Intratumoral concentration of sex steroids and expression of sex steroid-producing enzymes in ductal carcinoma in situ of human breast

Rie Shibuya, Takashi Suzuki, Yasuhiro Miki, Kimako Yoshida, Takuya Moriya, Katsuhiko Ono, Jun-ichi Akahira, Takanori Ishida, Hisashi Hirakawa, Dean B Evans and Hironobu Sasano

Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
1Department of Surgery, Tohoku University School of Medicine, 980-575 Sendai, Japan
2Department of Surgery, Tohoku Kosai Hospital, 980-0803 Sendai, Japan
3Novartis Institutes for BioMedical Research Basel, Oncology Research, 4002 Basel, Switzerland

(Correspondence should be addressed to T Suzuki; Email: t-suzuki@patholo2.med.tohoku.ac.jp)

Abstract

It is well known that sex steroids play important roles in the development of invasive ductal carcinoma (IDC) of the human breast. However, biological significance of sex steroids remains largely unclear in ductal carcinoma in situ (DCIS), regarded as a precursor lesion of IDC, which is partly due to the fact that the intratumoral concentration of sex steroids has not been examined in DCIS. Therefore, in this study, we first examined the intratumoral concentrations of estradiol and 5α-dihydrotestosterone (DHT) using liquid chromatography/electrospray tandem mass spectrometry in DCIS. Intratumoral concentrations of both estradiol and DHT were threefold higher in DCIS than non-neoplastic breast tissues and estrogen-producing enzymes (aromatase, steroid sulfatase, and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1)), and androgen-producing enzymes (17βHSD5 and 5α-reductase type 1 (5αRed1)) were abundantly expressed in DCIS by real-time PCR and immunohistochemical analyses. The intratumoral concentration of DHT was significantly lower in IDC than DCIS, while the expression of aromatase mRNA in carcinoma cells and intratumoral stromal cells was significantly higher in IDC than those in DCIS. Immunohistochemistry for sex steroid-producing enzymes in DCIS demonstrated that 5αRed1 immunoreactivity was positively correlated with Ki-67 labeling index and histological grade and was also associated with an increased risk of recurrence in patients with DCIS examined. Results of our study suggest that intratumoral concentrations of estradiol and DHT are increased in DCIS, which is possibly due to intratumoral production of these steroids. Therefore, estradiol and DHT may play important roles in the development of DCIS of the human breast.

Introduction

Breast carcinoma is the most common malignant neoplasm in women worldwide. Among breast carcinomas, the incidence of ductal carcinoma in situ (DCIS) has been markedly increasing during the past two decades due to advancements in detection by mammographic screening (Li et al. 2005) such that DCIS now comprise ~20% of all human breast carcinomas diagnosed (Kepple et al. 2006, Tsikitis & Chung 2006). DCIS is regarded as a precursor lesion of invasive ductal carcinoma (IDC) and the risk of IDC developing was reported to be four to ten times higher after the diagnosis of DCIS compared to women without DCIS (Franceschi et al. 1998, Wannberg et al. 2000). Therefore, it is very important to examine the biological features of DCIS in order to improve clinical outcome of breast carcinoma patients.

It is well known that breast tissue is a target for sex steroids. Among the sex steroids, estrogens greatly contribute to the growth of IDC (Vihko & Apter 1989) and anti-estrogens such as tamoxifen, aromatase inhibitors, or luteinizing hormone-releasing hormone (LH-RH) agonists are currently used in patients...
with IDC to block the intratumoral estrogen actions. A great majority of DCIS cases express sex-steroid receptors, i.e., estrogen (ER), progesterone (PR), and androgen (AR) receptors (Selim et al. 2002, Baqai & Shousha 2003, Moinfar et al. 2003, Barnes et al. 2005, Rody et al. 2005, Kepple et al. 2006), which suggest an important role of sex steroids in both DCIS and IDC. Tamoxifen was reported to inhibit the growth of premalignant mammary lesions and the progression to invasive carcinoma in a transplantable mouse model of DCIS (Namba et al. 2005). The National Surgical Adjuvant Breast Project (NSABP) P-1 trial demonstrated that tamoxifen significantly reduced the risk of noninvasive breast cancer by 50% (Dunn et al. 2005) and results of the NSABP B-24 trial indicated that adjuvant tamoxifen therapy was clinically effective in ER-positive DCIS and reduced the recurrence of non-invasive breast carcinomas by 27% (Cuzick 2003).

