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Expression of enzymes involved in synthesis and metabolism of estradiol in human breast as studied by immunocytochemistry and *in situ* hybridization

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Summary. It is well documented that human breast is actively involved in the local formation of estrogens. To determine the site(s) of action of enzymes involved in synthesis and metabolism of the most potent estrogen estradiol (E_2), we have studied the expression of the following enzymes: 3ß-hydroxysteroid dehydrogenase (3-HSD), 17ß-HSD types 1, 2, 5, 7 and 12, aromatase, steroid sulfatase (STS) and estrogen sulfotransferase (EST) 1E1 at the cellular level in breast. Both in situ hybridization and immunocytochemistry were used for enzyme localization in normal breast tissues. For immunocytochemistry, we used rabbit antibodies, while in situ hybridization studies were performed using (^{35}S)-labeled cRNA probes. Similar results were obtained with both approaches. All the enzymes (3ß-HSD; 17ß-HSD types 1, 5, 7 and 12; aromatase) involved in the conversion of circulating dehydroepiandrosterone (DHEA) to E2 as well as STS which converts estradiol sulfate (E_2-S) to E_2 have been found to be expressed in epithelial cells of acini and/or ducts as well as the stromal cells. Moreover, 17ß-HSD type 2 and EST1E1, two enzymes which inactivate E_2, have been also localized in the same cell types. The present results indicate the enzymes which play a role in the synthesis and metabolism of E_2 are expressed in both epithelial and stromal cells in human breast.

Key words: Estrogen, Human breast, Steroidogenic enzymes

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Introduction

Estrogens play a predominant role in the growth of mammary glands. They originate from two important sources in premenopausal women, namely the ovaries and the adrenals, through the secretion of dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) which are ultimately converted into active sex steroids (estrogens and androgens) in peripheral target tissues (Labrie et al., 1995, 2001). In fact, plasma DHEA-S levels in adult women are 10,000 times higher than those of testosterone and 3,000 to 30,000 times higher than those of estradiol, thus providing a large reservoir of substrate for conversion into androgens and/or estrogens in the peripheral intracrine tissues (Labrie et al., 1995). The local biosynthesis and action of sex steroids in peripheral tissues has been named intracrinology (Labrie, 1991; Labrie et al., 1995). In women it is estimated that intracrine formation of estrogens is the order of 75% before menopause and 100% after menopause (Labrie et al., 2000). The existence of local biosynthesis of the most potent estrogen E2 has been supported by the demonstration of the expression of enzymes involved in the conversion of the inactive adrenal precursor DHEA and DHEA-S to E2 (Fig. 1) (Martel et al., 1994; Labrie et al., 2000). The enzyme 3ß-hydroxysteroid dehydrogenase (3ß-HSD) converts DHEA into androstenedione (Simard et al., 2005) while 17ß-HSD type 5 transforms androstenedione into testosterone (Dufort et al., 1999). Aromatase converts testosterone into E2 and androstenedione into the weak estrogen estrone (E1) (Miller and O'Neill, 1987). 17ß-HSD type 1, 7 and 12 are all involved in the conversion of E1 to E2 while 17ß-HSD type 2 converts the transformation of E2 to E1 (Mindnich et al., 2004). Estrogen sulfotransferase 1E1 (EST 1E1) sulfonates estrogens (E1, E2) to biologically

inactive estrogen sulfates (Song, 2001; Pasqualini and Chetrite, 2005). The inactive estrogens (E1-S, E2-S) can be transformed into the active estrogens by the sulfatase pathway involving the enzyme steroid sulfatase (STS) which converts E1-S into E1 and E2-S into E2 (Pasqualini and Chetrite, 2005; Reed et al., 2005).

All the enzymes involved in the synthesis and metabolism of estrogens have been found to be expressed in human breast cancer and some of them (17ß-HSD type 1, 5, 7, 12, aromatase and STS) have been also detected in apparently normal tissues adjacent to neoplastic tissues (Pasqualini and Chetrite, 2005; Suzuki et al., 2005; Song et al., 2006). Since the majority of these previous studies have been performed in postmenopausal patients, very little is known about the expression of enzymes involved in synthesis and metabolism of estrogens in normal mammary tissue in premenopausal women. We have then studied the expression and cellular localization of 3ß-HSD, aromatase, three reductive 17ß-HSDs, 17ß-HSD type 1, 7 and 12, the oxidative 17ß-HSD, 17ß-HSD type 2, as well as STS and EST 1E1, in normal breast tissues from premenopausal women using immunocytochemistry and in situ hybridization.

Materials and methods

Tissue of the patients

This study was approved by the institutional review board at Laval University Medical Center. All of the patients signed informed consent forms before participation in this research project. Twenty-five adult human mammary tissues were obtained from premenopausal patients (age of 20-40 years old) who had breast reduction. Immediately following surgical removal, the specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PH 7.4). After 48-hr fixation at 4°C, the samples were dehydrated through increasing concentrations of ethanol, cleared in toluene, and embedded in paraffin or frozen on dry ice in support medium (OCT, Bayer Corp., Elkhart, IN, USA) and kept at -80°C until use.

Immunocytochemistry

Paraffin sections (5µm) were deparaffinised, hydrated, and then treated with 3% H_2O_2 in PBS (pH 7.6) for 15 min. They were incubated overnight at 4°C with the antisera to 3ß-HSD, aromatase, 17ß-HSD type 1, 2, 5, 7, 12 diluted at 1:500 and to STS and EST 1E1 diluted at 1:250.

