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The estrogen receptor as a mediator of the pathological actions of cholesterol in breast cancer

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Abstract

Despite increased survivorship among patients, breast cancer remains the most common cancer among women, and is the second leading cause of cancer death in women. The magnitude of this problem provides a strong impetus for new chemopreventative strategies and/or lifestyle changes that reduce cancer incidence. It is of significance, therefore, that several studies positively correlate obesity to the development of breast cancer. Importantly, obesity is also highly associated with elevated cholesterol, and cholesterol itself is a risk factor for breast cancer. Furthermore, patients taking statins demonstrate a lower breast cancer incidence, and decreased recurrence. The recent observation that 27-Hydroxycholesterol (27HC) is produced in a stoichiometric manner from cholesterol, together with our recent demonstration that it exerts partial agonist activity on both the estrogen and liver X receptors (ERs and LXRs), suggested a potential mechanistic link between hypercholesterolemia and breast cancer incidence. Using genetic and pharmacological approaches we have recently shown that elevation of circulating 27HC significantly increases tumor growth and metastasis in murine models of breast cancer. Further we have demonstrated in appropriate animal models that the impact of high fat diet on tumor pathogenesis can be mitigated by statins or by small molecule inhibitors of CYP27A1. These findings suggest that pharmacological or dietary modifications that lower total cholesterol, and by inference 27HC, are likely to reduce the impact of obesity/metabolic syndrome on breast cancer incidence.

Keywords

SERM; 27-Hydroxycholesterol; Cyp27A1; Breast cancer; Statins

Introduction

Breast cancer is the most commonly diagnosed cancer in women, and is currently the second highest cause of cancer deaths. Fortunately, advances in early detection and the advent of effective targeted therapeutics have had a significant positive effect on overall survival in breast cancer patients. However, the lifetime risk of breast cancer continues to increase

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highlighting the need to define the genetic and environmental factors that predispose women to this disease. It is anticipated that the exploitation of this information will enable the development of approaches to attenuate the impact of the risks identified. For instance the identification of BRCA1 and BRCA2 loss of function mutations as highly penetrant genetic risk factors for breast cancers has led to the exploration of chemopreventative strategies that may prevent the disease in BRCA1/BRCA2 carriers or which may defer mastectomy (1). However, somatic mutations in BRCA1 and BRCA2 account for only a small percentage of all breast cancers and it now appears unlikely that any one mutation will be identified as a primary driver of this disease. This realization has focused considerable attention on defining other factors that impact breast cancer pathogenesis.

The mammary gland is an estrogen target organ and the majority of breast cancers express the alpha subtype of the estrogen receptor (ER α). This is significant given the large amount of epidemiological data that suggests that lifetime exposure to estrogens is a significant risk factor for ER-positive breast cancer. Thus, early menarche, later age of first pregnancy each likely contribute in a small but significant manner to overall risk, and are thus components of most breast cancer risk assessment tools (2). These robust observations and the success of anti-hormonal therapy in the adjuvant setting in breast cancer led to the successful development of tamoxifen and raloxifene, Selective Estrogen Receptor Modulators (SERMs) that exhibit ER antagonist activity in the breast, as breast cancer chemopreventives in women at high risk for breast cancer (1,3). Intervention with either of these SERMs reduced the incidence of both non-invasive and invasive breast cancer in high risk women by ~50% (3-5). The success of this chemopreventative approach has provided the impetus to identify additional modifiable risk factors for breast cancer.

Recently, obesity and the metabolic syndrome have emerged as important “environmental” risk factors for several different cancers (6,7). Of particular note is the observation that obesity increases the risk of post-menopausal ER-positive breast cancer by over 50% (8). The potential impact of this observation is highlighted by the fact that ~40% of the US population is clinically obese and that obesity is rising most rapidly in women over 60 years of age (9). The mechanisms linking obesity and breast cancer are complex although there are compelling data to suggest that increases in circulating insulin and insulin-like growth factors, local production of estrogens in adipose tissue, and the influence of adipokines and inflammatory cytokines, are causally involved in disease pathogenesis (6,10). Interestingly, there is a wealth of data linking these factors to ER signaling and thus it is not surprising that obesity related breast cancer is most apparent in postmenopausal women where ER-positive tumors are most common (11). Finally, hypercholesterolemia, a comorbidity of obesity, has also been established as an independent risk factor breast cancer (12-15).

