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Review

Role of Caveolae and Lipid Rafts in Cancer: Workshop Summary and Future Needs[1]

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Introduction

The "Caveolae and Lipid Rafts in Cancer" Workshop was held March 13–15, 2002 in Washington, DC to discuss how caveolae and lipid raft research is impacting, and can impact, the field of cancer biology. The goal of this meeting was to present some of the key issues in the field that need to be resolved and what research opportunities exist to better understand the role of caveolae/rafts in cancer progression and therapeutics. The format of the meeting was the presentation of brief reports that focused on concepts rather than specifics, with extensive discussion periods to identify the issues and barriers hindering progress in this area. This report summarizes the findings of this meeting. Highlighted are the recent advances in our understanding of the existence, organization, composition, and function of caveolae and lipid rafts as well as their relationship to each other, possible function in signaling, trafficking, and cancer immunology, and the role of caveolin-1 in tumor growth and progression. Finally, the workshop addressed opportunities for future study to define further how these lipid domains (and their constituents) participate in the pathogenesis of human cancers and what their possible utility may be for the detection and treatment of prostate, lung, and other cancers. Several specific recommendations are made to advance knowledge and progress in this field.

Existence and Structure of Lipid Rafts

The meeting began with a discussion of biophysical studies investigating the structure and physical basis of caveolae and lipid rafts in a session chaired by Gerrit van Meer (Utrecht University, the Netherlands). Ken Jacobson (University of North Carolina, Chapel Hill, NC) began the session by addressing the difficulties associated with defining the size of lipid domains. For flat domains, such as lipid rafts, different methods of microscopic examination give different results, ranging from very small (<1 nm), transient structures using conventional FRET to >700 nm (600,000 molecules) when using single dye tracking. Because of their distinct morphology as invaginations, caveolae are more amenable to ultrastructural and biochemical characterization. In addition, he discussed the shell hypothesis for sorting proteins to specific lipid domains whereby each protein is surrounded by a phospholipid/cholesterol "condensed complex" that confers lipid buoyancy to the protein independently of caveolae/raft association (1). These protein/lipid shells are dynamic within the membrane and are attracted to lipid domains that have a l_o phase in which they are miscible.

Michael Edidin (Johns Hopkins University, Baltimore, MD) made the point that part of the difficulty in defining and studying lipid domains is that their definition is operational (2) and that our understanding of these structures is constrained by the methods (microscopy, detergent solubility), model systems (biological membranes *versus* liposomes), and probes used to visualize and characterize them. Although the biophysical analyses performed on lipid phases in reconstituted liposomes have provided important insights into these structures, there are important differences between these lipid phases (the static, stable structures found in model liposomes) and lipid domains (dynamic, transient structures found in biological membranes), and care must be taken when interpreting the data and applying it to living membranes *in vivo*.

Finally, Gerrit van Meer addressed the mechanisms that generate and maintain differences in lipid composition and organization within organellar and plasma membranes, including asymmetry in transbilayer distribution (3). The formation of lipid domains requires both lipid-driven aggregation as well as protein–lipid interactions. These two processes are interrelated, such that specific lipid aggregates attract specific proteins whereas protein–lipid interactions may direct lipid sorting into microdomains.

Trafficking in Lipid Rafts and Caveolae

The second session, chaired by Jan Schnitzer (Sidney Kimmel Cancer Center, San Diego, CA), explored the role of caveolae in cellular trafficking. Ivan R. Nabi (University of Montreal, Montreal, Quebec) reported that the rate of internalization of AMF,[3] whose receptor is stably expressed in caveolae and is, in part, internalized by caveolae, is increased in ras- and abl-transformed NIH 3T3 cells that have reduced levels of caveolin-1 and fewer flask-shaped caveolae compared with wild-type cells (4). Conversely, increased expression of caveolin-1 increases the number of flask-shaped caveolae but decreases the rate of AMF internalization. Uptake of AMF is also inhibited, and the number of flask-shaped caveolae is also dramatically increased in low caveolin-1-expressing cells that have been transfected with dominant negative dynamin (K44A), consistent with inhibition of budding (5). Dr. Nabi postulates that caveolin-1 stabi-

