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Cholesterol and Prostate Cancer

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Abstract Cholesterol is a neutral lipid that accumulates in liquid-ordered, detergent-resistant membrane domains called lipid rafts. Lipid rafts serve as membrane platforms for signal transduction mechanisms that mediate cell growth, survival, and a variety of other processes relevant to cancer. A number of studies, going back many years, demonstrate that cholesterol accumulates in solid tumors and that cholesterol homeostasis breaks down in the prostate with aging and with the transition to the malignant state. This review summarizes the established links between cholesterol and prostate cancer (PCa), with a focus on how accumulation of cholesterol within the lipid raft component of the plasma membrane may stimulate signaling pathways that promote progression to hormone refractory disease. We propose that increases in cholesterol in prostate tumor cell membranes, resulting from increases in circulating levels or from dysregulation of endogenous synthesis, results in the coalescence of raft domains. This would have the effect of sequestering positive regulators of oncogenic signaling within rafts, while maintaining negative regulators in the liquid-disordered membrane fraction. This approach toward examining the function of lipid rafts in prostate cancer cells may provide insight into the role of circulating cholesterol in malignant growth and on the potential relationship between diet and aggressive disease. Large-scale characterization of proteins that localize to cholesterol-rich domains may help unveil signaling networks and pathways that will lead to identification of new biomarkers for disease progression and potentially to novel targets for therapeutic intervention. J. Cell. Biochem. 91: 54–69, 2004.

Key words: caveolae; lipid raft; HMG CoA-reductase inhibitor; chemoprevention; signal transduction

It has been known for about a century that cholesterol and other fatty deposits accumulate in solid tumors [White, 1909]. Increases in cholesterol content of prostatic adenomas relative to normal tissue was reported by Swyer 60 years ago [Swyer, 1942]. Since then, many studies of human subjects and animal models have supported the existence of a relationship between cholesterol in prostate tissues or secretions and benign and malignant prostate growth. Despite this long history, there have been few recent studies on the role of cholesterol in prostate cancer (PCa). Consequently, the physiological consequences, if any, of the accumulation of fat, and specifically of cholesterol, in relation to prostate carcinogenesis or progression are still poorly understood. This review will summarize the basic research and clinical observations that may relate to a functional role for cholesterol in PCa. We also present a testable model that attempts to unify many of the published observations pertaining to cholesterol and PCa progression. We propose that

Abbreviations used: BPH, benign prostatic hyperplasia; cdk2, cyclin dependent kinase 2; CI, confidence interval; DIGs, detergent-insoluble, glycolipid enriched complexes; DRMs, detergent resistant membranes; FDA, US Government Food and Drug Administration; GPI, glycophosphatidylinositol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IL-6, interleukin-6; LDL, low density lipoprotein; NSE, neuron specific enolase; PCa, prostate cancer; PI3K, phosphoinositide-3-kinase; PrEC, normal, primary culture prostate epithelial cells; PTEN, phosphatase and tensin homolog on chromosome 10; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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this model provides a new approach toward the identification of novel molecular targets for PCa therapy.

PROSTATE CANCER INCIDENCE AND CHOLESTEROL

A variety of studies, beginning in the early 1980s, have linked increased risk of aggressive PCa to the consumption of animal products and/or fatty food. This association is still tentative, however, and the specific dietary components that may underlie such risks remain unknown. Nevertheless, it has been suspected for many years that life-style factors play a significant role in the rates of appearance and in the aggressiveness of clinically relevant PCa. Immigrants to the US and other Western nations from Asian countries, where the incidence of clinical PCa is typically low, show a dramatic increase in PCa detected clinically [Cook et al., 1999]. This increase in cancer incidence has been related to time of arrival, with increased cancer risk associated with early arrival in comparison to individuals who migrated later in life [Shimizu et al., 1991]. Because autopsy studies have shown that the incidence of occult PCa is similar in Asian and Western societies [Yatani et al., 1988; Pienta, 1994], the studies on immigrants point to an important role for exogenous factors, most probably diet, in PCa progression. Consistent with this, PCa incidence rates have recently risen in Asian countries that have been undergoing Westernization [Weisburger, 1997; Yang et al., 1999a]. Michaud et al. [2001] have reviewed the literature on diet and PCa incidence and have reported that, while association of PCa risk with the consumption of meat products has been relatively consistent (15 out of 19 studies reporting an association), studies examining fat intake have been less consistent. In a recent review [Kolonel et al., 1999], it was concluded that, while dietary fat may be related to PCa risk, "the specific fat components that are responsible are not yet clear."

As of this writing, the Michaud et al. prospective study is the most comprehensive analysis of the relationship between consumption of animal products and PCa risk. It involved 47,780 subjects in the Health Professionals Follow-up Study and demonstrated an elevated risk specifically of metastatic PCa and consumption of red meat and dairy products. In that analysis, there was no demonstrable association between animal products in the diet and total PCa, suggesting that the association is specifically with progression to metastatic disease. These investigators concluded that nutrients such as calcium and fatty acids explain much of the association between dairy products and metastatic PCa risk, but that the association with meat products cannot be explained by intakes of calcium, saturated fat, or α-linoleic fatty acids.

Cholesterol, a neutral lipid that plays an essential role in the maintenance of the integrity of biological membranes, is a prominent component of a diet containing animal products. In addition to its role in membrane structure, cholesterol also serves as a precursor in the synthesis of bile acids and many endocrine signaling mediators, such as the steroid hormones. Cholesterol is synthesized in mammalian cells via the mevalonate pathway (Fig. 1), which also produces a number of other important biochemical end-products. Isoprene units, produced by the mevalonate pathway, are precursors in the synthesis of a variety of molecules, including proteins, which are modified post-translationally. Isoprenoid modification of signaling proteins, such as Ras and Rho family members, are essential for proper membrane targeting of these molecules. Isoprenylated proteins participate in signal transduction pathways that regulate diverse processes such