The characteristics of the antisera against human 3ß-HSD, aromatase, 17ß-HSD types 1, 2, 5, 7, 12 and EST 1E1, have already been described (Luu-The et al., 1989; Almadhidi et al., 1995; Pelletier et al., 2001; Song et al., 2006; Takase et al., 2006, 2007). For preparation of antibodies to STS the peptide sequence 28 to 165 which was overproduced in E. Coli BL-21, in fusion with a His Tag at the carboxy-terminal, using PET23a expression vector (EMD Biosciences, San Diego, CA). The purified protein was subsequently diluted in phosphate saline buffer containing 50% complete Freund's adjuvant (concentration: 3 mg/ml) and injected sc with 1 ml at

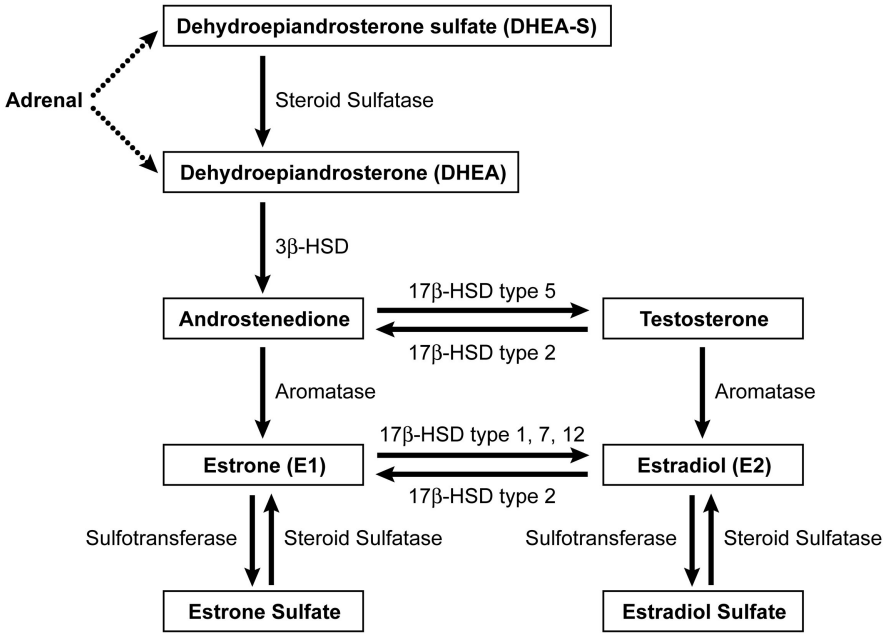


Fig. 1. Representation of the pathways involved in the biosynthesis and metabolism of estradiol from circulating dehydroepiandrosterone (DHEA).

multiple sites on four New Zealand rabbits. The animals were treated twice with the same amounts of proteins in 50% of Freund's adjuvant at 1 month intervals. Antisera were analyzed by immunoblot using HEK-293 cells non-transfected and stably transfected with STS, as negative and positive controls, respectively. The proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose gel for analysis with the protein A-purified antibody to EST 1E1 (diluted 1: 6000). Horse anti-rabbit IgG antibody conjugated with horseradish peroxidase (Amersham Biosciences, Inc., Baie d'Urfé) was used as secondary antibody (dilution: 1: 10 000) and the resulting immunocomplexes were then visualized using enhanced chemiluminescence kit (Perkin Elmer Life Science) and exposed on a X-OMAT blue film for 30 sec. As shown in Figure 2, the antibodies react only with the overexpressed protein. The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC; Ottawa, Ontario, Canada) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC; Rockville, MD). The study was performed in accordance with the CCAC Guide for Care and Use of Experiment Animals. Control experiments were performed on adjacent sections by substituting nonimmune rabbit serum diluted 1:500 or 1:250 or preabsorbed with an excess of the corresponding enzyme (10^{-6}M). All the sections were then washed and incubated at room temperature for 4 hours with peroxidase-labeled goat anti-rabbit γ-globulin (Hyclone; Logan, UT) diluted 1:200, as previously described (Pelletier et al., 1995). Peroxidase was revealed during incubation with 10 mg of 3,3'-diaminobenzidine (DAB) in 100 ml of Tris- saline buffer containing 0.03% H_2O_2. The intensity of the staining was controlled under the microscope. Thereafter, the sections were counterstained with hematoxylin.

In situ hybridization

Frozen sections (10 µm thick) were serially cut and mounted onto glass slides. The vector used for production of the cRNA probe was constructed by insertion into a pCR-Blunt II-TOPO (Invitrogen; Ontario, Canada) of cDNA fragments. The characteristics of the fragments of the human enzyme cDNAs that have been studied are listed in Table 1. In situ hybridization with the antisense and sense ^{35}S-labeled cRNA probes was performed as previously described (Givalois et al., 1997). Briefly, the sections were prehybridized at room temprerature in a humid chamber for 2 hrs in 450 µl/slide of a pre-hybridization buffer containing 50% formamide, 5 x SSPE (1 x SSPE = 0.1 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 0.8 mM EDTA), 5 x Denhart's buffer, yeast-t-RNA 200 µg/ml, Poly A (Boehringer-Mannheim; Montreal, Canada), and 4% dextran sulphate. After prehybridization treatment, 100 µl hybridization mixture (prehybridization buffer containing 10 mM dithiothreitol and the ^{35}S-labeled cRNA probe at a concentration of $10x10^6$ cpm/ml) was spotted on each slide, sealed under a coverslip, and incubated at 60°C overnight (15-20 hr) in a humid chamber.

