Endocrine Response Phenotypes Are Altered by Charcoal-Stripped Serum Variability

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Charcoal-stripped bovine serum (CSS) is a critical reagent in the study of steroid hormones. However, CSS has high lot-to-lot variability, including residual growth factor and steroid hormone content. Assessing and reporting this variability is challenging but may affect experimental outcomes and data reproducibility. We hypothesized that CSS lot variability would affect endocrine response phenotypes in breast cancer cells, and we tested the effects of five individual CSS lots on endocrine response in MCF-7 and MDA MB 134VI (MM134) cells. Based on the effects of antiestrogens on MCF-7 cell proliferation, we defined CSS lots as having complete vs partial hormone deprivation. In partial deprivation CSS, the absolute effects of residual estrogens on cell proliferation were modest, but these effects masked the partial agonist activity of 4-hydroxytamoxifen in MM134 cells. Importantly, this effectively reversed the interpretation of tamoxifen-resistance in MM134 cells. Variable effects of CSS lots on endocrine resistance phenotypes were also observed in MCF-7 cells. In this context, we observed that partial vs complete deprivation CSS allowed for the development of unique early endocrine resistance phenotypes that correlated with the presence or absence of residual estrogenic hormones. We evaluated the methods of CSS preparation and identified factors contributing to the extent of hormone deprivation. Our observations suggest that CSS lot-to-lot variability has substantial effects on endocrine response phenotypes and that this ubiquitous factor in study methodology may confound reproducibility. Renewed vigilance in testing and reporting CSS phenotypes will greatly aid in interpreting and reproducing endocrine response and resistance data by the community. (Endocrinology 157: 3760–3766, 2016)

Modeling the effects of steroid hormones in cell culture is subject to the effects of residual exogenous estrogens and estrogen-like compounds. The archetype of these effects is phenol red, which confounded the study of estrogens in breast cancer models until components of phenol red were found to act as estrogen receptor (ER) agonists (1). ER agonists are also pervasive in the cell culture plastics, from liquid handling plastics (2, 3) to dishes and plates used to culture cells (4), with substantial intermanufacturer variability. Another underlying source of exogenous estrogens is the hormone-depleted bovine serum used to supplement medium, ie, dextran-treated charcoal stripped serum (CSS). Charcoal treatment of serum depletes a wide range of peptides and small molecules, including lipophilic molecules like steroid hormones, eg, androgens and estrogens. After short-term maintenance of cells in medium containing CSS (ie, hormone deprivation), exogenous hormones can be applied, and phenotypes can be assessed after treatment. However, CSS is an agricultural product with the potential for high lot-to-lot variability, stemming from the source material and from charcoal-stripping methods. Fetal bovine serum (FBS) or calf serum typically serve as the source material, but these have broadly variable hormone and growth factor content (5) and may require different extents of charcoal stripping to adequately deplete steroid hormones. Variability in the

Abbreviations: CSS, charcoal-stripped bovine serum; E2, estradiol; ER, estrogen receptor; FBS, fetal bovine serum; ICI, ICI 182,780 or fulvestrant; MM134, MDA MB 134VI; 4OHT, 4-hydroxytamoxifen;
source material is compounded by differences in charcoal preparation (eg, grade, washing), serum processing (eg, sulfatase treatment), and specific stripping protocols (eg, temperature, time) that vary across institutions and commercial sources, are not standardized, and are frequently proprietary. These factors lead to extensive variability in steroid hormone and growth factor content of CSS, and, coupled with challenges in reporting these factors in study methodology, this has the potential to affect experimental outcomes and reproducibility. We recently experienced problems with repeating well-established experiments while testing a new serum purchase and further explored the potential for lot variability to affect endocrine response phenotypes.

