Cellular Metabolism of Estradiol in Models for Select Molecular Subtypes of Clinical Breast Cancer

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Abstract

The mitogenic ovarian steroid hormone 17β-estradiol (E2) is associated with progression of Estrogen Receptor Positive (ER+) breast cancer, and ER represents a major therapeutic target for endocrine therapy. In addition to the ER dependent signal transduction, cellular oxidative metabolism of the ER ligand E2 generates several metabolites with pleotropic growth modulatory effects on breast cancer cells, providing valuable leads for novel therapeutic approach. The molecular classification of clinical breast cancer has defined cancer subtypes based on differential expression of the genes for hormones and growth factor receptors, thereby facilitating subtype targeted therapeutic interventions. However, de novo or acquired resistance to conventional endocrine and targeted small molecule based treatment limits the therapeutic efficacy and promotes resistance disease progression. These aspects emphasize the need for identification of new efficacious non-toxic lead compounds. The present review summarizes critical experiments conducted to i) develop and optimize human tissue derived cell culture models for select molecular subtypes of clinical breast cancer, ii) determine the status of homeostatic growth control, cellular metabolism of 17β-estradiol (E2) and cancer risk in the developed models, and iii) evaluate the therapeutic efficacy and identify possible mechanisms of action of select herbal extracts/phyto-chemical. Additionally, this review discusses the evidence for the role of E2 metabolism in breast carcinogenesis and therapy. The data generated from the cell culture experiments demonstrate that the models for select molecular sub-types exhibit aberrant hyper-proliferation, altered cellular metabolism of E2 and enhanced cancer risk. Select mechanistically distinct herbal extracts and natural phyto-chemical at their respective maximum cytostatic concentrations modulate cellular metabolism of E2 favoring generation of anti-proliferative metabolites and inhibit anchorage independent growth, thus reducing cancer risk. Collectively, these data validate the present mechanism based cell culture approach to identify and prioritize novel efficacious lead compounds for subtype targeted therapy of clinical breast cancer.

Keywords: Breast cancer models; Estradiol metabolism; Therapeutic target

Introduction

The ovarian steroid hormone 17β-estradiol (E2) represents a major hormone involved in promotion of breast cancer. E2 functions as a physiological ligand for Estrogen Receptor (ER), a well-established nuclear transcription factor that via a complex signaling cascade drives the expression of several E2 responsive genes, promoting proliferation of normal and tumor derived breast epithelial cells. In addition to the ER dependent biological functions of E2, cellular Cyp450 mediated oxidative metabolism of E2 is involved in various biological functions including normal mammary cell proliferation and modulation of breast cancer growth [1,2].

Global gene expression profiling based on differential expression of hormone and growth factor receptor genes has classified the molecular subtypes of clinical breast cancer, and thereby, has provided important molecular insights in breast cancer pathogenesis and identified mechanism based, subtype targeted therapeutic interventions [3].

Conventional targeted therapy for hormone responsive breast cancer includes selective estrogen receptor modulators and aromatase inhibitors for the Luminal A subtype, and small molecule inhibitors of growth factor function together with the anti-estrogens for the Luminal B subtype. HER-2 targeted therapy represents the treatment of choice for the endocrine resistant HER-2 enriched subtype, while the triple negative subtype responds to anthracyline/taxol based chemotherapy and to PARP inhibitors [4-8]. However, long-term treatment with conventional chemo-endocrine therapeutics as well as with newer targeted therapeutics is frequently associated with de novo or acquired resistance that limits the efficacy of therapeutic intervention, in part due to progression of drug resistant cancer stem cells [4,7,9]. These aspects emphasize a need to identify and evaluate promising novel lead compounds for their efficacy on select molecular subtypes of clinical breast cancer.

Experimental Models, Biomarkers and Test Agents

Models

The human mammary carcinoma derived MCF-7 cells express the receptors for Estrogen (ER) and Progesterone (PR) but lack the expression of Human Epidermal Growth Factor-2 (HER-2). The MCF-7 cells represented the model for the Luminal A molecular...
subtype of clinical breast cancer [10]. The human mammary epithelial cell line 184-B5/HER is stably transfected with HER-2 oncogene and unlike the parental 184-B5 cells, exhibits tumorigenic transformation [11]. This cell line represented a model for the HER-2 enriched molecular subtype of clinical breast cancer. The human mammary carcinoma derived MDA-MB-231 cell line lacks the expression of ER, PR and HER-2 [10,12]. This cell line represented the model for the triple negative molecular subtype of clinical breast cancer.