Cholesterol as an environmental risk factor for breast cancer

Studies probing potential links between serum cholesterol and breast cancer risk have yielded equivocal results, with some finding no relationship, some indicating a protective effect and others implicating cholesterol as a significant risk factor (16-18). Much of this uncertainty likely relates to the small size of the studies performed to date and to differences in breast cancer risk within the populations studied (premenopausal vs. menopausal).

However, a recent analysis of the ACALM (Algorithm for Comorbidities, Associations, and Length of stay and Mortality) study population revealed that breast cancer risk was increased 1.64 fold (95% CI 1.5-1.79) in patients with hyperlipidemia (13). Intriguing also were the recent results of a cohort study in which it was demonstrated that patients with established breast cancer had higher LDL-cholesterol and VLDL-cholesterol, although no associations with HDL or total cholesterol and breast cancer was evident (19).

It has been demonstrated that, when corrected for obesity, dietary consumption of cholesterol is strongly associated with increased breast cancer risk in postmenopausal but not in premenopausal women (20). This finding was corroborated by other studies that have also revealed an association between increased dietary consumption of cholesterol and breast cancer risk (21). The importance of cholesterol as a breast cancer risk factor has also been confirmed in two large prospective studies (22,23).

The data linking increased serum cholesterol and increased dietary cholesterol to breast cancer risk suggests that agents that (a) block cholesterol biosynthesis by inhibiting HMG-CoA reductase (statins), or (b) inhibit cholesterol absorption (NPC1L1 inhibitors or agents that sequester intestinal bile acids) should reduce the incidence of this disease. Most of the published studies that have addressed this issue have focused on statins although no consensus has emerged as to their impact on the onset of breast cancer. Indeed a recent meta-analysis suggested that statins can have either a positive or a negative effect on breast cancer incidence (16). However, interpretation of these studies is confounded by the fact that the assumption is made that all statins are equivalent. Further, given the patient demographics of most of the studies where statins have been used it is not clear if the expected cases of breast cancer were high enough to allow a protective effect of statins to be observed. Indeed, it is likely considering what we have learned from the BCPT (tamoxifen) and STAR (tamoxifen vs. raloxifene) breast cancer prevention trials that a definitive study of the chemopreventative activities of statins would have to be performed in a population of women who are at elevated risk for ER-positive breast cancer (3-5). There is however, compelling data indicating that statin use decreases recurrence in patients with breast cancer (24) (25,26).

Collectively, these studies highlight the need to define the potential mechanisms by which cholesterol impacts the biology of breast cancer as a means to focus further clinical studies that more specifically evaluate the impact of cholesterol on the development and progression of breast cancer.

Cholesterol is implicated as a risk factor in animal models of breast cancer

It has been shown in several studies that high fat, high cholesterol (Western) diets decrease the latency and increase the growth and metastasis of tumors in murine models of mammary cancer (27) (18,28). However, in wild-type mice, high fat diets (HFDs) do not in and of themselves increase serum cholesterol and thus this model does not allow an accurate modeling of the effects of Western diets on tumor biology. This limitation can be circumvented by deleting the *ApoE* gene in mice creating a model in which dietary fat results in a dramatic increase in LDL-cholesterol. In *APOE*^{-/-} mice the effects of a Western

diet on tumor growth and metastasis are greatly accentuated suggesting that cholesterol is pathogenic in breast cancer. However, there are two significant caveats to this conclusion. The first is that in most of the key studies performed in this model utilize diets high in both fat and cholesterol and thus the specific contributions of dietary cholesterol, endogenously produced cholesterol, and dietary fat could not be determined (18). A more significant issue is the fact that the APOE^{-/-} mice display a very substantial generalized inflammatory response and this could, independent of cholesterol status, impact tumor pathogenesis. To circumvent these issues we evaluated the specific effects of hypercholesterolemia on tumor growth and metastasis in the MMTV-PyMT mouse model of mammary cancer (29). It was noted in this study that increased dietary intake of cholesterol alone resulted in a significantly decreased tumor latency and increased tumor growth, supporting the idea that cholesterol itself can impact tumor pathophysiology (**Figure 1**).

Realizing the problems with the APOE^{-/-} mouse model we took advantage of a related model in which the mouse *ApoE* locus is replaced with the human APOE allele (30). In this model dietary fat results in increased serum cholesterol absent a generalized inflammatory response. As expected the growth of breast tumors propagated in this model were significantly increased when the mice were fed a high fat diet and importantly this effect could be attenuated by administering a statin (29). When taken together it appears as if cholesterol is a pathogenic agent in breast cancer and that the impact of HFD on breast cancer risk can be attributed to increased serum cholesterol.

