Loss of Anti-proliferative Effect of All-trans Retinoic Acid in Advanced Stage of Breast Carcinogenesis

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Abstract. Background: Mechanisms by which the inhibitory effect of retinoic acid on tumor growth is attenuated as tumors progress to more advanced stages are unclear. Materials and Methods: This study utilizes a novel cell culture system of human breast epithelial cells (HBEC). Immortal (M13SV1), weakly tumorigenic (M13SV1-R2), and highly tumorigenic (M13SV1-R2N1) transformed Type I HBEC were derived sequentially from the same parental Type I HBEC (stem cells) developed from reduction mammoplasty of healthy women. Effects of all-trans retinoic acid (AT-RA) on the growth, protein expression of RAR-α, β and γ, and RARE transcriptional activation were determined. Results and Conclusion: AT-RA reduces proliferation rates of immortal and weakly tumorigenic cells, but not highly tumorigenic cells. This loss of response of highly tumorigenic cells to AT-RA is associated with overexpression of p185c-erbB2/neu. It is not associated with decreased RAR-α, β or γ expression, or activation by AT-RA; RAR-α, β and γ are expressed and AT-RA increases RARE transcriptional activity in all cell lines tested in this study.

Retinoids, natural and synthetic vitamin A compounds, gained much attention for their anti-tumorigenic effects against cancers including breast cancer (1). Early studies with vitamin A-deficient animals demonstrated a link between vitamin A status and susceptibility to cancer. Retinoic acid, the active metabolite of vitamin A, inhibits proliferation of estrogen receptor-positive (ER⁺) human breast cancer cells, but has no effect on most estrogen receptor-negative (ER⁻) human breast cancer cells (2, 3). Although effective at reversing premalignant lesions, vitamin A, and its derivatives have been unsuccessful as chemotherapeutic agents for advanced stages of cancer (1).

Therefore it is important to understand why advanced tumors are resistant to retinoic acid. Studies have attempted to answer this question by looking at different and unrelated cell lines with different genetic backgrounds. This approach makes it difficult to compare the effect of retinoic acid over different cell lines and to translate in vitro findings to in vivo and clinical studies.

In the present study, we investigated the sensitivity of progressive stages of breast carcinogenesis to all-trans retinoic acid (AT-RA) using a novel system of human breast epithelial cells (HBEC). This cell culture system recapitulates multiple stages of breast carcinogenesis: normal mammary epithelial cells, immortal/non-tumorigenic cells (preneoplastic stage), weakly tumorigenic cells, and highly tumorigenic cells. These HBECs were developed from reduction mammoplasty tissues of healthy women (Type I HBEC with stem cell characteristics and Type II HBEC with basal epithelial cell phenotypes) (4). Type I HBEC express estrogen receptors and are deficient in gap junctional intercellular communication and more susceptible to immortalization and neoplastic transformation. Immortal (M13SV1), weakly tumorigenic (M13SV1-R2), and highly tumorigenic (M13SV1-R2N1) HBEC were derived sequentially from the same parental type I HBEC stem cells, after ectopic expression of SV40 large T-antigen, X-ray irradiation, and expression of c-erbB2/neu oncogene (4-6, for review: 7, 8) (Figure 1).

Materials and Methods

Derivation of in vitro neoplastically transformed HBEC lines from Type I HBEC. Previously Kao et al. (4) and Kang et al. (5) sequentially derived in vitro neoplastically transformed HBEC lines (M13SV1, M13SV1-R2, M13SV1-N6, and M13SV1-R2N1) from Type I HBEC (for review: 7, 8).
**Culture of in vitro neoplastically transformed tumorigenic Type I HBEC.** The MSU-1 medium with supplements (called ‘MSU-1+S medium’ hereafter) was prepared as described previously (4). All transformed Type I HBEC lines (M13SV1, M13SV1-R2, M13SV1-N6, and M13SV1-R2N1) were grown in ‘MSU-1+S medium’ with 5% FBS throughout (4). Cells were grown in 5% CO₂ cell culture incubator at 37°C.

