

Summer 2003

Regulation of Steroid Sulfatase in Human Breast Cancer Cells and Screening of Human Tissues for Steroid Sulfatase Status

Abhinav Chandra

Follow this and additional works at: <https://dsc.duq.edu/etd>

Recommended Citation

Chandra, A. (2003). Regulation of Steroid Sulfatase in Human Breast Cancer Cells and Screening of Human Tissues for Steroid Sulfatase Status (Master's thesis, Duquesne University). Retrieved from <https://dsc.duq.edu/etd/391>

This Immediate Access is brought to you for free and open access by Duquesne Scholarship Collection. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Duquesne Scholarship Collection. For more information, please contact phillips@duq.edu.

**Regulation of Steroid Sulfatase in Human Breast
Cancer Cells and Screening of Human Tissues for
Steroid Sulfatase Status**

A Thesis Presented to the Bayer School of Natural and
Environmental Sciences
Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements for the
Degree of Master of Science

by

Abhinav B. Chandra
M.B.,B.S., (University of Mumbai, India)

Thesis Advisor: Kyle W. Selcer, Ph.D.

Thesis Committee:
Richard P. Elinson, Ph.D.
John S. Doctor, Ph.D.
Kyle W. Selcer, Ph.D.

©Copyright Abhinav B. Chandra, 2003

Signature page

Name: Abhinav B. Chandra
Thesis Title: Regulation of Steroid Sulfatase in Human Breast Cancer
Cells and Screening of Human Tissues for Steroid
Sulfatase Status
Degree: Master of Science
Date: July 16, 2003

Approved: _____
Dr. Kyle W. Selcer, Advisor
Department of Biological Sciences

Approved: _____
Dr. Richard P. Elinson, Committee Member and Chairman
Department of Biological Sciences

Approved: _____
Dr. John S. Doctor, Committee Member
Department of Biological Sciences

Approved: _____
Dr. David W. Seybert, Dean
Bayer School of Natural and Environmental Sciences

ACKNOWLEDGEMENTS

I would like to thank Dr. Selcer for his guidance and support throughout. He has been a true inspiration and a great mentor. I would also like to thank Dr. Elinson and Dr. Doctor for agreeing to be on my committee and providing valuable feedback. I would like to thank Lisa Nespoli for her companionship and encouragement and also other members of the Selcer lab. I appreciate Suman Barua's help in carrying out the scoring of the slides. I am also grateful to my fellow graduate students who all have made this journey a memorable one. Last but not the least, I am thankful to my family for everything.

ABSTRACT

Breast cancer is the most frequently occurring cancer in women and is the second leading cause of death. About 40% of breast cancers are hormone-dependent and require estrogen for their growth. The steroid sulfatase pathway is an important means of converting local inactive estrogens to their active forms, thus supporting tumor growth. A substantial amount of research has been performed in the design of steroid sulfatase inhibitors, but the regulation of steroid sulfatase has been largely overlooked. My goal was to study the regulation of steroid sulfatase by different steroid hormones, using two human breast cancer cell lines the hormone-dependent MCF-7, and the hormone-independent MDA-MB-231. I also studied the effect of various growth factors (oxytocin, prolactin, IGF-1 and EGF) on steroid sulfatase activity of MDA-MB-231 cells. MDA-MB-231 cells treated in whole growth medium showed a decrease in steroid sulfatase activity, whereas, those treated with cortisol had an increase in steroid sulfatase activity. Estradiol and estrone sulfate had no effect on steroid sulfatase activity in MDA-MB-231 cells. No differences in steroid sulfatase activity were found for any treatments in hormone-dependent MCF-7 cells. Growth factors also did not have any effect on the steroid sulfatase activity in MDA-MB-231 cells. Recent evidence shows that steroid sulfatase immunoreactivity is associated with breast cancer relapse. Hence, steroid sulfatase could be an independent predictive marker for breast cancer. My other aim was to evaluate different human tissues for steroid sulfatase status by immunohistochemistry. The results showed that placenta, liver, normal breast, breast carcinoma (ER+/ PR+) and skeletal muscle had high immunoreactivity for steroid sulfatase. Skin, lung, brain and

uterine carcinoma showed moderate steroid sulfatase activity, whereas, adrenal gland, breast carcinoma (ER-/ PR-), ovarian carcinoma and prostate had very low steroid sulfatase activity. The study of regulation and inhibition of steroid sulfatase may be important in treatment, prognosis and reduction of mortality due to breast cancer.

TABLE OF CONTENTS

Signature page	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
TABLE OF CONTENTS	vi
LIST OF FIGURES	vii
CHAPTER 1	1
Background.....	1
Breast cancer.....	1
Steroid sulfatase inhibition	4
Steroid hormones	7
Steroid hormone receptors.....	8
Steroid hormone synthesis.....	10
Specific Aims.....	12
CHAPTER 2	13
Experimental Design.....	13
Methods	15
Chemicals and Reagents.....	15
Cell Culture and Media	15
Steryl Sulfatase Assay of Intact Cells after treatment with steroid hormones	16
Steroid sulfatase assay of MDA-MB-231 cells after treatment with oxytocin, prolactin, IGF-1 and EGF	17
BCA Assay	18
MTT Assay	18
Statistical analysis	19
Results	20
Discussion.....	26
CHAPTER 3	30
Experimental design.....	30
Model systems	30
Antibody	30
Assay design	30
Methods	31
Steroid sulfatase immunohistochemistry.....	31
Protein A chromatography.....	32
Results	34
Discussion.....	43
References	46

LIST OF FIGURES

Figure 1. Synthesis of steroid hormones.....	9
Figure 2. Steroid sulfatase activity in MDA-MB-231 cells after treatment with 1 μ M cortisol, estradiol, estrone sulfate, EMATE or whole growth medium.....	22
Figure 3. Steroid sulfatase activity in MDA-MB-231 cells after treatment with 10 μ M cortisol, estradiol, estrone sulfate or whole growth medium.....	23
Figure 4. Steroid sulfatase activity in MCF-7 cells after treatment with 10 μ M cortisol, estradiol, estrone sulfate, EMATE or whole growth medium.....	24
Figure 5. Steroid sulfatase activity in MDA-MB-231 cells treated with 0.2 μ M of oxytocin, 500 ng/ml of prolactin, 2 μ g/ml of IGF-1 or 1 μ g/ml of EGF.....	25
Figure 6. Immunohistochemical analysis for steroid sulfatase of human tissues.....	35
Figure 7. Slides showing immunohistochemical analysis for steroid sulfatase of various human tissues.....	36

CHAPTER 1

Background

Breast cancer

Breast cancer is the most commonly occurring cancer among women and it is the second leading cause of death due to cancers in women. During 2002, about 203,500 new cases of invasive breast cancer are expected to occur among women in United States and about 40,000 persons are expected to die of breast cancer this year (American Cancer Society, 2002). Although men can have breast cancer, it is much less common than in women.

Breast cancer can be categorized as either hormone-dependent or hormone-independent. About 40% of breast cancers are hormone-dependent, meaning that growth of these cells require hormones. The most important hormones in this respect are estradiol and androstenediol (Ahmed et al., 2002). Hormone-dependent tumor cells express estrogen receptor (ER). In contrast, hormone-independent breast cancer cells do not express ER and steroid hormone is not required for tumor growth. Before selecting treatment, it becomes important to determine if a breast tumor is hormone-dependent or hormone-independent, because treatment methods are different.

Effective ways of treating hormone-dependent cancers can be to block the hormone-receptor or to inhibit hormone synthesis. Several clinical treatments use one of these strategies. For example, tamoxifen is an anti-estrogen used as an endocrine treatment for breast cancer. Tamoxifen blocks the binding of estrogens to estrogen receptor of human breast cancer cells (Jordan, 1997). In an alternative strategy,

formestane, exemestane and anastrozole (Brodie, 2002) are inhibitors of aromatase, which is important in estrogen synthesis.

Steroid sulfatase is an enzyme in another synthetic pathway for estrogens with potential for use in clinical treatment of hormone-dependent cancers. Steroid sulfatase converts various inactive hormones into their active forms by cleaving their sulfate group. This enzyme is implicated in support of hormone-dependent cancers by providing the estrogen locally for the growth of hormone-dependent breast cancer tissue (Santner et al., 1986; Reed and Purohit, 1997).

Aromatase is the enzyme that forms estrogens from androgens, but it appears that steroid sulfatase may play a more important role in formation of estrogens within cancerous tissue. Steroid sulfatase is found in a greater percentage of breast cancers than aromatase and its activity is about 10-times greater than aromatase activity (Santner et al., 1984). Also, steroid sulfatase mRNA levels are markedly increased in breast cancer tissues (Utsumi et al., 2000). Thus, the steroid sulfatase pathway appears to be quantitatively more important than the aromatase pathway in local production of estrogens in cancer cells (Pasqualini et al., 1996).