After hybridization, coverslips were removed and slides were rinsed in 2 x SSC at room temperature for 30 min. Sections were then digested by RNase A (20 µg/ml

Table 1. Characteristics of the cDNA fragments used for cRNA probe production.

Enzyme	Enzymatic activity	GenBank number	cDNA fragment
3ß-HSD type 1	E_2 Formation	NM_000862	1294-1339
17ß-HSD type 1	E_2 Formation	NM_000413	505-760
17ß-HSD type 2	E_2 Inactivation	NM_002153	308-838
17ß-HSD type 5	E_2 Formation	NM_003739	422-456
17ß-HSD type 7	E_2 Formation	NM_016371	565-1023
17ß-HSD type 12	E_2 Formation	NM_016142	177-747
Aromatase	E_2 Formation	NM_000103	565-1108
Sulfatase	E_2 Formation	NM_000351	1293-1763
Sult1E1	E_2 Inactivation	NM_005420	205-971

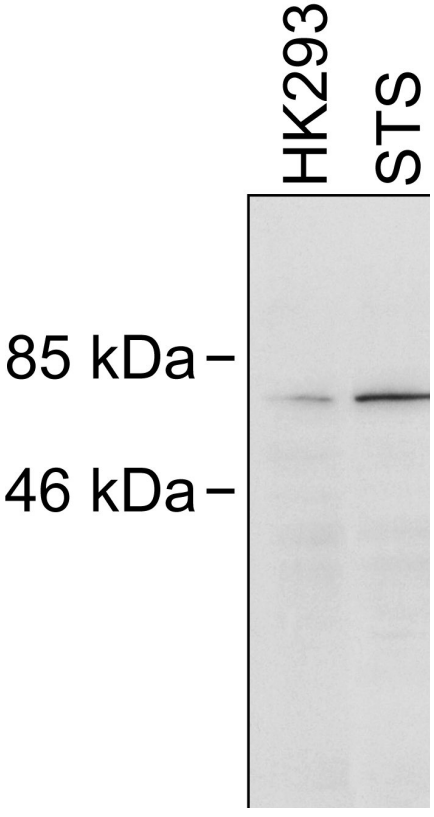


Fig. 2. Western blot analysis of proteins from untransfected or transfected HK293 cells stably overexpressing steroid sulfatase (STS) (MW: 65.5 kDa). The antiserum specifically reacts with the overpressed enzyme.

in 2 x SSC) at 37°C for 30 min, rinsed in decreasing concentrations of SSC (2 x SSC and 2 x SSC) for 30 min at room temperature, washed in 0.5 x SSC for 30 min at 37°C, followed by 90 min at room temperature in 0.5 x SSC, and finally for 30 min at room temperature in 0.1 x SSC.

The sections were then exposed to Hyperfilm (Kodak; Rochester, NY) for 4-5 days and subsequently coated with liquid photographic emulsion (Kodak-NTB2; diluted 1:1 with water). Slides were exposed for 14-42 days, developed in Dektol developer (Kodak) for 2 min, and fixed in rapid fixer (Kodak) for 4 min. Thereafter, the sections were rinsed and stained with hematoxylin and eosin.

Results

Immunocytochemistry

In mammary glands of premenopausal women, immunostaining for 3ß-HSD was weak and inconsistently observed in the cytoplasm of epithelial cells in alveoli and ducts, while a strong reaction could be detected in stroma cells in close proximity of ducts and alveoli (not shown) (Table 2). Labeling was also detected in blood vessel walls and adipocytes. 17ß-HSD type 1 was found in stromal cells associated with the alveoli and ducts as well as in blood vessel walls and adipocytes. Staining was detected in epithelial cells in only one case (out of 25) (not shown). With antibodies to 17ß-HSD type 2, specific reaction was detected on the cytoplasm of epithelial cells in alveoli and ducts as well as in stromal cells, blood vessel walls and adipocytes (Fig. 3). Immunostaining for 17ß-HSD type 5 was localized in the cytoplasm of ductal and alveolar epithelial cells, as well as in the blood vessel walls (Fig. 4). 17ß-HSD type 7 immunoreactivity was found in the cytoplasm and occasionally in nuclei in both epithelial and stromal cells (Fig. 5). 17ß-HSD type 12 was located in both the cytoplasm and nucleus of ductal and alveolar cells as well as stromal cells, adipocytes and blood vessel walls (Fig. 6). In a very few cases, the staining was restricted to the the basal cells of ducts and alveoli. Aromatase was largely distributed throughout the mammary gland tissue, the labeling being particularly intense in the cytoplasm and nucleus of basal cells in ducts and alveoli with labeling also occurring in stromal cells as well as in adipocytes and blood vessel walls (not shown). Strong STS immunostaining was observed in the cytoplasm and occasionally in the nuclei of epithelial cells especially those located on the outer layer of ducts and acini (Fig. 7) as well as in the cytoplasm of stromal cells. Immunolabeling for EST 1E1 was similar to that observed for STS with cytoplasmic localization in epithelial cells in the outer zone of ducts and alveoli and in stromal cells (Fig. 8).

When we used antisera immunoabsorbed with the corresponding antigen or preimmune antisera, no reaction could be detected (not shown).

