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How retinoids regulate breast cancer cell proliferation and apoptosis

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Abstract. Breast cancer still remains a major problem in its incidence, morbidity and mortality; therefore, more effective strategies for its prevention are urgently needed. Retinoids, natural and synthetic derivatives of vitamin A, possess antiproliferative and proapoptotic properties, making them a promising class of chemopreventive agents against breast cancer. The efficacy of all-trans retinoic acid, 9-cis-retinoic acid, LGD1069 (Targretin, bexarotene), and N-(4-hydroxyphenyl)retinamide (fenretinide) as breast cancer chemopreventive agents is being studied. A better understanding of the molecular mechanisms of action of these agents should lead to improvements in their clinical application. In this review, we discuss the mechanisms by which retinoids exert their antiproliferative and apoptotic effects in breast cancer cells.

Key words. Breast cancer; retinoids; apoptosis; chemoprevention; all-trans retinoic acid; 9-cis-retinoic acid (altretinoin); 4-HPR (fenretinide); LGD1069 (Targretin, bexarotene).

Introduction

Breast cancer has the highest incidence rate and the second highest mortality rate of all cancers in American women. Efforts to lower these rates have focused on the development of chemopreventive strategies. Chemoprevention, which is the administration of natural or pharmacologic agents to reverse or suppress carcinogenesis, represents a novel approach to controlling secondary breast malignancies. Results from the National Surgical Adjuvant Breast and Bowel Project P-1 Breast Cancer Prevention Trial (BCPT) and the more recent Multiple Outcomes of Raloxifene Evaluation (MORE) trial demonstrated the effectiveness of selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene in preventing the development of secondary breast tumors [1]. However, the use of these agents is limited by their lack of efficacy against estrogen receptor (ER)-negative breast tumors. There is a major need to further develop methods for the prevention of breast cancer. In particular, chemopreventive strategies for ER-negative breast cancer are of critical importance because patients with these malignancies generally have a poor prognosis. Retinoids, natural or synthetic vitamin A analogues, can regulate cell growth, differentiation and apoptosis in various cell types [2]. The regulation of cell growth by retinoids is thought to result from direct and indirect effects on gene expression. These effects are mediated by the nuclear receptors retinoic acid receptor (RAR)-α, -β and -γ and retinoid X receptor (RXR)-α, -β and -γ, which are ligand-activated transcription factors and members of the steroid hormone receptor superfamily. Retinoid receptors activate transcription in a ligand-dependent manner by binding as RAR/RXR heterodimers to retinoic acid response elements (RAREs) located in the promoter regions of target genes or as RXR homodimers to retinoic X response elements (RXREs) in gene promoters.

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Both naturally occurring and synthetic retinoids have been shown to inhibit the growth of breast cancer cells. The naturally occurring retinoid 9-cis retinoic acid (9-cis RA, alitretinoin) transactivates both RARs and RXRs [3, 4]. All-trans retinoic acid (ATRA) is a naturally occurring retinoid that binds RAR with high affinity but does not bind RXR [5]. The use of these natural retinoids to treat patients with advanced breast cancer has been limited by their lack of clinical efficacy and their toxic side effects, including hyperlipidemia and mucocutaneous and liver toxicity [6–8]. To increase the potency and reduce the toxicity of naturally occurring retinoids, synthetic derivatives have been developed. LGD1069 (bexarotene, Targretin), a synthetic derivative of 9-cis RA, and N-(4-hydroxyphenyl)retinamide (4-HPR, fenretinide), a synthetic derivative of ATRA, have been shown to be more potent and less toxic than their parent compounds. In contrast to 9-cis RA, LGD1069 displays selective binding and activation of the three known RXRs and does not have significant RAR binding or transactivation of RAR-responsive genes [9]. The specific activation of RXRs and not RARs has been shown to reduce the mucocutaneous toxicity typically associated with retinoid treatment in both preclinical and clinical trials [10–12]. Unlike ATRA, 4-HPR appears to induce its inhibitory effects mainly via retinoid receptor-independent mechanisms [13–16]. 4-HPR effectively inhibits the proliferation of breast cancer cells that do not express RARs [13–15]. 4-HPR has very poor affinity to RARs [13]. 4-HPR could induce RAR transcriptional activation and repression in breast cancer cells, but is >100-fold less potent than ATRA [14, 15]. 4-HPR preferentially accumulates in breast tissue, and this may account for its reduced toxicity compared with other retinoids [17–19]. Despite favorable preclinical data, 4-HPR and LGD1069 have shown limited therapeutic efficacy in patients with advanced breast cancer [20, 21].