Not only do estrogens themselves, but also estrogenic androgens, play a crucial role in supporting hormone-dependent tumors. Dehydroepiandrosterone sulfate (DHEAS) is acted upon by steroid sulfatase to form dehydroepiandrosterone (DHEA). Thereafter, DHEA is converted to androstenediol (ADIOL) by 17-ketoreductase. ADIOL is an androgen that mimics estradiol by binding to estrogen receptor, causing estrogenic effects, such as increasing the synthesis of estrogen and progesterone receptors (Adams, 1998). These effects are not caused by conversion of ADIOL to estradiol (van Doorn et

al., 1981). At concentrations present in normal women, ADIOL can act as estrogen in MCF-7 cells (Adams et al., 1981). ADIOL stimulates proliferation of human breast cancer tissue at concentrations normally present in women (Poulin and Labrie, 1986).

Another important aspect of steroid sulfatase is its relationship compared to prognosis of cancer. Steroid sulfatase activity is about three times higher in human breast cancer than in nonmalignant breast tissue (Utsumi et al., 2000). Also, high levels of steroid sulfatase activity are associated with decrease in relapse-free interval. In other words, increase in steroid sulfatase level is associated with relapse (Utsumi et al., 1999). These data suggest that steroid sulfatase could be an independent indicator of prognosis of breast cancer. At present, various prognostic factors are used clinically to help in treatments, such as tumor size, lymph node involvement, distant metastasis and presence of estrogen and progesterone receptor. Use of additional indicators, such as steroid sulfatase status, may further help in assessment and treatment. Recent studies show that steroid sulfatase mRNA levels are elevated in ER-positive breast cancer but not in ER-negative breast cancers (Miyoshi et al., 2003). Another group (Suzuki et al., 2003) studied the steroid sulfatase immunoreactivity in human breast cancer and found that steroid sulfatase expression was present only in carcinoma cells and was associated with increased recurrence and poor prognosis. Hence, it would be worthwhile to test breast cancers for steroid sulfatase status and to assess them as a marker for diagnosis and relapse.

To perform such a study requires a steroid sulfatase antibody, such as the one that has been prepared in Dr. Selcers' laboratory (Selcer et al., 2001). Earlier, a few researchers had used steroid sulfatase antibody but those were prepared by purifying the

steroid sulfatase to some extent from placenta and then injecting it into a rabbit or mouse so as to get the antiserum. Dr. Selcer's laboratory used a different approach. Here, the cDNA sequences of human, rat and mouse steroid sulfatases were aligned, and a conserved region was selected, taking into consideration the following factors – homology among the species, lack of homology to other proteins, high antigenicity, location away from steroid sulfatase active site, and the membrane spanning region. A peptide was synthesized using the sequence selected, coupled with keyhole limpet hemocyanin and then injected into rabbits in order to obtain the antisera. Sera obtained from the rabbits before injecting the antigen are also available to be used as negative control. The immune antisera have been shown by Western blotting to bind to steroid sulfatase in various tissues; namely, human placenta, human breast cancer cells (MCF-7 and MDA-MB-231), rat liver, rat granulosa cells, porcine ovaries and monkey ovaries (Selcer et al., 2001). This antiserum could be used as a tool for carrying out immunohistochemical studies.

Steroid sulfatase inhibition

Given the potential importance of steroid sulfatase in support of breast cancer, inhibition of this enzyme may be useful in therapy (Reed and Purohit, 1993). A variety of inhibitors have been developed. Danazol, a compound used for treating endometriosis, was one of the first compounds to show inhibition of steroid sulfatase, but it was not very potent (James et al., 1992). Estrone-3-methylthiophosphonate (E1-3-MTP) mildly inhibited estrone sulfatase activity in MCF-7 breast cancer cells, human placental and breast tumor tissues (Duncan et al., 1993). The next compound developed was EMATE, (estrone-3-O-sulfamate) which is an extremely potent inhibitor of steroid sulfatase

activity (Reed et al., 1995) and virtually eliminates steroid sulfatase activity in breast cancer cell lines MDA-MB-231 and MCF-7 (Kolli et al., 1999). The disadvantage of EMATE is that it is estrogenic (Elger et al., 1995). Hence, new non-estrogenic steroid sulfatase inhibitors have been designed and synthesized. One of these, COUMATE (4-methylcoumarin-7-O-sulfamate), is active *in vitro* (Purohit et al., 1996). Other compounds synthesized were 3,4-dimethylcoumarin-3-O-sulfamate, 667 COUMATE and 2-methoxyestrone-3-O-sulfamate (2-MeOEMATE) (Woo et al., 1998; Purohit et al., 2000). 667 COUMATE inhibits DHEAS conversion about 25-times more than EMATE in a placental microsomal preparation (Woo et al., 2000). Another group of compounds, the (p-O-sulfamoyl)-N-alkanoyl tyramines, are also potent inhibitors of estrone sulfatase (Selcer et al., 1997). Additionally, the 17 β -(N-alkylcarbamoyl) and 17 β -(N-alkanoyl)-estra-1,3,5,(10)-trien-3-o-sulfamates show anti-steroid sulfatase activity. These compounds have increased affinity for the steroid sulfatase enzyme and decreased estrogenic properties (Li et al., 1998). Many of these compounds are under clinical trial for evaluation as treatments for breast cancer.

Whether a particular inhibitor will be effective in reducing steroid sulfatase activity depends upon whether the steroid sulfatase enzyme is active at the time when the inhibitor is being used. Thus, it becomes important to study what factors regulate the activity of steroid sulfatase, under physiological conditions. At present, not much is known about the regulation of steroid sulfatase.

Different peptide hormones alter steroid sulfatase activity in the placenta (Braverman and Gurpide, 1986). Oxytocin is synthesized in the cell bodies of supraoptic and paraventricular nuclei of the hypothalamus, transported down the axon and released

from the posterior pituitary. Oxytocin stimulates contraction of myoepithelial cells of lactating mammary gland and also stimulates contraction of smooth muscles of the uterus during labor (Robertson, 1995). Prolactin is a hormone that is secreted by the anterior pituitary. Along with other hormones, prolactin helps in normal breast development and lactation, by causing synthesis of milk proteins, lipids and carbohydrates (Frohman, 1995). Oxytocin and prolactin stimulate the activity steroid sulfatase in the placenta before the onset of labor, and inhibit it after labor is initiated (Braverman and Gurpide, 1986).

Various growth factors seem to be involved in regulation of steroid sulfatase. Insulin-like growth factor 1 (IGF-1) is a mitogen that has anti-apoptotic activity. In a prospective case-control study, premenopausal women with increased IGF-1 levels (>256 ng/ml) had increased risk of breast cancer (Toniolo et al., 2000). Epidermal growth factor receptor (EGFR) is expressed by a number of tumors and corresponds to poor prognosis. EGF, binding to EGFR, leads to activation of a second messenger system that eventually leads to proliferation and differentiation of the tumor. In breast cancer, a high level of expression of EGFR is associated with hormone-resistant disease (Morris, 2002). Antibodies against EGFR, namely anti-HER-2 antibody, are now used in combination with chemotherapy to modify cellular response and treat breast cancer (Puztai et al., 1999). Tumor necrosis factor alpha and interleukin-6 may also increase the activity of steroid sulfatase in breast cancer by post-translational modification, or by increasing substrate availability (Newman et al., 2000).

Retinoic acid and carotenoids stimulate the activity of estrone sulfatase in MCF-7 breast cancer cells, but have no effect on its activity in the MDA-MB-231 breast cancer

cell line (Ng et al., 2000). Synthetic progestins, such as medrogestone, promegestone, normegestrol acetate and tibolone, inhibit steroid sulfatase in breast cancer cells (Chetrite et al., 1996; Chetrite et al., 1997; Chetrite et al., 1999). Also, there is evidence that estradiol-17 β inhibits steroid sulfatase activity in estrogen-dependent MCF-7 and T-47D cells (Pasqualini and Chetrite, 2001). Hence, it seems that steroids may also play an important role in regulation of steroid sulfatase.

Steroid hormones

Steroid hormones are a class of signaling molecules that have a variety of functions associated with maintenance of homeostasis. All are derived from cholesterol and share the same basic ring structure, the cyclopentanoperhydrophenanthrene nucleus. This consists of 3 six-carbon cyclohexane rings designated as A, B and C rings and a five-carbon cyclopentane ring denoted as the D ring. On these four rings are attached a variety of side-groups which confer specificity to the different steroid hormones.

Based on their action, steroid hormones are classified into six groups (Duke et al., 1995). Glucocorticoids, produced by the adrenal fasciculata and reticularis, play an important role in carbohydrate metabolism, immunity and stress. Mineralocorticoids, synthesized in the adrenal glomerulosa, maintain sodium and potassium homeostasis. Androgens, produced in the adrenal glands and in the testes, are important in male sexual and reproductive functions. Estrogens, synthesized by the ovarian follicle, are responsible for a number of female sexual and reproductive functions. Progestins, synthesized by the ovarian corpus luteum, are responsible for some female sexual and reproductive functions. Vitamin D, derived from cholecalciferol, has important functions in calcium metabolism.