The concentration of the biologically active estrogen, estradiol, is significantly higher in IDC than in the areas considered as morphologically normal (Chetrite et al. 2000), and estradiol is locally produced from circulating inactive steroids by estrogen-producing enzymes such as aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol), steroid sulfatase (STS; hydrolysis of circulating estrone sulfate to estrone), and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1; conversion of estrone to estradiol) in IDC (Suzuki et al. 2005a; Fig. 1). Intratumoral concentrations of the biologically active androgen, 5α-dihydrotestosterone (DHT), were also significantly higher in IDC than in plasma (Recchione et al. 1995) and androgen-producing enzymes, such as 17βHSD5 (conversion of circulating androstenedione to testosterone) and 5α-reductase (5αRed; reduction of testosterone to DHT) were frequently expressed in IDC (Suzuki et al. 2005a; Fig. 1). Immunolocalization of aromatase (Zhang et al. 2002, Oliveira et al. 2006) and 17βHSD1 (Ariga et al. 2000) has been previously reported in DCIS, suggesting the possible importance of in situ production of sex steroids in DCIS. However, to the best of our knowledge, the intratumoral concentration of sex steroids has not been reported at all in DCIS and no information is available regarding the expression of androgen-producing enzymes in DCIS. Information on sex steroids is very limited in DCIS compared with that in IDC and so the clinical and/or biological significance of sex steroids in DCIS remains largely unclear. Therefore, in this study, we examined the intratumoral concentrations of estradiol and DHT and expression of sex steroid-producing enzymes in DCIS, and compared these findings with those in non-neoplastic breast and IDC tissues. In addition, we immunolocalized sex steroid-producing enzymes in 83 DCIS cases, and correlated these findings with various

**Figure 1** Schema representing intratumoral production of sex steroids in human invasive ductal carcinoma (IDC), which is currently postulated. STS, steroid sulfatase; 17βHSD1, 17β-hydroxysteroid dehydrogenase type 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; and 5αRed, 5α-reductase.
Materials and methods

Patients and tissues

Specimens of pure DCIS (n = 12) and IDC (n = 12) were obtained from female patients who underwent breast cancer surgical treatment from 2001 to 2004 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Non-neoplastic breast tissues were also obtained in 8 out of the 12 IDC patients, who underwent mastectomy and were examined in this study. Specimens for sex-steroid extraction or RNA isolation were snap-frozen and stored at −80°C and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin-wax. The histological grade of each specimen was evaluated based on the Van Nuys classification (Silverstein et al. 1995) in DCIS and by the method of Elston & Ellis (1991) in IDC. Informed consent was obtained from all patients prior to their surgery and the examination of the specimens used in this study.

Eighty-three pure DCIS specimens were obtained by surgical excision from 1990 to 2005 in Department of Surgery, Tohoku University Hospital, Sendai, Japan. The mean age of the patients was 57.0 years (ranges 30–80 years). All of the patients did not receive irradiation, chemotherapy, or hormonal therapy prior to the surgery. Disease-free survival data were available in 78 patients, and the mean follow-up time was 54 months (ranges 8–117 months). All specimens were fixed with 10% formalin and embedded in paraffin wax.

Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

Liquid chromatography/electrospray tandem mass spectrometry (LC–MS/MS)

Concentrations of estradiol and DHT were measured by LC–MS/MS analysis in Teizo Medical Co. (Kawasaki, Japan), as described previously (Miki et al. 2007, Suzuki et al. 2007, Yamashita et al. 2007). Briefly, the weights of the breast carcinoma specimens (32–89 mg for each sample) were measured by an electronic balance to a reasonable accuracy (AEK-200B (Shimadzu, Kyoto, Japan); readability, 0.1 mg; and capacity, 200 g), and these were then homogenized in 1 ml distilled water. After addition of 100 pg estradiol-13C4 (Hayashi Junyaku, Tokyo, Japan) or DHT-2H3 (CDN Isotope, Pointe-Claire Quebec, Canada) as internal standard, steroids were extracted with diethyl ether from the homogenate. The separated organic layer was evaporated, and then dissolved in picoline anhydride in tetrahydrofran solution (100 μl) with triethylamine (20 μl). After application to a Bond Elut C18 column, steroid derivatives were eluted with 80% acetonitrile solution. The derivative estradiol and DHT fraction were dissolved in the elution solvent of LC.

In this study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated with electrospray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3×150 mm, 3.5 μm, Imtakt, Kyoto, Japan). The injection volume was 20 μl. The mobile phase consisted of solvents A (0.1% formic acid in water (v/v)) and B (acetonitrile) and delivered at a flow rate of 0.4 ml/min. Total run time was 10 min. We used a mixture of solvents A and B (30:70 (v/v)) as an initial condition. After injection, it was followed by a linear gradient to 100% solvent B for 4 min, and this condition was maintained for 3 min. The system was returned to the initial proportion within 0.05 min, and maintained for the final 2.95 min of each run. The retention times for the derived estradiol and DHT were 5.3 and 5.8 min respectively. Ion spray voltage was 4.5 kV and turbo gas temperature was 450 °C in ionization conditions. For multiple reaction monitoring mode, the instrument monitored the m/z 255.3 (I.S.: 258.3) as ion produced from 396.4 (I.S.: 399.4) and the m/z 262 (I.S.:268) from 383.3 (I.S.: 487.2) respectively for estradiol and DHT derivatives.