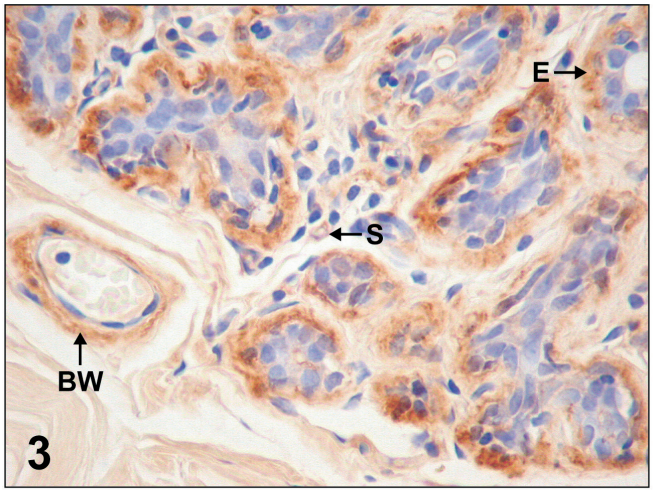
In situ hybridization

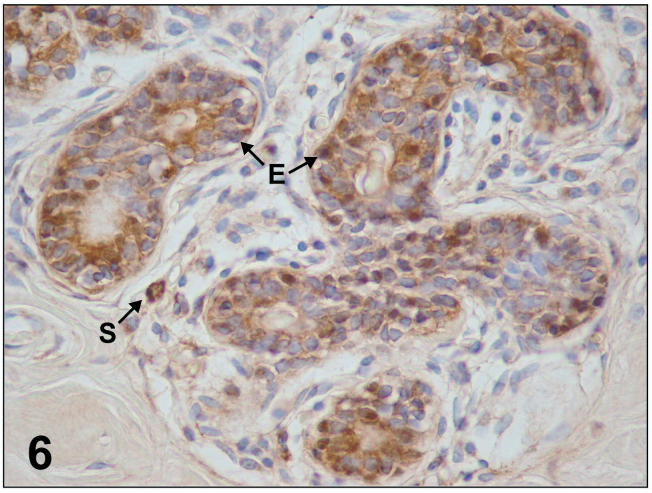
After 4-5 days of exposure to the films, specific hybridization signals could be obtained for all the cRNA probes with the exception of 3ß-HSD probe which could not generate any signals. As observed at the light microscopic level after 14-42 days of exposure, the localization of enzyme mRNAs was similar to that observed by immunocytochemistry. Specific labeling was detected in both epithelial and stromal cells for the following enzymes: 17ß-HSD type 1, 2, 5, 7 and 12, aromatase, EST 1E1 and STS (Figs. 9-14; Table 2). The use of frozen sections did not allow to clearly identify the layers of the epithelial cells which were labeled in alveoli and ducts. No specific signal could be detected in blood vessel walls or adipocytes. When hybridization was performed using the sense probes, only weak diffuse labeling could be seen (Figs. 9-14).

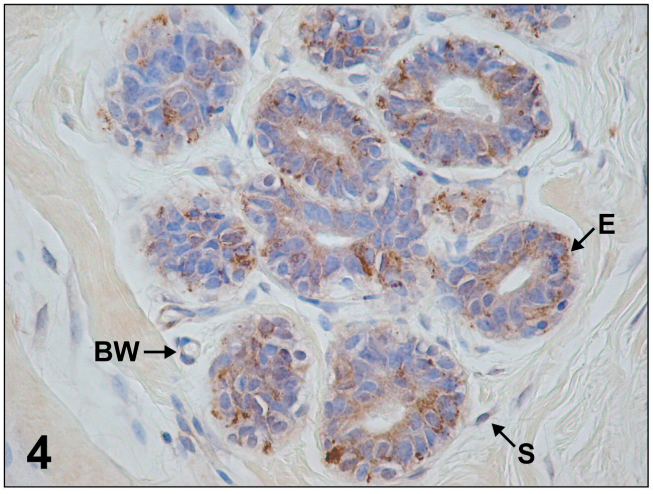
Table 2. Mammary gland cell types expressing the enzymes involved in the synthesis and metabolism of E2.

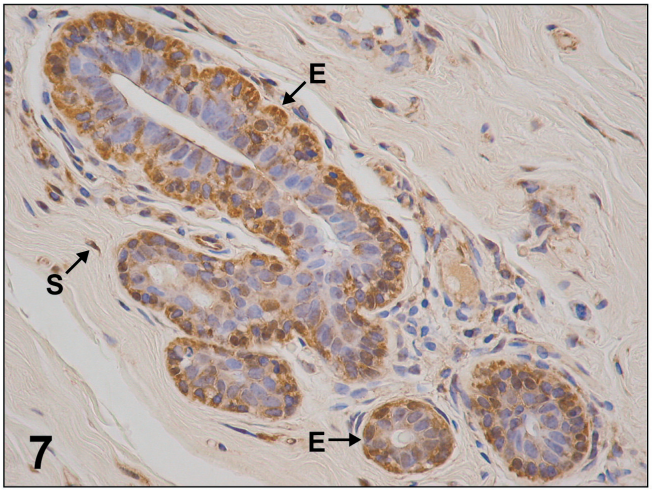
Enzyme/Technique	Labeled cell types	
	Epithelial	Stromal
3ß-HSD type 1		
Immunocytochemistry	+/-	+++
In situ hybridization	-	-
17ß-HSD type 1		
Immunocytochemistry	-	++
In situ hybridization	+++	++
17ß-HSD type 2		
Immunocytochemistry	++	+
In situ hybridization	+/-	+
17ß-HSD type 5		
Immunocytochemistry	++	+
In situ hybridization	++	+
17ß-HSD type 7		
Immunocytochemistry	++	+/-
In situ hybridization	+	+
17ß-HSD type 12		
Immunocytochemistry	+++	+
In situ hybridization	++	+
Aromatase		
Immunocytochemistry	+++	++
In situ hybridization	+	+/-
Steroid sulfatase		
Immunocytochemistry	+++	++
In situ hybridization	+++	+
Estrogen Sulfotransferase 1E1		
Immunocytochemistry	+++	+
In situ hybridization	++	+