Mechanisms of retinoid-induced antiproliferation and apoptosis

ATRA

RARS

The main mechanism by which ATRA inhibits the proliferation of breast cancer cells is by inducing G1 cell cycle arrest [22–25]. ER-positive breast cancer cells are sensitive to the growth inhibitory effects of ATRA, while the majority of ER-negative cells are resistant [26, 27]. ER-positive cells respond to ATRA because they express RARα, whereas the majority of ER-negative cells are refractory toward ATRA treatment because they have little or no RARα [27, 28]. Overexpression of RARα in ER-negative MDA-MB-231 cells sensitized the cells to the antiproliferative effects of ATRA [26]. Increased ATRA sensitivity in HER2/neu-overexpressing breast cancer cells treated with the anti-HER2/neu antibody trastuzumab was correlated with increased RARα protein levels and increased RARE binding activity [29, 30].

Retinoids are known to cause changes in the expression of genes in target cells. The RARβ gene may act as a tumor suppressor, and loss of RARβ2 messenger RNA (mRNA) expression may be an important event in tumorigenesis. Reduced RARβ2 mRNA expression has been observed in a number of solid tumor cells, including lung carcinoma [31–33], squamous cell carcinoma of the head and neck [34], and breast cancer [33, 35–38]. RARβ transcription has been shown to be downregulated in breast cancer cell lines and tumors and upregulated in normal mammary epithelial cells [33, 35–38]. Evidence suggests that the inducibility of RARβ expression plays a role in mediating the growth inhibitory effects of retinoids. Growth inhibition in response to ATRA has been associated with RARβ2 mRNA induction, while resistance to ATRA has been associated with a failure in RARβ2 inducibility in lung, stomach and breast cancer cells in vitro [33, 39, 40]. ATRA sensitivity of ER-positive cells was inhibited by an RARβ antagonist and the expression of RARβ antisense [39, 41]. Furthermore, introduction of RARβ in ER-negative cells by stable transfection or through an RARβ expression vector restored their sensitivity to ATRA [33, 39, 42]. ATRA-mediated induction of RARβ has also been demonstrated in vivo; RARβ was induced in 33% of breast cancer patients treated with ATRA for 3 weeks [43].

Cell cycle effects

Alterations in the expression and activity of cell cycle regulators have been associated with breast cancer [44]. Several cell cycle modulators are important in controlling the G1 transition to S phase, including retinoblastoma protein (pRb), cyclins D and E, cyclin-dependent kinases (cdks) 2, 4 and 6, and their inhibitors, p15, p16 and p21. Phosphorylation of the tumor suppressor pRb inactivates its growth suppressive function and is important for the progression from the G1 to the S phase of the cell cycle [22, 45, 46].

Alterations in the expression and activity of cell cycle regulators have been associated with the antiproliferative effects of ATRA in breast cancer cells. Growth inhibition induced by ATRA in breast cancer cells has been correlated with its ability to decrease expression of cyclin D1 and D3 [47–49], the activity of cdk2 and cdk4 [47–50], and the expression and phosphorylation of pRb [47, 49–51]. Cdk inhibitors may also be a target of ATRA. ATRA increased p21 levels, which were associated with decreased cdk2 activity in normal breast epithelial cells [51]. Thus, ATRA appears to induce its antiproliferative effects predominantly by blocking the transition from G1 to S phase.

AP-1

AP-1 is made up of the protooncogenes jun and fos, and its activity is associated with breast cancer cell proliferation and transformation [52]. AP-1 activity is inhibited by ATRA and has been associated with ATRA-mediated growth inhibition in breast cancer cells [53–56]. Inhibition of AP-1 activity by ATRA may involve either direct interaction between ATRA-activated RARs and components of AP-1 or competition for common coactivators, such as cyclic AMP (cAMP) response element-binding protein (CBP) [57, 58]. ATRA has been shown to inhibit AP-1 activation by regulating CBP recruitment in cervical cancer cells [59]. ATRA regulates the expression of CBP and the homologous protein p300 in breast cancer cells and may therefore make these proteins unavailable for AP-1 activation [60].

