsequent studies performed with time-pulse DNA ladder fragmentation assays and Western blotting demonstrated that cell death occurred *via* the caspase-9/caspase-3 apoptotic pathway (Figure 2). In their animal studies, the authors validated the potential anti-cancer effect of sugar-cholestanol derivates when administered intraperitoneally at different time intervals: Balb/c mice showed a significant reduction in tumor growth and had prolonged survival. This is one of the first CRC *in vivo* studies that unambiguously demonstrated the importance of membrane microdomains as a molecular target for cancer therapy (see also next section).

Other studies have shown that food-derived biochemical compounds can induce substantial cell death in CRC. In 2003, resveratrol - a polyphenol found in various food products - was reported to trigger apoptosis in SW480 human CRC cells[42]. By combining microscopy, cell sorting and protein blotting, the authors established the direct involvement of the caspase-8/caspase-3-mediated apoptotic cascade (Figure 2). Furthermore, resveratrol exposure induced a specific redistribution of the cell death receptor Fas (i.e. CD95) within membrane microdomains, and caused formation of the death-inducing signaling complex. Intriguingly, no interaction between the cell death receptor ligand (i.e. FasL) and Fas was required for the resveratrol-induced cell death. The authors, therefore, postulated that resveratrol, which is abundantly found in grape skin, holds strong potential as a chemoprotective and therapeutic agent for CRC and other malignant tumors. In another study, quercetin - a plant-derived flavonoid, plentiful in apples and red onions - was reported to induce apoptosis in HT-29 and SW-620 cells, although by a different apoptosis signaling pathway[40]. It was found that quercetin enhanced apoptosis caused by the TNF-related apoptosis-inducing ligand (TRIAL) through redistributing the death receptors (DR) DR4 and DR5 into membrane microdomains (Figure 2). The application of nystatin, a cholesterol-sequestering agent, prevented (1) quercetin-induced clustering of death receptors; and (2) sensitization to TRIAL-induced apoptosis in CRC cells. The involvement of the mitochondrial-dependent death pathway was demonstrated by the activation of related pro-apoptotic molecules and the subsequent release of cytochrome C to the cytosol. These data suggest that membrane microdomain localization of death receptors is probably required for optimal cytotoxicity of quercetin and/or TRIAL.

Cisplatinum or cisplatin is a well-known chemotherapeutic drug, widely used to treat various types of cancers. Rebillard *et al*[32] demonstrated that cisplatin-induced apoptosis in human CRC cells involves cell membrane fluidification *via* the inhibition of the Na^+/H^+ membrane exchanger-1. Inhibition leads to an overall intracellular acidification and the subsequent activation of acidic sphingomyelinase, which generate ceramides that finally affect membrane fluidity. The team also found that this