**Assessment of cell proliferation.** Cells were seeded at 3.4×10⁴ cells per well in 6-well plate 24 h prior to the treatment with AT-RA (Sigma-Aldrich, Inc., St. Louis, MO, USA). Fresh ‘MSU-1+S medium’ with 5% FBS and AT-RA treatments were renewed after 3 and 6 days. Well in 6-well plate 24 h prior to the treatment with AT-RA (Sigma-Aldrich, Inc., St. Louis, MO, USA). Fresh ‘MSU-1+S medium’ with 5% FBS and AT-RA treatments were renewed after 3 and 6 days.

**Isolation and quantitation of cellular protein.** Cells were seeded at 3.9×10⁵ cells per 100 mm dish 24 h prior to the treatment with AT-RA. After treatment with AT-RA for 3 days, cells were lysed in 20 mM Tris containing 450 mM NaCl, 10 mM Na₂HPO₄, 100 μM Na₃VO₄, 100 μM ammonium molybdate, 10% glycerol, 1% Igepal CA-630 (NP-40), 1 mM PMFS, 10 mM DTT, 1% protease inhibitor cocktail (Sigma-Aldrich Inc.). Cellular protein was quantified with Coomassie Plus protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA).

**Immunoblot analysis of retinoic acid receptors-α, β and γ (RARα, RARβ, RARγ), p185 c-erbB2/neu, and ERK1/ERK2.** Protein samples were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes at 30 V overnight. The blots were incubated with appropriate primary antibodies (RAR-α β γ, p185 c-erbB2/neu, and phospho-tyrosine (PY99) from Santa Cruz Biotechnologies, Inc. Santa Cruz, CA, USA; ERK1/ERK2 from Cell Signaling/New England Biolabs, Beverly, MA, USA) followed by secondary antibodies (all were with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Cell Signaling), except phospho-tyrosine with mouse secondary antibody (Cell Signaling). Immunocomplexes were visualized with Supersignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Inc.) and exposed to X-ray film for 1-10 min.

**Luciferase assay.** Cells were seeded at 2.8×10⁴/cm² in either 60 mm dish or 6-well plate and cultured for 24 h to 30 h. Then cells were transfected with tk-RARE-luciferase (a gift from Dr. David Mangelsdorf, The University of Texas Southwestern Medical Center at Dallas) for 12 h using calcium phosphate. The media were removed and cultured with AT-RA for 2 days (0, 1, 10, 100 nM, and 1 μM) and retinoic acid receptor element (RARE) transactivation activity was determined using the Steady-Glo luciferase assay (Promega Co., Madison, WI, USA).

**Statistical analysis.** Data were analyzed by two-way ANOVA with Bonferroni’s method for multiple comparisons. Differences were considered significant at p<0.05.

**Results**

**Anti-proliferative effect of all-trans retinoic acid (AT-RA) is lost in highly tumorigenic transformed Type I HBEC (M13SV1-R2N1).** Total nucleic acids in control cultures of immortal cells (M13SV1) increased 3-fold in 3 days, 8.5-fold in 6 days, and 15-fold in 8 days. In comparison, control cultures of weakly tumorigenic cells (M13SV1-R2) and highly tumorigenic cells (M13SV1-R2N1) increased 5-fold in 3 days, 22- to 23-fold in 6 days, 45- to 47-fold in 8 days, indicating that both weakly and highly tumorigenic cells proliferate faster than immortal cells. AT-RA at 1 nM, 10 nM, 100nM, and 1 μM significantly reduced proliferation of immortal cells (Figure 1A) and weakly tumorigenic cells (Figure 1B), but had little effect on the growth of highly tumorigenic cells (Figure 1C) at days 6 and 8. Specifically, AT-RA at 10 nM, 100 nM, or 1 μM significantly reduced total nucleic acids at day 8 by approximately 30% (p<0.05), compared to the corresponding controls in both immortal cells (Figure 1A) and weakly tumorigenic cells (Figure 1B). In contrast, AT-RA at 10 nM, 100 nM, or 1 μM reduced total nucleic acids at day 8 by approximately 6% compared to the corresponding controls in highly tumorigenic cells (Figure 1C).