Regardless of the type of steroid hormone, all act through a similar mechanism. Steroid hormones are present in blood both in free and protein-bound forms. The free hormone is considered the active form. About 95% of steroid hormones are bound to plasma transport proteins. These bound hormones are inactive and thus act as reservoir of steroid.

Steroid hormone receptors

Being hydrophobic, steroids diffuse into the interior of the cell passively. A cell's ability to respond to a particular steroid hormone is based on the presence or absence of specific steroid receptors. Steroids bind to the inactive receptor either in the cytoplasm or in the nucleus.

The steroid receptors are a family of similar proteins which can be divided into 3 functional domains. Domain I acts to activate gene transcription. Domain II, which contains a zinc finger motif, is a domain for DNA binding. Domain III is a steroid binding domain (O'Malley et al., 1991). The steroid receptors are normally associated with chaperone proteins such as heat shock protein 90. Once the receptor binds with hormone, it disassociates from the chaperone protein and gains the ability to bind to DNA. This is known as activation or transformation.

After this, two steroid receptors often dimerize and bind to DNA sequences in the nucleus, usually in the proximity of genes regulated by that hormone. There are cis-elements present at the 5' upstream region of the gene which control the expression of steroid-regulated genes. These are promoters, steroid-responsive enhancers, silencers and hormone-independent enhancers (O'Malley et al., 1991).

Promoters include the TATA box and USP (Upstream promoter) and are responsible for setting the basal rate of transcription. Enhancers are the DNA-binding sites for the activated steroid receptor complexes and are called steroid response elements (SRE). SRE are base pair sequences which are arranged palindromically with respect to each other with a spacer region in between. The hormone-receptor complex identifies this specific sequence and binds to each of the two half sites as a dimer. Silencers decrease the rate of transcription. Hormone-independent enhancers aid the function of SRE. These interactions affect the transcription of genes in a positive or negative way. Thus, the steroid hormone receptors can be viewed as ligand-activated transcription factors.

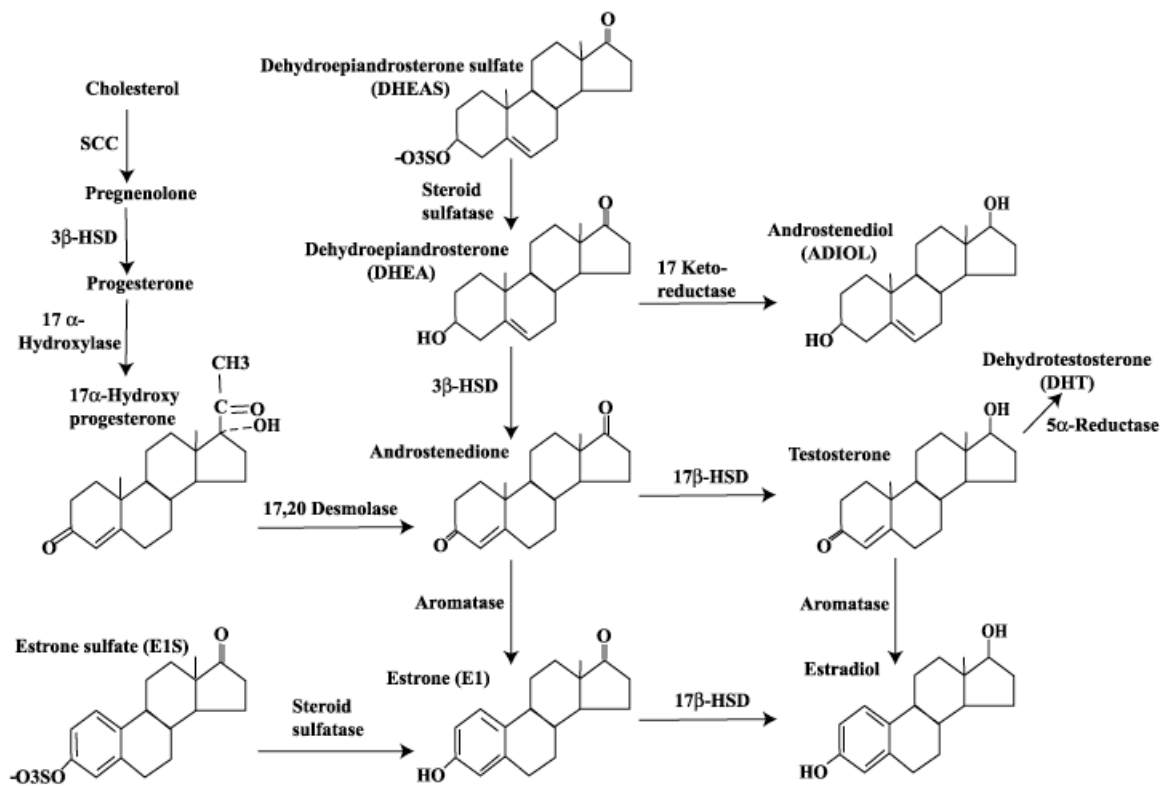


Figure 1 : Synthesis of steroid hormones (adapted from O'Malley et al., 1991)

Steroid hormone synthesis

Steroid hormones are synthesized from a common precursor, cholesterol (Figure 1). In the first step cholesterol (C27) is converted to pregnenolone (C21) by the enzyme CYP 450- scc (side chain cleavage) which is present on the inner mitochondrial membrane (Gill, 1995). This step is the enzymatic rate limiting step in the production of steroids. In the next step, pregnenolone is converted to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β HSD). Further conversions takes place through either the pregnenolone (Δ 5) or the progesterone (Δ 4) pathways. These lead to formation of C21 steroids such as cortisol and aldosterone, C19 hormones such as testosterone and dihydrotestosterone, and C18 steroids such as estrone and estradiol.

The particular steroid hormone that a given tissue produces depends upon the nature of the tissue and the activity of the enzymes present in that tissue. The adrenal glands produce significant amount of cortisol and aldosterone, but lesser amounts of estrogens and testosterone. The testes produce primarily the sex steroid testosterone and lesser quantities of estrogen. In the ovary, the main pathway is the Δ 4 pathway, which forms progesterone, androgens and, more importantly, estrogens from androgens. Aromatase is a cytochrome P450 (CYP19) present in ovarian granulosa cells, adipose tissue and placenta (Gill, 1995). It converts androstenedione and testosterone to estrone (E1) and estradiol (E2), respectively, by aromatization of the A ring.

Steroids produced by one tissue are often modified in other tissues. Steroids can be conjugated with glucuronic acid by the enzyme glucuronyl transferase. This reaction takes place in the liver and uridine diphosphoglucuronic acid is the glucuronide donor. Glucuronidation makes the steroid more water-soluble and is a process of

inactivation for excretion of steroids (O'Malley et al., 1991). Another peripheral conversion is testosterone to dihydrotestosterone, which is a more potent form of testosterone, by the enzyme 5α reductase.

Steroid sulfatase (EC 3.1.6.2) is an enzyme that removes the sulfate group from 3-hydroxysteroid sulfates, such as cholesterol-3-sulfate, dehydroepiandrosterone-3-sulfate and estrone-3-sulfate. It converts dehydroepiandrosterone sulfate (DHEAS) to dehydroepiandrosterone and estrone sulfate to estrone (Purohit et al., 1994). It is primarily localized to the endoplasmic reticulum (Chang et al., 1986). Liver and placenta express steroid sulfatase (Shankaran et al., 1991). Munroe and Chang (1987), showed expression of steroid sulfatase in adrenal gland, muscle, spleen, thyroid gland, heart, lung, intestine and ovary. The steroid sulfatase gene *Sts* is present on the distal portion of the short arm of the X-chromosome and is non-functional on the Y-chromosome (Yen et al., 1987).

Specific Aims

1) (a) To determine the effect of steroid hormones (cortisol, estradiol, estrone sulfate, EMATE and those in whole growth media) on steroid sulfatase activity in human breast cancer cell lines (MDA-MB-231 and MCF-7).

(b) To determine the effect of various growth factors (prolactin, oxytocin IGF-1 and EGF) on steroid sulfatase activity in the hormone-independent breast cancer cell line MDA-MB-231.

2) To screen various human tissues for steroid sulfatase status using immunohistochemistry.

CHAPTER 2

Experimental Design

Specific aim 1) (a) To determine the effect of steroid hormones (cortisol, estradiol, estrone sulfate, EMATE and those in whole growth media) on steroid sulfatase activity in human breast cancer cell lines (MDA-MB-231 and MCF-7).

Model systems

The experiments were conducted on two human breast cancer cell lines, namely, the hormone-dependent breast cancer cell line, MCF-7 and the hormone-independent breast cancer cell line, MDA-MB-231. These two cell lines are widely used in breast cancer research and our lab has extensive experience in using these two cell lines to measure steroid sulfatase activity.