In our present study, the lower limit of quantification (LLOQ) was 0.2 pg for both the estradiol and the DHT. It was determined by combination of determination validation, reproducibility, accuracy, and precision. The reproducibility was evaluated by intra- and inter-assays (n = 3) and their coefficient variations (CVs) were 11.8 and 2.4% for estradiol, and 15.0 and 13.4% for DHT respectively. The accuracy and precision were measured using five different concentrations (0.2, 0.5, 1.0, 10, and 50 pg) of estradiol or DHT and five determinations for each concentration. The LLOQ did not exceed 20% of the CV. The recovery for estradiol and DHT was 80–85%.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies Inc.), and a reverse transcription kit (SUPERSCRIPT II Preamplification system (Gibco-BRL) was used in the synthesis of cDNA. The Light Cycler system (Roche Diagnostics GmbH)
Aromatase (aromatase; X13589) FWD: 691–712 116

RPL13A (RPL13A; NM012423) FWD: 487–509 125

Vandesompele

STS (STS; M16505) FWD: 1550–1569 153

Red1 (SRD5A1; NM001047) FWD: 658–677 158

Red2 (SRD5A2; NM000348) FWD: 500–520 315

HSD1 (HSD17B1; NM000413) FWD: 1300–1321 326

HSD5 (AKR1C3; NM003739) FWD: 969–992 103

5αRed1 (SRD5A1; NM001047) FWD: 796–815 158

5αRed2 (SRD5A2; NM000348) FWD: 794–814 158

RPL13A (RPL13A; NM012423) FWD: 487–509 125

Vandesompele et al. (2002)

<table>
<thead>
<tr>
<th>cDNA (gene symbol; accession no.)</th>
<th>Sequence (position in cDNA)</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatasea</td>
<td>FWD: 691–712 REV: 786–806</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>STSa</td>
<td>FWD: 1550–1569 REV: 1683–1702</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>17βHSD1a (HSD17B1; NM000413)</td>
<td>FWD: 1300–1321 REV: 1604–1625</td>
<td>326</td>
<td></td>
</tr>
<tr>
<td>17βHSD5a (AKR1C3; NM003739)</td>
<td>FWD: 969–992 REV: 1052–1071</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>5αRed1a (SRD5A1; NM001047)</td>
<td>FWD: 658–677 REV: 796–815</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>5αRed2 (SRD5A2; NM000348)</td>
<td>FWD: 500–520 REV: 794–814</td>
<td>315</td>
<td>Suzuki et al. (2001)</td>
</tr>
</tbody>
</table>

aOligonucleotide primers used in this study were designed in the different exons.

was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin et al. 2000). Characteristics of the primer sequences used in this study were summarized in Table 1 (Suzuki et al. 2001, Vandesompele et al. 2002). Settings for the PCR thermal profile were as follows: initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 10 s, annealing at 60 °C (17βHSD1), 64 °C (17βHSD5 and STS), 68 °C (aromatase, 5αRed1, 5αRed2 and ribosomal protein L 13a (RPL13A)) for 10 s, and elongation at 72 °C for 12 s. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. The mRNA level of the steroidogenic enzymes was summarized as a ratio (%) of that of RPL13A. The results of real-time PCR analyses were similar when we used other internal standards, such as glyceraldehyde-3-phosphate dehydrogenase (Suzuki et al. 2001), instead of RPL13A as used in this study (data not shown).

**Laser capture microdissection (LCM)/real-time PCR for aromatase**

Seven specimens of non-neoplastic breast, eight of DCIS and nine of IDC were available for LCM/real-time PCR analysis in this study. LCM was conducted using the Laser Scissors CRI-337 (Cell Robotics Inc., Albuquerque, NM, USA) and ~ 5000 epithelial cells or stromal cells were collected under the microscope from frozen sections of breast tissues. Total RNA was extracted according to a RNA microisolation protocol described by Niino et al. (2001). The real-time PCR protocol for aromatase was described above.

**Immunohistochemistry**

The characteristics of primary antibodies for steroidogenic enzymes, such as aromatase (Miki et al. 2007), STS (Suzuki et al. 2003), 17βHSD1 (Suzuki et al. 2000), 17βHSD5 (Suzuki et al. 2001), 5αRed1 (Suzuki et al. 2001), and 5αRed2 (Suzuki et al. 2001), used in this study were described previously. Monoclonal antibodies for ERα (ER1D5), PR (MAB429), AR (AR441), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), DAKO (Carpinteria, CA, USA) and DAKO respectively.