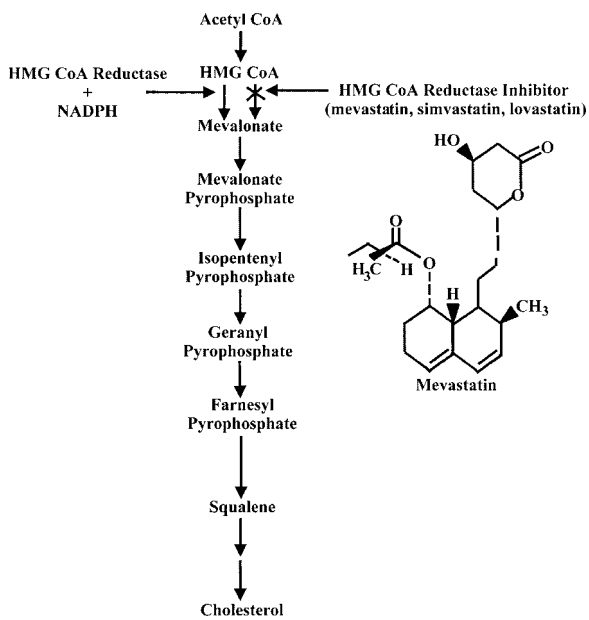

Fig. 1. The mevalonate pathway.

as the cell cycle, cell survival mechanisms and cell motility. Mevalonate products are thus essential for a wide-range of biological activities, from hormonal regulation of endocrine target organs to electron transport. The complexity and diversity of products originating from the mevalonate pathway have confounded studies focused on potential relationships between circulating cholesterol levels, cholesterol intake by diet or pharmacologic management of circulating cholesterol in cancer incidence or progression.

Most epidemiological studies have not found an association between circulating cholesterol levels, whether or not linked to diet, and cancer risk [Wu et al., 1994; Veierod et al., 1997; Chen et al., 2002; Smith-Warner et al., 2002]. This is consistent with the current state of the literature in which links to intake of fat and cancer incidence at most organ sites are modest. However, there are exceptions to this general rule. Several studies have reported statistically significant correlations between cholesterol intake and cancer risk [De Stefani et al., 1997; Horn-Ross et al., 1997; Jarvinen et al., 2001]. These findings are consistent with the possibility that prolonged consumption of cholesterol-rich foods might promote progression of certain cancer types or cancer growth in select tissues.

Some studies have reported an inverse association between cancer incidence and cholesterol levels for certain neoplasms [Kaplan et al., 1997]. Evidence suggests that this negative relationship is likely attributable in many cases to hypocholesteremic effects of pre-existing cancer [Knekt et al., 1988; Wald et al., 1989]. Although the question of the effect of undetected, pre-existing cancer on circulating cholesterol can be debated [Vatten and Foss, 1990], it is clear that frank cancer is indeed associated with lower circulating cholesterol levels in human patients [Umeki, 1993; Eichholzer et al., 2000; Fiorenza et al., 2000]. This negative association provoked long-term studies designed to identify potential health risks to patients on cholesterol-lowering therapy for cardiovascular disease. The results of several such studies indicate that chronically lowered cholesterol does not increase cancer risk [Waters, 2001; Heart protection study collaborative group, 2002] and may, in fact, lower cancer incidence at many organ sites [Blais et al., 2000; Pedersen et al., 2000].

HMG-CoA REDUCTASE INHIBITORS AND CANCER

The above discussion makes it clear that attempts to use epidemiological tools to assess any potential association between dietary or circulating cholesterol and risk of clinical PCa are confronted with significant challenges. Several older studies that attempted to establish a link between serum cholesterol levels and PCa risk did not report an association [Hiatt and Fireman, 1986; Knekt et al., 1988; Smith et al., 1992]. Another approach is to ask whether long-term treatment with cholesterol-lowering drugs affects PCa detection rates, incidence of aggressive disease, or disease-specific survival. These questions are only now beginning to be addressed.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly known as "statins," are cholesterol-lowering drugs that have been widely used for many years to reduce the incidence of adverse cardiovascular events. HMG-CoA reductase catalyzes the rate-limiting step in the mevalonate pathway (Fig. 1) and these agents lower cholesterol by inhibiting its synthesis in the liver and in peripheral tissues [Koga et al., 1990]. HMG-CoA reductase inhibitors function at an early step in the synthesis of cholesterol; as a consequence, the levels of cholesterol, and its upstream precursor isoprenoids, geranylgeranylpyrophosphate and farnesylpyrophosphate, are reduced. Thus, essential cell components that require isoprenoids, e.g., dolichols and ubiquinone (a polyisoprenylated quinoid cofactor of the electron transport chain), are affected by HMG-CoA reductase inhibitors. Statin drugs (e.g., pravastatin, lovastatin, simvastatin) now have a sufficiently long clinical history so that safety concerns for many of them can be definitively evaluated. Toxicity during long-term therapy with most statins is minor and recent studies have reported significant general health benefits with prolonged statin therapy [Pedersen et al., 2000; Waters, 2001].

A prospective analysis of the effect of long-term HMG-CoA-reductase inhibitor therapy on PCa incidence or progression rates using modern methods of study design has not yet appeared in the peer-reviewed literature. However, the results of a large-scale study evaluating the effect of long-term statin therapy specifically on cancer incidence rates was reported at the

annual meeting of the American Society for Clinical Oncology in 2003 [Graaf et al., 2003]. That study, the report of which has to date only appeared in abstract form, was conducted by researchers from the Academic Medical Center at the University of Amsterdam. The study examined 20,000 patients, comparing those taking statins with those taking other cardiovascular protective drugs within the period 1983–1998. These investigators found a 20% reduction in total cancer incidence (adjusted odds ratio = 0.80; 95% CI = 0.66–0.96) in the statin cohort with the largest reductions in the incidence of prostate and kidney cancer. Graaf et al. found statins to be protective when used longer than 4 years (adjusted OR 0.64; 95% CI: 0.44–0.93) or when more than 1350 Defined Daily Doses were taken (adjusted OR 0.60; 95% CI: 0.40–0.91). Interestingly, patients that terminated statin therapy returned to a baseline level of risk within 6 months. At this writing, the evaluation of these data awaits peer review; however, if verified, this finding suggests the possibility that inhibiting HMG-CoA-reductase may have clinical benefit in the chemoprevention of PCa.