4-HPR

As with ATRA, induction of gene expression and cell cycle effects have been implicated in 4-HPR-mediated growth inhibition in breast cancer cells. Growth inhibition by 4-HPR has been correlated with the induction of the RARβ2 gene in lung adenocarcinoma cells [40], stomach adenocarcinoma cells [40] and ovarian cancer cells [61, 62]. Whether induction of RARβ represents a mechanism of 4-HPR-induced growth inhibition in breast cancer cells has not been determined. However, in our own unpublished observations we found that 4-HPR did not induce RARβ expression in breast cancer cells. The antiproliferative effects of 4-HPR have been associated with its ability to decrease cyclin D1 expression and cdk2 and cdk4 activity and to induce the dephosphorylation of pRb in breast cancer cells [63]. However, our data suggest that 4-HPR does not affect cell cycle distribution [64]. In contrast to ATRA, induction of apoptosis is an almost uniform response of cancer cells to 4-HPR [65]. We and others have reported that 4-HPR is a potent inducer of apoptosis in breast cancer cells [13, 14, 66, 67]. Caspase activation has been shown to be an integral event in 4-HPR-induced apoptosis [68–70]. ATRA may induce apoptosis in breast cancer cells but only when the cells have been continuously exposed to ATRA for 6 or more days, with replenishment of ATRA in the medium every other day [24]. Despite the quantity of research that has been done to elucidate the mechanisms of 4-HPR-induced apoptosis in cancer cells, these mechanisms are still not well defined. What has become evident from these studies is that the mechanisms of 4-HPR-mediated apoptosis appear to be tissue specific, and furthermore, it appears that multiple mechanisms may operate within specific tissues.

Nitric oxide

Nitric oxide (NO), a free radical produced from L-arginine by nitric oxide synthases (NOS), can be a proapoptotic or an antiapoptotic molecule. In breast cancer cells, NO mainly serves as an antiproliferation and apoptosis inducer [71–75]. The production of NO by NOSII was found to be essential for pharmacologically achievable doses of 4-HPR (1–3 μM) to induce apoptosis in breast cancer cells [64]. Furthermore, NO production may be an important mechanism in 4-HPR-based therapies in breast cancer. One potential mechanism by which tamoxifen and interferon-γ enhance the potency of 4-HPR in breast cancer cells is by increasing NO production [64]. Interferon-γ enhanced 4-HPR-mediated NO production in both ER-positive and ER-negative breast cancer cells, while tamoxifen increased 4-HPR-mediated NO production only in ER-positive cells [64]. Lim et al. [76] reported that cyclosporin A increased the ability of 4-HPR to induce apoptosis in breast cancer cells, and this effect was correlated with increased NO production. Recently, NOSII-mediated NO production was found to be essential for the combination of 4-HPR and trastuzumab to induce apoptosis in HER2/neu-overexpressing breast cancer cells [77]. HER2/neu suppresses 4-HPR-mediated apoptosis in breast cancer cells predominantly by suppressing NO production mediated by NOSII [77].

Ceramide

Elevations in the levels of the sphingolipid ceramide have also been implicated in 4-HPR-induced apoptosis. 4-HPR increases ceramide levels in neuroblastoma cells [78, 79], leukemia cells [80, 81] and prostate cancer cells [82]. The blockage of ceramide synthesis [79, 80] or the addition of agents that block the metabolism of ceramide [82] were shown to reduce and enhance 4-HPR-induced apoptosis, respectively, in leukemia and prostate cancer cells. 4-HPR has also been reported to increase ceramide levels in breast cancer cells, and inhibitors of ceramide metabolism enhanced 4-HPR-induced apoptosis [83].