Materials and Methods

Cell culture

MCF-7 cells were obtained from the Georgetown University-Lombardi Comprehensive Cancer Center Shared Tissue Resource and were routinely maintained in DMEM/Gibco; 11965) + 10% FBS (Gibco; 26140). MDA MB 134VI (MM134; American Type Culture Collection) was maintained in equal parts DMEM/L-15 (Gibco; 11415) + 10% FBS. Cell lines are authenticated annually by PCR restriction fragment length polymorphism analyses and confirmed to be mycoplasma negative. Cells were hormone deprived in Improved minimum essential medium (Gibco; A10488) + 10% CSS (FBS derived CSS, referred to as CSS below) by washing twice daily with serum-free Improved minimum essential medium for 3 days prior to plating (6). Five individual CSS lots from two commercial sources were assessed (Gibco, 12676; Hyclone, SH30068); lot information is available on request but has been omitted to remove potential bias regarding the supplier.

17β-Estradiol (E2) and 4-hydroxytamoxifen (4OHT) were obtained from Sigma; ICI 182780 (ICI; fulvestrant) was obtained from Tocris Biosciences. All compounds were dissolved in ethanol, and vehicle treatments are using 0.1% EtOH.

Cell proliferation assays

Cellular proliferation assays used the FluoReporter dsDNA quantitation kit (Thermo Fisher) as described (6). Points/bars represent the mean of five to six biological replicates ± SD.

Early endocrine resistance phenotypes assay

MCF-7 cells were hormone depleted as above in each of four CSS lots and plated at 75 k/well in a 12-well plate in triplicate; medium was changed every 3–4 days. After 2 weeks, cells were imaged using an inverted microscope. Twenty-one days after beginning hormone deprivation, cell phenotypes were assessed. Cellular metabolism was assessed using Alamar Blue (Thermo Scientific) per the manufacturer’s instructions. From the same samples, DNA/RNA were harvested using the Allprep Mini kit (QIAGEN) according to the manufacturer’s instructions; crude protein was collected by acetone precipitation of DNA/RNA harvest supernatants. Cell number was determined by DNA quantification (FluoReporter), relative to a standard curve of parental MCF-7 cells. Relative transcription and metabolism were determined by normalizing the relative cell number determined by total RNA yield or Alamar Blue signal (each vs parental MCF-7 standard curve) vs total cell number (from DNA quantification).

Preparation of charcoal-stripped FBS

Using a single lot of FBS, serum stripping methods based on a series of published protocols (7–11) were evaluated. Complete methodology is described in Supplemental Methods.

Statistical analyses

Curve-fitting and statistical analyses were performed using GraphPad Prism, version 5.04 (GraphPad Inc). Clustering (Manhattan complete) was performed with MeV software (TM4 Software Suite).

Methods supplement

Methods for immunoblotting and mRNA expression analyses are described in Supplemental Methods.

Results

Residual estrogenic compounds in CSS alter endocrine response phenotypes

To manage variability in CSS, we routinely test new serum lots for residual estrogenic potential using MCF-7 breast cancer cells. MCF-7 cells are hormone depleted in test CSS lots, and proliferation is assessed after treatment with E2 or the selective ER degrader ICI vs vehicle. Although some variability in proliferation can be attributed to differences in other growth factors, a decrease in growth in ICI vs vehicle treatment is interpreted as being due to residual estrogenic compounds. Shown in Figure 1A are results from two representative CSS lots. With lot 1 (blue), E2 robustly induced proliferation, but ICI treatment caused no decrease in growth vs vehicle; we thus define CSS lot #1 as having complete steroid hormone deprivation. Conversely, with lot 2 (red), ICI decreased growth vs the vehicle (37% decrease at d 6), and we define CSS lot 2 as having partial hormone deprivation. These data are representative of five lots from two suppliers; based on the above definitions, two lots had complete deprivation, two had partial deprivation, and one had a mixed phenotype (discussed below).

Although the absolute magnitude of the effect of partial deprivation CSS on proliferation is modest in this setting, another model revealed more serious implications for the effects of CSS variability. We next assessed the proliferation of MM134 breast cancer cells because we previously reported that these cells recognize 4OHT as a partial ER agonist (6) and included this phenotype in our testing. MM134 cells were hormone deprived as above and treated with 4OHT or ICI vs vehicle (Figure 1B). Using lot
1 (blue), similar to MCF-7 cells, ICI did not reduce growth vs vehicle, confirming complete deprivation; 4OHT induced growth as expected. Using lot 2 (red), ICI decreased MM134 growth vs vehicle, consistent with partial hormone deprivation. However, 4OHT did not induce growth vs vehicle and appears to act instead as a pure antagonist. This reversal of the 4OHT response was observed in both of the CSS lots that were defined as having partial deprivation (data not shown for second partial deprivation lot). 4OHT-induced growth was not observed in MCF-7 in complete or partial deprivation (Figure 1C).