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin–biotin amplification method was used for immunohistochemistry in this study. Antigen retrieval for ERα, PR, AR, and Ki-67 immunostaining was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilution of primary antibodies used in this study was as follows: aromatase; 1/6000, STS; 1/9000, 17βHSD1; 1/500, 17βHSD5; 1/1000, 5αRed1; 1/1000, 5αRed2; 1/1000, ERα; 1/50, PR; 1/30; AR; 1/100, and Ki-67; 1/50. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H2O2) and counterstained with hematoxylin. As a negative control, normal rabbit or mouse immunoglobulin G (IgG) was used instead of the primary antibody.

Immunoreactivity of steroidogenic enzymes was detected in the cytoplasm and cases that had more than 10% of positive carcinoma cells staining were considered positive (Suzuki et al. 2007). Immunoreactivity of ERα, PR, AR, and Ki-67 was detected in more than 1000 carcinoma cells for each case.
and subsequently the percentage of immunoreactivity, i.e., labeling index (LI), was determined (Suzuki et al. 2007).

Statistical analysis
The statistical analyses between two groups were performed using a Mann–Whitney U test, and P values <0.05 were considered significant. The relative ratio between two groups was evaluated by their median values. Disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using a log-rank test.

Results
Intratumoral concentration of sex steroids in DCIS
We first examined the tissue concentration of sex steroids in the non-neoplastic breast, DCIS, and IDC tissues by LC–MS/MS. The median with min–max value of tissue concentration of estradiol was 16 (5–83) pg/g in non-neoplastic breast, 52 (10–494) pg/g in DCIS, and 206 (11–1586) pg/g in IDC. The median value in DCIS was 3.3-fold higher than that in non-neoplastic breast tissues (Fig. 2A). The intratumoral concentration of estradiol was 4.0-fold higher in IDC than DCIS, but no significant association was detected (P = 0.20).

The median with min–max value of tissue concentration of DHT was 100 (63–128) pg/g in the non-neoplastic breast, 323 (140–1593) pg/g in DCIS, and 162 (41–990) pg/g in IDC. The tissue concentration of DHT was 3.2-fold higher in DCIS than non-neoplastic breast (Fig. 2B). The intratumoral concentration of DHT was significantly higher in DCIS than IDC (P = 0.04 and 2.0-fold).

The intratumoral concentration of estradiol in DCIS was 2.2-fold higher in premenopausal women (93 (10–494) pg/g (n = 5)) than in postmenopausal women (42 (13–70) pg/g (n = 7)), but no significant association was detected (P = 0.46). The median of the intratumoral concentration of DHT in DCIS was 260 (253–380) pg/g in premenopausal women and 326 (140–1593) pg/g in postmenopausal women (P = 0.52).

mRNA expression of sex steroid-producing enzymes in DCIS
We next examined mRNA expression of sex steroid-producing enzymes in non-neoplastic breast, DCIS, and IDC using real-time PCR. As shown in Table 2, mRNA levels of aromatase, STS, 17βHSD1, 17βHSD5, and 5αRed1 were significantly higher in DCIS than non-neoplastic breast (P = 0.03 and 4.0-fold in aromatase, P = 0.01 and 9.5-fold in STS, P = 0.04 (relative ratio could not be evaluated) in 17βHSD1, P = 0.01 and 18-fold in 17βHSD5, and P = 0.02 and 5.1-fold in 5αRed1). The expression level of 5αRed2 mRNA was negligible and no significant difference was detected between DCIS and non-neoplastic breast (P = 0.67). The expression level of aromatase mRNA was significantly higher in IDC than DCIS (P = 0.046 and 5.9-fold), but mRNA levels of other sex steroid-producing enzymes were not significantly changed between these two breast carcinoma groups.

Previous studies demonstrated that aromatase was immunolocalized in carcinoma and stromal cells in human breast carcinoma (Zhang et al. 2002, Suzuki et al. 2005a). Therefore, we further examined the expression of aromatase mRNA according to cell type in the non-neoplastic breast, DCIS, and IDC using LCM/real-time PCR. In the epithelial cells, the median
with min–max value of aromatase mRNA was 0.00 (0.00–0.30) × 10⁻²% in epithelial cells of non-neoplastic breast, 0.06 (0.00–0.29) × 10⁻²% in carcinoma cells of DCIS, and 1.00 (0.01–3.00) × 10⁻²% in carcinoma cells of IDC and its expression level was significantly higher in IDC than non-neoplastic breast (P = 0.01 and relative ratio could not be evaluated) or DCIS (P = 0.03 and 17-fold; Fig. 3A). The expression level of aromatase mRNA in the stromal cells was 0.00 (0.00–0.04) × 10⁻²% in non-neoplastic breast, 0.04 (0.00–0.10) × 10⁻²% in DCIS, and 4.00 (1.00–6.23) × 10⁻²% in IDC, and the expression level was significantly higher in IDC than non-neoplastic breast (P = 0.001, and relative ratio could not be evaluated) or DCIS (P = 0.001, and 100-fold; Fig. 3B).

