Cholesterol-rich Lipid Rafts Mediate Akt-regulated Survival in Prostate Cancer Cells

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Abstract

Although cholesterol accumulation in tumors was first reported in the early 20th century, the mechanistic implications of this observation are still obscure. Here we report that caveolin-negative human prostate cancer (LNCaP) cells contain cholesterol-rich lipid rafts that mediate epidermal growth factor (EGF)-induced and constitutive signaling through the Akt1 serine-threonine kinase. EGF receptor and Akt1 phosphorylation were inhibited and autonomous cell survival was reduced when the rafts were disrupted. Reconstitution of the rafts with cholesterol restored EGF receptor→Akt1 axis signaling and cytoprotection from a phosphoinositide 3-kinase-dependent apoptotic signal. These results suggest that cholesterol present in membrane microdomains is a prominent mediator of survival in prostate cancer cells.

Introduction

PCa incidence and progression have been linked to high-fat “Western” diets, including intake of animal products (1, 2). The mechanism by which PCa cell growth and/or cell survival might be promoted by dietary fat is poorly understood. White (3) reported in 1909 and subsequent studies have also demonstrated that solid tumors (4, 5), including those of the prostate (6), accumulate cholesterol and other fatty deposits. Several older reports have suggested an association between malignancy and cholesterol levels specifically in prostatic tissue and secretions (6, 7). More recently, caveolin-1, the major structural protein of caveolar lipid membrane microdomains, generally known as lipid rafts (8), was identified as a protein that may be functionally associated with tumor progression and metastasis in PCa (9–11). Caveolae and other types of lipid rafts are now known to serve as membrane platforms for cell signaling (12, 13). Importantly, lipid raft microdomains are enriched in cholesterol relative to the liquid-disordered component of the cell membrane, suggesting a role for membrane cholesterol as a determinant of growth factor-mediated cell survival mechanisms.

To test the hypothesis that membrane cholesterol plays a role in cell survival in PCa cells, we used LNCaP human PCa cells that do not express caveolins. The absence of these proteins, which sequester survival mechanisms. PCa cells are also useful as a model system because they express prostate-specific properties and are dependent for cell survival on a constitutive signal through the PI3K/Akt phosphorylation cascade (14). The essential role of this signaling mechanism in this cell line may be related to the presence of an inactivating mutation in the gene encoding the PTEN phosphatase, a negative regulator of this pathway. Loss of PTEN is a frequent event in human PCa (15, 16), and up-regulation of the PI3K/Akt pathway has been linked to PCa progression (17).

Materials and Methods

Cell Culture. Human PCa cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (Rockville, MD). Both cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. LNCaP cells transfected with the plasmid pcDNA-Cav-1 or with an empty vector were cultured in medium containing 300 µg/ml G418 as described (18). Details of specific cell treatments are described in the figure legends.

Antibodies and Reagents. The following mAbs and pAbs were used: anti-EGFR pAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated EGFR mAb, anti-Gi3 pAb (Calbiochem, La Jolla, CA); anti-caveolin-1 mAb (clone 2297), anti-caveolin pAb, anti-Fyn mAb (clone 25; Transduction Labs, San Diego, CA); anti-Akt pAb, anti-phosphorylated Akt pAb (Cell Signaling, Beverly, MA). Human recombinant EGF and HB-EGF were purchased from R&D (Minneapolis, MN). Filipin, cholesterol, and cyclodextrin were from Sigma Chemical Co. (St. Louis, MO).

Successful Detergent Extraction of Lipid Rafts. Extraction of Triton-soluble and -insoluble membrane constituents was performed essentially as described (19). In brief, cells were resuspended in buffer A [25 mM 2-(N-morpholino)-ethanesulfonic acid, 150 mM NaCl, pH 6.5]. To this, an equal volume of the same buffer with 2% Triton X-100, 1 mM NaN3 and, 2 mM phenylmethylsulfonyl fluoride was added, and the cells were incubated on ice for 30 min. Insoluble fractions were pelleted in a microcentrifuge (14,000 × g) for 20 min at 4°C. The supernatant was removed (“S” [soluble] fraction), and the insoluble pellet was resuspended in buffer B [1% Triton X-100, 10 mM Tris (pH 7.6), 500 mM NaCl, 2 mM NaN3]. For Triton extraction, cells were collected by scraping. Cells were fixed in 4% paraformaldehyde and permeabilized, and DNA was labeled with fluorescein using the TUNEL reaction mix. The percentage of apoptotic cells was determined by flow cytometry. Apoptosis induced by filipin was confirmed by the ladder genomic DNA fragmentation assay as described (14).