**Biomarkers**

Population doubling time and saturation density represented the quantitative endpoint biomarkers for the status of homeostatic growth control. Estrogen metabolite ratios represented the biomarkers for endocrine responsiveness. Anchorage independent (AI) growth in vitro and tumor development in vivo represented the quantitative endpoint biomarkers for the risk for cancer development.

The cellular metabolism of \( E_2 \) was evaluated by determining the formation of select metabolites including Estrone (\( E_1 \)), 2-hydroxyestrone (2-OHE\(_1\)), 16a-hydroxyestrone (16α-OHE\(_2\)) and estriol (\( E_3 \)), using stable isotope dilution and GC-MS analysis or tritium exchange assay [13]. Since \( E_2 \) functions as the common precursor for 2-OHE\(_1\) and 16α-OHE\(_2\), and since 16α-OHE\(_1\) is converted to \( E_3 \) [2,13], the data on the cellular metabolism of \( E_2 \) is expressed as 2-OHE\(_1\):16α-OHE\(_2\) and \( E_3 \):16α-OHE\(_2\) ratios.

**Test Agents**

Non-fractionated herbal extracts were prepared from the bark of Lycium barbarum (LBB) and the fruit of Lycium barbarum (LBF), from the fruit of Cornus officinalis (CO), and from the leaf and stem of Epimedium grandiflorum (EG) according to the published protocol [14,15]. The naturally occurring phyto-chemical Indole-3-Carbinol (I3C, Sigma Chemical Co., St. Louis, MO) was solubilized in Dimethyl Sulfoxide (DMSO). Initial dose response experiments using the herbal extracts and I3C identified the respective maximum cytostatic concentrations for individual test agent. Each test agent at its respective maximum cytostatic concentration was used for the experiments on \( E_2 \) metabolism and AI growth.

Status of homeostatic growth control and risk for cancer development: The data presented in Table 1 compares the modulation in population doubling time, saturation density, \( E_2 \) metabolism, AI growth and tumor development in the models for select molecular subtypes of clinical breast cancer. Relative to the non-tumorigenic 184-B5 cells the models for luminal A, HER-2 enriched and triple negative subtypes exhibited about a 55.6% decrease in population doubling times, and a 19.3 to 47.3% increase in saturation density. Overall, these data are consistent with the published data on aberrant hyper-proliferation, accelerated cell cycle progression, down-regulated cellular apoptosis and enhanced cancer risk in oncogene transfected mouse and human mammary epithelial cell culture models, as well as in human mammary carcinoma derived cell culture models [16].

The data on the \( E_2 \) metabolite ratio demonstrated that relative to the non-tumorigenic 184-B5 cells, the models for the three molecular subtypes exhibited an 85.7%, 88.1% and 90.5% decrease in the 2-OHE\(_1\):16α-OHE\(_2\) ratio. This down-regulation was predominantly due to increased formation of 16α-OHE\(_2\). In this context it is noteworthy that similar alteration in \( E_2 \) metabolism has been documented in mammary epithelial cells that have been initiated for tumorigenic transformation using chemical carcinogens, retrovirus or oncogenes [17].

| Biomarker End Point                  | Cell Culture Model | 184-B5 | MCF-7 | 184-B5/HER | MDA-MB-231 |
|-------------------------------------|-------------------|--------|-------|------------|------------|
| Population Doubling (hr.)\(^a\)     | 34                | 15.2   | 15    | 15         | 15         |
| Saturation Density (x10\(^6\))\(^b\)| 22.3 ± 1.2        | 26.6 ± 1.7 | 32.8 ± 1.5 | 32.9 ± 2.3 |
| 2-OHE\(_1\):16α-OHE\(_2\) Ratio\(^c\)| 4.2 ± 0.3        | 0.8 ± 0.2 | 0.5 ± 0.1 | 0.4 ± 0.2 |
| AI Colony Formation\(^d\)           | 0/18              | 18/18  | 18/18 | 18/18      |            |
| Mean Colony Number                  | 30.9 ± 2.4        | 23.0 ± 2.6 | 38.9 ± 1.6 |            |
| Tumor Formation\(^e\)               | 0/10              | 10-Oct | 10-Oct | 10-Oct     |            |
| Latency                             | 24 weeks          | 3-5 weeks | 3-5 weeks | 3-5 weeks |

**Table 1:** Homeostatic Growth Control and Cancer Risk in Cell Culture Models for Molecular Subtypes of Clinical Breast Cancer. \(^a\)Determined from the exponential growth phase. \(^b\)Determined at day 7 post-seeding of \( 1.0 \times 10^5 \) cells. \(^c\)Determined at day 3 post-seeding of \( 3.0 \times 10^6 \) cells. \(^d\)Anchorage Independent (AI) colonies counted at day 21 post-seeding of 1,000 cells. \(^e\)Palpable tumors after sub-cutaneous transplantation.