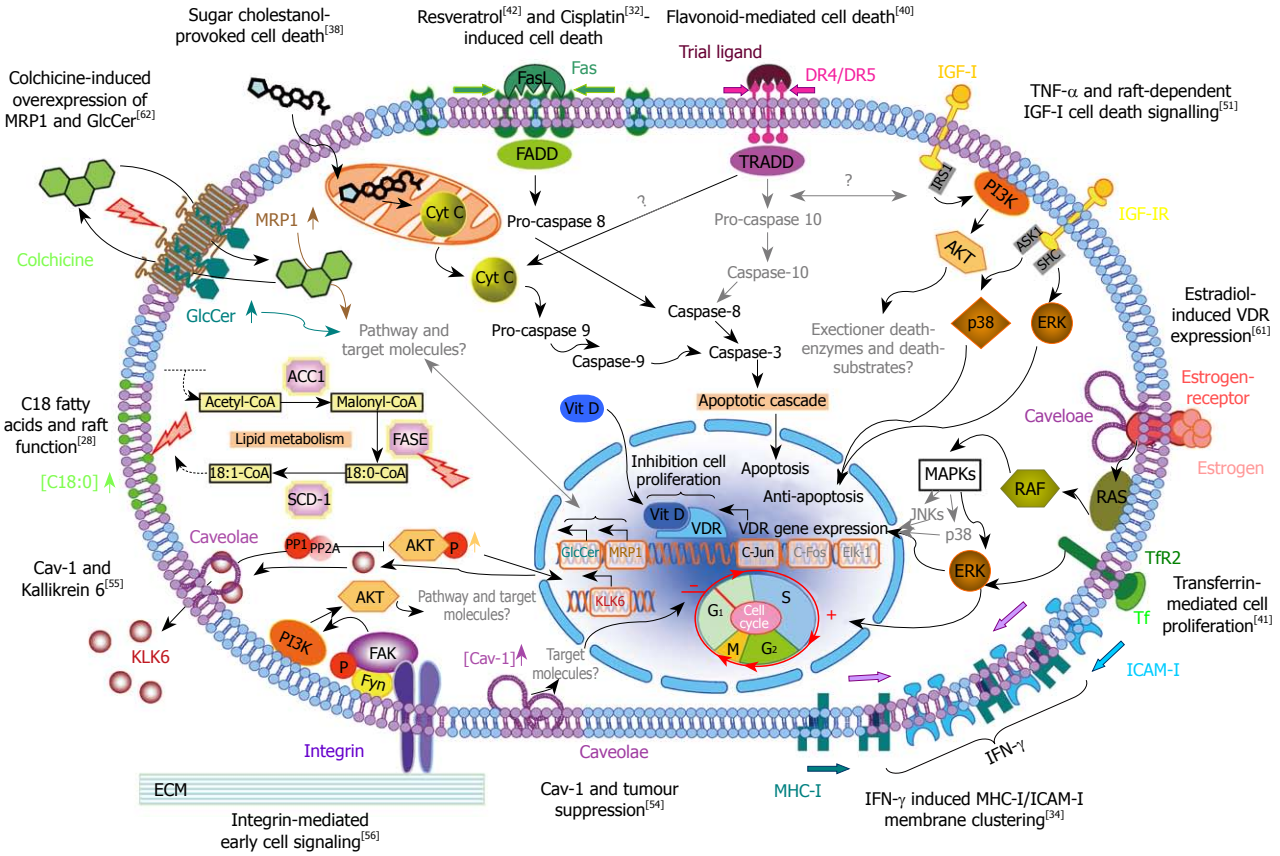

Figure 2 Scheme outlining the various membrane microdomain-mediated intracellular signaling pathways in colorectal cancer. This diagram summarizes what has been reported to date in the literature about the different intracellular signaling pathways that are mediated by lipid rafts and the implications of these paths for the colon cancer cells' state and fate. Briefly, the different observations of colorectal cancer (CRC) lipid rafts can be generally categorized under the following main topics of investigation: cell death-mediated mechanisms, caveolae in cancer cell growth and function, unique structure-function molecular associations, and intervention studies with bioactive compounds. Note that the text and connector arrows as shown in black are confirmed observations, whereas the gray denotes postulated signaling pathways and/or unknown molecular targets. The lipid bilayer of the cell membrane is depicted in light blue, membrane microdomains or lipid rafts in light purple, and the pear-shaped caveolae associated with these rafts in dark purple. For detailed descriptions of each of the individual cell signaling pathways, refer to the corresponding sections in this paper. The numbers in superscript refer directly to the original published papers. MRP: Multidrug-resistance protein; GlcCer: Glucosylceramide; FADD: Fas-associated protein with death domain; TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain protein; PI3K: Phosphoinositide 3-kinase; AKT: Serine/threonine protein kinase; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; IRS1: Insulin receptor substrate 1; ASK1: Apoptosis signal-regulating kinase 1; SHC: Src homology 2 domain; TNF-α: Tumor necrosis factor-α; IGF-Ⅰ: Insulin-like growth factor-Ⅰ; VDR: Vitamin D receptor; Vit D: Vitamin D; RAF: Proto-oncogene serine/threonine-protein kinase; RAS: RAt sarcoma; TfR2: The second transferrin receptor; Tf: Transferrin; JNKs: c-Jun N-terminal kinases; ICAM-Ⅰ: Intercellular adhesion molecule Ⅰ; IFN-γ: Interferon-γ; MHC-Ⅰ: Major histocompatibility complex Ⅰ; FAK: Focal adhesion kinase; ECM: Extracellular matrix; FASE: Fatty acid synthase; SCD-1: Stearoyl-coenzyme A desaturase 1; ACC1: Acetyl-CoA carboxylase; Cav: Caveolin.