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[1] This workshop was held from March 13 to 15, 2002 at Holiday Inn–Georgetown in Washington, D.C. It was organized by J. E. S., R. G. W. A., and S. M. and supported by the National Cancer Institute, Division of Cancer Biology, Tumor Biology, and Metastasis Branch. In addition to the organizers, the meeting attendees included Drs. Grace Ault, Lucy A. Carver, Malgorzata Czarny, Harold F. Dvorak, Michael A. Edidin, Colette Freeman, Filippo G. Giancotti, Ari Helenius, Ken Jacobson, Teymuras Kurzchalia, Mordechai Liscovitch, Michael L. Lu, Susan A. McCarthy, M. Carrie Miceli, Barbara Mueller, Dorothy Mundy, Ivan R. Nabi, Diane Palmieri, Richard Pagano, Susan K. Pierce, Linda Pike, Christopher E. Rudd, Alan R. Saltiel, Neeraja Sathyamoorthy, Dinah Singer, John Sogn, Barbara A. Spalholz, Peter Stralfors, Alex Strongin, Betty Tarnowski, Timothy C. Thompson, Gerrit van Meer, and Christine Siemon.

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[3] The abbreviations used are: AMF, autocrine motility factor; LacCer, lactosylceramide; DFCR, detergent-free caveolae/raft; ER, estrogen receptor; GPI, glycosylphosphatidyl inositol; TCR, T-cell receptor; MDR, multidrug resistance; MAP, mitogen-activated protein; BCR, B-cell receptor; APC, antigen-presenting cell; ERK, extracellular signal-regulated kinase; N-SMase, neutral sphingomyelinase.

lizes caveolae at the plasma membrane and functions as a negative regulator of caveolae-mediated AMF endocytosis. He also hypothesizes that endocytosis competent caveolae may still form in the absence of caveolin-1 in part because the formation of the invagination may be a lipid-, not protein (caveolin-1)-, driven event.

Richard Pagano (Mayo Clinic and Foundation, Rochester, MN) discussed caveolae-mediated trafficking of glycosphingolipid, LacCer, from the plasma membrane to Golgi. Using Bodipy-labeled LacCer, he showed that increasing caveolin-1 expression causes an increase in LacCer uptake and that internalized LacCer transiently colocalizes with transferrin in EEA1-containing early endosomes. Later, it separates from these endosomes as transferrin recycles back to the cell surface. Increasing cell cholesterol levels inhibited Golgi targeting of LacCer. Finally, he showed that Rab7 and Rab9, but not Rab 11, are involved in cholesterol-dependent Golgi targeting of LacCer (6).

Jan Schnitzer presented new antibodies targeting caveolae directly as a means to dissect trafficking in caveolae *versus* lipid rafts. Antibodies targeting rafts (GPI-anchored proteins) distributed in very small punctae that were dynamic but stable at the cell surface over 2 h with little internalization. Only with cross-linking secondary antibodies did the GPI-anchored proteins begin to sequester into larger, yet still caveolin-1 free, rafts and then after 30–40 min show caveolin-1 colocalization followed within minutes by internalization to perinuclear intracellular compartments. In contrast, antibodies targeting caveolae directly were rapidly internalized within 5–10 min to similar intracellular compartments even without cross-linking. Novel strategies for exploiting the trafficking function of caveolae clinically were also discussed. A new monoclonal antibody recognizing a caveolar antigen expressed selectively in lung microvascular endothelium not only targets lung tissue after i.v. injection (75% of the injected dose within 30 min) but, perhaps more importantly, is rapidly and selectively transcytosed through endothelial cells to reach the underlying tissue cells *in vivo* (7). A comparative proteomic analysis of silica-isolated caveolar proteins (see comments in Ref. 8) from normal and tumor tissues shows distinct molecular fingerprints with several apparently tumor- and caveolae-specific molecules. Antibodies to new target proteins showing restricted tumor-induced expression improved tumor-specific delivery after i.v. injection. These discoveries uncover the possibility of imaging primary and metastatic solid tumors *in vivo* and facilitating tumor-directed pharmacodelivery that overcomes *in vivo* barriers ultimately to improve localized cancer therapy that limits damage to bystander tissues (9).

Finally, Ari Helenius (Swiss Institute of Technology, Zurich, Switzerland) described how SV40 virus enters cells via caveolae. Viruses are internalized after binding MHC1 and travel to a unique endosomal structure called the caveosome that differs from classic endosomes of the clathrin-dependent pathway. They are caveolin-positive compartments with a neutral pH and lack standard Golgi, lysosome, ER, or endosomal markers. In addition to accumulating SV40, the caveosomes accumulate cholera toxin, LacCer, and GPI-anchored green fluorescent protein but not transferrin or fluid phase markers. The viruses in the caveosomes then migrate through tubular extensions to the ER and onto the nucleus (10). In addition, he showed that on SV40 entry into caveolae of CV-1 cells, actin stress fibers were transiently disassembled followed by recruitment of dynamin to caveolae before internalization. Dynamin has been shown to mediate fission of caveolae through GTP hydrolysis (5, 11, 12). SV40 internalization in cultured cells appears to occur in a cholesterol- and actin-dependent manner.