Other potential mechanisms

Both the generation of reactive oxygen species (ROS) and mitochondrial permeability transition (MPT) have been implicated in 4-HPR-mediated apoptosis in some cancer cell types; however, whether these mechanisms are involved in the proapoptotic effects of 4-HPR in breast cancer cells has not been investigated. The generation of ROS, such as hydrogen peroxide and superoxide, has been linked to 4-HPR-induced apoptosis in a variety of cancer cell types, including cutaneous squamous cell carcinoma [84], leukemia [68, 85, 86], cervical carcinoma [87, 88] and retinoblastoma cells [89]. Exogenous antioxidants have been shown to inhibit 4-HPR-induced ROS production and apoptosis, demonstrating that ROS are critical in mediating apoptosis in these cancer cell types [68, 84–87, 89]. Other types of cancer cells produce ROS, including prostate carcinoma, non-small cell lung carcinoma, and neuroblastoma cells, but it does not

appear to be the main or sole mechanism of apoptotic induction by 4-HPR [78, 90, 91]. ROS production appears to be specific to 4-HPR; other retinoids such as ATRA and 9-cis-RA do not induce ROS [85, 87, 92].

MPT also appears to play a central role in 4-HPR-induced apoptosis in some cancer cells [84, 88]. MPT is the opening of mitochondrial megachannels leading to the disruption of the mitochondrial membrane inner transmembrane potential [93]. In cutaneous squamous carcinoma cell lines, MPT is the main coordinating event of 4-HPR-induced apoptosis [84]. MPT antagonists, such as cyclosporin A, rescued squamous carcinoma cells from the proapoptotic effects of 4-HPR [84]. In contrast, cyclosporin A increased the ability of 4-HPR to induce apoptosis in ER-positive and ER-negative breast cancer cells [76]. The role of MPT in 4-HPR-induced apoptosis in breast cancer cells has not been determined. However, given that the MPT inhibitor cyclosporin A has such markedly different effects on 4-HPR in breast cancer cells and in cutaneous squamous carcinoma cells, MPT may not be involved in 4-HPR's induction of apoptosis in breast cancer cells.

While NO production, ceramide, ROS production and MPT represent separate events in 4-HPR's induction of apoptosis, these events may be interrelated. ROS can activate ceramide, and conversely, ceramide can induce ROS production [94]. NO production can be upstream or downstream of ceramide [94]. MPT can be triggered by ROS or result in ROS production [93]. 4-HPR-induced ROS generation was shown to be required for MPT induction in cervical carcinoma cells [88]. A linkage of 4-HPR's induction of ROS, release of cytochrome c, induction of MPT and activation of caspase 3 has been reported in some cancer cell types, such as cervical carcinoma and leukemia cells [86, 88]. NO-mediated induction of apoptosis in breast cancer cells has been shown to require mitochondrial damage, in particular, cytochrome c release, disruption of mitochondrial transmembrane potential and ROS generation, as well as activation of caspases 1, 3 and 6 [75].

Given that 4-HPR is an analogue of ATRA, some overlap in the mechanisms of action of these two retinoids is expected. In fact, increased NO production and alterations in the levels of growth factors have been implicated in both ATRA and 4-HPR-mediated growth inhibition in breast cancer cells. The antiproliferative effects of ATRA have been correlated with increased NO production in a breast cancer cell line [95]. While we have also observed that ATRA can induce NO production in breast cancer cells, its induction of NO is less potent and of shorter duration than that observed in response to 4-HPR [77].

It has been proposed that retinoids inhibit cell growth by inhibiting the expression of growth-stimulating factors or by inducing the expression of growth inhibitory factors, but whether alterations in growth factors in response to retinoid treatment are a mechanism or a marker of activity remains to be determined. Insulin-like growth factor-I (IGF-I) has been associated with mammary gland development and is a potent stimulator of mammary epithelial cell growth in vitro [96]. Elevated circulating levels of IGF-I have been associated with increased breast cancer risk in premenopausal women [97–101]. IGF-binding proteins (IGFBPs) regulate IGF bioavailibility and IGF-I receptor responsiveness to IGF-I [100]. ATRA-mediated antiproliferative effects were correlated with the increased secretion of the IGFBPs [102]. IGFBP-3 expression is induced by ATRA, and inhibition of IGFBP-3 expression decreased ATRA-induced antiproliferative effects in breast cancer cells [103–106]. 4-HPR has been shown to significantly decrease the levels of IGF-I, IGFBP-4, IGFBP-5 and type I IGF-receptor mRNA in both ER-positive and ER-negative breast cancer cell lines in vitro, and these reductions were associated with 4-HPR-induced growth inhibition [107]. Decreases in IGF-I have been implicated in the chemopreventive effects of 4-HPR in breast cancer patients. Clinically, decreased plasma levels of IGF-I were observed in premenopausal patients with stage I breast cancer in response to a 1-year treatment with 4-HPR [108, 109]. The decline in IGF-I induced by 4-HPR appears to be a durable response, with reductions maintained for up to 5 years in premenopausal women [110].