Apoptosis Assays. A quantitative sandwich ELISA was performed to measure mon- and oligonucleosomes in the cytoplasmic fraction of cell lysates according to the manufacturer’s manual (cell death detection ELISA; Roche, Indianapolis, IN). Briefly, 1.5–2.0 × 10^6 cells/well were seeded in six-well plates for 24 h, cell lysates were collected after various treatments as indicated, and the amount of histone-associated DNA fragments was quantified by spectrophotometric measurement of peroxidase activity retained in the immunocomplex (415 nm) against the substrate solution as a blank (490 nm). Apoptosis was also evaluated by the TUNEL method, using the In Situ Cell Death Detection kit (Roche). Briefly, 1.5–2.0 × 10^6 cells/well were seeded in six-well plates for 24 h, and cells were collected by scraping. Cells were fixed in 4% paraformaldehyde and permeabilized, and DNA was labeled with fluorescein using the TUNEL reaction mix. The percentage of apoptotic cells was determined by flow cytometry. Apoptosis induced by filipin was confirmed by the ladder genomic DNA fragmentation assay as described (14). Briefly, cells (8.5 × 10^5) seeded in 6-cm dishes for 3 days in 10% FBS RPMI 1640 were cultured in serum-free medium and subjected to various treatments.
I-fraction. After reconstitution of the raft domains with cholesterol, 95% into the Triton-insoluble/octylglucoside-soluble fraction in both cell types (Fig. 1). Caveolin-1 partitioned nearly completely (13, 19, 20), were similarly distributed and enriched in the I-fraction with some previous reports (9, 18). [LNCaP cell variants expressing caveolin-1 have also been reported (21).] These findings indicate that LNCaP cells possess a lipid raft compartment that is not dependent on the presence of caveolin-1.

EGFR-mediated activation of the PI3K/Akt signaling pathway has been shown to promote cell survival in LNCaP and other cell types, suggesting an important role for this signaling system in PCa progression (22). Because EGFR activation was demonstrated to be regulated by lipid rafts in other cell types (23), we investigated their possible biological function in regulating EGFR signaling in LNCaP cells. Serum-starved cells were treated with EGF, and levels of total and phosphorylated forms of the EGFR were examined in S- and I-fractions. EGFR was predominantly located in the I-fraction (Fig. 2). In fact, the EGFR was nearly undetectable in the I-fraction [on overexposed immunoblots a small amount of EGFR could be visualized in the I-fraction (data not shown)]. Importantly, ligand-induced phosphorylation of EGFR (on Tyr-1173) was predominantly seen in the S-fraction (Fig. 2), suggesting that EGFR treatment did not appear to cause detectable redistribution of EGFR.

To further investigate the involvement of lipid rafts in EGFR signaling, LNCaP cells were treated with the raft-disrupting agent, filipin, a polyene macrolide that binds cholesterol with high specificity (24–26). Filipin has been shown repeatedly to disrupt lipid raft-dependent signaling and transport events (27–29). Filipin pretreatment (2 μg/ml) suppressed ligand-dependent EGFR phosphorylation in the I-fraction. After reconstitution of the raft domains with cholesterol, EGFR phosphorylation recovered to the levels observed in cells not treated with filipin (Fig. 2), suggesting that EGFR signaling is mediated by lipid rafts.

EGFR signaling occurs in a lipid raft membrane compartment. LNCaP cells were seeded on poly-l-lysine-coated six-well plates overnight and starved for 16–20 h in serum-free medium. A, mock- or filipin-pretreated (2 μg/ml) cells were incubated in serum-free medium with or without cholesterol/cyclodextrin (Cholesterol) complexes for 1 h at 37°C, followed by EGF treatment for 20 min. All cells were subjected to SDEM, and the I- and S-fractions were analyzed by immunoblot. p-EGRF, EGFR phosphorylated on Y1173 detected with a phospho-specific Ab. B, cell viability measured by trypan blue exclusion 1 h after treatment of cells with filipin.

To determine whether EGFR/lipid raft signaling can mediate a prosurvival effect in LNCaP cells, EGFR-dependent cell survival was evaluated. Previous studies have shown that cell survival in LNCaP cells is enhanced by EGFR activation when apoptosis is stimulated by PI3K inhibitors (14). Consistent with published data, the PI3K inhibitor LY294002 triggered apoptosis in LNCaP cells, and the apoptotic effect of this drug was reversed by treatment with EGF (Fig. 3). Surprisingly, filipin alone stimulated apoptosis to a similar extent as the PI3K inhibitor, suggesting that disrupting the cholesterol-rich rafts not only interferes with EGFR signaling but also inhibits a critical cell survival pathway that is operating constitutively. The filipin effect was not attributable to membrane permeabilization because, at the doses used in these experiments, 100% of the cells excluded trypan blue 1 h after treatment (Fig. 2). Under conditions in which cells were treated with filipin, EGF pretreatment did not protect the cells from the apoptotic stimulus. In contrast, when membrane cholesterol was repleted after filipin treatment, the protective effect of EGF was again observed.