MCF-7 cells are epithelial-like cells obtained from the pleural effusion of a 69-year old Caucasian woman with metastatic breast adenocarcinoma. These cells grow as monolayers and were described to be positive for cytoplasmic estrogen receptors (Soule et al., 1973). Cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and retain several characteristics of differentiated mammary epithelium (ATCC, 2003). Tumor necrosis factor alpha inhibits growth of MCF-7 cells (Sugarman et al., 1985). MCF-7 cells secrete insulin-like growth factor binding proteins (IGFBP) that can be modulated by estrogens and antiestrogens (Pratt and Pollak, 1993).

MDA-MB-231 cells were obtained from a 51-year old Caucasian female with metastatic breast adenocarcinoma. They are epithelial-like cells and are estrogen receptor negative (Cailleau et al., 1974). MDA-MB-231 cells express high levels of transforming growth factor alpha and epidermal growth factor receptor mRNAs (Bates et al., 1990).

Test compounds

Several steroid hormones and agonists/antagonists, were tested for their effect on steroid sulfatase activity, such as, estradiol, cortisol, estrone sulfate, EMATE and whole growth media.

Assay design

Steroid sulfatase activity was determined by using conversion of [^3H]E₁S to [^3H]E₁ and was slightly different as described by MacIndoe, 1988. The activity was measured in estrogen-free media, after steroid hormone treatment.

Specific aim 1) (b) To determine the effect of various growth factors (prolactin, oxytocin, IGF-1 and EGF) on steroid sulfatase activity in the hormone-independent breast cancer cell line MDA-MB-231.

Model systems

The experiments were conducted on a human breast cancer cell line, MDA-MB-231.

Test compounds

Oxytocin (0.2 μM), prolactin (500 ng/ml), IGF-1 (2 $\mu\text{g}/\text{ml}$) and EGF (1 $\mu\text{g}/\text{ml}$) were tested (Braverman and Gurpide, 1986; Toniolo et al., 2000).

Assay design

Steroid sulfatase activity was determined by using conversion of [^3H]E₁S to [^3H]E₁ (MacIndoe, 1988). The activity was measured in estrogen-free serum-free media, after treatment with the various compounds.

Methods

Chemicals and Reagents

³H-estrone sulfate (ammonium salt, [6,7-³H(N)]-; 49 Ci/mmol) and ³H-dehydroepiandrosterone sulfate (sodium salt, [7-³H]-; 16 Ci/mmol) were obtained from DuPont/ New England Nuclear (Boston, MA). Radioinert steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT). EMATE was produced by the method of Purohit et al. (1995). Toluene and Tris were acquired through Fisher Scientific (Pittsburgh, PA). Trypsin-EDTA and Dulbecco's Phosphate Buffered Saline (PBS) were purchased through Sigma (St. Louis, MO).

Cell Culture and Media

MDA-MB-231 (hormone-independent) and MCF-7 (hormone-dependent) human breast cancer cells were acquired from the American Type Culture Collection (Manassas, VA). Cells were plated in 100 x 20 mm tissue culture dishes and medium was changed every other day. Growth medium, which was used for routine cultures and cell growth assays, was purchased from Sigma Chemical Co. (St. Louis, MO). Growth medium consisted of RPMI-1640 (pH 6.9) supplemented with L-Glutamine (0.5mM), 25mM HEPES (pH 7.5), 7.5% sodium bicarbonate (0.2% v/v), 5% fetal calf serum (FCS), 10mg/ml gentamicin (0.2% v/v) and antibiotic/antimycotic (10,000 units penicillin, 10 mg streptomycin and 25µg amphoterecin-B in one ml) (1% v/v). Cells were routinely grown in 100mm tissue culture dishes in 10ml growth medium.

Steryl Sulfatase Assay of Intact Cells after treatment with steroid hormones

MDA-MB-231 and MCF-7 cells in 100 x 20 mm tissue-culture dishes were washed with 10 ml PBS for 1 min. After removing the PBS, 0.5 ml trypsin-EDTA was added to the cells for 30 sec. The trypsin-EDTA was then removed and the dishes were tapped in order to dislodge the cells. Cells were resuspended in 10 ml growth media. Cells were counted using a hemocytometer. Five-hundred thousand cells were plated in six-well plates and incubated overnight in whole growth media at 37° C. After the incubation, the medium was removed and the cells were washed with PBS three times. After that, the cells were incubated in 10µM or 1µM concentrations of cortisol, estradiol, estrone sulfate or EMATE for 48 hours in estrogen-free medium. One molar stock solution of these hormones was prepared in ethanol. These concentrations of steroid hormones used were near physiological levels. One well per six-well plate containing cells was left untreated. One well per six-well plate with no cells was used as a negative control. After two days, the treatments were removed and the cells were washed three times with PBS. The cells were then incubated with estrogen-free medium containing [³H]E₁S at 37° C for 18 h, after which 500µl medium from each well was pipetted into 13 x 100 mm borosilicate glass tubes in duplicate. Three milliliters of toluene were added to each tube for extraction of unconjugated steroids. The mixture was vortexed for one min and then centrifuged at 1000xg for five min at 4°C to separate the aqueous and organic phases. One milliliter of the organic phase was removed from the tubes and placed into scintillation vials in duplicate. Six milliliters of scintillation cocktail was added to each vial. The radioactivity was counted using a Packard Tri-carb scintillation counter at 50% efficiency for ³H. The conversion values obtained for all treatments were adjusted for

spontaneous product formation by subtracting the value obtained for wells containing medium but no cells. Product formation for samples containing test compounds was compared to those of the control samples (no test compounds) and expressed as a percentage of the control. Cells remaining after sulfatase assay were counted by MTT assay.

Steroid sulfatase assay of MDA-MB-231 cells after treatment with oxytocin, prolactin, IGF-1 and EGF

Five-hundred thousand cells were plated in six-well plates and incubated overnight in whole growth media at 37° C. After the incubation, the medium was removed and the cells were washed with PBS three times. After that, the cells were incubated in the desired concentration of the respective treatments [0.2µM oxytocin, (10mg stock); 500 ng/ml prolactin (50µg stock); 2µg/ ml IGF-1 (50µg) and 1µg/ml EGF (0.2mg)] Sigma Chemical Co. (St. Louis, MO) for 48 hours in estrogen-free, serum-free medium. One well per six-well plate containing cells was left untreated. One well per six-well plate with no cells was used as a negative control. After two days, the treatments were removed and the cells were washed three times with PBS. The cells were then incubated with estrogen-free, serum-free medium containing [³H]E₁S at 37° C. After 18 h incubation, 500µl medium from each well was pipetted into 13 x 100 mm borosilicate glass tubes in duplicate. Three milliliters of toluene was added to each tube for extraction of unconjugated steroids. The mixture was vortexed for 1 min and then centrifuged at 1000xg for 5 min at 4°C to separate the aqueous and organic phases. One milliliter of the organic phase was removed from the tubes and placed into scintillation vials in duplicate. Six milliliters of scintillation cocktail was added to each vial. The radioactivity was

counted using a Packard Tri-carb scintillation counter at 50% efficiency for ^3H . The conversion values obtained for all treatments were adjusted for spontaneous product formation by subtracting the value obtained for wells containing medium but no cells. Product formation for samples containing test compounds was compared to those of the control samples (no test compounds) and expressed as a percentage of the control. Cells remaining after sulfatase assay were counted by MTT assay.

BCA Assay

The Pierce (Rockville, IL) BCA Protein Assay was used according to the manufacturer's instructions. A standard curve of bovine serum albumin was prepared with water as the diluent. Duplicate concentrations (in $\mu\text{g}/\text{tube}$) were 200, 150, 125, 100, 75, 50, 30, 20, 10 and 5. A blank of only water was prepared. Membranes were prepared as duplicates at differing amounts such that the volume of membrane and water equal 100 μl . Two milliliters of BCA working reagent was added to each tube, vortexed and incubated in a 37°C waterbath for 30 min. Tubes were then allowed to be cooled to room temperature and absorbances were read at 562 nm using a spectrophotometer. Standards were plotted and the relationship between absorbance and concentration was determined by linear regression. Unknown protein concentration was calculated from the regression equation.

MTT Assay

Two hundred μl of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) solution (5 mg/ml in RPMI-1640) was added to each well of a six-well plate containing cells (media removed) and incubated for three hours at 37°C. At end of the incubation period, the converted dye was solubilized with one ml of acidic

isopropanol. Aliquots of 100 μ l of the acidic isopropanol from a single well on the six-well plate, were then transferred to each of eight consecutive wells on a 96-well microtiter plate. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. Absorbance of converted dye corresponded to the number of cells. To generate standard curves, MDA-MB-231 and MCF-7 cells were plated into six-well plates with each well containing a different number of cells obtained by serial dilution. Cells were incubated in whole medium overnight prior to the MTT assay to allow them to adhere to the plates. The relationship between cell number and absorbances was determined by linear regression.

Statistical analysis

The various treatment groups were compared by one way analysis of variance (ANOVA) with repeated measures. If a significant difference was found, then, Student-Newman-Keuls test was used as a posteriori test. The statistical program used was Prism (GraphPad Inc.).