Deprivation status did not shift E2 concentration-responses (Figure 1D). However, MCF-7 cells had greater growth induction in partial deprivation (1.76-fold vs complete); conversely, MM134 had decreased growth induction in partial deprivation (0.82-fold vs complete) because maximal growth was constant. These observations demonstrate that data interpretation and experimental reproducibility may differ based on the CSS lots used in individual experiments.

**CSS variability drives unique modes of developing endocrine resistance**

Maintenance of breast cancer cells in CSS-supplemented medium is also used to mimic estrogen-depleted conditions in patients receiving aromatase inhibitor therapy, so we next assessed MCF-7 proliferation during hormone depletion in test CSS lots. Shown in Figure 2A, short-term proliferation reflected the relative estrogenic content as defined in Figure 1; lots 1 and 3 (complete deprivation) showed minimal growth in CSS, whereas lot 2 (partial deprivation) maintained proliferation, consistent with exogenous estrogenic stimuli. Lot 4 had an intermediate growth phenotype, but because this lot was defined as
Hormone depletion in different CSS lots yields unique proliferative and signaling phenotypes. A, MCF-7 proliferation was assessed as in Figure 1. *, Two-way ANOVA vs lot 1 (P < .05 for cell line effect.) B–E, Methodology for assessing short-term endocrine resistance phenotypes are described in Materials and Methods. B, Representative phase-contrast images at magnification ×100 after 2 weeks of hormone deprivation. C, Immunoblotting from hormone-deprived MCF-7 cells was performed using crude protein lysates after DNA/RNA extraction as described in Materials and Methods. Panels A–C represent biological triplicate samples; protein could not be extracted from lot 4-A. D, Left panel, Cell number was determined by DNA quantification relative to a standard curve of parental MCF-7 cells. D, Right panel, Relative transcription (total RNA) and metabolism (Alamar Blue) vs parental MCF-7 cells were determined as described in Materials and Methods. E, Quantitative PCR for target genes was normalized to ribosomal protein lateral stalk subunit P0. Data are normalized to lot 3 because this lot was used by us previously (6). A–C represent biological triplicate samples.

Methods of charcoal stripping modify serum hormone deprivation

The effects of differing serum-stripping protocols on hormone deprivation are not well characterized. We tested CSS generated from a single FBS lot after varying charcoal type/preparation, charcoal mass per serum volume, and stripping time. MCF-7 cells in each test CSS were treated with E2 or ICI to assess relative growth induction or decrease vs control, respectively. Shown in Figure 3, each stripping condition variable contributed to hormone deprivation. Charcoal preparation had a modest effect (Figure 3A), whereas mass/volume and stripping time had stronger effects; less than 10 g/L charcoal or less than 16 hours stripping time were insufficient for complete deprivation (Figure 3, B and C). Strikingly, no condition produced the severity of deprivation observed in commercial lot 1 because E2-induced growth was comparable in all test conditions and greatly surpassed that in commercial CSS (Figure 3D). In parallel, we measured estrogen content by mass spec-
trometry (Figure 3E). Although several test lots were defined as having partial deprivation, estradiol was undetectable, and estrone was detected in only one sample. These observations demonstrate that stripping conditions have widely variable effects on relative hormone deprivation of serum and highlight the importance of phenotypic endocrine response assays in characterizing CSS.