rapid increase in membrane fluidity after cisplatin treatment was inhibited by membrane stabilizing agents, such as excess cholesterol or monosialoganglioside-1 treatment. Furthermore, these lipid-interfering compounds prevented the early aggregation of the Fas receptor and of membrane microdomains on the cell surface of HT-29 cells. As a result, significant inhibition of cisplatin-induced apoptosis was observed, without altering the intracellular drug uptake or the formation of cisplatin-DNA adducts. Hence, cisplatin-induced cell death in CRC cells seems to be mediated, in part, *via* the Fas-signaling pathway, the cell death receptors of which reside within lipid rafts (Figure 2). This concept was elegantly demonstrated through a variety of analytical tools, including GM1 labeling studies, in the course of lipid fluidity studies on isolated lipid rafts, and biochemical assays to detect caspase-3 activity and determine the extent of apoptosis.

Caveolin at the raft interface

There is widespread evidence that caveolin proteins associate structurally and functionally with membrane microdomains, interacting closely with numerous microdomain-associated molecules and thereby regulating cell signaling pathways that control cell function and cell fate[23]. Although all types of caveolins (Cav-1, Cav-2 and Cav-3) are structurally similar and associate with cholesterol and sphingolipids in parts of the cell membrane to form caveolae, Cav-1 and Cav-2 are most prominently upregulated and/or downregulated in oncogene-transformed cells[35]. However, a certain degree of variability in Cav-1 and Cav-2 expression seems to occur within various classes of cancer and across different types of cells or tissues. Furthermore, the amount of Cav-1 and Cav-2, together with the ultrastructural presence of caveolae, not only depends on the tumor model studied, but also on the stage and grade of

the cancer. This is particularly relevant for CRC in which the expression of Cav-1 mRNA is four to five times lower or two times lower, respectively, for Cav-2 mRNA in HT-29 or in COLO-205 CRC cell lines. These data were obtained by directly comparing the relative expression of caveolins in more than 55 commonly-used human cancer cell lines from different tissues (for an overview[35]). However, others have demonstrated, *via* classical immunocytochemical staining[52] and reverse transcriptase-polymerase chain reaction (RT-PCR)[53] in human colon tumor tissue, that Cav-1 was significantly enhanced compared to normal colon epithelium. In addition, caveolin-mediated raft signaling has been demonstrated to be pivotal in various experimental CRC models, especially in the strong anti-proliferative action of Cav-1. Bender *et al*[54] showed that Cav-1 possesses a strong tumor suppression activity in CRC cells, but the exact signaling mechanism responsible is not yet clear (Figure 2). Experiments involving immunoblotting, RT-PCR and microarrays disclosed that expression of Cav-1 in HT-29 and DLD-1 cells delayed or blocked tumor formation in nude mice. Likewise, Cav-1 levels were significantly reduced in colon tumors from human patients. Another interesting finding was that increased levels of Cav-1 were observed in multidrug-resistant HT-29 cells. The authors concluded from their studies that Cav-1 modulates a variety of signaling pathways, but that we still require a better understanding of target molecules affected by the expression of this all-round protein in malignant cells.