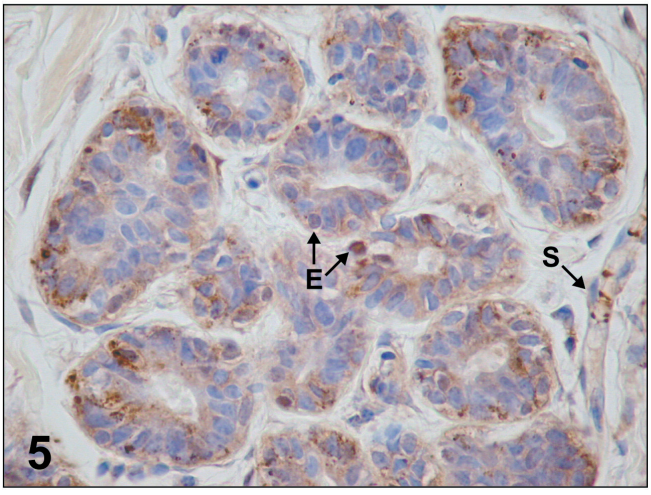
negative: <1% positive cells; +/-: 1-5% positive cells; +: 5-25% positive cells; ++: 25-50% positive cells; +++: >50% positive cells.

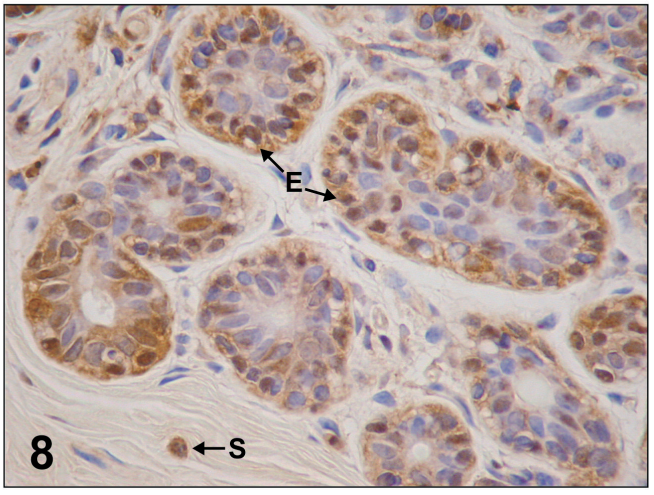




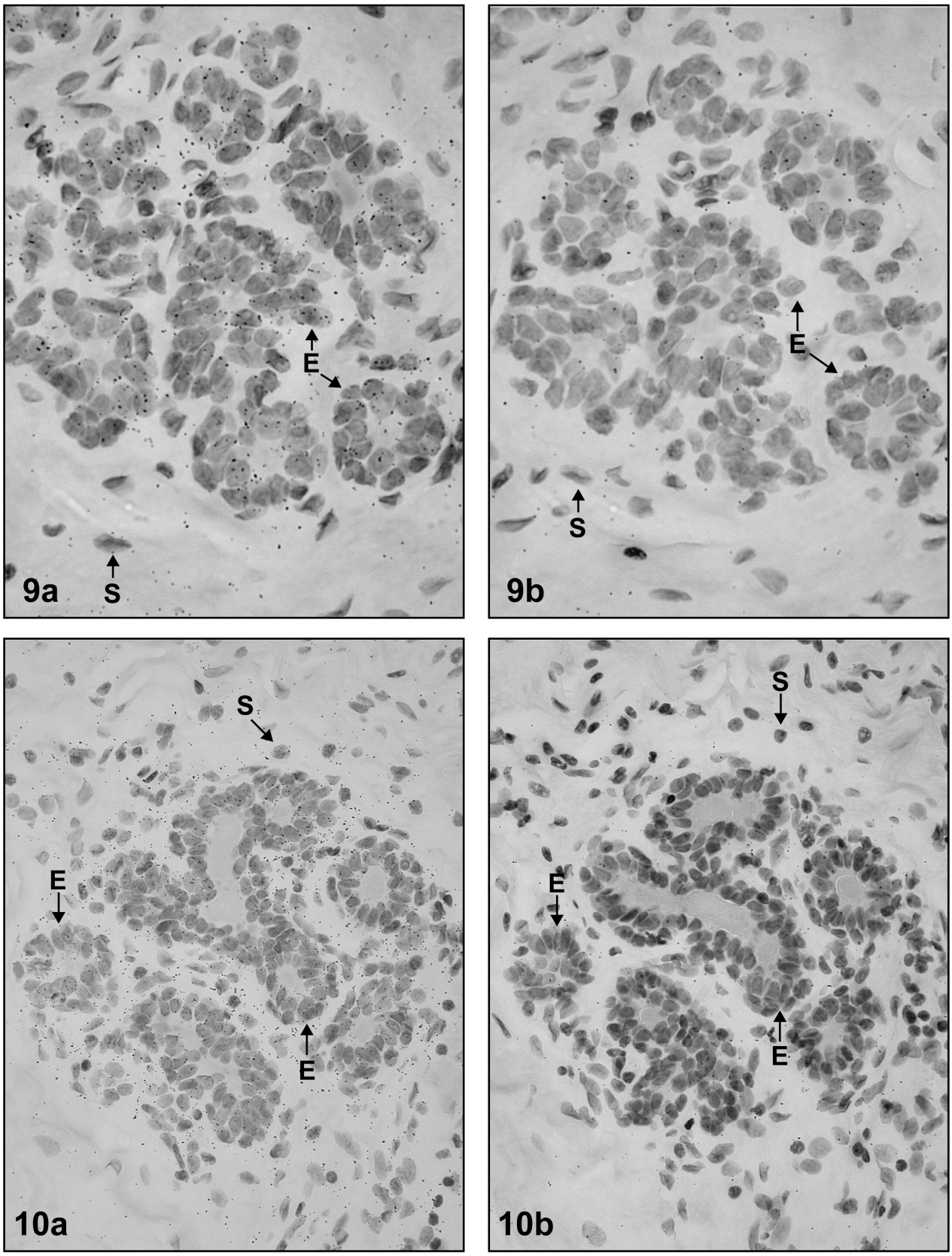








Figs. 3-8. Representative micrographs illustrating the localization of the enzymes as studied by immunocytochemistry. **Fig. 3.** 17ß-HSD type 2. Specific reaction is observed in the cytoplasm of epithelial cells (E) in alveoli and stromal cells (S) as well as blood vessel walls (BW). x 600. **Fig. 4.** 7ß-HSD type 5. Positive labeling is localized in the cytoplasm of epithelial cells (E) in alveoli and ducts as well as stromal cells (S). A blood vessel (BW) wall is also labeled. x 400. **Fig. 5.** Immunostaining for 17ß-HSD type 7. Labeling is seen in the cytoplasm and nuclei in epithelial cells (E) in alveoli and ducts and stromal cells (S). x 600. **Fig. 6.** Immunostaining for 17ß-HSD type 12. Specific staining is observed in both cytoplasm and nuclei of alveolar and ductal epithelial cells (E) as well as stromal cells (S). x 600. **Fig. 7.** Immunostaining for steroid sulfatase. Strong immunolabeling is observed in the cytoplasm and occasionally in the nuclei of epithelial cells (E) especially those located on the outer layer of ducts and alveoli, as well as in the cytoplasm of stromal cells (S). x 600. **Fig. 8.** Immunostaining for estrogen sulfotransferase 1E1. Staining is found in the cytoplasm and nuclei in epithelial cells (E) of ducts and alveoli and also in the cytoplasm of stromal cells (S). x 600



Figs. 9-14. Representative micrographs illustrating the localization of the enzymes as studied by in situ hybridization. **Fig. 9.** 17ß-HSD type 2. **a.** Silver grains are overlying both epithelial (E) and stromal (S) cells. **b.** Consecutive section hybridized with the sense probe. Few dispersed silver grains can be observed. x 600. Exposure time: 42 days. **Fig. 10.** 17ß-HSD type 5. **a.** Numerous silver grains can be observed over epithelial cells (E) in ducts and alveoli as well as stromal cells (S). **b.** Consecutive section hybridized with the sense probe. Only a few silver grains can be detected. x 600. Exposure time: 21 days.