Pedersen et al. [2000] conducted a randomized, placebo-controlled study of cause-specific mortality rates in patients on long-term (up to 8 years) simvastatin therapy. These investigators reported fewer deaths from cancer in the simvastatin group in comparison to the placebo group, although the difference was not considered significant. In a recent nested case–control study addressing potential risks of HMG-CoA-reductase inhibitor therapy with respect to potential increases in cancer incidence, nearly all cancer sites examined were either not associated, or were inversely associated, with statin therapy [Blais et al., 2000]. Interestingly, in this study PCa incidence declined in the HMG-CoA-reductase inhibitor group (adjusted rate ratio = 0.74; CI = 0.36–1.51) in comparison to the referent group (patients taking bile acid-binding resins). Anti-cancer efficacy of statins in comparison to other methods of cholesterol lowering may arise from the fact that these agents not only lower serum cholesterol but, in addition, reduce cholesterol synthesis in peripheral tissues as well as in the liver. This may be of considerable benefit in the case of prostatic neoplasms because the prostate has been reported to synthesize cholesterol at a rate even higher than the liver [Schaffner, 1981].

HMG-CoA-reductase inhibitors have been demonstrated to exert potent anti-cancer effects in model systems. A recent review has summarized the relevant publications on this topic [Chan et al., 2003]. Studies with cell culture models indicate that statin drugs can inhibit cancer cell growth and motility [Jani et al., 1993; Farina et al., 2002], induce apoptosis [Wong et al., 2001; van de Donk et al., 2002] and inhibit endothelial cell migration and tube formation, properties associated with angiogenesis [Vincent et al., 2001; Park et al., 2002]. Mevastatin, for example, has been shown to inhibit cell cycle progression in PC-3 human PCa cells by inhibiting cyclin dependent kinase (cdk2) phosphorylation [Ukomadu and Dutta, 2003]. Animal studies have verified that this class of agents has a substantial capability to retard tumor growth [Narisawa et al., 1994; Alonso et al., 1998], in vivo angiogenesis [Park et al., 2002] and tumor metastasis [Jani et al., 1993; Alonso et al., 1998; Farina et al., 2002]. In general, the statins also exhibit a robust selectivity for tumor cells over normal cells [Wong et al., 2001], an essential attribute for successful cancer therapy. Their ability to enhance the efficacy of conventional chemotherapeutic agents has also been demonstrated [Lishner et al., 2001; Wachtershauser et al., 2001]. Because most of the statins are now known to be well-tolerated by patients, and because they affect many processes governing the behavior of malignant cells (via multiple downstream effects on the mevalonate pathway), continued evaluation of these compounds in clinical trials as potential chemopreventive agents or as adjuvants to standard therapy is warranted. However, general conclusions about the anti-cancer effectiveness of the statins is not advised because the different compounds can exhibit significantly different activity profiles against tumor cells [Wong et al., 2001]. This difference in potency between various statins may account for reports claiming no effect of statin use on cancer incidence [Coogan et al., 2002].

CHOLESTEROL CONTENT OF PROSTATE CANCER CELLS AND TUMORS

Cells in the prostate, as is the case with other tissues, synthesize cholesterol endogenously via the mevalonate pathway. However, much of the cholesterol residing in cell membranes originates from the uptake of circulating lipoproteins [Simons and Ikonen, 2000]. Consequently,

cellular cholesterol content is a balance between metabolic mechanisms intrinsic to the cell and the regulatory functions of cholesterol distribution in the organism. Cholesterol content of cell membranes is under tight homeostatic regulation and involves synthetic pathways in the endoplasmic reticulum, transfer of cholesterol from lipoproteins to the exoplasmic leaflet, receptor-mediated internalization, several intracellular transport mechanisms, and extensive efflux from the cell via secretion of lipoprotein complexes. Extensive evidence indicates that this complex homeostatic mechanism breaks down in cancer and also in the aging prostate.

Swyer, using a histologic test, was the first to report that the cholesterol content of BPH tissues was higher (approximately double) than that of normal prostatic tissues [Swyer, 1942]. He also noted a spatial relationship between presumptive cholesterol accumulation and cellular hyperplasia, a finding similar to that reported by White in the early 20th century in an analysis of non-prostatic tumors [White, 1909]. Subsequent studies of human and animal prostate tissues also reported increases in cholesterol content in the prostate and in prostatic secretions correlating with disease, age, or the presence of malignancy [Schaffner, 1981]. These older observations are in agreement with recent studies of the relative cholesterol content of human breast cancers as evaluated by Raman spectroscopy (M. Feld, A. Haka, personal communication). Cholesterol accumulation may be a more general property of cancer and has been reported in a variety of tumor types [Dessi et al., 1992, 1994; Rudling and Collins, 1996; Yoshioka et al., 2000; Kolanjiappan et al., 2003]. Cancer-associated increases in tissue cholesterol content have also been reported to affect normal tissues surrounding malignant tumors [Nygren et al., 1997]. Cholesterol increases in tumor tissues likely occur by multiple mechanisms, including increased absorption from the circulation [Graziani et al., 2002; Tatidis et al., 2002], loss of feedback regulation through down-regulation of low density lipoprotein (LDL) receptors [Caruso et al., 1999] and up-regulation of components of the mevalonate pathway, particularly HMG-CoA reductase [Caruso et al., 1999; Caruso et al., 2002]. Androgen also stimulates lipogenesis in human PCa cells directly by increasing transcription of the fatty acid synthase and HMG-CoA-reductase genes [Heemers et al., 2001]. Other components of the mevalonate pathway, such as farnesyl diphosphate synthase, are also regulated by androgen and may play a role in accumulation of cholesterol and other lipid products in the prostate [Jiang et al., 2001]. Because cholesterol uptake and synthesis are coupled to the cell cycle [Wadsack et al., 2003], the link between cholesterol, other lipogenic mechanisms and androgen action suggests the possibility that lipid products of these pathways are involved in androgenic stimulation of PCa cell growth.