Henkhaus *et al*[55] demonstrated a direct link between Cav-1-mediated expression and secretion of kallikrein 6 (KLK6) in HCT116 human CRC cells. Sucrose-gradient subcellular fraction analysis revealed that Cav-1 and KLK6 co-localize to lipid rafts. Deactivation of Cav-1 - through interference in the Src-mediated phosphorylation pathway - decreases KLK6. In addition, immunoblotting, ELISA and RT-PCR studies revealed that Cav-1 controls KLK6 expression *via* the Src, Akt and phosphatases (i.e. PP1/PP2A) signaling pathway (Figure 2). Kallikreins are serine proteases, and the various subtypes of kallikreins are considered to hold promise as specific biomarkers for different cancers. Furthermore, once secreted by the cell, these serine proteinases have the ability to degrade the surrounding extracellular matrix (ECM). The authors postulated that KLK6-mediated degradation of the ECM enhances CRC cell invasiveness.

Raft-associated molecular expression

Besides the presence of cell death receptors and caveolar proteins within membrane microdomains, other key molecules have been shown to have close structure-function associations with rafts, thereby controlling a variety of other vital cellular processes in CRC, such as cell adhesion and motility, intracellular transport and cellular exchange, immune tolerance, and numerous hormone-mediated cellular responses[23]. For example, Baillat *et al*[56] demonstrated how focal adhesion kinase (FAK) and Src family protein tyrosine kinases (SFKs) work together in lipid rafts during the initial stage of CRC cell adhesion. It is well known that elevated expression and activity of SFKs in CRC often accompany disease progression. In cancer development, tumor cells acquire migration capability and effective homing ability in the body's host environment. Cell adhesion molecules, such as integrins that link components of the ECM with the cytoskeleton, are pivotal during this process. By applying a combination of cell transfection methods, protein-blotting assays and membrane raft ultracentrifugation to Me-β-CD-treated and/or cholesterol-treated SW480 cells, the team found that the formation of raft-associated FAK/Fyn complexes and the activation of Akt-1 *via* PI3K occur simultaneously during early contact with the ECM (Figure 2). Fyn is a tyrosine-specific phospho-transferase and member of the SFKs. Akt-1 is a serine threonine kinase that is considered to be an oncogene and is often activated in human cancers, thereby contributing to tumor progression and metastasis. The team concluded that, during the very early stage of cell adhesion, FAK is transiently co-located with Fyn thereby inducing raft-dependent Akt-1 signaling. This study also showed, for the first time, that FAK in membrane microdomains can act as a signaling intermediate to control various aspects of tumor cell behavior during cell adhesion.

Major histocompatibility complex I (MHC- I) and intercellular adhesion molecule I (ICAM- I) are crucially involved in the functioning of the immune system and are implicated in inflammatory bowel diseases and CRC. Cytokines used in therapy, such as interferon γ (IFN-γ), are known to modulate the expression of MHC- I and/or ICAM- I , so it is no surprise that these cell surface receptors are under investigation in an attempt to cure various gastrointestinal diseases. Bacsó *et al*[34] showed that exposing LS-174-T colon carcinoma cells to IFN-γ significantly increased the cell surface density of MHC- I and ICAM- I . Flow cytometric fluorescence energy-transfer measurements of immunolabeled LS-174-T cells and confocal microscopy of GM1-labeled LS-174-T cells revealed that both receptor types cluster in the nanometer range and that amounts of both substantially increase, within GM1-positive membrane microdomains, upon IFN-γ treatment (Figure 2). Moreover, the team found that the relative size of the lipid rafts increased, while the total cell size and membrane surface remained unchanged. Another interesting observation was that MHC- I and ICAM- I form sterically tight hetero-associates such that ICAM-1, with its long protrusion above the cell membrane, can readily bind to a cytotoxic lymphocyte (CTL) and simultaneously MHC- I can favorably present its peptide directly to the CTL. As both receptors are co-localized in lipid rafts, which are considered as pre-formed cell-signaling sites, all steric conditions are present for rapid trans-membrane signal transduction. These data imply that IFN-γ treatment can alter the surfaces of CRC cells to make them better target for CTLs.