Results

Effect on growth of the cells by the various compounds

One way analysis of variance showed that cells grown in steroid hormones at 1 μ M concentration had significant differences in cell number when assayed by MTT (F=5.755, 4, 14 df, p<0.001). Cells grown in whole growth media were higher than control cells (Student-Newman-Keuls test, p<0.05). One way analysis of variance also showed significant differences in cell number in MDA-MB-231 cells treated with 10 μ M concentration of steroid hormones (F=6.410, 4,19, df, p<0.001). Again, cells grown in whole growth media showed higher cell number (Student-Newman-Keuls test, p<0.05).

One way analysis of variance on MCF-7 cells treated with 10 μ M steroid hormones showed significant differences in cell number (F=3.769, 4,14, df, p<0.001). Again, there was increase in cell number in MCF-7 cells treated with whole growth medium (Student-Newman-Keuls test, p<0.05). One way analysis of variance (ANOVA) did not reveal any significant differences in cell number in MDA-MB-231 cells treated with growth factors (F= 0.4553, 4, 19 df, p<0.001).

As a result of these differences in cell number, data were normalized for cell number prior to statistical analysis.

Steroid sulfatase assay of MDA-MB-231 cells after treatment with steroid hormones

MDA-MB-231 cells were treated with 1 μ M cortisol, estradiol, estrone sulfate or EMATE. Steroid sulfatase activity was assayed by conversion of [³H]E₁S to unconjugated forms. The readings were expressed as percentage of control (Figure 2). One way analysis of variance (ANOVA) showed significant differences among the treatments (F= 11.26, 4,14 df, p<0.001). Cells treated with whole growth media showed a

significant decrease in steroid sulfatase activity over control, cortisol, estradiol and estrone sulfate (Student-Newman-Keuls test, $p < 0.05$).

MDA-MB-231 cells were treated with 10 μ M cortisol, estradiol or estrone sulfate. Steroid sulfatase activity was assayed by conversion of [^3H]E₁S to unconjugated forms. The readings were expressed as percentage of control (Figure 3). . One way analysis of variance (ANOVA) indicated significant differences in steroid sulfatase activity among groups ($F = 8.638$, 4, 19 df, $p < 0.001$). Cortisol showed a significant decrease in steroid sulfatase activity over control but not over estradiol and estrone sulfate (Student-Newman-Keuls test, $p < 0.05$). Cells grown in whole growth media also showed a decrease in activity over control and all other treatment groups (Student-Newman-Keuls test, $p < 0.05$).

Steroid sulfatase activity in MCF-7 cells treated with steroid hormones

MCF-7 cells were treated with 10 μ M concentration of cortisol, estradiol, estrone sulfate or EMATE. Steroid sulfatase activity was assayed by conversion of [^3H]E₁S to unconjugated forms. The readings were expressed as percentage of control (Figure 4). One way analysis of variance (ANOVA) did not reveal any significant differences among treatment groups ($F = 1.897$, 4, 14 df, $p < 0.001$).

Steroid sulfatase activity in MDA-MB-231 cells treated with growth factors

MDA-MB-231 cells were treated with 0.2 μ M of oxytocin, 500 ng/ml of prolactin, 2 μ g/ml of IGF-1 or 1 μ g/ml of EGF for 48 hours. Steroid sulfatase activity was assayed by conversion of [^3H]E₁S to unconjugated forms. The readings were expressed as percentage of control (Figure 5). One way analysis of variance (ANOVA) showed no statistical differences among the treatment groups ($F = 11.8$, 4, 19 df, $p < 0.001$).

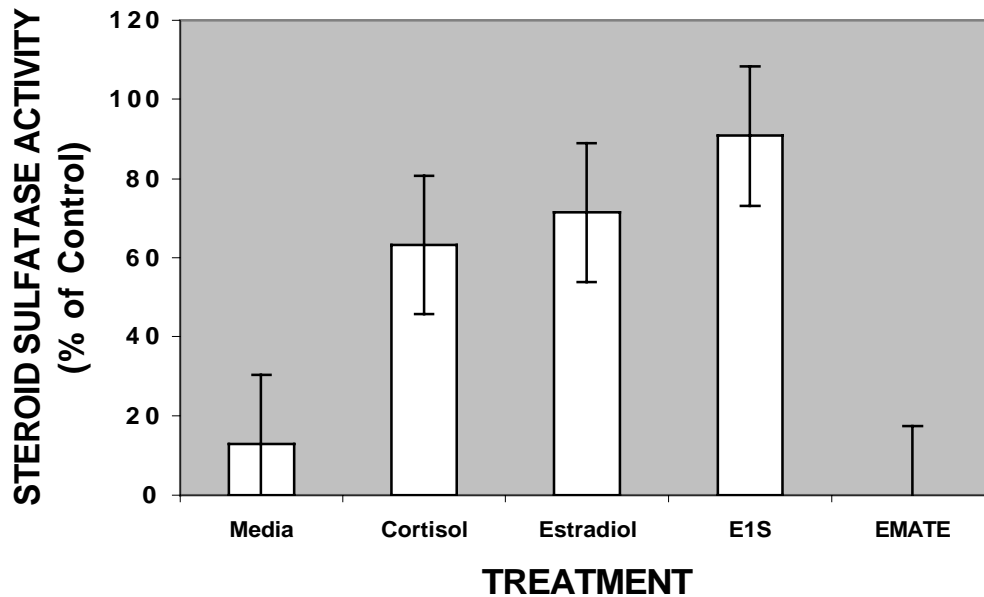


Figure 2: Steroid sulfatase activity in MDA-MB-231 cells after treatment with 1 μ M cortisol, estradiol, estrone sulfate, EMATE or whole growth medium. MDA-MB-231 cells (0.5 million) were plated in six-well plates and incubated overnight in growth medium. The growth medium was removed, cells were washed with PBS and incubated for 48 hours in 1 μ M concentration of cortisol, estradiol, estrone sulfate and EMATE in estrogen-free medium. Wells containing no cells were used as a blank and cells treated with only estrogen-free medium were used as a control. Three wells were incubated in whole growth medium. After 48 hours, the treatments were removed, cells were washed with PBS and incubated in [³H]E₁S for 18 hours. Steroid sulfatase activity was determined and represented as percentage of control. Data are average of 3 experiments with each experiment run in triplicate. Bars are +/- 1 standard error.

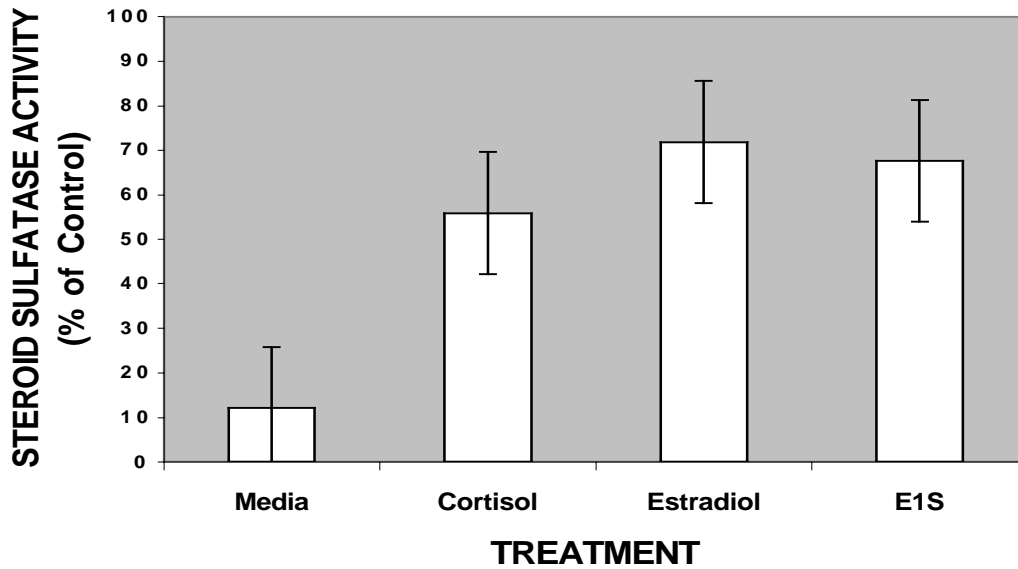


Figure 3: Steroid sulfatase activity in MDA-MB-231 cells after treatment with 10 μ M cortisol, estradiol, estrone sulfate or whole growth medium. MDA-MB-231 cells (0.5 million) were plated in six-well plates and incubated overnight in growth medium. The growth medium was removed, cells were washed with PBS and incubated for 48 hours in 10 μ M concentration of cortisol, estradiol and estrone sulfate in estrogen-free medium. Wells containing no cells were used as a blank and cells treated with only estrogen-free medium were used as a control. Three wells were incubated in whole growth medium. After 48 hours, the treatments were removed, cells were washed with PBS and incubated in [3 H]E $_1$ S for 18 hours. Steroid sulfatase activity was determined and represented as percentage of control. Data are average of 4 experiments with each experiment run in triplicate. Bars are +/- 1 standard error.

