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Cell Density is a Critical Determinant of Aromatase Expression in Adipose Stromal Cells

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Abstract

Obesity is associated with an increased risk of breast cancer among post-menopausal women. This is at least partly due to excessive estrogen production in adipose tissue of obese women. Aromatase, the key enzyme in estrogen biosynthesis, is an important target in endocrine therapy for estrogen receptor (ER)-positive postmenopausal breast cancer. In this study we show that high confluency of human adipose stromal cells (ASCs) cultured in vitro can significantly stimulate aromatase gene expression and reduce the expression of breast tumor suppressor BRCA1 and members of the NR4A orphan nuclear family. Furthermore, small interfering RNA (siRNA)-mediated knockdown of Nurr1, a member of the NR4A family, substantially increased aromatase expression. Lastly, we found that the cell density-triggered inducibility of aromatase expression varies in ASCs isolated from different disease-free individuals. Our finding highlights the impact of increased cell number on estrogen biosynthesis as in the case of excessive adiposity.

INTRODUCTION

Obesity represents one of the most alarming public health problems in this country and worldwide [1]. Increasing prevalence of obesity in the last three decades has reduced the life expectancy in the world population [2]. The mortality risk arises from a higher prevalence of a number of obesity-associated diseases including type II diabetes, hyperlipidemia, osteoarthritis, hypertension, gall bladder diseases, and certain types of cancer [3]. Numerous studies indicate that growth and progression of breast as well as other tumor cells depend not only on their malignant potentials but also on stromal factors present in the tumor microenvironment [4–6]. Epidemiologic studies have linked obesity to an increased number of postmenopausal breast cancer cases, with 30–50% of postmenopausal breast cancer deaths in the US attributed to obesity [7].

A recent study indicates a strong association of weight gain and estrogen receptor (ER)/progesterone receptor (PR)-positive breast tumors [8], suggesting the importance of estrogen biosynthesis and actions in obesity-induced breast cancer risk. Indeed, it has been shown that estrogen production in adipose tissue of overly obese postmenopausal women could be 10-fold higher than the lean controls [9]. It is believed that the increase in cancer risk predominantly results from increased aromatization of plasma androstenedione to estrone in adipose tissue [10,11]. Estrogens produced in distal subcutaneous adipose tissue and within the breast tissue

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microenvironment influence the growth of breast epithelial cells in an endocrine and paracrine fashion, respectively [12].

Aromatase P450 (*CYP19*), which converts androgen to estrogen, catalyzes the final and rate-limiting step in estrogen biosynthesis [13]. As aromatase abundance is a critical determinant for local and circulating estrogen levels, regulation of tissue-specific aromatase expression has a significant impact on various estrogen target tissues under both physiological and pathological conditions. Indeed, Breast adipose tissue adjacent to tumor has a striking increase in aromatase expression and activity [14–16]. Aromatase inhibitors (AIs) such as letrozole inhibit the enzymatic activity of aromatase and thus dampen the estrogenic capability throughout the body. The clinically proven efficacy of AIs in treating postmenopausal breast cancer unequivocally demonstrates the important role of extra-gonadally expressed aromatase in breast cancer development [14,17]. Despite the importance of adipose tissue in estrogen production, relatively little is known about how aromatase expression and estrogen production are regulated in adipose tissue. A comprehensive understanding of the mechanism(s) through which adipose tissue-derived estrogen synthesis is de-regulated will go a long way to reduce obesity-associated breast cancer risk.

In the current study, we investigated the impact of cell confluency on aromatase expression in adipose stromal cells (ASCs). We explored the relationship between the NR4A orphan nuclear receptor family and aromatase expression. Using a cohort of ASCs samples, we also examined variation of aromatase expression in a cohort of ASC samples from cancer-free individuals.

MATERIALS AND METHODS

2.1 Cell culture

Primary human adipose stromal cells (ASCs) were isolated from individuals undergoing elective surgical procedures at the University of Virginia, using methods previously published and approved by the University of Virginia's Human Investigation Committee [18]. The cells were cultured in DMEM/F-12 medium with 10% FBS and 1% antibiotic-antimycotic solution, using previously described methods [18]. Sub-confluent and confluent ASC cultures were plated at 0.75×10^5 and 2×10^5 /well in 6 well plates, respectively. Cells under sub-confluent condition were treated for 18h with phorbol ester 12-O-tetradecanoyl phorbol 13 acetate (TPA, Sigma) or dexamethasone (Dex, Sigma) at 2nM and 250nM concentration respectively.

2.2 Aromatase activity assay

Aromatase activity assay was performed as previously described [19].