The first evidence that lowering cholesterol levels systemically might have the capability to alter prostate cell growth and/or survival was first presented by Schaffner and colleagues in a series of innovative studies. These investigators demonstrated that prostate regression could be selectively induced in dogs and rodents by oral application of hypocholesteremic agents, such as the polyene macrolide candicidin [Gordon and Schaffner, 1968; Schaffner and Gordon, 1968; Fisher et al., 1975; Schaffner, 1981]. Candicidin and structurally similar agents, such as amphoteracin B, exert biological effects by binding to cholesterol and closely related sterols [Charbonneau et al., 2001]. Oral administration of candicidin and similar agents likely lowers circulating cholesterol by inhibiting its absorption from the gut [Schaffner and Gordon, 1968]. Several human trials of oral candicidin for BPH in the 1970s reported symptomatic improvement [Keshin, 1973; Orkin, 1974; Sporer et al., 1975], with no alteration in hormonal status [Orkin, 1974], indicating that changes in the prostate, which included regressive histomorphologic changes within the gland [Keshin, 1973], were likely not the result of suppression of androgen production or utilization. Collectively, these studies suggest the intriguing possibility of manipulating prostate cell growth or homeostasis in situ by lowering circulating cholesterol levels pharmacologically.

CHOLESTEROL AND LIPID RAFTS

In the plasma membrane and other intracellular membranes, cholesterol accumulates in specialized structures known by various names, such as lipid rafts, detergent-resistant membrane domains (DRMs), and detergent-insoluble, glycolipid-enriched complexes (DIGs). Evidence for the existence of such cholesterol-rich membrane domains was first developed

from studies of glycosylphosphatidylinositol (GPI)-anchored proteins on lymphocytes and brush border membranes of the kidney and gut [Gunter et al., 1984; Hooper and Turner, 1987, 1988a,b]. These early studies described GPI-anchored proteins as being lipid-anchored and 'detergent-insoluble', yet capable of delivering cell signals when cross-linked. These findings were, however, paradoxical: how could proteins incapable of spanning the lipid bilayer transduce signals? This dilemma began to clarify when it was subsequently demonstrated that GPI-anchored proteins co-immunoprecipitated with both Src family tyrosine kinases [Stefanova et al., 1991; Shenoy-Scaria et al., 1992; Thomas and Samelson, 1992] and heterotrimeric G proteins [Solomon et al., 1996]. However, these observations led to a second paradox: how do proteins that do not span the bilayer interact with inner-leaflet signaling molecules? In the last decade this latter paradox has been largely resolved with the recognition that detergent-insoluble membrane domains serve as important nodes for signal transduction and other essential processes, such as cholesterol transport.

Studies into the composition of biological membranes that are resistant to solubilization in cold non-ionic detergents, such as Triton X-100 and Nonidet P-40, but which are not associated with the insoluble cytoskeleton, resulted in the discovery that discrete membrane subfractions contain high concentrations of cholesterol and fatty acids with long saturated acyl chains. Based on the apical vs. basolateral sorting properties of these domains, Simons and colleagues began to refer to these regions as 'patches,' and later as 'lipid rafts' [Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990]. These and subsequent studies established that biological and artificial membranes that contain high concentrations of cholesterol and saturated fatty acid chains will spontaneously form "liquid ordered" aggregates, a heretofore theoretical condition that had been hypothesized to exist between the common liquid disordered state and the non-biological gel state [Pike, 2003]. In biological membranes, lipid rafts are enriched in sphingolipids (e.g., sphingomyelin and glycosphingolipids) relative to the majority of the membrane. Rafts are formed by self-aggregation of these lipids during their transport from the *trans*-Golgi network to the cell surface. These membrane patches are rich in proteins but likely represent only 10–15% of the plasma membrane area.

At least two morphologically distinguishable varieties of lipid raft exist on cell surfaces. The more familiar type has been named caveolae ("little caves") and are identifiable in electron micrographs as striated 50–100 nm invaginations in the plasma membrane [van Deurs et al., 2003]. Caveolae also exist as intracellular vesicles. Their invaginated and vesicular architecture is conferred by members of the caveolin protein family [Rothberg et al., 1992]. Caveolins, structural proteins that bind cholesterol, are necessary for caveolae formation but, because the biological function(s) of caveolae are still not well understood, their wider function is unclear. All three members of the mammalian caveolin family (caveolin-1, -2, and -3) have been knocked out in the mouse and, surprisingly, the functional deficits in these animals are relatively minor, given that loss of caveolin expression results in the complete ablation of an intracellular organelle [Galbiati et al., 2001a; Razani et al., 2001]. The second variety of raft has been named the flat raft or G domain. Flat rafts do not contain caveolin proteins and thus do not form a recognizable membrane structure identifiable by electron microscopy. Both type of lipid raft are isolated biochemically using similar approaches and have been shown to contain GPI-anchored proteins, Src family kinases, heterotrimeric G protein subunits, and other cell signaling molecules, such as receptor tyrosine kinases (RTKs) [Li et al., 1996; Solomon et al., 1996; Liu et al., 1997b; Rietveld et al., 1999]. Raft composition is likely to be dependent on cell type, although large-scale characterization of raft-resident proteins using proteomics approaches is just beginning [Bini et al., 2003; Foster et al., 2003]. Caveolae, which are present in adipocytes, myocytes, osteoblasts, endothelials as well as other cell types, are the most studied form of lipid raft to date.

Although the mechanisms of protein localization to rafts are still poorly understood, many involve post-translational modifications. Targeting mechanisms to rafts include the presence of a GPI-anchor, dual acylation (Src kinases and heterotrimeric G protein subunits) [Moffett et al., 2000] and linkage to cholesterol (Hedgehog) [Rietveld et al., 1999]. In contrast, prenylated proteins (e.g., Rap1, Rab5) may be excluded from rafts [Rietveld et al., 1999; Zacharias et al., 2002]. Other signaling mole-

cules identified in rafts in certain cell types include the endothelin receptor, thrombin receptors, multiple growth factor receptors, ion channels and pumps, an inositol 1,4,5-trisphosphate receptor, phosphoinositide-3-kinase (PI3K), and protein kinase C (PKC) isoforms [Chun et al., 1994; Schnitzer et al., 1995; Couet et al., 1997; Liu et al., 1997a,b; Bi et al., 2001].