*Overexpression of c-erbB2/neu attenuates the anti-proliferative effect of all-trans retinoic acid (AT-RA).* As shown in Figure 2, highly tumorigenic cells overexpress tyrosine phosphorylated p185 c-erbB2/neu. To determine whether the resistance to AT-RA was a feature of the highly tumorigenic state or resulted from c-erbB2 overexpression per se, we measured the effect of AT-RA on proliferation of M13SV1-N6 cells. This cell line (M13SV1-N6) is established by transfecting immortal cells (M13SV1) with c-erbB2/neu, but it is not tumorigenic (5). As seen in Figure 1D, the M13SV1-N6 cell line is resistant to the anti-proliferative effect of AT-RA.

*Expression and activation of retinoic acid receptors-α, β, γ (RARα, RARβ, RARγ) in immortal (M13SV1), weakly tumorigenic (M13SV1-R2), and highly tumorigenic (M13SV1-R2N1) transformed Type I HBEC.** In order to determine whether RAR-α, β and γ expression and/or functionality is responsible for loss of the anti-proliferative effect of AT-RA on highly tumorigenic cells, the three different transformed cells (immortal cells, weakly tumorigenic cells, and highly tumorigenic cells) were treated with 1μM AT-RA for 3 days and RAR-α, β and γ protein expression was measured by immunoblotting. All cell lines expressed RARα, RARβ, and RARγ, and AT-RA had little effect on RAR protein levels (Figure 3). This indicates that resistance to the anti-proliferative effects of AT-RA in highly tumorigenic cells is not associated with silencing of RAR expression.

The ability of AT-RA to transactivate RARs was tested by transfecting cells with retinoic acid receptor element (RARE)-luciferase reporter. As shown in Figure 4, treatments with AT-RA at 1, 10, 100 nM, and 1 μM for 2 days increased RARE-luciferase activity in all cell lines. This suggests that the loss of general RAR activity did not cause the resistance to the anti-proliferative action of AT-RA.
Discussion

This study systematically examined the anti-proliferative activity of AT-RA during breast carcinogenesis using a novel cell culture model of human breast epithelial cells (HBEC). In the current study, AT-RA at 1 nM, 10 nM, 100 nM, and 1 μM inhibited the growth of immortal (M13SV1) cells and weakly tumorigenic (M13SV1-R2) cells; whereas it had little effect on highly tumorigenic transformed (M13SV1-R2N1) type I HBEC. This loss of response of highly tumorigenic cells to AT-RA is associated with overexpression of p185c-erbB2/neu. This was confirmed by the observation that AT-RA had little effect on the growth of M13SV1-N6 cells, a line of immortalized with overexpression of p185c-erbB2/neu, but not tumorigenic cells.

The human epidermal growth factor-2 (HER2, also known as c-erbB2, neu, and NGL) oncogene is amplified and overexpressed in 20 to 30% of invasive breast carcinomas (11, 12). Overexpression of HER2 in human breast carcinomas has been associated with increased metastatic potential and a lower survival rate (12, 13). The HER2/c-erbB2/neu gene encodes a 185-kDa transmembrane glycoprotein that is a
member of the epidermal growth factor (EGF)/erbB receptor family. The current finding suggests that AT-RA will not be efficient for treating breast tumors overexpressing c-erbB2.