2.3 RNA isolation, cDNA preparation, and quantitative RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using the ImPrompII kit (Promega). Real-time PCR was carried out using the fluorescent dye SYBR-Green and an ABI 7900 Real-Time PCR System (Applied Biosystems). Primers used for GAPDH, BRCA1, and aromatase were previously reported [20]. Primers to detect Nurr1 and Nur77 genes were reported earlier [21]. RT-PCR primers used for PPAR γ are 5'TGACAGCGACTTGGCAATATTT3' and 5'TGTAGCAGGTTGTCTTGAATGTCTTC3'; and for CEBP α are 5'AAGAACAGCAACGCGTACCGG3' and 5'CATTGTCAGTGGTCAGCTCCA3'.

2.4 Gene Knockdown with siRNA

Gene-specific knockdown by siRNA oligonucleotides were conducted using Lipofectamine reagent RNAiMAX (Invitrogen). The knockdown experiment was performed as previously described [22]. Briefly, cells at a density of 60% were first transfected with siRNA oligos at a

final concentration of 10nM and incubated for 72h before they were harvested for RNA isolation.

2.5 Adipogenesis and Oil-Red-O staining

Adipogenesis of ASCs was induced by seeding them confluent and treating with DMEM/F-12 medium containing 1 μ M dexamethasone (Sigma), 10 μ M insulin (Sigma), 200 μ M indomethacin (Sigma), 0.5mM 3-isobutyl-1-methylxanthine (Sigma). Differentiation along the adipogenic lineage was assessed based on cell morphology, Oil-Red-O staining, and expression of adipogenic marker genes.

RESULTS

3.1 High cell density significantly enhances aromatase expression in ASCs

Stromal cells were isolated from the fat tissue excised from cancer-free individuals. Upon seeding confluent and growing for 2 days in regular growth medium, ASCs were either cultured continuously in the same medium or switched to the adipogenic medium. As shown in Fig 1A, a large percentage of confluent cells in the adipogenic medium contained Oil-Red-O positive fat droplets, indicating terminal differentiation of ASCs into mature adipocytes. This is confirmed by the expression of known markers for adipogenesis C/EBP α and PPAR γ (Fig. 1B and 1C). In contrast, confluent ASCs growing in the regular medium were not stained positive for Oil-Red-O, nor did they expressed significant amounts of either adipogenic markers (Fig. 1B and 1C). To our surprise, there was a striking elevation of aromatase mRNA level in confluent culture under normal growth medium condition (Fig 1D). We also observed an induction of aromatase transcript in mature adipocytes, although the level of induction is much less robust than confluent culture with regular medium (Fig. 1D). Thus, cell density alone is sufficient to induce aromatase gene expression in ASCs.

3.2 Nurr1 modulates aromatase expression in ASCs

Our previous work in ovarian granulosa cells has implicated BRCA1 and the NR4A orphan nuclear receptor family in modulation of aromatase transcription [20,21]. Therefore, we also examined expression of these regulators of aromatase expression in confluent ASCs cultured for 2 and 12 days in regular medium. As shown in Fig. 2A, BRCA1 mRNA level was transiently reduced in the high-density cells, with a significant reduction at day 2 and partial recovery at day 12 (Fig 2A). Nurr1 and Nur77, two members of the NR4A family, were down-regulated at both time points (Fig 2B and 2C). Given our earlier finding that the NR4A family represses aromatase transcription in an ovarian granulosa tumor cell line [21], we further examined its role in ASCs. As shown in Fig. 3, siRNA-mediated knockdown of Nurr1 led to a marked increase in the aromatase mRNA transcript level (Fig 3B). This result therefore corroborates and extends the earlier finding that the NR4A nuclear receptor family plays a repressive role in aromatase gene regulation.

3.3 Differential expression of density-driven aromatase expression in various individuals

Given the well-documented link between elevated estrogen levels and breast cancer risk, we also examined the cell density-triggered aromatase inducibility in a cohort of ASC samples isolated from different cancer-free individuals (Fig. 4A and 4B). While both aromatase mRNA and enzymatic activity can be induced by high cell confluency in a number of samples examined, the induced levels varied by several orders of magnitude. The relatively low degree of aromatase induction in some primary ASCs is unlikely due to low percentages of ASCs in the clinical samples, as all samples in the cohort displayed similar degrees of adipogenesis when cultured in adipogenic medium (Fig 4C).