Examination of resected tumor tissue has demonstrated that expression of the second transferrin receptor (TfR2) occurs in human colon carcinomas[41]. These authors also showed the presence of TfR2 in three different CRC cell lines (HT29, HCT116 and SKCO1) as assessed by immunolabeling, flow cytometry and Western blot analysis. This is intriguing given that TfR2 expression in

normal tissues is restricted to the liver, where it mediates cellular uptake of transferrin (Tf)-bound iron. The authors also found evidence that TfR2 expression induces a rapid and pronounced ERK1/ERK2 phosphorylation, indicating involvement of the Ras-dependent ERK1/ERK2 MAP-kinase signaling pathway (Figure 2). This pathway has a central role in controlling cell proliferation and is frequently activated in cancers, including CRC. It was concluded that TfR2 present within lipid rafts of CRC cells might contribute to the growth advantage of these cells.

Recently, Taïeb *et al*[57] demonstrated the presence of prominin 1 (CD133) within Caco-2 and HT-29-D4 cells. CD133 is a trans-membrane protein that has been shown, in different experimental tumor models, to make cancer cells transplantable, resistant against radiation therapy, and highly likely to initiate tumors. Researchers also believe CD133-ganglioside interactions are crucial for the recruitment and/or the phenotype of cancer stem cells. For this reason, there is obvious interest in understanding the molecular biology of $CD133^+$ cancer cells. With the aid of a novel anti-CD133 antibody, Taïeb *et al*[57] demonstrated that CD133-immunolabeling progressively decreased to undetectable levels in postconfluent CRC cell cultures, possibly through ganglioside-mediated epitope masking, because the staining was partially recovered after chemical disruption of lipid rafts. It is noteworthy that the N-terminal epitope of CD133 belongs to a ganglioside-binding domain and that blocking experiments with various gangliosides, including purified GM1, resulted in negative labeling. The authors proposed that synthetic soluble ganglioside analogues, which act as competitors and specifically affect $CD133^+$-mediated signaling pathways, deserve thorough evaluation in the development of new therapeutic approaches to CRC.

Bioactive compounds and raft function

Since the mid-1990s, hundreds of papers have examined the interference of drugs, pharmacological agents and other chemical or natural compounds with membrane microdomains[26]. Recent headway in animal disease models has provided knowledge as to how compounds affect membrane raft function, which might help to cure diseases such as ischemic heart impairment[58], keratitis[59] and colitis[60]. In colorectal studies, findings are limited so far to experimental *in vitro* models (see below). However, despite the limited data, the results all indicate that CRC membrane microdomains appear to be an important entryway for anti-cancer drugs, hormone(-like) molecules and dietary components. As reviewed above (see "Raft-Mediated Cell Death"), the bioactive compounds cisplatin[32], sugar cholestanol[38], flavoids[40] and resveratrol[42] have all been shown to possess a strong adverse cellular effect that is mediated *via* membrane raft signaling. Besides these classes of cell death-inducing compounds, other molecules have been described that control CRC cell function and fate.

For instance, in a study of the estrogen-induced vitamin D receptor (VDR) expression model, Gilad *et al*[61] elegantly illustrated how vitamin D (Vit D) controls CRC cell proliferation. By combining agents that interfere with lipid and intracellular signaling with subsequent protein immunoblotting studies, the authors unraveled that the estrogen 17β-estradiol (E2) binds to estrogen receptors (ERs) confined to lipid rafts or caveolae in HT29 cells, thereby activating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway *via* the protein kinases Ras/Raf. This affected transcriptional activity and finally resulted in upregulation of expression of the *VDR* gene. The MAPK/ERK pathway links intracellular responses to the binding of hormones and/or cell growth factors to cell membrane receptors (Figure 2). A direct functional association of ERs with lipid rafts was shown by using the cholesterol-binding agent Me-β-CD that blocked ERK phosphorylation concomitantly with VDR upregulation. E2 treatment did not affect proliferation of HT29 cells, while Vit D exposure significantly inhibited cell proliferation, and the combined treatment resulted in potentiation of Vit D activity. This anti-proliferative effect of Vit D, mediated *via* membrane microdomain receptor signaling, again illustrates the significance of lipid rafts in the regulation of tumor growth. It also emphasizes the importance of a well-balanced diet, including the daily intake of essential vitamins such as Vit D abundant in dairy products and fish oils, for controlling health.