Mechanisms underlying the pathological actions of cholesterol in breast cancer

The intracellular level of free cholesterol within most cells is kept relatively constant by a series of tightly regulated homeostatic processes. Excess free cholesterol can be accommodated by its partitioning into membranes and/or its esterification and subsequent storage in lipid droplets. Whereas these latter processes enable a somewhat passive regulation of intracellular levels of cholesterol, it is alterations in the processes that control the efflux, influx, and *de novo* synthesis of this lipid that have the most impact. These latter processes are coordinately regulated by Sterol Regulatory Element Binding Protein-2 (SREBP2), a transcription factor whose activity is both transcriptionally and post-transcriptionally regulated by cholesterol (31). When cholesterol homeostasis is achieved this protein is maintained in an inactive state within a large multiprotein complex associated with the endoplasmic reticulum. When cholesterol levels fall a cascade of events is initiated that enables SREBP2 to enter the nucleus and upregulate the expression of genes responsible for cholesterol synthesis (HMGCoA Reductase (HMGCR)) and cholesterol import (LDLR) (31,32). In addition to SREBP2, the liver X receptors (LXRs) are also involved in maintaining intracellular cholesterol homeostasis. The transcriptional activity of these receptors is regulated by oxysterol ligands that are derived from cholesterol within cells (33). Among the genes regulated by LXR are the reverse cholesterol transporters (ABCA1 and ABCG1), and IDOL, an E3 ligase responsible for the degradation of LDLR (34,35).

Given the complexity of the mechanisms that regulate intracellular cholesterol homeostasis it is unclear how increases in circulating cholesterol could impact cancer pathogenesis. One

widely held hypothesis is that increases in cholesterol content results in changes in the biophysical properties of cell membranes and that this impacts signaling events initiated at the membrane. Indeed, a recent study demonstrated increased PI3K activity (and AKT phosphorylation) activity in mammary tumors propagated in hyperlipidemic APOE^{-/-} mice (36). However, the plasma cholesterol levels in this mouse model exceed 2000mg/dL, far greater than would be considered “hypercholesterolemic” in humans (~240 mg/dL). Further, in studies performed *in vitro* it has been demonstrated that the level of exogenous cholesterol needed to stimulate cell proliferation is far lower than that required for lipid raft formation and AKT phosphorylation. Taken together these data argue against a direct role for cholesterol in tumor pathogenesis but suggest the possibility that cholesterol, or a derivative, is functioning as a signaling molecule in cancer cells. It is significant therefore that 27-hydroxycholesterol (27HC), a primary metabolite of cholesterol has been shown to function as both a SERM and a liver X receptor (LXR) agonist (37,38). Notably 27HC inhibits estrogen receptor (ER) antagonist in the cardiovascular system but functions as an agonist in osteoblasts and in models of ER-positive breast cancers (39-42). These findings suggest that the pathogenic actions of cholesterol require its metabolism to 27HC a molecule that functions as a mitogen in ER-positive tumors.

Hormonal actions of 27-hydroxycholesterol in breast cancer

The oxysterol 27HC is synthesized from cholesterol by CYP27A1 a rate limiting enzyme in the ‘alternative’ or ‘acidic’ pathway for bile acid synthesis. In both humans and in animals, the circulating levels of 27HC closely mirror those of cholesterol and hypercholesterolemia results in commensurately high levels of 27HC (43). Whereas 27HC may have several functions within cells its role as an LXR agonist which inhibits cholesterol production and increases cholesterol efflux from cells is very well established. Thus, it would be expected, as has been observed for other LXR agonists, that 27HC would inhibit the growth of cancer cells (44-46). However, we and others have shown that 27HC actually promotes the proliferation of ER-positive, but not ER-negative, breast cancer cell lines *in vitro* (29). This paradox was resolved by the finding that, in addition to functioning as an LXR agonist, 27HC functions also as an endogenous SERM that exhibits ER-agonist activity. Recently, we have also demonstrated that ER can, by an as yet to be determined mechanism, inhibit LXR transcriptional activity explaining how the antiproliferative effects of LXR activation are circumvented (29). Importantly, we and others have shown that the growth of ER-positive tumors in several different animal models of breast cancer can be stimulated by 27HC administration (29,47). The ability to reverse the growth promoting effects of 27HC by coadministration of an ER-antagonist confirmed that ER is the primary target of this oxysterol in tumors. It was also demonstrated that deletion of the enzyme responsible for the catabolism of 27HC (CYP7B1^{-/-}) increased circulating levels of 27HC, and this resulted in increased tumor growth (29). Given these data we hypothesized that the effects of hypercholesterolemia on breast cancer may require its metabolism to 27HC. In support of this hypothesis we demonstrated that although a high cholesterol diet increased mammary tumor growth in the MMTV-PyMT mouse model, it was without effect in the same mouse model when the enzyme responsible for the conversion of cholesterol to 27HC (CYP27A1) was ablated (**Figure 2**).