Lipid rafts appear to serve a number of functions, such as intracellular transport and sorting of molecules, receptor down-regulation and recycling, and targeted export of proteins and lipids. In this review, which deals with the potential link between cholesterol and PCa, we will emphasize the role that lipid rafts are likely to play as essential platforms for signal transduction.

In cell signaling, rafts appear to act as a means of assembling components of specific pathways in ways that provide a regulatory architecture for transmission of signal. Rafts are believed to accomplish this by co-localizing cognate proteins so as to facilitate interactions and by excluding proteins capable of degrading signal, such as protein or lipid phosphatases. These functional properties of lipid rafts result in their ability to organize downstream signaling components in close proximity to surface receptors. They also create local environments in which signal propagation, amplification and cross-talk between pathways can occur. Some of the mechanisms by which lipid rafts may conceivably regulate signal transduction events are diagrammed in Figure 2 and include protein sequestration, assembly of pre-formed signaling complexes and intracellular and intramembrane trafficking and sorting. Proteins that have been implicated in signaling through lipid rafts include the T-cell receptor [Horejsi, 2003], the B-cell receptor [Saeki et al., 2003], integrins [Wary et al., 1998], ephrins [Bruckner et al., 1999], and the EGF receptor [Couet et al., 1997; Zhuang et al., 2002].

LIPID RAFTS AND PROSTATE CANCER

The first evidence linking lipid rafts to PCa was published by Thompson and colleagues, who identified caveolin-1 as a marker for aggressive PCa [Yang et al., 1998, 1999b; Tahir et al., 2001]. Subsequent studies from this group indicated that caveolin-1 is a predictor of poor outcome following surgery in lymph node-negative PCa patients [Satoh et al., 2003]. This literature has recently been reviewed [Mouraviev et al., 2002]. The relevance of these observations to our topic lies in the realization that caveolins localize essentially exclusively to lipid raft microdomains and are, in fact, the structural basis for the invaginated appearance of the caveolar form of raft. Consequently, a prominent marker of disease progression in PCa is also a marker for a cholesterol-rich membrane compartment. In addition, because caveolins may be involved in cholesterol transport to the cell membrane [Simons and Ikonen, 2000], higher caveolin levels may coincide with higher membrane cholesterol.

The link to caveolin-1 implicates the lipid raft microdomain as a potential site for signal transduction events relevant to PCa progression. The possibility that this association is functional, as opposed to simply correlative, is supported by the demonstration that anti-caveolin-1 antibodies suppressed PCa metastasis in mice, suggesting that caveolin-1 may play a direct role in metastatic dissemination [Tahir et al., 2001]. Caveolin-1 has also been shown to interact directly with the androgen receptor (AR) and appears to be capable of participating in the mediation of androgen-dependent signals in PCa cells [Lu et al., 2001]. Recent reports have demonstrated that members of the steroid hormone superfamily, including the androgen receptor [Sun et al., 2003], can function by a mechanism that is independent of their traditional role as transcriptional regulators (so-called "non-genomic" functions for these molecules), and that they can localize to rafts. These findings suggest the possibility that lipid rafts may regulate PCa cell growth and survival functions by compartmentalizing signaling proteins involved in hormonally responsive or dependent pathways, e.g., steroid hormone receptors. Although this possibility has only begun to be explored, recently published papers suggest this is going to be an extremely fruitful area of inquiry in studies of signal transduction by steroid hormones [Boonyaratanakornkit et al., 2001; Lu et al., 2001; Chambliss et al., 2002; Sun et al., 2003].

Because raft domains are known to be involved in cell signaling in caveolin-negative cells [Magee et al., 2002; Horejsi, 2003; Saeki et al., 2003], signal transduction through rafts in cancer may be caveolin-independent. Down-regulation of caveolins is a common characteristic of malignant cells [Wiechen et al., 2001];