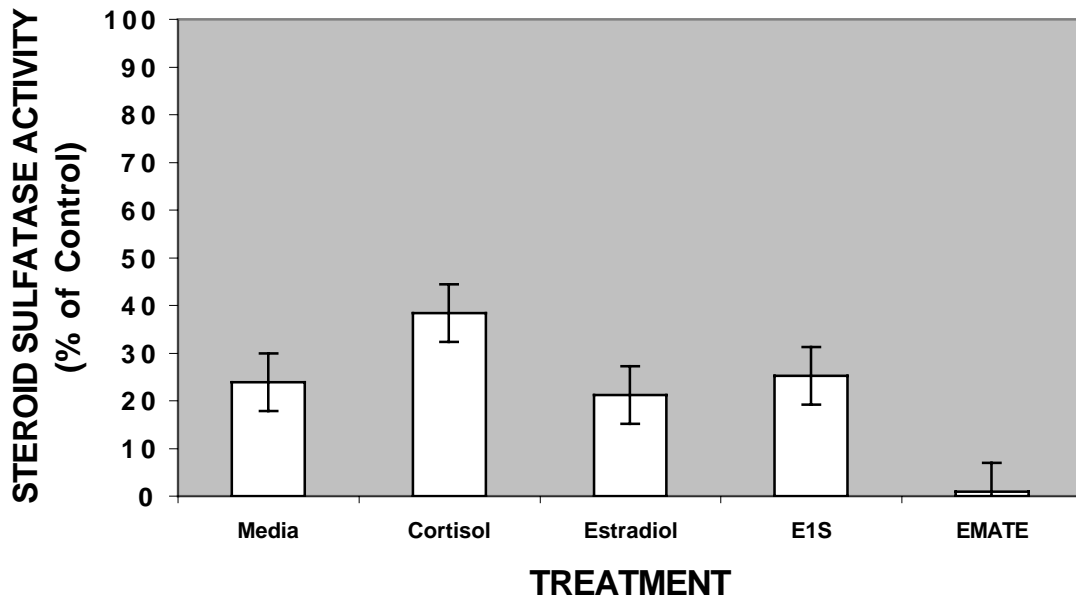


Figure 4: Steroid sulfatase activity in MCF-7 cells after treatment with 10 μ M cortisol, estradiol, estrone sulfate, EMATE or whole growth medium. MCF-7 cells (0.5 million) were plated in six-well plates and incubated overnight in growth medium. The growth medium was removed, cells were washed with PBS and incubated for 48 hours in 10 μ M concentration of cortisol, estradiol, estrone sulfate and EMATE in estrogen-free medium. Wells containing no cells were used as a blank and cells treated with only estrogen-free medium were used as a control. Three wells were incubated in whole growth medium. After 48 hours, the treatments were removed, cells were washed with PBS and incubated in [3 H]E $_1$ S for 18 hours. Steroid sulfatase activity was determined and represented as percentage of control. Data are average of 3 experiments with each experiment run in triplicate. Bars are +/- 1 standard error.

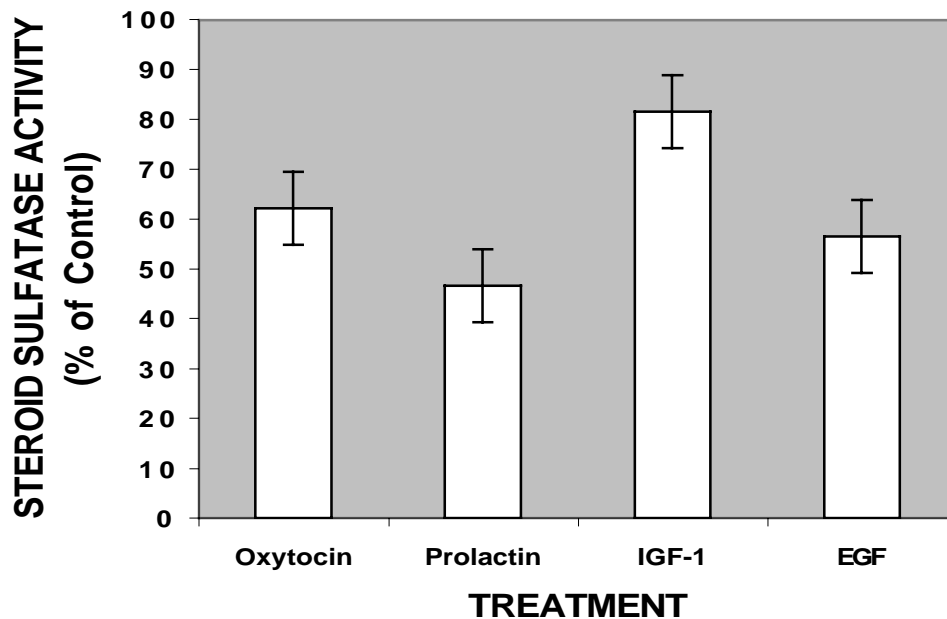


Figure 5: Steroid sulfatase activity in MDA-MB-231 cells treated with 0.2 μ M of oxytocin, 500 ng/ml of prolactin, 2 μ g/ml of IGF-1 or 1 μ g/ml of EGF. MDA-MB-231 cells (0.5 million) were plated in six-well plates and incubated overnight in growth medium. The growth medium was removed, cells were washed with PBS and incubated for 48 hours in 0.2 μ M of oxytocin, 500 ng/ml of prolactin, 2 μ g/ml of IGF-1 or 1 μ g/ml of EGF in estrogen-free, serum-free medium. Wells containing no cells were used as a blank and cells treated with only estrogen-free, serum-free medium were used as a control. After 48 hours, the treatments were removed, cells were washed with PBS and incubated in [3 H]E $_1$ S for 18 hours. Steroid sulfatase activity was determined and expressed as percentage of control. Data are average of 4 experiments with each run in triplicate. Bars are +/- 1 standard error.

Discussion

The steroid sulfatase pathway is an important means of converting local inactive estrogens to their active forms, thereby supporting tumor growth (Santner et al., 1986; Reed and Purohit, 1997). A substantial amount of research has been performed in the design of steroid sulfatase inhibitors, but the regulation of steroid sulfatase has been largely overlooked. My research was to determine whether certain steroid hormones or growth factors are involved in regulation of steroid sulfatase in human breast cancer cell lines.

Two breast cancer cell lines were used for my study: the hormone-independent MDA-MB-231 cells and the hormone-dependent MCF-7 cells. Both of these cell lines are widely used in research of breast cancer and have been used in studies of steroid sulfatase. MDA-MB-231 cells, used extensively by our laboratory, have more steroid sulfatase activity than MCF-7 cells (Selcer et al., 1997). However, in the presence of estrone sulfate as their only source of estrogenic steroid, the level of steroid sulfatase in MCF-7 cells is sufficient enough to sustain their growth (Selcer et al., 1996).

The steroid sulfatase assay that I used has been previously shown to be an effective method for determining steroid sulfatase activity in MCF-7 and MDA-MB-231 cells (Selcer et al., 2002; Kolli et al., 1999; Clemens et al., 2000). EMATE is an extremely potent inhibitor of steroid sulfatase activity (Reed et al., 1995) and virtually eliminates steroid sulfatase activity in breast cancer cell lines MDA-MB-231 and MCF-7 (Kolli et al., 1999). EMATE was added to the assay as a control. As expected, EMATE at 1 μ M concentration eliminated steroid sulfatase activity, indicating that the sulfatase assay was working as expected.

In the first set of experiments, steroid sulfatase activity was assayed after treatment of MDA-MB-231 cells with 1 μ M cortisol, estradiol, estrone sulfate or EMATE. Subsequently, 10 μ M cortisol, estradiol or estrone sulfate were used.

Cortisol decreased steroid sulfatase activity at 10 μ M concentration but not at 1 μ M concentration when compared with control and other treatment groups. Earlier work in collaboration with J. Clemens showed that a potent synthetic glucocorticoid, dexamethasone, decreased steroid sulfatase activity in rat granulosa cell line (POGRS-5) (Clemens, J.W., unpublished data). Also, J. Clemens laboratory studied the steroid sulfatase activity in an immortalized committed osteoprogenitor cell line OPC1. They found approximately six-fold decrease in steroid sulfatase activity in OPC1 cells treated with osteogenic supplement that contains dexamethasone (Clemens, J.W., Doctor, J.S., Olshansky, A., Sero, J., unpublished data). Thus, synthetic glucocorticoids seem to decrease steroid sulfatase activity in a granulosa cell line and in OPC1 cells. My data also supports that cortisol decreases the steroid sulfatase activity in human breast cancer cell line, MDA-MB-231, at least at a 10 μ M concentration. This needs to be further evaluated by determining the effect at different time intervals and using different concentrations of steroid hormones. Further studies can be carried out by doing protein analysis using ELISA or Western blotting. Also determining mRNA levels by RT-PCR can be done.