### Immunoreactivity of sex steroid-producing enzymes in DCIS

Aromatase immunoreactivity was detected in the cytoplasm of carcinoma cells in 45 out of 83 DCIS cases (54%; Fig. 4A) and was also detected in some intratumoral stromal cells (Fig. 4B). Immunoreactivity of other sex steroid-producing enzymes was detected in the cytoplasm of carcinoma cells and the number of positive cases was as follows: STS; 45/83 (54%; Fig. 4C), 17βHSD1; 54/83 (65%; Fig. 4D), 17βHSD5; 59/83 (71%; Fig. 4E), 5αRed1; 52/83 (63%; Fig. 4F), and 5αRed2; 13/83 (16%). Associations between immunoreactivity of sex steroid-producing enzymes and clinicopathological parameters in the 83 DCIS cases are summarized in Tables 3 and 4. Among the estrogen-producing enzymes, STS immunoreactivity was significantly associated with the histological grade (Van Nuys classification; P = 0.01), while no significant association was detected between aromatase or

### Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>mRNA expression level (median (min–max)) × (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (n = 8)</td>
<td>DCIS (n = 12)</td>
</tr>
<tr>
<td>Estrogen-producing enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aromatase</td>
<td>3.5 (0.0–8.0)</td>
<td>14.1 (1.5–113.3)</td>
</tr>
<tr>
<td>STS</td>
<td>0.4 (0.0–1.5)</td>
<td>3.8 (0.0–93.1)</td>
</tr>
<tr>
<td>17βHSD1</td>
<td>0.0 (0.0–0.5)</td>
<td>0.6 (0.0–4.0)</td>
</tr>
<tr>
<td>Androgen-producing enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17βHSD5</td>
<td>0.6 (0.0–3.1)</td>
<td>10.8 (1.0–57.3)</td>
</tr>
<tr>
<td>5αRed1</td>
<td>11.5 (0.0–19.8)</td>
<td>58.6 (3.0–223.6)</td>
</tr>
<tr>
<td>5αRed2</td>
<td>0.0 (0.0–15.9)</td>
<td>0.2 (0.0–49.0)</td>
</tr>
</tbody>
</table>

Normal; non-neoplastic breast tissues. The statistical analyses between two groups were performed using a Mann–Whitney’s U test. P values < 0.05 were considered significant and are indicated in boldface. The mRNA level of the steroidogenic enzymes was summarized as a ratio (%) of that of RPL13A.

Figure 3

Expression of aromatase mRNA in (A) epithelial cells or (B) stromal cells in non-neoplastic breast (normal), DCIS, and IDC tissues. Epithelial cells and the adjacent stromal cells in non-neoplastic breast tissues, or carcinoma cells and intratumoral stromal cells in DCIS and IDC were collected separately by LCM. Data are represented as box and whisker plots. The statistical analyses were performed using a Mann–Whitney U test between indicated two groups. P values < 0.05 were considered significant and are indicated in boldface.
17\(\beta\)HSD1 immunoreactivity and the clinicopathological factors examined (Table 3).

Among the androgen-producing enzymes, 5\(\alpha\)Red1 immunoreactivity was positively associated with the Van Nuys classification \((P = 0.001)\) or Ki-67 LI \((P = 0.02)\), but 17\(\beta\)HSD5 immunoreactivity was not significantly correlated with the clinicopathological parameters examined (Table 4). No significant association was detected between 5\(\alpha\)Red2 immunoreactivity and the clinicopathological parameters examined (data not shown). There was no significant association among the immunoreactivity of these five sex steroid-producing enzymes in DCIS. The significant correlations described above were confirmed in increased rankings of the positivity to three groups (0–9, 10–49, and 50–100% positive cells; STS and Van Nuys classification, \(P = 0.04\); 5\(\alpha\)Red1 and Van Nuys classification, \(P = 0.01\); and 5\(\alpha\)Red1 and Ki-67 LI, \(P = 0.04\)).

As summarized in Fig. 5, the status of 5\(\alpha\)Red1 immunoreactivity was associated with an increased risk of recurrence in 78 DCIS patients examined, although \(P\) value was not available because no patients were associated with clinical recurrence in a group of 5\(\alpha\)Red1-negative breast carcinomas. On the other hand, no significant association was detected between the status of other steroidogenic enzyme immunoreactivity and risk of recurrence in these DCIS patients (aromatase, \(P = 0.87\); STS, \(P = 0.47\); 17\(\beta\)HSD1, \(P = 0.83\); 17\(\beta\)HSD5, \(P = 0.98\); and 5\(\alpha\)Red2, \(P = 0.45\)).
Discussion