Several epidemiological studies have suggested that obesity and elevated cholesterol, increase the risk of breast cancer metastases (48,49). Initially, we thought that this could be explained by the fact that 27HC was an ER-ligand that processes regulated by this receptor facilitated metastasis. However, in following up on this hypothesis we observed that estradiol, while having the expected effect on the growth of ER-positive tumors, was without noticeable effect on metastasis (29). Further studies revealed that 27HC-mediated increases in metastasis likely involve the LXR as the effects of this oxysterol mirrored those of synthetic LXR agonists. Given the inhibitory effect of ER on LXR signaling we are currently exploring the possibility that estrogens, while stimulating the growth of primary tumors, may actually inhibit LXR-dependent increases in metastasis. Regardless, it is clear that 27HC, through its actions on both ER and LXR, functions as an important biochemical link between hypercholesterolemia and breast cancer.

Autocrine/paracrine roles for 27HC in breast cancer

The majority of the 27HC found in circulation is derived from the liver, a tissue where CYP27A1 is highly expressed. Given that macrophages also express CYP27A1 it is likely that they also support the production of 27HC. Of specific importance to breast cancer is the observation that CYP27A1 is abundantly expressed within tumor associated macrophages in human breast tumors (29). There is abundant evidence that breast tumor infiltration by macrophages is associated with a poor prognosis in breast cancer and, although they may impact tumor pathophysiology in several ways, their ability to produce 27HC within tumors and activate ER α activity is likely to be important. In addition to macrophages, we have recently shown that CYP27A1 is also expressed within the malignant cancer cells themselves and that higher expression of this enzyme is found in higher grade tumors (29). Significant also was the observation that the expression of CYP7B1, the enzyme responsible for the catabolism of 27HC, is decreased in human breast tumors compared to normal tissue (29,47). This suggests that 27HC metabolism, both anabolism and catabolism, and resulting differences in the local concentrations of 27HC may play an important role in breast tumor pathophysiology. Interestingly, breast tumors have significantly higher 27HC concentrations than normal breast tissue (47). This suggests that the development of therapeutic approaches to decrease the impact of 27HC on tumor growth will have to accomplish a lowering of both circulating and intratumoral 27HC.

Conclusion

Using a series of genetic and pharmacological approaches we have established that the oxysterol 27HC is a biochemical link between cholesterol and tumor pathology and that these activities require both ER and LXR. Further we have demonstrated in appropriate animal models that statins and/or small molecule inhibitors of CYP27A1 inhibit HFD-dependent increases in tumor growth suggesting that drugs of this class may have utility in the treatment and/or prevention of breast cancer. These data highlight additional unexpected benefits of lifestyle interventions aimed at managing hyperlipidemia.

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Figure 1. Cholesterol stimulates tumor growth in a mouse model of ER-positive breast cancer

The impact of hypercholesterolemia on breast cancer pathogenesis was evaluated in mice genetically engineered to express the PyMT oncogene in the mammary gland. In this well-validated model of ER-positive breast cancer tumors arise spontaneously. For this study mice were placed on either a control diet or a high cholesterol diet (2% cholesterol), ad libitum from weaning. At 5 weeks of age mice were ovariectomized and monitored for the appearance of the first palpable tumors, the growth of which was then recorded through time. (Figure adapted from *Science* 342: 1094-1098, 2013)

Figure 2. 27HC levels affect tumor growth in the MMTV-PyMT mouse model of breast cancer
 The growth of tumors in the PyMT tumor model was evaluated in wild-type mice or in those which the enzyme responsible for (a) synthesis of 27HC from cholesterol (CYP27A1), or (b) metabolism of 27HC (CYP7B1) was genetically ablated. The complete details of this study have been published previously (29). (Figure adapted from *Science* 342: 1094-1098, 2013)