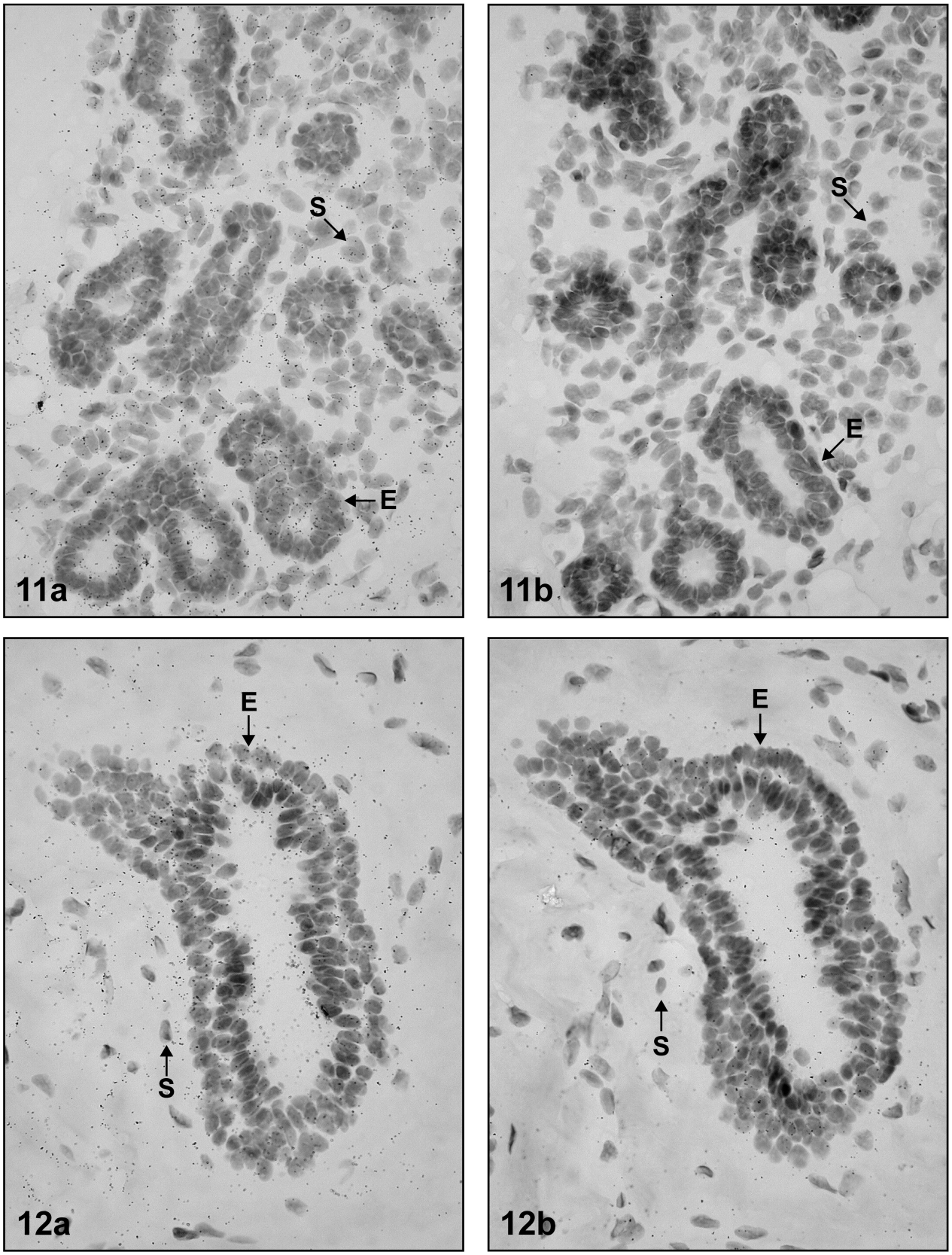


Fig. 11. 17ß-HSD type 7. A. Silver grains are shown overlying the epithelial cells (E) of alveoli and stromal cells (S). Control section hybridized with the sense probe. Very weak labeling can be seen. x 600. Exposure time: 42 days. **Fig. 12.** 17ß-HSD type 12. **a.** Labeling is observed over epithelial cells (E) of a duct. Stromal cells (S) are also specifically labeled. **b.** Consecutive section hybridized with the sense probe. Dispersed silver grains can be detected. x 600. Exposure time: 35 days.