Caveolae in Signaling

The third session, chaired by Richard Anderson (University of Texas Southwestern Medical Center, Dallas, TX), focused on the role of caveolae in cell signaling. Caveolae/rafts are generally considered to be cholesterol-sphingolipid rich membranes (13). Linda Pike (Washington University School of Medicine, St. Louis, MO) compared the lipid composition of a light buoyant density, caveolae/rafts fraction prepared with (DRM) or without (DFCR) Triton X-100. Presumably, the two methods are enriching for the same piece of membrane, yet the lipid compositions were dramatically different. DRMs had a markedly higher cholesterol and phospholipid content compared with DFCRs yet lower sphingolipid content. Membranes prepared by both methods were high in phosphatidylserine and ethanolamine plasmalogens. DRMs were found to have few EGF receptors whereas DCFRs were substantially enriched in this receptor tyrosine kinase. Therefore, the physical and functional properties of light buoyant density membranes depend on the method of isolation.

Malgorzata Czarny (Sidney Kimmel Cancer Center, San Diego, CA) talked about mechano-signaling from caveolae. Caveolae in lung endothelial cells contain multiple signaling molecules, such as eNOS, src-like tyrosine kinases, and N-SMase, that are involved in mediating acute responses to changes in blood flow and shear stress (14–16). Mechanical stimulation causes tyrosine phosphorylation of caveolar proteins and the activation and dissociation of eNOS from caveolin-1 (14, 15). Hemodynamic stress rapidly and transiently stimulates the activity of neutral- but not acidic-sphingomyelinase in a time- and flow rate-dependent manner to produce the lipid second messenger, ceramide. This activity can be inhibited by scyphostatin, a specific inhibitor of N-SMase. The ceramide produced, in turn, stimulates phosphorylation of a number of proteins, including ERK1/2 (16). Thus, N-SMase was proposed to be a newly discovered mechanosensor at the endothelial cell surface in caveolae that functions to mediate acute responses to mechanical stimulation by initiating downstream signaling pathways. Dr. Czarny also described how mechanical forces are different in tumors (low fluid shear, high interstitial pressures), which may contribute toward altered tissue phenotype and even metastasis.

Richard Anderson also discussed the effects of shear-stress on caveolae function. Fluid flowing over cultured endothelial cells causes caveolae to redistribute at the cell surface and move in the direction of fluid flow. As the cells migrate, the caveolae accumulate at the trailing edge. The heterotrimeric G protein, $G_{\alpha q}$, is carried with these caveolae, and ATP-stimulated calcium release from the ER is now initiated exclusively at these sites instead of randomly at the cell surface. He concluded that caveolae appear to function as containers that carry signaling modules to different cellular locations.

Cell migration depends on integrins that attach cells to their substrata and regulate the organization of the cytoskeleton. Filippo Giancotti (Sloan-Kettering Institute, New York, NY) discussed his work on the role of caveolin-1 in integrin signaling. His laboratory has found that after ligands bind to β1 integrin, a complex forms that contains caveolin-1, Shc, and fyn. This complex is linked to the mitogen-activated protein kinase pathway, which controls progression through the cell cycle. Caveolin-1 is difficult to see at sites of cell adhesion, but an antibody that recognizes tyrosine phosphorylated caveolin-1 stains focal adhesions, thereby implicating a new regulatory function for caveolin.

An important receptor tyrosine kinase is the insulin receptor. Peter Stralfors (Linkoping University, Linkoping, Sweden) presented his studies on insulin signal transduction and caveolae using 3T3-L1 adipocytes grown in culture. Cholesterol binding agents that disrupt the structure of caveolae inhibit insulin-dependent glucose transport without altering insulin-stimulated receptor autophosphorylation. Caveolae disruption seems to affect the ability of insulin to stimulate the downstream IRS-1 signaling pathway but does not prevent signaling through the ERK1/2 pathway. Caveolae disassembly can also prevent downstream tyrosine phosphorylation in other cells via plate-

let-derived growth factor and mechanical stimulation. But in these cases, ERK pathway is also inhibited (17). Although it is clear that more investigation is needed to understand the molecular basis of these differences, a role for caveolae in organizing signaling molecules into functional units initiating selective downstream signaling appears likely.