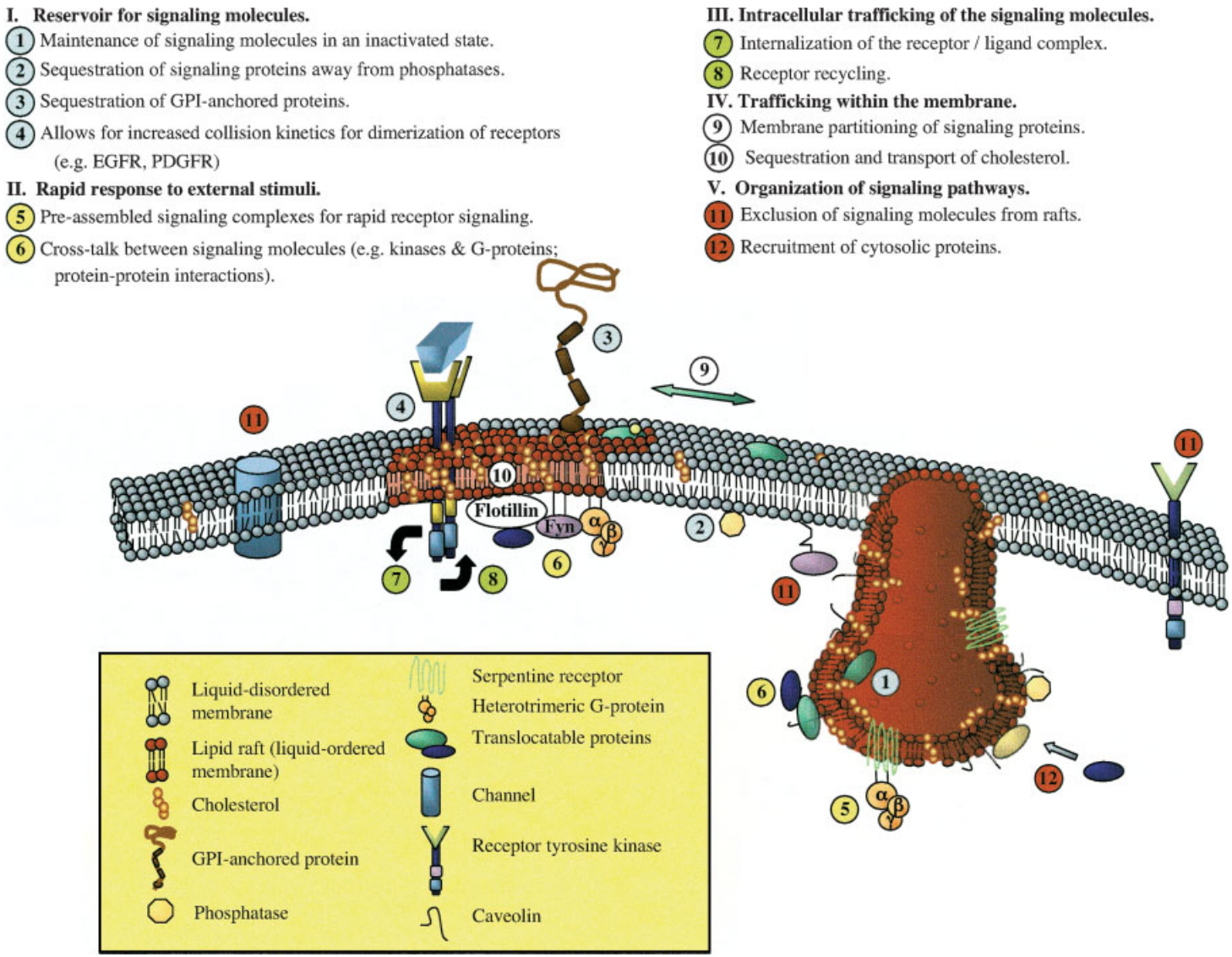

Fig. 2. Caveolae and flat lipid rafts. The diagram illustrates known and hypothetical mechanisms for control of signal transduction by caveolar and non-caveolar rafts. It is not intended that most of the mechanisms attributed to caveolae cannot be mediated by flat rafts, and vice versa.

consequently, despite the apparent connection between caveolin-1 and PCa progression, it is important to realize that, in cancer cells, rafts might not require caveolins for the performance of signaling functions relevant to tumor progression. The reader is directed to several recent reviews for in-depth discussions of raft-dependent signal transduction mechanisms [Simons and Toomre, 2000; Galbiati et al., 2001b; Magee et al., 2002; Zajchowski and Robbins, 2002]. Our own view is that in cancer cells, caveolar and non-caveolar rafts may be equally important in sequestering signaling molecules and/or for signal processing necessary for cancer cell growth and survival in the face of apoptotic triggers present in the tumor environment.

Addition of exogenous LDL to cultures of PC-3 human PCa cells has been reported to stimulate cell growth [Hughes-Fulford et al., 2001]. At least one older study reported that addition of LDL to cultured cells was sufficient to "transform" them, using in vitro criteria [Zwijsen, 1992]. These results are intriguing when one considers the aforementioned literature demonstrating that solid tumors can accumulate cholesterol. How might we interpret these findings in the context of lipid raft-mediated signaling? Cholesterol has been demonstrated in a large number of studies to be a lipid raft component that is essential for the functional integrity of caveolar and non-caveolar rafts. This literature persuasively illustrates that raft-dependent signaling events can be inhibited by dispersing cholesterol or removing it from the membrane with cholesterol-binding compounds [Liu et al., 1997a; Pike and Miller, 1998; Peiro et al., 2000; Parpal et al., 2001]. On the other hand, experiments with artificial membranes have demonstrated that liquid-ordered, sphingomyelin-enriched lipid microdomains can exist in the absence of cholesterol [Milhiet et al., 2002]. These cholesterol-poor rafts can actually be disrupted by cholesterol addition [Milhiet et al., 2002]. Collectively, this information suggests that lipid microdomains in living cells might be heterogeneous in structure and function and might respond in a variety of ways to changes in steady-state cholesterol levels in the membrane. Thus, from first principles one can conclude that the accumulation of cholesterol that can occur in tumors, in concert with other tumor-associated alterations in normal mechanisms of cholesterol homeostasis, is likely to alter raft-dependent signaling in tumor cells. But which signal transduction mechanisms might be altered by changes in cholesterol metabolism in tumors and how might they be affected by these changes?

The possibility that some signaling mechanisms relevant to PCa cancer progression may be dependent on cholesterol present in the plasma membrane is currently under study in our laboratories. Zhuang et al. [2002] recently demonstrated that LNCaP androgen-responsive human PCa cells can be stimulated to undergo apoptosis in response to treatment with filipin, a polyene macrolide that binds cholesterol and disperses it in the plane of the membrane. That study showed that signaling through the Akt serine–threonine kinase is partly dependent on the integrity of plasma membrane rafts and that the effects of filipin on Akt signaling and apoptosis can be attenuated by repletion of the membrane with cholesterol. Akt is an important node for cell survival and growth signals in PCa and in other solid tumors [Paez and Sellers, 2003]. Akt is also believed to be physiologically relevant to clinical PCa because PTEN, a lipid phosphatase that is an important negative regulator of this pathway, is inactivated in a significant fraction of aggressive PCa [McMenamin et al., 1999]. In another study that we have recently submitted for publication, Zhuang, Kim and colleagues go on to show that this dependence on lipid rafts for ligand-activated signaling through Akt extends even to normal prostate epithelial cells (PrEC). Unlike the situation in LNCaP cells, cholesterol-binding agents did not stimulate apoptosis in PrEC, indicating that the cancer cells may have become dependent on a cholesterol-mediated cell survival pathway. Interestingly, the Zhuang, Kim et al. study (unpublished results) also showed that simvastatin, which has been demonstrated previously to stimulate apoptosis in cancer cells, also inhibits Akt signaling in LNCaP cells. Furthermore, the cholesterol content of lipid rafts in these cells was shown to be dramatically decreased with simvastatin treatment. This result indicates that it may be possible to target raft-dependent cell survival mechanisms in PCa cells by pharmacologic intervention using FDA-approved drugs that have been demonstrated in clinical trials to be well tolerated with long-term therapy. The finding that cholesterol-binding polyene macrolides can be potent stimulators of cancer cell death and that, in contrast, normal cells are

relatively resistant to this treatment, was actually demonstrated and reported over 25 years ago [Fisher et al., 1975]. This was before the phenomenon of apoptosis as we know it today was established as a cellular process, although these older data are consistent with our own studies of cholesterol-dependent survival mechanisms in prostatic cells.