The Akt1 serine-threonine kinase is a prominent prosurvival signaling protein that is both downstream from EGFR activation and constitutively up-regulated in LNCaP cells. Given the strong inhibitory effects of filipin on EGFR activation and its ability to stimulate apoptosis, we hypothesized that filipin exerts a negative effect on Akt1 activity. Akt1 phosphorylation increased in response to 20 ng/ml EGF (Fig. 4A, consistent with a previous report from our group (14). EGFR-induced Akt1 phosphorylation was substantially reduced in cells treated with 2 μg/ml filipin. Significantly, both constitutive and EGF-induced EGFR and Akt1 phosphorylation were decreased with filipin treatment in a dose- and time-dependent fashion (Fig. 4, A, C, D).
consistent with the apoptosis results shown in Fig. 3. Similar inhibitory effects on Akt1 phosphorylation were also demonstrated after membrane cholesterol was depleted with cyclodextrin, another cholesterol-binding drug (Fig. 4B). After cell membranes were loaded with cholesterol to reconstitute the raft microdomains, constitutive and EGF-induced levels of phosphorylated Akt1 were restored in filipin-treated (Fig. 4A, compare Lanes 7–9 versus Lanes 10–12) and cyclodextrin-treated (not shown) cells. Cholesterol repletion alone (in the absence of EGF and/or filipin) did not induce changes in Akt1 phosphorylation, and total Akt1 levels did not change with any of the treatments described above. These experiments were repeated with another EGFR ligand, HB-EGF, and similar results were obtained (data not shown). These findings indicate that EGF→Akt1 signaling, as well as constitutive signaling through Akt1, appear to be under partial control of a cholesterol-rich membrane domain in LNCaP cells. This result is intriguing because Akt1 is believed to be stably activated in cells (such as LNCaP) that do not express a functional PTEN phosphatase. Our findings here indicate that despite stable up-regulation of Akt1 activity in a PTEN-null background, this kinase still remains partly dependent on an upstream, cholesterol-dependent signaling mechanism.

Caveolin-negative LNCaP cells stably transfected with caveolin-1 were used to determine whether expression of this protein, which is functionally involved in structural organization and cell signaling through caveolar lipid rafts (13), alters the apparent regulatory role of membrane cholesterol demonstrated above. Transfected caveolin-1 partitioned into the I-fraction as anticipated (Fig. 4E). Filipin treatment suppressed ligand-induced EGFR and Akt1 phosphorylation in both caveolin-transfected LNCaP cells and in PC-3 cells (Fig. 4F). In PC-3 cells, which express high levels of endogenous caveolin (Fig. 1), the levels of total and phosphorylated EGFR and Akt1 were very similar to those seen in the wild-type LNCaP cells, which do not express caveolins. In addition, the inhibitory effect of filipin treatment was also indistinguishable between the caveolin-expressing cells and unmodified LNCaP cells. These findings indicate that caveolin-1 does not regulate, nor does the presence of caveolin in the I-fraction alter, the effects of lipid raft disruption on the EGFR→Akt pathway in these cell types.

Our findings are the first to identify an important role for membrane cholesterol in the transmission of cell survival signals through the EGFR→PI3K/Akt1 pathway. We show that, in LNCaP cells, cholesterol-rich lipid rafts appear to be important for constitutive signaling through the Akt1 kinase, which is up-regulated in this cell line because the PTEN phosphatase is inactive. Because activation of PI3K/Akt signaling is thought to be an important, clinically relevant attenuator of apoptotic signals in PCs and other human malignancies, our current study suggests that, despite the absence of PTEN, signaling through Akt1 is still subject to down-regulation via alteration of membrane composition. This result suggests the possibility that targeting membrane cholesterol is a rational means for therapeutically down-regulating this pathway. This hypothesis is supported by published evidence demonstrating that polyene macrolide sterol-binding compounds, including filipin, significantly reduced prostate glandular hyperplasia in dogs by up to 75% with no toxicity (30). This effect, which was highly tissue specific (possibly because the prostate accumulates high levels of cholesterol), may be the result of disruption of cholesterol-mediated cell survival mechanisms. The presence or absence of caveolin-1 did not detectably alter the dependence of EGFR and Akt phosphorylation on cholesterol intact lipid rafts, suggesting that the EGFR/PI3K/Akt cell survival axis is not dependent on the expression of caveolin proteins, and further, that down-regulation of this mechanism can be accomplished in caveolin-positive cells. Caveolin-1 expression has been linked recently to aggressive PCs (9, 31). Our studies provide a new mechanistic framework for the exploitation of cholesterol-mediated pathways.

and D). Therefore, although the EGFR axis was affected by this agent, the inhibitory effect of filipin on Akt1 phosphorylation was not dependent on stimulation with exogenous EGF, a result that is consistent with the apoptosis results shown in Fig. 3. Similar inhibitory
ration of a role for cholesterol as a mediator of PCA development and progression.

Observations of cholesterol and other lipids accumulating in solid tumors, including PCa, have a long history (3). In this regard, it is interesting to point out that circulating cholesterol is a major source of plasma membrane cholesterol as a result of cellular absorption of lipoprotein from serum, and furthermore, that membrane levels of cholesterol can be substantially modified by diet (32). Rates of PCa progression are significantly affected by exogenous factors, including a Western diet and consumption of red meat and/or dietary fat (2). These observations may be related to the present findings that cholesterol-rich membrane microdomains regulate a survival function in human PCa cells. Furthermore, our observations may help provide a mechanistic link between cholesterol-rich diets and certain other diseases in which high-cholesterol, high-fat diets have been historically and epidemiologically associated.

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