Transforming growth factor-β (TGF-β) is a potent growth inhibitor of epithelium-derived cells [111]. ATRA has been shown to increase active TGF-β in breast cancer cells; however, a TGF-β antibody was unable to block ATRA-induced antiproliferative effects [51]. TGF-β mRNA levels increased in tumors from breast cancer patients after 3 weeks of ATRA treatment [43]; however, the correlation between ATRA-induced increases in TGF-β mRNA and therapeutic activity was not determined. TGF-β involvement in 4-HPR-induced apoptosis in breast cancer cells was demonstrated by the blockage of 4-HPR-induced apoptosis with neutralizing TGF-β antibodies, the transient transfection of cells with antisense oligomers to TGF-β1 or TGF-βII receptor, and the inhibition of latent TGF-β activation [67]. Furthermore, it has been shown that breast cancer cells defective in TGF-β1 signaling are resistant to 4-HPR-induced apoptosis [67], and TGF-β1 was not increased in cells resistant to 4-HPR [112].

Despite in vitro evidence for the involvement of TGF-β in 4-HPR-induced apoptosis, increases in TGF-β have not been observed in preclinical animal models or clinical trials. This discrepancy may be explained by the fact that TGF-β acts on both the tumor cell and its environment. Despite the growth inhibitory effects of TGF-β on early breast cancer cells, TGF-β predominantly possesses oncogenic activities [113, 114]. TGF-β mRNA has been detected in breast cancer cell lines and tumors, and its ex-

pression has been associated with tumor aggressiveness, increased metastatic potential, increased drug resistance and immunosuppression [115]. Despite demonstrated chemopreventive efficacy, 4-HPR alone or in combination with tamoxifen had no significant impact on TGF-β expression in mammary epithelial or stromal cells in a rat model of mammary tumorigenesis [116]. Plasma concentrations of TGF-β1 were not significantly different in untreated controls or women with stage I breast cancer treated with 4-HPR for 1 year [117] and in women with metastatic breast cancer treated with 4-HPR and tamoxifen for 3 months [118].

9-cis RA and LGD1069

Cell cycle effects

9-cis RA decreased cyclin D1 and D3 expression levels as well as the expression and activity of cdk2 and cdk4 in breast cancer cells; changes in these cell cycle regulators were correlated with the antiproliferative activity of 9-cis RA in vitro [48].

Induction of gene expression

LGD1069 has been shown to increase the expression of adipocyte-related genes both in vitro and in vivo [119]. Expression of aP2, adipsin, Peroxisome Proliferator activated receptor γ (PPARγ) and lipoprotein lipase was higher in LGD1069-responding tumors than in controls or nonresponding rat mammary tumors [119]. Similar changes in gene expression and inhibition in growth were seen in tumor cells exposed to LGD1069 in vitro. These studies demonstrate a correlation between the increased expression of the adipocyte-related genes aP2, adipsin and PPARγ, and the LGD1069-mediated regression of rat mammary carcinomas. Importantly, aP2 protein was also more highly expressed in regressing tumors than in control tumors and was found in the tumor cells as well as in the adipocytes present in the tumor. Agarwal et al. [119] have proposed that LGD1069 causes tumor regression by inducing adipocyte differentiation in the tumor cells, which is followed by terminal cell division and cell death.