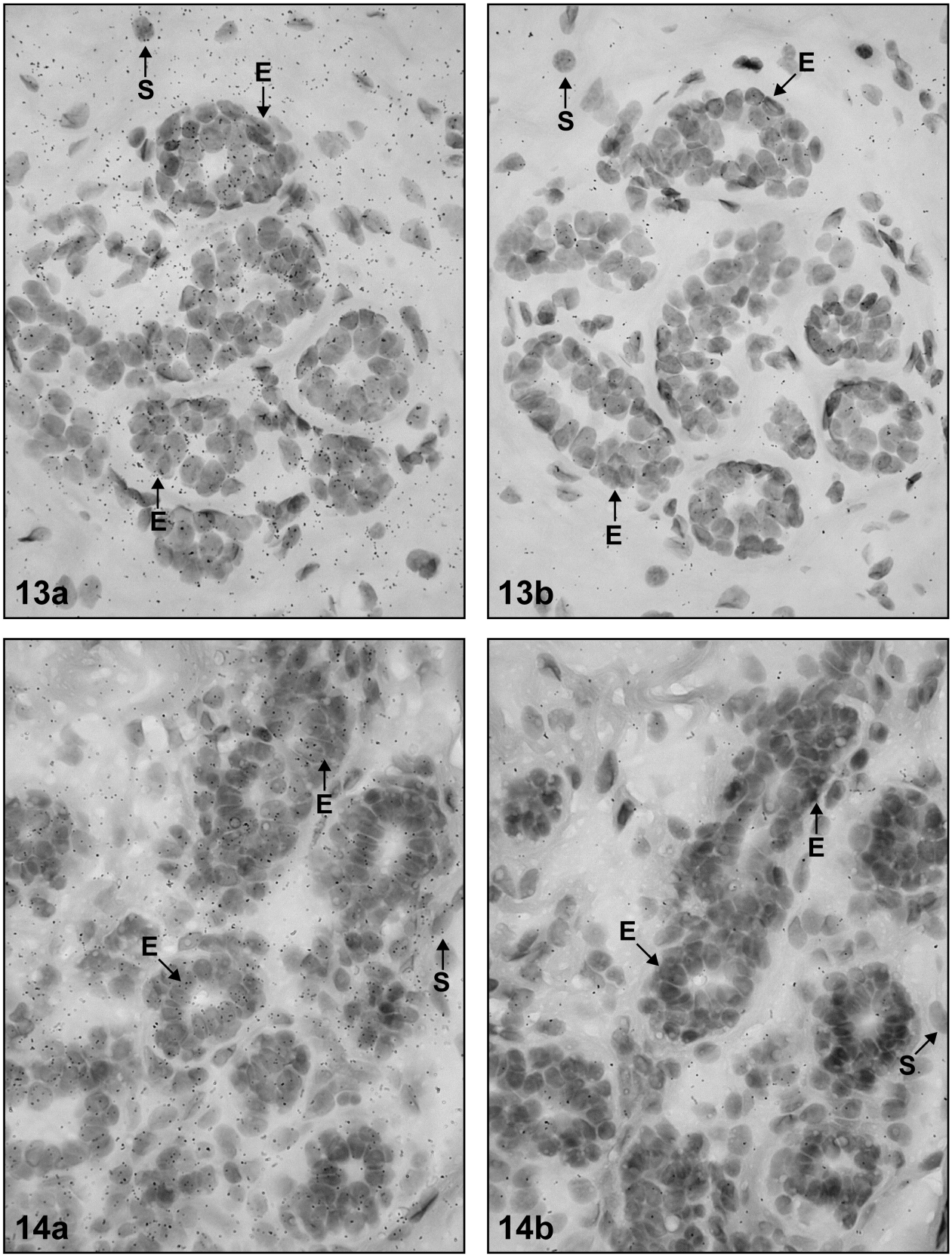


Fig. 13. Steroid sulfatase. **a.** Silver grains are overlying epithelial cells (E) of alveoli as well as stromal cells (S). **b.** Consecutive section hybridized with the sense probe. Few dispersed silver grains are seen. x 600. Exposure time: 35 days. **Fig. 14.** Estrogen sulfotransferase E1E. **a.** Labeling is observed over epithelial cells (E) of alveoli and stromal cells (S). **b.** Consecutive section hybridized with the sense probe. Few silver grains can be detected. x 600. Exposure time: 14 days.

Discussion

The results obtained by a combination of immunocytochemistry and in situ hybridization techniques indicate that the enzymes involved in the synthesis of E2 from DHEA as well as two enzymes involved in the inactivation of E2 (EST1E1 and 17ß-HSD type 2) are expressed in normal breast tissues from premenopausal women.

3ß-HSD which converts DHEA to androstenedione has already been shown to be active in several peripheral intracrine tissues including the mammary gland (Lachance et al., 1990; Martel et al., 1994). The present data indicate that immunostaining for 3ß-HSD is located in stromal cells surrounding the acini and ducts and also to a lesser extent in epithelial cells. These results are in agreement with our previous results obtained by immunoelectron microscopy showing the localization of immunoreactive 3ß-HSD to the epithelial and stromal cells (Pelletier et al., 2001).

17ß-HSD type 5, the enzyme which converts androstenedione to testosterone has been detected by immunocytochemistry and in situ hybridization in both epithelial and stromal cells with the highest expression being found (by both approaches) in the epithelial cells. This confirms our previous immunocytochemical results showing high expression of the enzyme in acini cells in human mammary gland (Pelletier et al., 1999, 2001). We have also recently observed using immunocytochemistry and in situ hybridization that 17ß-HSD type 5 was expressed in high percentage of human breast cancer (unpublished data).

Aromatase which is a key enzyme in the biosynthesis of estrogens (Fig. 1) has been found by immunocytochemistry the cytoplasm and nuclei of epithelial cells and the cytoplasm of stromal cells. By in situ hybridization aromatase mRNA was also detected in both cell types. We have previously reported that aromatase was expressed in epithelial cells in acini and ducts as well as in stromal cells in normal breast tissue adjacent to breast carcinoma (Song et al., 2006). In this previous study, the vast majority of the tissues were from postmenopausal patients. It thus appears that the localization of aromatase is similar in both pre and postmenopausal women.