Several other groups have recently reported findings in other cell systems consistent with a role for rafts in signal transduction through Akt [Bauer et al., 2003; Podar et al., 2003]. Interestingly, the Hemmings laboratory has identified an enzymatic activity capable of phosphorylating Akt on Ser-473 as a protein that resides in the lipid raft subcellular compartment [Hill et al., 2002]. The findings reported in that study are significant because, although it is known that translocation to the plasma membrane from the cytosol is a feature of Akt phosphorylation by upstream activators, there is still considerable controversy about the mechanism by which Akt becomes phosphorylated on its two principal regulatory sites (Thr-308 and Ser-473) [Scheid and Woodgett, 2003]. The presence of an Akt kinase in the raft compartment suggests that cells may employ raft microdomains as a means to rapidly mobilize or, alternatively, repress the enzymatic or binding functions of the molecule.

In a third study from our laboratories, Kim and colleagues demonstrated that signaling to the transcription factor, STAT3, by IL-6 also involves lipid rafts in LNCaP cells [Kim et al., 2003]. Increases in circulating IL-6 are associated with PCa progression [Nakashima et al., 2000], and IL-6 has been shown in cell culture models to be an inducer of neuroendocrine characteristics in PCa cells [Deeble et al., 2001]. Neuroendocrine properties in prostate and other solid tumors, and in animal models of PCa, have been associated with more aggressive disease [Abrahamsson, 1999]. Steady-state increases in STAT3 activation are also associated with advanced PCa [Mora et al., 2002]. In our study by Kim et al., IL-6 induced phosphorylation of STAT3, its translocation from the cytoplasm to the nucleus, as well as promoter activity of the neuroendocrine marker, neuron specific enolase (NSE) and accumulation of NSE protein, were partly dependent on intact plasma membrane rafts. Phosphorylated STAT3 also predominantly localized to the raft compartment after stimulation of the cells with IL-6. Consequently, these findings represent another demonstration of a cholesterol-dependent signal transduction mechanism underlying a process that is potentially relevant to disease progression in humans. Interestingly, neuroendocrine differentiation in PCa cells most likely occurs independently of androgenic signaling [Adam et al., 2002], suggesting the possibility that raft-dependent signals may operate promiscuously (i.e., without the influence of androgen) in hormone-refractory disease and in the androgen-depleted state. This hypothesis is potentially all the more relevant because of the demonstration in animals [Cinci et al., 1993] and humans [Moorjani et al., 1988] that androgen suppression can induce hypercholesterolemia.

A MODEL INTEGRATING THE PHENOMENON OF INCREASED MEMBRANE CHOLESTEROL IN TUMOR CELLS WITH LIPID RAFT SIGNALING

Inspection of Figure 2 makes it abundantly clear that both caveolar and non-caveolar rafts might alter signal transduction processes in cancer cells in a multitude of ways. Is there a simpler model that would allow testing of the hypothesis that elevation of cholesterol content in tumor cell membranes promotes disease progression? If so, how might this model be applied toward the identification of new targets for disease therapy? In imagining such a model, it is useful to understand how the concept of the lipid microdomain explains the phenomenon of signal transmission by GPI-anchored proteins. It is now well established that GPI-anchored proteins reside in lipid rafts along with an array of other signal transducing molecules. Although in isolation individual GPI-anchored proteins do not appear capable of generating signals, when cross-linked by antibodies they are able to generate many different types of signals, including Ca^{2+} mobilization, inositol phosphate production, as well as a range of cellular responses such as proliferation, growth factor production and apoptosis. It is reasonable to speculate that what is occurring when the GPI-anchored proteins are cross-linked is that as the proteins are being pulled together, their associated raft domains are also being brought together as well. Thus, the small, isolated rafts coalesce to form substantially larger rafts (think: island). This concept is illustrated in Figure 3. Isolated rafts are likely to be relatively small, with limited compositional complexity.