It is also noteworthy that the formation of 16α-OHE\(_2\) is up-regulated in patients with breast and endometrial cancer [18-20], and that the human breast organoids of intact Terminal Duct Lobular Units (TDLU) obtained from the non-involved areas of breast cancer patients exhibit a higher extent of 16α-OHE\(_2\) formation relative to the TDLU obtained from the patients undergoing reduction mammoplasty [21,22]. Collectively, these observations suggest that altered cellular metabolism of \( E_2 \) may represent a cause and/or consequence of tumorigenic transformation in \( E_2 \) responsive target
cells, and more importantly, suggest a potential “field effect” for the relative risk for breast cancer progression.

Furthermore, unlike the 184-B5 cells the three models exhibited a 23.0 to 38.9 fold increase in the number of AI colonies in vitro, and retained their tumorigenic potential in vivo as evidenced by a 100% tumor incidence with a latency of 3-5 weeks, thus demonstrating that AI colony formation represents an in vitro surrogate end point biomarker for cancer risk. These data from the three models demonstrate cancer cell phenotype specific expression of the end point biomarkers, thereby suggesting that the models for the three molecular subtypes of clinical breast cancer have lost homeostatic growth control, have altered cellular metabolism of E$_2$, and have retained the risk of developing cancer.

**Modulation of end point biomarkers by herbal extracts**

The data presented in Table 2 compares the status of E$_2$ metabolite ratio and AI growth in response to treatment with herbal extracts in the model for Luminal A molecular subtype. The 2-OHE$_1$: 16α-OHE$_2$ ratio was increased by 3.7 fold, 12 fold and 16 fold respectively in response to treatment with EG, LBB and CO. In addition, the E$_2$: 16α-OHE$_2$ ratio was increased by about 25 fold in response to treatment with LBF and LBB. These data suggest that EG and CO function predominantly via favoring the formation of anti-proliferative 2-OHE$_1$ [14,23]. On the other hand, LBB and LBF function via increased formation of 2-OHE$_1$ as well as via accelerated conversion of the pro-mitogenic 16α-OHE$_2$ to relatively inert E$_2$ [15].

| Model: MCF-7 | End Point Biomarker | Treatment | Concentration | 2-OHE$_1$: 16α-OHE$_2$ | E$_2$: 16α-OHE$_2$ | Al Colonies |
|--------------|---------------------|-----------|---------------|----------------------|------------------|-------------|
| E$_2$        | 20 nM               | 0.4 ± 0.1$^d$ | 0.1 ± 0.1$^h$ | 36.0 ± 1.5$^b$      |                  |
| E$_2$+LBB    | 0.05%               | 5.2 ± 1.3$^a$ | 2.3 ± 0.3$^i$ | 2.5 ± 1.0$^f$       |                  |
| E$_2$+LBF    | 1.0%                | 0.1 ± 0.09  | 2.6 ± 0.3$^i$ | 4.8 ± 1.2$^m$       |                  |
| E$_2$+CO     | 0.1%                | 6.8 ± 1.7$^f$ | 0.4 ± 0.1     | 4.0 ± 1.2$^b$       |                  |
| E$_2$+EG     | 1.0%                | 1.9 ± 0.5$^g$ | 0.8 ± 0.2     | 4.9 ± 1.5$^p$       |                  |

**Table 2: Modulation of End Point Biomarkers in Model for Luminal A Molecular Subtype of Breast Cancer.**

E$_2$ is a well-established physiological ligand for ER, a ligand activated nuclear transcription factor that dictates cellular metabolism and represents a therapeutic target for selective estrogen receptor modulators [4]. In addition to ER dependent functions, cellular metabolism of E$_2$ impacts on cell proliferation [2]. Thus, experimental modulation of E$_2$ metabolic profile may provide novel mechanistic leads to identify potential therapeutic targets. In this context it is noteworthy that E$_2$ metabolites exhibit pleotropic growth modulatory effects. Thus, 2-OHE$_2$, 2-OHE$_1$ and E$_2$ inhibit cellular proliferation, while 4-OHE$_1$ and 16α-OHE$_1$ are pro-mitogenic and induce DNA adduct formation and/or DNA damage/repair in mammary epithelial cells, as well as in tumor derived epithelial cells [2,24-27].

Treatment with the herbal extracts resulted in an 87% to a 93% reduction in the number of AI colonies. The observed reduction in the number of AI colonies in response to treatment with herbal extracts provides evidence for the efficacy of these extracts towards reduction of breast cancer risk [14,15,23].