Estradiol and estrone sulfate did not change steroid sulfatase activity at 1 μ M or at 10 μ M concentrations in MDA-MB-231 cells. However, cells that were grown in whole growth medium showed decrease in their steroid sulfatase activity. This growth media contained fetal calf serum, which has a mixture of various different hormones. Hence, one cannot pinpoint exactly which compounds are causing the decrease in the activity of

steroid sulfatase. Possibly, there may be competitive inhibition due to the total amount of sulfated steroids.

Hormone-dependent breast cancer MCF-7 cells were treated with 10 μ M cortisol, estradiol or estrone sulfate, but no difference was observed among the treatment groups. In the presence of estrone sulfate as their only source of estrogenic steroid, the level of steroid sulfatase in MCF-7 cells is sufficient enough to sustain their growth, indicating a possible role of steroid sulfatase in tumor growth (Selcer et al., 1996). The absence of any effects might be because the cells have been grown in culture for many generations and have lost their ability to respond to the treatment compounds. Alternatively, steroid hormones may not regulate steroid sulfatase activity in this breast cancer cell line.

Hormone-independent human breast cancer cells MDA-MB-231 were treated with various growth factors, including 0.2 μ M of oxytocin, 500 ng/ml of prolactin, 2 μ g/ml of IGF-1 or 1 μ g/ml of EGF. These concentrations were based on previously published research on these compounds (Braverman and Gurpide, 1986; Toniolo et al., 2000). My results showed no significant difference among the treatment groups. Earlier report has shown that there is increased conversion of estrone sulfate to estrone via steroid sulfatase in vaginal deliveries than in cesarean deliveries and also that oxytocin inhibits this activity once labor has started (Mitchell et al., 1984). Also, Braverman and Gurpide (1986) showed that oxytocin and prolactin stimulate the activity of steroid sulfatase in the placenta before the onset of labor and inhibit it after labor has started. These data suggest a role of oxytocin and prolactin in the regulation of steroid sulfatase. However, my results showed no effect in the hormone-independent MDA-MB-231 cells after treatment with

oxytocin and prolactin. Further studies could be carried using different concentrations of these growth factors.

CHAPTER 3

Experimental design

Specific aim 2) To screen various human tissues for steroid sulfatase status using immunohistochemistry.

Model systems

The experiment was conducted on various human tissues, including placenta, skin, skeletal muscle, liver, normal breast, breast carcinoma (ER+/PR+), breast carcinoma (ER-/PR-), uterine carcinoma, adrenal gland, lung, brain, ovarian carcinoma and prostate that were obtained from Spring Bioscience (Fremont, CA).

Antibody

Steroid sulfatase antibody was prepared by aligning cDNA sequences of human, rat and mouse steroid sulfatases and selecting a conserved region. A peptide was synthesized with this sequence and injected in rabbits as an antigen. The antisera that was collected was used as primary antibody. This antisera has been shown by Western blot to bind to steroid sulfatase in various tissues, including human placenta, human breast cancer cells (MCF-7 and MDA-MB-231), rat liver, rat granulosa cells, porcine ovaries and monkey ovaries (Selcer et al., 2001).

Assay design

Screening of various tissues for steroid sulfatase presence was carried out by immunohistochemistry. Later, these tissues were scored for steroid sulfatase immunoreactivity on a scale of 0–3, with 0 representing no steroid sulfatase immunoreactivity and 3 representing the highest immunoreactivity.

Methods

Steroid sulfatase immunohistochemistry

Tissues were obtained from Spring Bioscience (Fremont, CA). Thirteen different human tissues were screened for steroid sulfatase presence, including placenta, skin, skeletal muscle, liver, normal breast, breast carcinoma (ER+/PR+), breast carcinoma (ER-/PR-), uterus carcinoma, adrenal gland, lung, brain, ovarian carcinoma and prostate. Tissues were deparaffinized in xylenes for five min, two times. This was followed by incubating in dilutions of ethanol (100%, 95%, 70%, 50% and 30%) for two min. The slides were then immersed in tap water for five min. Antigen retrieval was done by immersing the slides in 10mM citrate buffer, pH 6.0, and followed by heating in a microwave at high, medium and low power for five min each. The slides were then allowed to cool to room temperature. This was followed by quenching of endogenous peroxidase by incubating the slides in 3% hydrogen peroxide for six min. The slides were then washed with PBS three times for five min followed by incubating in blocking solution (Vectastain Elite ABC kit, Vector laboratories, CA) for 30 min. Slides were then divided into two halves by tearing the tissue with a razor blade followed by using nail polish so as to keep the treatments separate. One half was incubated with primary antibody and the other with preimmune sera at room temperature for one hour or at 4°C overnight. After that, slides were washed with PBS three times for five min each. Slides were then incubated in secondary antibody (Vectastain biotinylated antirabbit IgG) for 30 min. This was followed by incubation in Vectastain Elite ABC Reagent for 30 min. After that slides were washed in PBS three times, for five min each. Slides were then incubated in DAB reagent for two min. The reaction was stopped by washing with tap water. Slides

were then counterstained with Hematoxylin QS (Vector laboratories, CA) for 30 s and washed with tap water to get rid of excess stain. Slides were later dehydrated in a 75%, 80%, 95% and 100% ethanol series for two min each. They were cleared by immersing in xylenes twice for five min each. Slides were mounted in Permount (Fisher Scientific, NJ).

After immunohistochemistry, slides were scored for steroid sulfatase status. A scale of 0 – 3 was used, with zero representing no steroid sulfatase immunoreactivity and three representing the highest immunoreactivity. Three slides of each tissue was read by a third party in a blind fashion. On a particular slide, four different regions were read and scored from 0 – 3. Average of the readings were taken and a final steroid sulfatase score was assigned to each tissue. Images of the tissues were also taken and permanently stored.

Protein A chromatography

The steroid sulfatase antiserum was thawed and sodium azide was added to a final concentration of 0.05%. The antiserum (2.5ml) was clarified by centrifugation at 15,000 x g for five min at 4° C. 500µl of this antiserum was saved. The remaining clarified antiserum was passed through the column twice at the rate of two ml per min. The wash was saved. The column was then washed with TBS (50mM Tris-HCl, pH 7.4; 150mM NaCl; 0.05% sodium azide) with 10-fold of the volume (25 ml) of antiserum was loaded onto the column. Centrifuge tubes (1.5ml) for collection of eluted sample were filled with 100µl of neutralization buffer (NB; 1M Tris-HCl, pH 8.0; 1.5M NaCl; 1mM EDTA; 0.5% sodium azide). The antibody was eluted (1 ml in each centrifuge tube) first using 15ml of elution buffer (50mM Glycine-HCl, pH 2.7) and later using 10ml of elution

buffer (50mM glycine-HCl, pH 1.9) at room temperature. Coomassie Bradford protein assay was carried out and the appropriate fractions were pooled. The purified antibody was stored at 2 - 8° C. The same procedure was used to purify the preimmune sera.

Results

Immunohistochemical analysis was carried out on various human tissues. Tissues were scored for steroid sulfatase immunoreactivity from 0 – 3, with zero being no steroid sulfatase immunoreactivity and three being the highest steroid sulfatase immunoreactivity detected. Tissues were then graded into three categories: high, moderate and low; where high is having steroid sulfatase immunoreactivity greater than two, moderate is having steroid sulfatase immunoreactivity between 1 –2 and low is having steroid sulfatase immunoreactivity less than one. Slides of placenta, liver, normal breast, breast carcinoma (ER+/ PR+) and skeletal muscle showed high immunoreactivity for steroid sulfatase. Skin, lung, brain and uterine carcinoma showed moderate steroid sulfatase immunoreactivity, whereas, adrenal gland, breast carcinoma (ER-/ PR-), ovarian carcinoma and prostate had very low steroid sulfatase immunoreactivity.