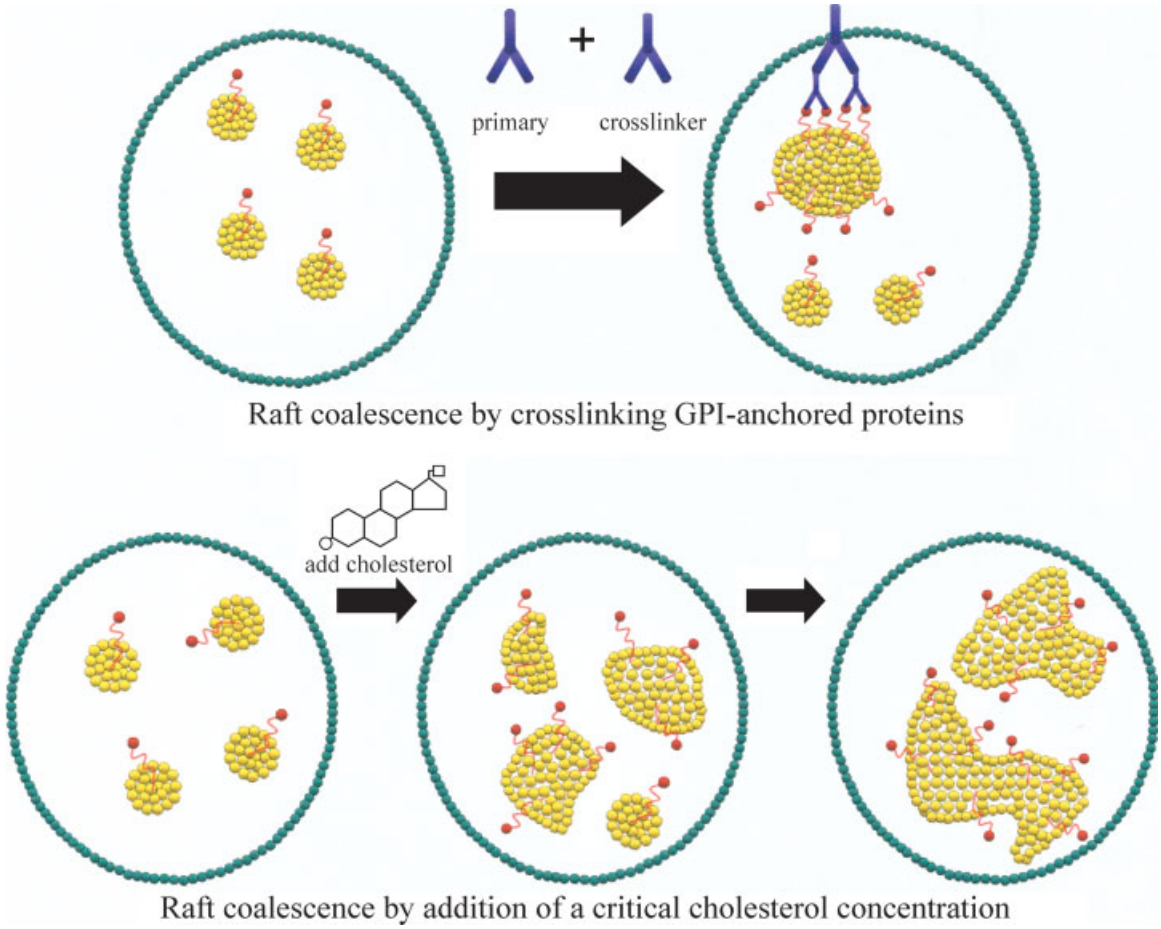

Fig. 3. A model for how increases in membrane cholesterol might alter signal transduction in cancer cells. Cross-linking of GPI-anchored proteins may induce the coalescence of lipid rafts, thereby activating signaling mechanisms. Similarly, increases in membrane cholesterol beyond some critical concentration may coalesce rafts, thereby sequestering oncogenic signaling molecules within rafts, increasing compositional complexity of individual rafts, and excluding negative regulators from the raft compartment.

As discussed above, although a variety of signaling molecules are found in rafts, it is probable that not all rafts are compositionally equivalent. Consequently, the process of raft cross-linking would not only create large rafts, but would probably increase raft complexity by assembling rafts with varying protein composition. In addition, because large, coalesced rafts maintain the same surface area as the sum of all the isolated rafts, but have a dramatically decreased circumferential length, fewer raft proteins will be present at the raft/non-raft (liquid-disordered) interface. Therefore, fewer raft proteins would be available to be regulated by moieties that are excluded from rafts but which may be abundant in the membrane-at-large. Solomon and co-workers have hypothesized for almost a decade that coalescence of rafts is the underlying mechanism for signaling induced by antibody cross-linking of GPI-anchored proteins [Solomon, 1996].

How does the cross-linking hypothesis apply to the observation that increases in cholesterol in tumor cell membranes, either from dietary or other factors, may promote PCa growth and disease progression? We know from experiments with model membranes that moderate increases in the level of membrane cholesterol (10–20%) reduces the number of isolated rafts and causes the formation of larger rafts [Lawrence et al., 2003]. Consequently, the literature already provides support for the idea that as membrane cholesterol levels increase, larger raft structures, with a smaller total perimeter, begin to form. The model illustrated in Figure 3 illustrates that raising cholesterol levels beyond some critical concentration may result in the coalescence of smaller raft domains, analogous

to the manner by which rafts might coalesce in response to cross-linking of GPI-anchored proteins. This may serve to sequester, and thereby stimulate, "on" signals to oncogenic pathways, as well as exclude negative regulators that contribute "off" signals in the normal environment. This model is consistent with the reported association between caveolin-1 expression and PCa progression [Yang et al., 1998] in the sense that higher caveolin levels may reflect an expansion of the raft compartment in aggressive tumor cells.

Importantly, the model illustrated in Figure 3 can be tested empirically. In unpublished studies from our group, Zhuang, Kim and colleagues have demonstrated that raising serum cholesterol in SCID mice harboring LNCaP PCa xenograft tumors results in an increase in cholesterol in lipid raft membranes. Importantly, this increase in raft cholesterol content was shown to correlate with alterations in several indices of oncogenic signal transduction, including an increase in the levels of raft proteins phosphorylated on tyrosine, an increase in phosphorylated Akt, and a decrease in apoptotic rates as evaluated by TUNEL.

CONCLUDING REMARKS

Cholesterol accumulation in PCa cells, in concert with alterations in cholesterol metabolism associated with age and malignancy in the prostate, is likely to alter signal transduction mechanisms underlying PCa progression in profound ways. We have proposed that one possible consequence of progressive increases in membrane cholesterol is the expansion of the tumor cell lipid raft compartment, a change in the plasma membrane that may potentiate oncogenic pathways of cell signaling. The ability to isolate lipid rafts from cells and tumors using established biochemical methods allows for raft proteins that respond to specific signals, such as soluble factors that promote cancer cell growth and survival, to be identified and characterized. Large-scale cataloging of lipid raft proteins using mass spectrometry is now ongoing by a number of groups [Bini et al., 2003; Foster et al., 2003]. This will allow the direct testing of the hypothesis diagrammed in Figure 3, as well as the identification of signaling proteins that may associate with rafts stably or transiently during the multiple processes illustrated in Figure 2. The identification of these raft-associated proteins, in combination with experiments designed to understand the functional implications of their association with cholesterol-rich membrane domains, will provide new insight into signal transduction processes related to cancer spread. We believe they will also provide a wealth of new targets for cancer therapy and possibly new biomarkers that will be useful in a clinical setting.

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