Mechanisms by which anti-proliferative activity of AT-RA is lost in HER2/c-erbB2-overexpressing transformed type I HBEC are unclear. Tari et al. (14) demonstrated that HER2/c-erbB2 interferes with the growth inhibitory effects of AT-RA based on the following findings: AT-RA inhibited the growth of MCF7 (ER+, and expressing low levels of c-erbB2) breast cancer cells, but did not inhibit the growth of MCF7 cells transfected with HER2 or its ligand neuregulin. Furthermore, when MDA-MB453 (ER–) and BT474 (ER+) breast cancer cells, which overexpress HER2/c-erbB2 and are resistant to growth inhibitory effects of AT-RA, were incubated with an inhibitor of HER2 pathway trastuzumab (Herceptin™) antibody, the growth was inhibited by AT-RA. When these AT-RA resistant cells were treated with trastuzumab (Herceptin™), RARα protein levels were higher in MDA-MB453 and BT474 cells. The growth of these AT-RA resistant cells was also inhibited by AT-RA when the expression of GRB2 and AKT were down-regulated by liposome-incorporated antisense oligonucleotides targeting GRB2 and a dominant negative AKT mutant. This suggested that HER2 utilizes GRB2 and AKT proteins to restore the anti-proliferative effects of AT-RA in the AT-RA-resistant cells and RARα protein levels are associated with AT-RA resistance.

In contrast to the finding of Tari et al. (14), the association of RARα with the loss of anti-proliferative effect of AT-RA, we found that RARα, RARβ, and RARγ levels were not significantly different in immortal, weakly tumorigenic, and highly tumorigenic transformed Type I HBEC. Moreover, AT-RA at 1 μM increased RARE transcriptional activity in all transformed type I HBEC tested in this study. This suggests that the loss of growth inhibitory effects of AT-RA in highly tumorigenic cells is not associated with RAR expression and/or activation by AT-RA. This discrepancy in roles of RARα between the current study and that of Tari et al. (14) may be due to differences in the origin of cells, biological characteristics, and gene expression profiling of the cells. Tari et al. (14) used MCF7 (ER– and low c-erbB2 expression), MDA-MB453 (ER– and c-erbB2 overexpression), and BT474 (ER+ and c-erbB2 overexpression) breast cancer cells, which were tumor-derived cells from unrelated and different breast cancer patients with disparate genetic backgrounds. In contrast, transformed Type I HBEC lines we used were derived from the same parental normal human stem cells (ER+) developed from reduction mammoplasty tissues of healthy women. Therefore, our current approach provides an advantage to systemically determine where in the stages of breast carcinogenesis the loss of anti-proliferative activity of AT-RA occurs at cellular levels.

An important common finding in both Tari et al. (14) and the current study is that anti-proliferative activity of AT-RA observed in ER+ and low c-erbB2 expressing human breast tumorigenic cells is lost in ER+ and c-erbB2-overexpressing human breast tumorigenic cells. In general, retinoic acid inhibits the growth of ER+ breast cancer cells; but has no effect on most ER– human breast cancer cells. ER status is a critical parameter in breast cancer because ER+ breast tumors have a better prognosis than ER– breast tumors (15). When breast tumors were subclassified into categories with distinct biological and clinical properties based on gene expression profiles, the strongest distinction was found...
between ER+ and ER- tumors (16-18). In particular, Perou et al. (16) reported that the tumors in the ER+ group were defined by the relatively high expression of many genes expressed by breast luminal cells. With one exception, the tumors in this ER+ group expressed low levels of c-erbB2 (16). Studies showed that most erbB2-amplified tumors are ER- and resistant to hormone therapy (19, 20).

Other factors contributing to different gene expression profiles for subtypes in breast cancer include: histological grade, lymph node status, c-erb2 (HER-2/neu) gene amplification, p53 mutational status, inflammatory breast cancer, and carcinoma-derived stromal signatures (17, 18, 21-34). Among these factors, c-erbB2 overexpression is associated with overexpression of genes involved in proliferation and undifferentiated stem cells and lack of expression of genes involved in hormone receptor-signaling pathway (12, 16, 22, 35).

In conclusion, we evaluated anti-proliferative effects of AT-RA in HBEC transformed at different stages of breast carcinogenesis representing immortalization, early tumorigenesis, and advanced tumorigenesis as a cell culture model. Our findings show that the overexpression of HER2/c-erbB2 causes resistance of highly tumorigenic cells to growth inhibitory effects of AT-RA. This resistance of highly tumorigenic cells to AT-RA is not associated with alterations in RAR expressions or the ability of RAR to be transactivated. The current study adds evidence that AT-RA is not an efficient chemotherapeutic agent against tumors overexpressing HER2/c-erbB2.

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References