To the best of our knowledge, this is a first report that demonstrates intratumoral concentrations of sex steroids in DCIS. Median values of both estradiol and DHT concentrations were (3.3-fold in estradiol and 3.2-fold in DHT) higher in DCIS than those in non-neoplastic breast tissues. Results of our present study also demonstrated that mRNA expression of both estrogen (aromatase, STS, and 17\(\beta\)HSD1)- and androgen (17\(\beta\)HSD5 and 5\(\alpha\)Red1)-producing enzymes was significantly higher in DCIS than the non-neoplastic breast tissues. Previous studies demonstrated that aromatase immunoreactivity was detected in the carcinoma and stromal cells (Zhang et al. 2002) in 70% of DCIS tissues (Oliveira et al. 2006) and 17\(\beta\)HSD1 immunoreactivity was positive in the carcinoma cells in 63% of DCIS cases (Ariga et al. 2000). In our present study, we detected aromatase and 17\(\beta\)HSD1 immunoreactivities in 54 and 65% of DCIS cases and these frequencies and localization were consistent with the previous reports. On the other hand, expression of STS, 17\(\beta\)HSD5, and 5\(\alpha\)Red has not been reported in DCIS. Our present results showed that immunoreactivity for STS, 17\(\beta\)HSD5, 5\(\alpha\)Red1, and 5\(\alpha\)Red2 was positive in 54, 71, 63, and 16% of DCIS cases respectively and these frequencies were similar in 70% of DCIS tissues (Zhang et al. 2002) in 70% of DCIS tissues (Oliveira et al. 2006) and 17\(\beta\)HSD1 immunoreactivity was positive in the carcinoma cells in 63% of DCIS cases (Ariga et al. 2000). In our present study, we detected aromatase and 17\(\beta\)HSD1 immunoreactivities in 54 and 65% of DCIS cases and these frequencies and localization were consistent with the previous reports. On the other hand, expression of STS, 17\(\beta\)HSD5, and 5\(\alpha\)Red has not been reported in DCIS. Our present results showed that immunoreactivity for STS, 17\(\beta\)HSD5, 5\(\alpha\)Red1, and 5\(\alpha\)Red2 was positive in 54, 71, 63, and 16% of DCIS cases respectively and these frequencies were similar

| Table 3 | Association between immunoreactivity of estrogen-producing enzymes and clinicopathological parameters in 83 ductal carcinoma in situ (DCIS) tissues |
|-------|---------------------------------|---------------------------------|
|        | **Aromatase immunoreactivity** | **STS immunoreactivity**        | **17\(\beta\)HSD1 immunoreactivity** |
| Value  | + (\(n=45\)) – (\(n=38\))    | + (\(n=45\)) – (\(n=38\))    | + (\(n=54\)) – (\(n=29\))    |
| Age* (years) | 60 (34–80) 56 (30–77) | 54 (34–77) 61 (30–80) | 57 (30–80) 61 (34–77) |
| Menopausal status |                         |                              |                              |
| Premenopausal | 13 (16%) 14 (17%) 16 (19%) 11 (13%) 14 (17%) 13 (16%) | 29 (35%) 27 (33%) 6.84 | 40 (48%) 16 (19%) 0.08 |
| Postmenopausal | 32 (39%) 24 (29%) | 8 (10%) 19 (23%) | 17 (20%) 10 (12%) |
| Van Nuys classification |               |                                |                                |
| Group 1 | 10 (12%) 6 (7%) 12 (14%) 4 (5%) 11 (13%) 5 (6%) | 78 (0–97) 66 (4–94) | 0.13 |
| Group 2 | 21 (25%) 19 (23%) | 25 (30%) 15 (18%) | 26 (31%) 14 (17%) |
| Group 3 | 10 (12%) 6 (7%) 12 (14%) 4 (5%) 11 (13%) 5 (6%) | 78 (0–97) 66 (4–94) | 0.13 |
| ERa LIa | 69 (0–97) 80 (0–97) | 67 (0–96) 80 (0–97) | 78 (0–97) 66 (4–94) | 0.13 |
| PR LIa | 52 (0–92) 47 (0–93) | 33 (6–92) 47 (0–93) | 38 (0–93) 31 (0–92) | 0.60 |
| AR LIa | 56 (0–97) 59 (4–97) | 53 (0–93) 59 (4–97) | 56 (2–97) 55 (0–93) | 0.86 |
| Ki-67 LIa | 16 (2–35) 13 (2–35) | 17 (2–35) 12 (2–32) | 13 (2–35) 17 (3–33) | 0.45 |

aData are presented as median with min–max values and were evaluated by a Mann–Whitney U test. All other values represent the number of cases and percentage.