Since long-term treatment with estrogen receptor modulators or aromatase inhibitors frequently gives rise to therapy resistant tumors [4,5], the present data provide potential clinically translatable leads that the herbal extracts from LBB, LBF, CO and EG may be efficacious for endocrine therapy resistant clinical breast cancer. Furthermore, the present experimental approach may also facilitate identification and prioritization of additional new herbal extracts with potential efficacy for endocrine therapy resistant clinical breast cancer.

**Modulation of end point biomarkers by Indole-3-Carbinol (I3C)**

The data presented in Table 3 examines the effect of I3C on the model for HER-2 enriched clinical breast cancer. I3C at its maximum cytostatic concentration induced an 18.4 fold increase in the 2-OHE$_1$: 16α-OHE$_2$ ratio, and a 55.1% decrease in the number of AI colonies.

| Model: 184-B5/HER | End Point Biomarker | Treatment | Concentration | 2-OHE$_1$: 16α-OHE$_2$ | E$_2$: 16α-OHE$_2$ | Al Colonies |
|-------------------|---------------------|-----------|---------------|----------------------|------------------|-------------|
| DMSO              | 0.1%                | 0.5 ± 0.2$^d$ | 20.5 ± 1.4$^f$ |                  |
| I3C               | 50 μM               | 9.7 ± 2.1$^a$ | 9.2 ± 3.3$^p$ |                  |

**Table 3: Modulation of End Point Biomarkers in Model for HER-2 Enriched Molecular Subtype of Breast Cancer.**

The present data provide potential data points to understand how herbal extracts may have potential efficacy in breast cancer prevention and treatment.
The data presented in Table 4 examines the effects of I3C in the model for triple negative clinical breast cancer. In response to the treatment with I3C at its maximum cytostatic concentrations, the MDA-MB-231 cells exhibited a 5 fold increase in the 2-OHE1: 16α-OHE1 ratio. In addition, I3C treatment induced a 62.2% decrease in the number of AI colonies. These data on the models for HER-2 enriched and the triple negative breast cancer essentially confirm and extend the previously published data [28] that demonstrate the anti-proliferative effects of I3C via cell cycle regulation and induction of cellular apoptosis. In this context it is noteworthy that I3C has been demonstrated to cooperate with the selective estrogen receptor modulator tamoxifen to induce cell cycle arrest in MCF-7 cells [29] and operates via inhibition of CDK-6 inducing G1 arrest in human breast cancer cells independent of ER signaling [30].

| Model: MDA-MB-231 | End Point Biomarker |
|--------------------|---------------------|
| Treatmenta | Concentration 2-OHE1: 16α-OHE1, Al Coloniesb |
| DMSO | 0.1% | 0.4 ± 0.1a | 23.8 ± 2.2a |
| I3C | 50 μM | 2.4 ± 0.3e | 9.0 ± 1.2f |

Table 4: Modulation of End Point Biomarkers in Model for Triple Negative Breast Cancer. aI3C at its maximum cytostatic concentration. bConcentration of individual E2 metabolites determined by the tritium exchange assay after a 48 hr treatment. Mean± SD, N=3 per treatment group. Data analyzed by the 2-sample t test comparing the data from the DMSO group (Control). cAnchorage independent (AI) colony counts at day 21 post-seeding of 1,000 cell per well of six well plates. Mean±SD, N=18 per treatment group. Data analyzed by the 2-sample t test comparing the data from the DMSO group (Control). dP=0.001. eP=0.01.

Effective up-regulation of the E1 metabolite ratio and inhibition of AI colony number by I3C in the models for HER-2 enriched and triple negative breast cancer together provide evidence for the efficacy of the natural phyto-chemical I3C independent of the status of ER, PR and/or HER-2 expression. Since long-term treatment with HER-2 targeted small molecule inhibitors or cytotoxic chemotherapeutic agents is associated with progression of therapy resistant tumors [4-8], the present data provides potential leads for the efficacy of I3C in HER-2 enriched and triple negative molecular subtypes of clinical breast cancer.

Conclusions

The data summarized in the present review permits the following conclusions.

- The cell culture models for select molecular subtypes of clinical breast cancer exhibit loss of homeostatic growth control, altered cellular metabolism of E2 and enhanced cancer risk.
- E2 metabolite ratio may represent a novel quantifiable surrogate end point biomarker for endocrine responsiveness of the cancer cell phenotype.
- Non-toxic herbal extracts and mechanistically distinct natural phyto-chemicals may represent promising alternatives for the treatment of chemo-endocrine therapy resistant clinical breast cancer.

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