The three reductive 17ß-HSDs, types 1, 7 and 12, which convert E1 to E2 have been found to be expressed in epithelial and stromal cells by the two approaches which have been used. In a previous study performed on normal breast tissue adjacent to cancer tissues from a majority postmenopausal women, we also observed that 17ß-HSD type 7 was localized in the cytoplasm of epithelial and stromal cells, while 17ß-HSD type 12 was found in both cytoplasm and nuclei of epithelial cells and a few stromal cells (Song et al., 2006). In the same study, we could not detect any immunostaining for 17ß-HSD type 1. The discrepancy between those previous results on 17ß-HSD type 1 and the present results obtained in women between 20 and 40 years of age could be explained by the age of the patients. It has also been reported that 17ß-HSD type 1 mRNA was not expressed in normal breast tissue from postmenopausal women (Oduwole et al., 2004).

EST1E1 converts estrogens into their sulfates (E1-S, E2-S) while the inactivated sulfated estrogens can be transformed into the active estrogens by the enzyme STS which converts E1-S into E1 and E2-S to E2. Both enzymes have been shown to be expressed in the cytoplasm of epithelial and stromal cells. The expression of the two enzymes have also been studied in human breast carcinoma by immunocytochemistry (Suzuki et al., 2003). In this previous study it was mentioned that both EST1E1 and STS were expressed in epithelial cells in normal breast tissue, the expression of STS being very weak. No reaction for either EST1E1 and STS could be detected in the stroma. The discrepancy between those previous results and the present ones could be explained by difference in the sensitivity and/or specificity of the antibodies used.

17ß-HSD type 2, the enzyme which converts E2 to E1, thus responsible for the peripheral inactivation of estrogens, was found to be expressed in epithelial cells in acini and ducts and stromal cells, as well as in adipocytes and blood vessel walls. By in situ hybridization, it was observed that 17ß-HSD type 2 mRNA was expressed at low levels in epithelial cells in ducts from normal breast of premenopausal women, while no signal could be detected in specimens from postmenopausal women (Miettinen et al., 1999; Oduwole et al., 2004). By immunocytochemistry, no 17ß HSD type 2 immunoreactivity could be detected in beast cancer and non cancerous breast tissue (Suzuki et al., 2000). The discrepancy between those previous results and those obtained in the present study could be explained by higher sensitivity of the antibodies developed in our laboratory and the cRNA probes which we used. Interestingly, it has been reported that 17ß-HSD type 2 play a predominant role in the conversion of E2 into E1 in normal breast tissue (Miettinen et al., 1999).

In summary, we report that the enzymes involved in E2 biosynthesis and metabolism are expressed in both epithelial and stromal cells in breasts of premenopausal women. These enzymes might contribute to the local regulation of E2 concentration in human breast tissue.

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