Telomerase

Telomerase is expressed in a vast majority of cancer cells, and telomerase activation may play a critical role in tumorigenesis by sustaining cellular immortality [120]. The presence of telomerase has been proposed as a biomarker of breast cancer development and progression [121]. 9-cis RA inhibited telomerase activity in a dose-dependent manner in MCF-7 breast cancer cells, which was correlated with its antiproliferative effects [122]. Reductions in telomerase activity are also a potential mechanism of ATRA- and 4-HPR-mediated growth inhibition in breast cancer cells. ATRA reduced human telomerase reverse transcriptase mRNA expression and subsequent telomerase activity in breast cancer cells, which was correlated with ATRA-mediated growth inhibition [122]. 4-HPR decreased telomerase activity in the bronchial epithelium of cigarette smokers [123, 124]. The suppressed growth of N-methyl-nitrosourea (NMU)-induced mammary tumors by 4-HPR was associated with decreased telomerase activity [125].

Retinoids as chemopreventive agents against breast cancer

4-HPR and LGD1069 hold promise as chemopreventive agents against breast cancer. Results of a phase III clinical trial suggest that 4-HPR may be effective in reducing local recurrent and contralateral breast cancer in premenopausal women [126]. LGD1069 has been shown to be superior to its parent molecule, 9-cis RA, in the prevention of breast cancer in both ER-positive and ER-negative animal models [4, 127]. LGD1069 has demonstrated chemopreventive efficacy in the ER-positive NMU-induced rat mammary tumor model [128, 129] and has chemopreventive effects in ER-negative animal models of breast cancer, including C3(1)-SV40 transgenic mice [12, 130] and mouse mammary tumor virus-erbB2 transgenic mice [131, 132]. The favorable toxicity profiles of these two synthetic retinoids also make them candidates for breast cancer chemoprevention [10–12, 133, 134].

To improve the clinical effectiveness of retinoids in breast cancer chemoprevention, their use in combination therapies has been investigated. The addition of tamoxifen enhanced the chemopreventive effects of 9-cis RA and 4-HPR in breast cancer rodent models [135, 136]. LGD1069 and other RXR-selective retinoids have also been tested in combination with antiestrogens. The combination of LGD1069 with tamoxifen had greater efficacy against the development of NMU-induced mammary tumors than did either agent alone [137]. In addition, mammary tumors that had become resistant to tamoxifen were sensitive to LGD1069 in this rat model [138]. Suh et al. [139] also recently reported that the combination of RXR selective retinoid LG 100268 with the SERM arzoxifene is more effective than either agent alone in preventing mammary tumor development.

The combination of retinoids and interferons has been shown to enhance the inhibition of breast cancer cell growth. Interferon-γ in combination with ATRA or 9-cis RA resulted in a synergistic inhibition of proliferation in ER-positive and ER-negative breast cancer cell lines [140–142]. Interferons have also been shown to enhance the growth inhibitory effects of 4-HPR in vitro [64, 143]. A novel synthetic retinoid 6-[3-(1-adamanty1)]-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437)

has been shown to induce growth arrest and apoptosis in breast cancer cells in vitro [144, 145]. Like 4-HPR, this retinoid appears to induce apoptosis in a retinoid receptor-independent manner in breast cancer cells [146, 147]. CD437-mediated growth arrest and apoptosis in breast cancer cells have been associated with elevated expression of p21 [144, 148], induction of growth arrest and DNA damage-inducible gene 45 (gadd45) [149], activation of caspases [148] and downregulation of Bcl-X_L expression [150]. Recently, a cell cycle and apoptosis regulatory protein (CARP)-1 was identified as a novel mediator of CD437 apoptotic signaling in breast cancer cells [145]. It remains to be determined whether CD437 has breast tumor chemopreventive activity in vivo.

Concluding remarks

Gaining a better understanding of the mechanisms by which retinoids induce their antiproliferative and apoptotic effects should improve the clinical application of these agents in breast cancer chemoprevention. Furthermore, the development of mechanism-based combinations of retinoids and other agents will be critical to improving the efficacy of retinoids as breast cancer chemopreventive agents. Determining the mode of action of retinoids will also help identify appropriate biomarkers with which to measure their efficacy and patients who will best respond to them. The clinical application of retinoids may also be improved by identifying genes and other factors in breast tumors that may decrease their activity. These genes may be used as biomarkers to predict the efficacy of retinoids against breast tumor development and may allow us to utilize retinoids more effectively by combining them with other drugs that can inhibit the function or expression of those particular genes.

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