Multidrug resistance (MRD) is a major challenge for drug designers and cancer cell biologists. When continuously exposed to chemotherapeutic compounds over long periods, cancer cells tend to acquire MDR by overexpressing proteins that belong to the superfamily of ATP-binding cassette (ABC) transporter proteins. Klappe *et al*[62] generated a new $HT29^{col}$ cell line that, during colchicine-induced acquisition of MDR, increases their glucosylceramide (GlcCer) content and upregulates multidrug-resistance protein I (MRP- I). The overexpression of sphingolipids, such as GlcCer, appears to be a rather general aspect of MDR cancers and has even been proposed as a candidate marker for MDR^+ malignant tumor cells. Furthermore, the tightly coordinated upregulation of MRP- I - a member of the ABC transporter proteins that displays strong drug-efflux properties - and GlcCer were both enriched in lipid rafts (Figure 2). The authors also demonstrated that GlcCer upregulation did not appear to be necessary for MRP- I function, given the absence of effects of inhibition of GlcCer biosynthesis on MRP- I -mediated drug efflux and cell survival. They, therefore, concluded that GlcCer appears to play a structural role in membrane microdomain organization instead and, as such, might help to accommodate the excess MRP- I expressed in membrane lipid islets in CRC cells.

The enzyme fatty acid synthase (FASE) synthesizes only saturated fatty acids and overexpression has been shown to be involved in numerous human malignancies, including CRC. Rakheja *et al*[28] demonstrated, through gas chromatography and mass spectrometry, a statistically significant increase in saturated C18:0 fatty acid (stearic acid) in colonic adenocarcinoma, compared to adjacent normal colonic mucosa tissue. None of the thirteen patients investigated had received any pre-operative chemotherapy and/or radiotherapy, thereby excluding artifactual read-

ings or false-positive measurements caused by treatment. Although the authors did not present any immunohistochemical data on normal or cancerous tissues, they postulated that the increase in the proportion of saturated fatty acids will most likely affect the functional properties of lipid rafts in CRC cells (Figure 2), and particularly might impair intracellular signaling mechanisms as discussed earlier (i.e. cell growth and cell death). In support of this proposal, it is known that the relative abundance of saturated fatty acids is a principle reason for the liquid-ordered state of lipid rafts and that the inhibition of FASE mainly affects the synthesis of raft-associated lipids. The authors concluded that dietary intervention to normalize the balance between saturated and unsaturated lipids within the cell membranes of colonic mucosa might be seen as a preventive or even therapeutic cure (see next section).

POTENTIAL DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS OF MEMBRANE MICRODOMAINS IN CRC

Many benign and malignant tumors synthesize and secrete compounds (i.e. tumor-associated proteins) that can be detected histopathologically on tissue sections or biochemically by chemical pathology analysis of blood or other bodily fluids. As the majority of these compounds are produced within tumors, they are said to be tumor-derived and so provide direct evidence of the tumor's existence (i.e. they are tumor-associated markers). It is highly probable that determination of raft composition or the detection of raft-associated markers, *via* proteomic and/or lipidomic approaches, could be extremely helpful in diagnosing malignancy in a patient with symptoms, preferably during the early stages of tumor formation[63,64]. The measured marker concentration or a combination of markers should directly correlate with the mass and/or activity of the tumor, and ideally might even help to fine-tune the formulation and dosages of anti-cancer drugs. Taking the currently available information on CRC rafts into account, the ratio of Cav-1/GM^+ and/or the presence of other molecules - such as TfR2, CD133 or KLK6 - have proven to be extremely useful candidate cancer markers that could aid in the diagnosis, staging and prognosis of CRC. However, despite initially high enthusiasm when the first experimental tumor marker-based assays appeared, only rarely does a marker exhibit sufficient specificity and sensitivity to be of any practical use in the clinic. While one single marker will not be sufficient to make a reliable diagnosis and prognosis, mass-spectrometry-based lipidomics[64] seems to hold great promise as a novel diagnostic approach to detect the unique "lipid fingerprint" of cancer cells. When combined with existing biomarker assays, lipidomics brings us one step closer to better monitoring and treating of cancer patients.