Alan Saltiel (University of Michigan School of Medicine, Ann Arbor, MI) has also mapped insulin receptor signal transduction to adipocyte caveolae. He described a unique rosette-like structure on the inside membrane surface of 3T3 L1 cells that is rich in both caveolin-1 and insulin receptors and probably represents sites of caveolae accumulation. These structures are reduced or absent in cells expressing a dominant-negative caveolin-3 (Cav3/dgv). Expression of cav3/dgv also inhibits insulin-stimulated glucose uptake but not signaling through the phosphatidylinositol 3′-kinase kinase pathway. He also presented evidence that caveolae contain a functional signaling complex composed of caveolin-1, insulin receptor, and adaptor proteins, such as flotillin, APS, CAP, CrkII, and Cbl.

Function of Caveolin, a Major Protein in Caveolae

The fourth session was chaired by Timothy Thompson (Baylor College of Medicine, Houston, TX) and focused on the role of caveolin-1 in tumor growth and progression. Teymuras Kurzchalia (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany) reported on the phenotype of his caveolin-1 knockout mice (18). These mice lacked caveolin-1 and caveolin-2 expression and all caveolae (quite readily apparent in microvascular endothelium, which usually are particularly abundant in caveolae). In rare cases, a small number of noncoated plasmalemmal vesicles similar in size to caveolae were observed in larger blood vessels. Moreover, these mice showed defects in NO and Ca^{2+}-dependent regulation of vascular tone as well as hyperproliferation of endothelial cell progenitors and alveolar epithelial cells in the lung. Mordechai Liscovitch (Weizmann Institute of Science, Rehovot, Israel) discussed the role of caveolin in cancer MDR. He described the changes in caveolin-1 expression in MDR cells and the role of peroxisome proliferator-activated receptorγ in regulating caveolin-1 expression during the acquisition of the MDR phenotype. He also presented data showing that in MCF-1 cells, caveolin-1 expression negatively affects cell growth and transformation possibly through inhibition of apoptosis (19). Harold Dvorak (Beth Israel Deaconess Medical Center, Boston, MA) described the development of a model for tumor vessel formation by ectopically expressing vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) *in vivo* in the mouse ear. Induction of tumor vessel development gives rise to mother vessels, abnormal vessels arising from small venules in induced tissues. These vessels differ from normal vessels in that they exhibit a disappearance of caveolin-1-positive vesiculo-vacuolar organelles, pericyte detachment, and partial digestion of the interstitium (20). Timothy Thompson outlined the development of a mouse model of transgenic metastatic prostate cancer. He presented data showing that metastatic cell lines generated from these mice exhibit increased caveolin-1 expression. The increased expression was associated with increased metastatic activity and promotion of cell survival possibly by protecting cell from apoptosis. This is consistent with the observation that human prostate cancers that are caveolin positive are associated with a poor prognosis and increased recurrence after surgery (21, 22). Moreover, caveolin-1 is found in the serum of prostate cancer patients and may be a good serum marker for potential metastatic prostate cancer in patients (23).

Signaling in Lipid Rafts

The fifth session chaired by Christopher Rudd (Dana-Farber Cancer Institute, Boston, MA) focused on signal transduction from lipid rafts in lymphocytes. The session began with Carrie Miceli (UCLA, Los Angles, CA) discussing her recent work on T-cell activation and lipid rafts. T-cell activation requires both a TCR and a costimulator. Dr. Miceli suggested that partitioning into lipid rafts is the molecular mechanism of a protein sorting process necessary to organize TCRs, costimulators, signal transducers, and the actin cytoskeleton at the interface (immunological synapse) between the T cell and the APC. She showed that rafts are recruited to the interface between T cells and APCs expressing the CD48 ligand for the costimulator CD2 and that disrupting the cytoskeleton blocks recruitment. Cells expressing a mutant Lck in which the SH3 domain was defective were impaired in sustained TCR tyrosine phosphorylation, raft recruitment, and the establishment of normal T-cell/APC junctions. In addition, recent results suggest that natural lectins like galectin-1 secreted by cells can modulate lipid raft organization, block costimulatory-dependant lipid raft migration to T-cell/APC contact sites, and alter T-cell activation.