To determine whether the low aromatase inducibility in some primary ASCs was due to a global defect in transcription at the aromatase gene locus, we chose ASCs that are responsive (h6-07L) and unresponsive (h6-06L) to density-mediated aromatase induction, and treated them with dexamethasone and 12-0-tetradecanoyl phorbol-13 acetate (TPA), two chemicals that are known to stimulate aromatase transcription from the adipose tissue-specific I.4 promoter and ovary/breast cancer-specific PII promoter, respectively [20]. As shown in Fig. 5A, dexamethasone induced aromatase expression to a comparable level in the two ASC samples. On the other hand, TPA exhibited a greater stimulatory effect on aromatase expression in h6-07L than h6-06L (Fig. 5B). However, the difference in TPA-induced aromatase expression between the two ASC samples (4 fold) is much smaller than what was observed in the cell density treatment (1000 fold) (Fig. 4A). Thus, the underlying mechanism for the density-mediated aromatase expression is most likely distinct from the actions of dexamethasone and TPA at the aromatase promoters.

DISCUSSION

Adipose tissue is a well-recognized source of extra-gonadal estrogen, excessive production of which is thought to be a major contributing factor in obesity-associated breast cancer risk. While adipose tissue in obese individuals undergoes substantial changes in tissue architecture and cellular compositions, little is known about their impact on aromatase expression and local estrogen biosynthesis. The findings from the current work uncover cell density as a critical determinant for the aromatase mRNA level in ASCs, an important resident cell population in adipose tissue. Our study also reveals a huge variation in density-triggered aromatase inducibility among the cancer-free population.

The high-density culturing condition used in our in vitro study most likely influences multiple aspects of cell physiology including increased cell-cell contact and altered cell shape. While the exact cellular trigger for the density-induced aromatase expression remains to be elucidated, our recent work indicates that cell shape change alone is sufficient to stimulate aromatase transcription in ASCs [23]. In this regard, it is worth pointing out that accumulating evidence supports an important role of tensional forces in the global gene expression in breast tumor cells and their invasive behaviors [24–27]. We propose that cell shape changes due to tensional forces in the stromal compartment of tumor microenvironment may have profound effects on the estrogen-producing capability of stromal cells, which in turn could promote estrogen-dependent tumor development and progression, on neighboring cells.

Our previous and present studies have identified a number of regulatory factors including BRCA1 and members of the NR4A family in modulation of aromatase expression in estrogen-producing cells [20,22]. It has been reported that liver receptor homologue-1 (LRH-1) is one of the prime factors that stimulates aromatase transcription in stromal cells of adipose tissue [29]. It is conceivable that NR4A may interfere with the transcriptional activity of LRH-1 at the aromatase promoter since they can compete with the similar binding motif (A/GGGTCA), which could explain why reduction of the Nurr1 level due to high cell density triggers the transcription process. Additional work is needed to shed further mechanistic light on the functional relationship between these transcription factors and aromatase gene regulation. Moreover, it will be of importance to determine the molecular basis for the down-regulation of these factors in response to high cell density, and in particular, whether variation of their expression levels in ASCs could contribute to the individual-based disparity in aromatase inducibility. A thorough understanding of the biological factors that determine the aromatase expression and estrogen synthetic potential of adipose tissue may lead to novel tools in assessing and reducing the obesity-associated breast cancer risks.

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Figure 1.

Cell density-induced aromatase expression is not associated with adipogenesis. ASCs following adipogenesis were stained with Oil-Red-O, together with ASCs growing under sub-confluent and confluent conditions in regular medium (A). mRNA levels of C/EBP α (B), PPAR γ (C) and aromatase (D) from ASCs under the three different culture conditions were analyzed by real time RT-PCR. In this and the following figures data represent mean of 3 individual experiments and error bars (standard deviation) are included in each graph.

Figure 2.

Differential expression of BRCA1, Nur77 and Nurr1 in confluent culture. ASCs were cultured under sub-confluent, 2 day and 12 day old confluent conditions and then harvested for RNA. Relative mRNA expression of BRCA1 (A), Nur77 (B) and Nurr1 (C) were measured using GAPDH as control.

Figure 3.

Effect of Nurr1 knockdown on aromatase expression. ASCs were transfected with Nurr1 siRNA for 72h and the relative expression level of Nurr1 (A) and aromatase (B) were measured by RT-PCR.

Figure 4.

Variability in density-induced aromatase expression among ASCs from different individuals. ASCs from different disease-free individuals were acquired and subjected to culturing under sub-confluent and confluent conditions. After 10 days of culturing aromatase mRNA (A) and activity assay (B) were measured. Due to undetectable levels of aromatase enzymatic activity in the sub-confluent samples, only the confluent samples were analyzed in the enzyme assay. Same ASCs were treated with adipogenic medium, stained with Oil-Red-O, and percentages of positively stained cells were presented (C).

Figure 5. Comparison of responsive and non-responsive ASCs in aromatase induction. Two ASC samples with different density-driven aromatase inducibility were subjected to treatments of dexamethasone (Dex) (50nM) (Fig. 5A) and TPA (10nM) (Fig 5B) for 18h. RNAs were isolated and aromatase expression levels were measured by RT-PCR.