As it stands, we still have a long way to go when it comes to implementing our practical knowledge of lipid rafts to treat patients. Although the first animal studies showed much promise - as briefly mentioned before - in helping find a cure for diverse diseases, we still await the first full translational (pre-)clinical studies for curing diseases by targeting lipid rafts and their associated molecules. This, however, does not mean that raft-mediated therapy is entirely impossible or merely wishful thinking. It is just a reflection of the early days of membrane raft biology. When it comes to cancer and membrane raft-mediated therapeutic intervention, commonalities in proposed therapeutic approaches can be found that are independent of the cancer model studied. The majority of work targets trans-membrane proteins present in lipid rafts that control cell death *via* the programmed pathway (i.e. apoptosis; for a review[65]). However, this enthusiasm must be tempered by caution, because cancer cells have the ability to change the expression pattern of membrane-associated cell death receptors, depending on their cellular environment and/or progression stage. CRC cells have the ability, for example, to switch between the cell death receptor Fas and its corresponding ligand (i.e. FasL), depending on their microenvironment or the chemokines they meet[66,67]. A therapeutic intervention that uses smart drug complexes to target several different cell death molecules, including membrane raft-disrupting compounds, therefore is the approach most likely to result in successful outcomes.

Another interesting field concerns the modulation of membrane raft composition *via* dietary intake, or altering lipid synthesis *via* pharmacologic intervention within malignant cells[68]. Both are meant to change the cholesterol content within the lipid membranes in the hope of impairing the membrane raft signaling that controls tumor development and growth. Altered levels of membrane cholesterol and cholesterol-rich membranes have been shown to influence the aggressiveness and progression of cancers[69]. In CRC, this approach has a considerable chance of success because of the cancer's position within the digestive tract, which allows clinicians to directly expose the cancer cells to significant amounts of dietary or pharmacological compounds. In animal studies of various tumor models, dietary supplementation with long-chain polyunsaturated fatty acids combined with anti-cancer drugs significantly decreased tumor size and resulted in prolonged survival (for a review[70]). These fatty acids have been shown to be beneficial in various colon-related diseases, so an important role has been postulated for membrane microdomains in controlling tumor growth; this is currently a topic of active investigation[29,71]. Other examples of potential dietary therapies, as previously discussed, include the reduction of CRC tumor growth mediated by sugar-cholestanols[38], or the beneficial effects of polyphenols, found in grape skin and various other food products[42], or of quercetin, which is present in apples and red onions[40]. Whatever the ultimate therapeutic approach will be, it is most likely that a combination of drugs, combining classic anti-cancer pharmacological agents with anti-membrane raft compounds, has the best chance for success against the cancer cells' unfortunate ability to change their cell membrane composition to resist anti-cancer drugs. Indeed, it is not inconceivable that the same molecular drift will occur in membrane microdomains of cancer cells after continuous exposure to a certain class of drugs. The quest to identify

stable membrane raft molecular markers and/or target molecules within the different stages of tumor development and progression is the greatest challenge that faces cancer membrane biologists today.

CONCLUSION

Membrane microdomains or lipid rafts in CRC cells appear to be involved in all key cellular regulatory processes that control tumor development, growth, progression and regression. Signaling pathways for lipid metabolism, drug resistance, cell death, cell division and many other processes all seem to diverge from those peculiar lipid islands, thereby orchestrating the cancer cells' state and fate. This demonstrates once more the multifaceted nature of lipid rafts. In the near future, we foresee that manipulating the structural and functional integrity of lipid rafts with anticancer drugs might result in the direct inhibition of CRC cell adhesion, the arrest of cancer cell division, or might even totally eliminate cancer cells. One can only dream.

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