Susan Pierce (National Institute of Allergy and Infectious Diseases, Bethesda, MD) presented work demonstrating that BCR signaling also involves sorting by lipid rafts. Cross-linking of BCR causes the complex to become detergent insoluble, suggesting operationally that it becomes associated with lipid rafts. Acquiring detergent insolubility did not depend on actin, although receptor polarization did. In contrast to TCR signaling, polarization of rafts was not required for signal transduction through the tyrosine kinase pathway. Coligation of coreceptors (CD19 and CD21) enhanced BCR signaling, which occurred coordinately with the appearance of the coreceptors in the detergent-resistant raft fraction. Stabilization of coreceptors in rafts was dependent on the tetraspanin membrane protein CD81. Interestingly, BCR signaling is blocked in cells infected with EBV, apparently because the viral protein LMP2A is targeted to lipid rafts (24).

Christopher Rudd raised the issue that lipid rafts may be able to explain how a costimulator can have both positive or negative effects. He presented evidence that expression of the positive costimulator CD28 correlates with an increase in the number of detectable GM1 ganglioside-positive lipid rafts. By contrast, increased expression of the negative costimulator CTLA-4 correlates with a reduction in the number of lipid rafts. The possibility was raised that there might be a reservoir of intracellular lipid rafts that can be mobilized at the cell surface by exocytosis and that mobilization is negatively and positively regulated by CTLA-4 and CD28, respectively.

Discussion and Conclusions

The workshop highlighted the numerous scientific challenges that are presently holding back progress in the field. Most of these stem from limitations in the technology available to study lipid domain structure. Simply defining what caveolae and lipid rafts are or even determining what distinguishes caveolae from lipid rafts remains difficult because of limitations of existing methodologies, model systems, and probes to visualize these microdomains in biological membranes. It is clear that new tools are needed to answer questions about the existence and characteristics of lipid domains that go beyond operational definitions, such as detergent solubility. New probes and biophysical and molecular methodologies to study sorting and maintenance of the biological lipid equilibrium (intramembrane and between subcellular structures) must be developed for the field to move forward.

This workshop highlighted the increasing appreciation in the field of the complex role played by caveolin-1 in cell growth and survival. It is clear from the emerging data that caveolin-1 has functions beyond its structural role in the formation of invaginated caveolae at the cell surface. Caveolin-1 has been detected not only in caveolae but also in other compartments, such as in vesiculo–vacuolar organelles (25), focal adhesions (26), and in lipoproteins secreted into exocrine and endocrine spaces (23, 27). Caveolin-1 may have an inhibitory role in

various cell signaling events, *e.g.*, regulation of vascular tone, integrin-mediated signaling, and apoptosis. Yet, because the knockout mice are clearly viable and function, in an overall sense, so normally, it is unclear how essential caveolin is in regulating the wide variety of signaling ascribed to it and caveolae of the last decade.

To complicate matters even further, important differences appear to exist for caveolin-1 function *in vivo versus* in cell culture. Oncogenic transfection of cultured cells causes a loss of caveolin-1 expression, and caveolin-1 overexpression in tumor cells suppresses the transformed phenotype and drastically slows cell growth (19, 28–31). Caveolin has been suggested by Lisanti *et al.* (29, 30, 32, 33) to be a tumor suppressor, and at least in cell culture, there is much supporting evidence. Yet, *in vivo* in tumor tissue, caveolin-1 expression is associated with increased tumor cell survival, aggression, and metastatic potential (23, 32, 34–39). Caveolin expression may be induced or increased at later stages of tumor progression leading to metastasis. However, caveolin-1-knockout mice, at least up to the time of this conference, do not develop any obvious tumors more frequently than wild-type animals (18). In particular, caveolin-1's protective function in apoptosis (19, 36) may help explain why caveolin-1 expression in tumor cells is associated with a higher virulence and metastatic activity *in vivo* despite the association *in vitro* between caveolin-1 expression and slowed cell proliferation. Finally, caveolin-1 knockout mice may provide a model system useful for investigating the various hypothesized functions of caveolin-1/caveolae, including signal transduction, mechanotransduction, membrane trafficking, and growth regulation. For cancer research, it should be interesting to induce spontaneous tumors in the caveolin-1 knockout mice and examine tumor development and progression. In this regard, it may be informative to cross caveolin-1-knockout mice with TRAMP mice that spontaneously develop primary tumors in the prostate with subsequent metastatic progression mimicking the human disease. Targeting caveolae and even caveolin-1 may prove to be very useful in treating cancer through vascular ablation or functional disruption of metastasis, tumorigenesis, angiogenesis, and tumor progression.

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