**Discussion**

CSS is a ubiquitous reagent in the study of steroid hormones, but lot-to-lot variability in CSS can have substantial effects on endocrine response and resistance phenotypes; these effects have important implications for model development and data interpretation. Based on our data, these concerns may be managed by characterization of CSS lots used in individual studies or experiments. Importantly, neither the complete nor partial deprivation CSS represents a better option for all research. The improved dynamic range in cell growth in partial deprivation CSS may be a boon for experiments on ER function (e.g., signaling, transcription, chromatin immunoprecipitation); in Figure 1A, using lot 2, E2 had a greater induction of growth than lot 1, perhaps owing to the lack of total hormone starvation initially and presence of residual estrogens (12, 13). Conversely, complete deprivation CSS is required to assess the effects of weak or partial ER agonists (14, 15) or potentially nonestrogenic hormones and...
growth factors. Notably, the low level proliferation caused by residual estrogens is consistent with the equivalent of subpicomolar/high-femtomolar concentrations of E2 (ref. 6 and Figure 1D), at or below the limits of detection of current mass spectrometry-based assays (16). Phenotypic assays using cell line models as described above will likely be necessary to assess the presence and contributions of residual estrogenic compounds from individual CSS lots.

Although the residual estrogens from partial deprivation CSS lots tested caused relatively modest absolute changes in cell proliferation, this was sufficient to mask the partial agonist effect of 4OHT in MM134 cells. In the presence of a low concentration of residual estrogenic compounds, 4OHT likely competes for ER binding but because it acts as a weak agonist, the net effect on growth is zero. This has a drastic effect on the interpretation of these data. In Figure 1B, with lot 1, MM134 are tamoxifen resistant (because growth is induced by the antiestrogen), but using lot 2, MM134 would not be considered tamoxifen resistant (no apparent effect on growth vs control). These observations demonstrate that in some scenarios, variability in CSS lots may not only alter endocrine response phenotypes but that this variability in fact has the potential to undermine study reproducibility.

The presence of residual estrogenic compounds in partial deprivation CSS may also alter the development of endocrine resistance. We previously reported that endocrine resistance phenotypes were modified by low concentrations of estrogenic steroids (17) and hypothesized that partial vs complete deprivation CSS represented an analogous scenario. Although the 3-week window used herein falls short of the 3–12 months typically used for acquired resistance models, our data suggest three unique programs, driven by CSS lot variability, during long-term hormone depletion 1) for lots 1 and 3, complete deprivation causes initial growth arrest, but cells increase metabolic activity independent of canonical ER activity; 2) for lot 2, low concentrations of estrogens maintain ER activity and low growth/metabolism; and 3) for lot 4, nonestrogenic factors maintain cells at low growth/metabolism, independent of ER. It is likely that each program will yield unique endocrine resistance phenotypes. Importantly, as above, either partial or complete deprivation CSS may be considered appropriate for the development of endocrine resistance models; the former may reflect residual aromatization, incomplete aromatase inhibition, or the contribution of alternative estrogens, and the latter may enrich for ER-independent modes of resistance, as suggested by our observations.

A potential solution to commercial CSS variability is to optimize laboratory FBS stripping. Selection of stripping parameters, as in Figure 3, would allow control over the extent of hormone deprivation. However, the conditions necessary to adequately deprive individual FBS lots will likely vary, as will the resulting endocrine response phenotypes. Similar endocrine response phenotyping of FBS lots prior to stripping may be needed to provide a baseline from which to apply selected stripping protocols.

Based on our observations, increased vigilance in manuscripts is necessary to properly provide context for endocrine response and resistance data. This includes reporting CSS source, supplier, and lot information with catalog numbers for medium components, thorough CSS testing and reporting of CSS phenotypes, and the use of single lots when possible for individual studies. Reporting these details routinely will greatly aid in interpreting and reproducing endocrine response and resistance data by the community.

### Appendix

<table>
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<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (if Known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised (Monoclonal or Polyclonal)</th>
<th>Dilution Used</th>
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<td>ER-α β-Actin</td>
<td>Full length recombinant β-actin N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Aasn-Gly-Ser-Gly-Lys, conjugated to KLH</td>
<td>ER 6F11 Clone AC-15, ascites fluid</td>
<td>Leica, ORG-8871 Sigma, A5441</td>
<td>Mouse monoclonal</td>
<td>1:1000 1:10 000</td>
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<tr>
<td>Mouse IgG</td>
<td>n/a</td>
<td>Mouse IgG, HRP-linked whole Ab</td>
<td>GE/Amersham, NA931</td>
<td>Sheep</td>
<td>1:10 000</td>
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</tbody>
</table>

Abbreviation: Ab, antibody; HRP, horseradish peroxidase.
Acknowledgments

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References