| Table 4 | Association between immunoreactivity of androgen-producing enzymes and clinicopathological parameters in 83 ductal carcinoma in situ (DCIS) tissues |
|-------|---------------------------------|---------------------------------|
|        | **17\(\beta\)HSD1 immunoreactivity** | **5\(\alpha\)Red1 immunoreactivity** |
| Value  | + (\(n=59\)) – (\(n=24\))    | + (\(n=52\)) – (\(n=31\))    |
| Age* (years) | 53 (30–80) 61 (42–69) | 55 (30–80) 61 (39–77) | 0.43 |
| Menopausal status |                         |                              |                              |
| Premenopausal | 23 (28%) 5 (6%) | 19 (23%) 8 (10%) | 0.19 |
| Postmenopausal | 36 (43%) 19 (23%) | 33 (40%) 23 (28%) | 0.44 |
| Van Nuys classification |               |                                |                                |
| Group 1 | 20 (24%) 7 (8%) | 11 (13%) 16 (19%) | 0.43 |
| Group 2 | 26 (3%) 14 (17%) | 26 (31%) 14 (17%) | 0.43 |
| Group 3 | 13 (16%) 3 (4%) | 15 (18%) 1 (1%) | 0.001 |
| ERa LIa | 71 (0–97) 87 (0–94) | 73 (0–97) 79 (0–97) | 0.12 |
| PR LIa | 53 (0–92) 57 (12–87) | 32 (0–92) 43 (0–93) | 0.06 |
| AR LIa | 73 (16–97) 83 (0–93) | 54 (2–93) 62 (0–97) | 0.12 |
| Ki-67 LIa | 20 (2–35) 13 (7–24) | 18 (3–35) 11 (2–32) | 0.02 |

P values <0.05 were considered significant and are indicated in boldface.

aData are presented as median with min–max values and were evaluated by a Mann–Whitney U test. All other values represent the number of cases and percentage and were statistically analyzed using a cross-table using the \(\chi^2\) test.
Aromatase in MCF-7 breast carcinoma cells were reported that mRNA level and enzymatic activity of carcinoma cells. Very recently, Miki stimulated aromatase activity in MCF-7 breast and keratinocyte growth factor (Zhang 1992), transforming growth factor (Ryde adipose fibroblasts (Zhou 1998), demonstrated that breast carcinoma cells secrete 100-fold respectively. Previous than DCIS in these two cellular components (17- and expression level was significantly higher in IDC in both carcinoma and intratumoral stromal cells and group of DCIS patients. Therefore, endocrine therapies, such as anti-estrogens, aromatase inhibitors, and/or LH-RH agonists, may be clinically effective in a selective anti-estrogens, aromatase inhibitors, and/or LH-RH antagonists. For instance, Birrell reported. For instance, Birrell et al. (1999) showed that both DHT and the synthetic non-metabolizable androgen, mibolerone, increased the cell proliferation of MCF-7 and MDA-MB-453 cells. In addition, Suzuki et al. (2007) demonstrated that aromatase expression was inversely associated with intratumoral DHT concentrations in IDC and aromatase inhibitors suppressed the DHT synthesis from androstenedione in coculture experiments. These findings all suggest that aromatase is a negative regulator of local DHT production in human breast carcinoma. In this study, the intratumoral DHT concentration was significantly lower in IDC than DCIS. On the other hand, aromatase expression was significantly higher in IDC than DCIS, while expression levels of androgen-producing enzymes were not significantly different between these two groups. Therefore, higher expression of aromatase in IDC may increase the conversion of androgens into estrogens with a subsequent decrease of intratumoral DHT concentrations.

In our present study, aromatase mRNA was detected in both carcinoma and intratumoral stromal cells and the expression level was significantly higher in IDC than DCIS in these two cellular components (17- and 100-fold respectively). Previous in vitro studies demonstrated that breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts (Zhou et al. 2001), including prostaglandin E2 (Zhao et al. 1996), interleukin (IL)-1, IL-6, IL-11, and tumor necrosis factorz (Reed & Purohit 2001, Simpson & Davis 2001). On the other hand, it has been also reported that exogenous growth factors such as epidermal growth factor (Ryde et al. 1992), transforming growth factor (Ryde et al. 1992), and keratinocyte growth factor (Zhang et al. 1998) stimulated aromatase activity in MCF-7 breast carcinoma cells. Very recently, Miki et al. (2007) reported that mRNA level and enzymatic activity of aromatase in MCF-7 breast carcinoma cells were significantly increased on coculture with primary stromal cells isolated from human breast carcinoma tissue. Therefore, aromatase expression is suggested to be, at least in a part, regulated by tumor–stromal interactions in breast carcinoma tissues, which may be promoted by invasion of the carcinoma cells into the stroma.

Intratumoral DHT level is associated with the testosterone level in IDC (Mistry et al. 1986, Recchione et al. 1995) and is considered to be mainly determined by amounts of the precursor. Aromatase catalyzes the conversion of androstenedione and testosterone, which are precursors of DHT, to estrone and estradiol respectively (Fig. 1). Spinola et al. (1988) previously showed that treatment with an aromatase inhibitor (4-hydroxyandrostenedione) markedly elevated intratumoral testosterone concentrations in dimethylbenz(a)anthracene-induced rat mammary tumors. In addition, Sonne-Hansen & Lykkesfeldt (2005) reported that aromatase preferred testosterone as a substrate in MCF-7 cells. Very recently, Suzuki et al. (2007) demonstrated that aromatase expression was inversely associated with intratumoral DHT concentrations in IDC and aromatase inhibitors suppressed the DHT synthesis from androstenedione in coculture experiments. These findings all suggest that aromatase is a negative regulator of local DHT production in human breast carcinoma. In this study, the intratumoral DHT concentration was significantly lower in IDC than DCIS. On the other hand, aromatase expression was significantly higher in IDC than DCIS, while expression levels of androgen-producing enzymes were not significantly different between these two groups. Therefore, higher expression of aromatase in IDC may increase the conversion of androgens into estrogens with a subsequent decrease of intratumoral DHT concentrations.

Various in vitro studies have shown that DHT inhibits the cell proliferation of breast carcinoma cells (de Launoit et al. 1991, Lapointe & Labrie 2001, Ando et al. 2002) and the proapoptotic effect of DHT was also reported in breast carcinoma cells (Kandouz et al. 1999). DHT treatment resulted in a rapid fall in tumor volume of ZR75-1 cells injected into athymic mice (Dauvois et al. 1991). However, it is also true that some divergent findings have been reported. For instance, Birrell et al. (1995) showed that both DHT and the synthetic non-metabolizable androgen, mibolerone, increased the cell proliferation of MCF-7 and MDA-MB-453 cells. In addition, Zhang et al. (2004) demonstrated that DHT-benzoate (DHT-B) induced growth of mouse mammary ductal cells, although it is much weaker than estradiol and

Figure 5 Disease-free survival curve of 78 DCIS patients according to 5αRed1 immunoreactivity (Kaplan–Meier method).

---

www.endocrinology-journals.org
treatment with both estradiol and DHT-B caused more pronounced hyperplasia of mammary ducts and alveoli, compared with the treatment with each hormone alone. In our present study, 5zRed1 immunoreactivity was significantly associated with Ki-67 LI and the Van Nuys classification in 83 DCIS cases and it was also associated with an increased risk of recurrence in the 78 DCIS patients. The Ki-67 antibody recognizes cells in all phases of the cell cycle except the G0 (resting) phase and the Ki-67 LI is closely correlated with the S-phase fraction and mitotic index (Vandesompele et al. 2002). The Van Nuys classification is known as a powerful prognostic classification for DCIS and Silverstein et al. (1995) reported that the incidence of local recurrence after breast-conservation surgery for DCIS in 238 patients was 4% in Group 1 (non-high-grade DCIS without comedo-type necrosis), 11% in Group 2 (non-high-grade DCIS with comedo-type necrosis), and 27% in Group 3 (high-grade DCIS with or without comedo-type necrosis). ER and PR status in DCIS was inversely associated with the histological differentiation or nuclear grade (Selim et al. 2002, Baqai & Shousha 2003). However, AR status was not correlated with ER status in DCIS (Rody et al. 2005) and a significant number of poorly differentiated DCIS was reported ER-negative, PR-negative, but AR-positive (Moinfar et al. 2003). Results of these previous and our present studies are indicative that DHT may be involved in the development of DCIS. However, no information is currently available on the effects of androgens in DCIS to our knowledge and so further examinations are required to clarify the significance of androgens in human DCIS.

In summary, intratumoral concentrations of estradiol and DHT were higher in DCIS than non-neoplastic breast tissues and estrogen-producing enzymes (aromatase, STS, and 17βHSD1) and androgen-producing enzymes (17βHSD5 and 5zRed1) were highly expressed in DCIS. The intratumoral concentration of DHT was significantly lower in IDC than DCIS and the expression of aromatase mRNA was significantly higher in IDC. Results of immunohistochemistry for the sex steroid-producing enzymes demonstrated that 5zRed1 immunoreactivity was associated with Ki-67 LI, histological grade, and increased risk of recurrence in DCIS patients. Results of our present study suggest that intratumoral concentrations of estradiol and DHT are increased in DCIS, which is possibly due to intratumoral production of these steroids. DCIS frequently expresses ER and/or AR in the carcinoma cells and therefore, both estradiol and DHT may play important roles in the development of DCIS.

Acknowledgements

We appreciate the skillful technical assistance of Ms Miki Mori (Department of Pathology, Tohoku University School of Medicine). This work was supported in part by a Grant-in-Aid from Kurokawa Cancer Research Foundation, Japan, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology Japan. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Suzuki T, Urano T, Tsuki T, Horie-Inoue K, Moriya T, Ishida T, Muramatsu M, Ouchi Y, Sasano H & Inoue S


Zhao Y, Agarwal VR, Mendelson CR & Simpson ER 1996 Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. *Endocrinology* **137** 5739–5742.