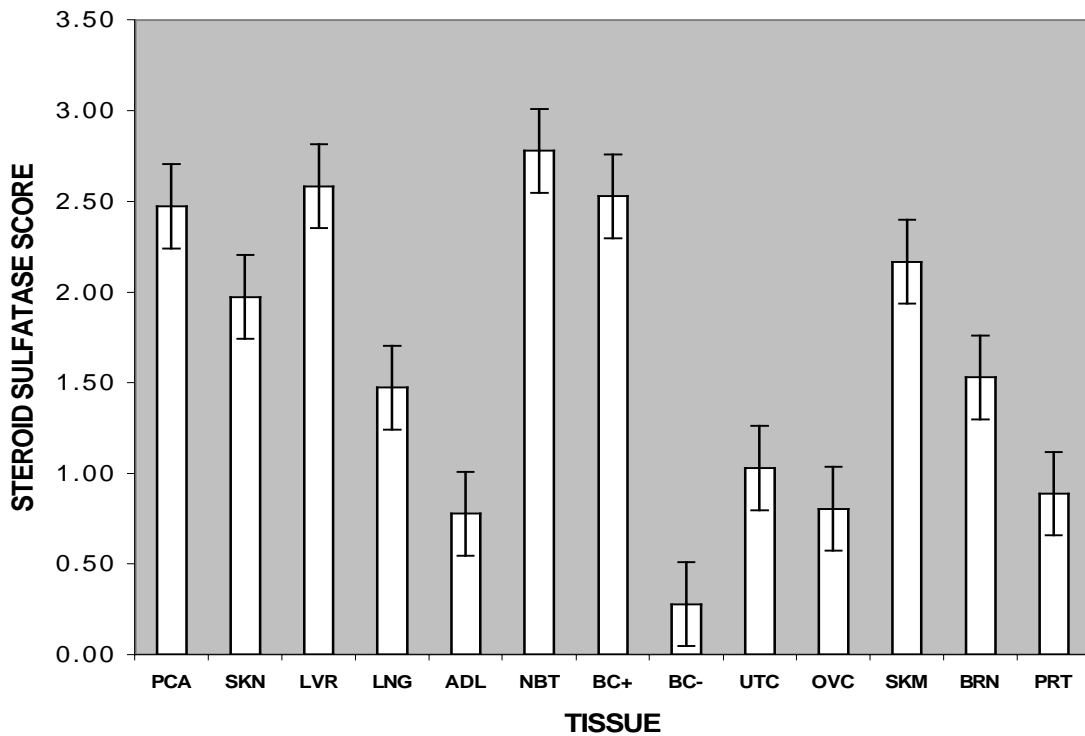
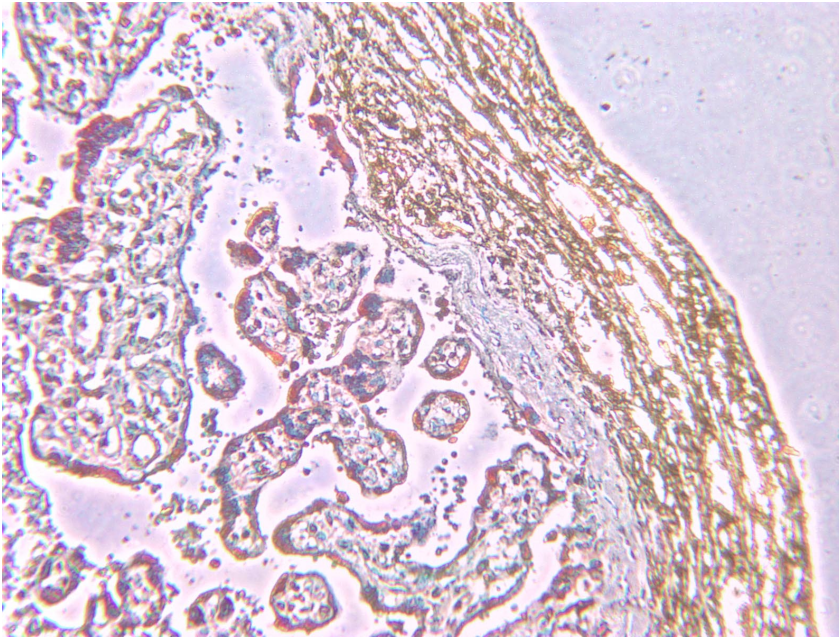
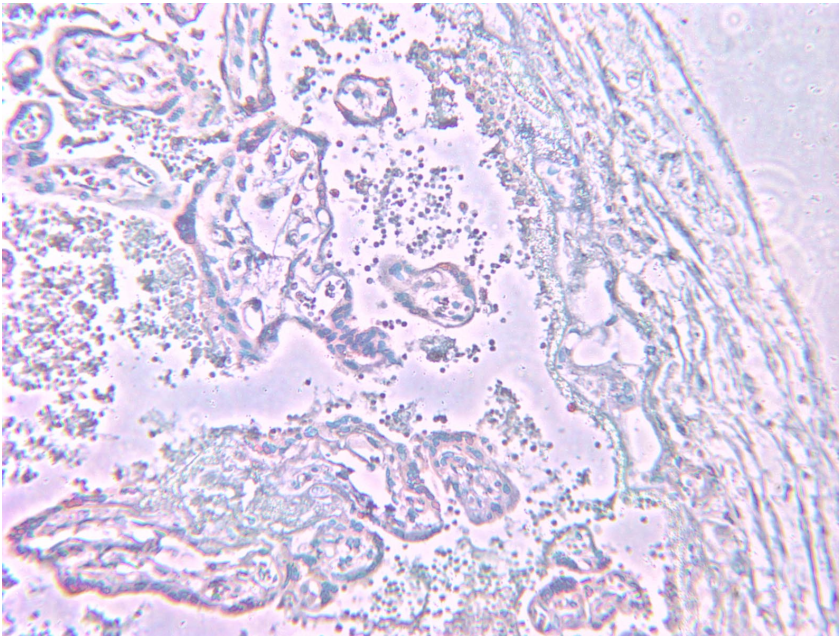


Figure 6: Immunohistochemical analysis for steroid sulfatase of various human tissues. See methods for the procedure. Slides were scored for steroid sulfatase status. A scale of 0 – 3 was used, with zero representing no activity and three representing the highest activity. Key to abbreviations: PCA – Placenta; SKN – Skin; LVR – Liver; LNG – Lung; ADL – Adrenal; NBT – Normal breast; BC+ - Breast carcinoma (ER +/ PR+); BC- - Breast carcinoma (ER -/ PR-); UTC – Uterus carcinoma; OVC – Ovarian carcinoma; SKM – Skeletal muscle; BRN – Brain; PRT – Prostate.

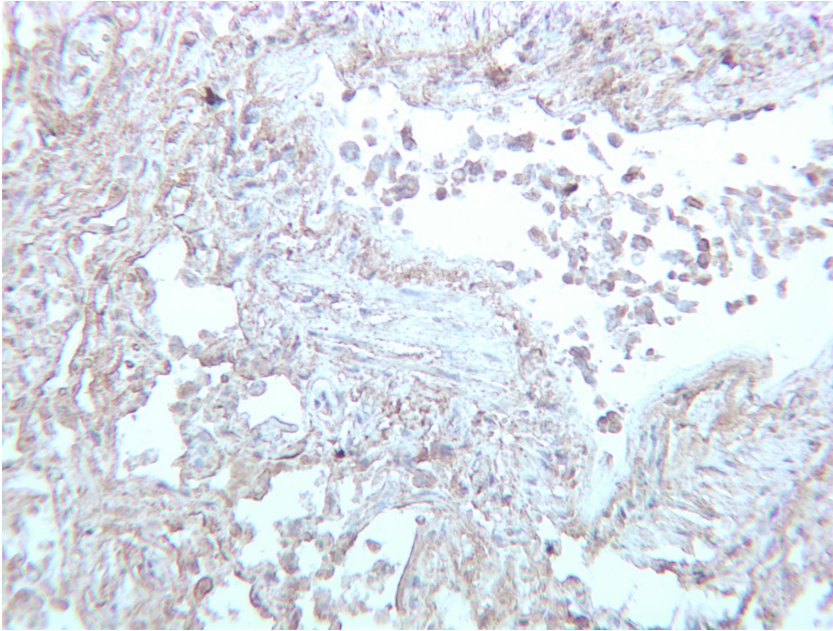
Placenta- steroid sulfatase antisera



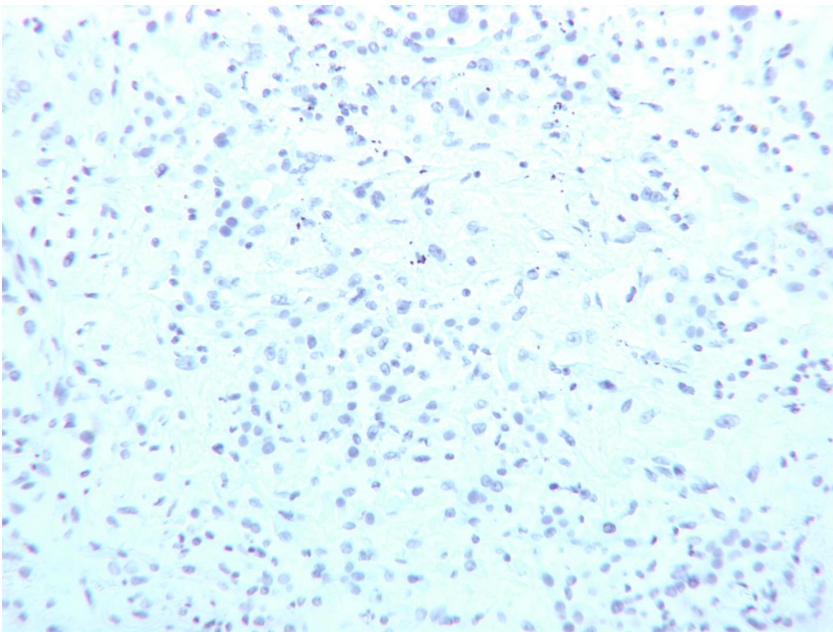
Placenta- preimmune sera



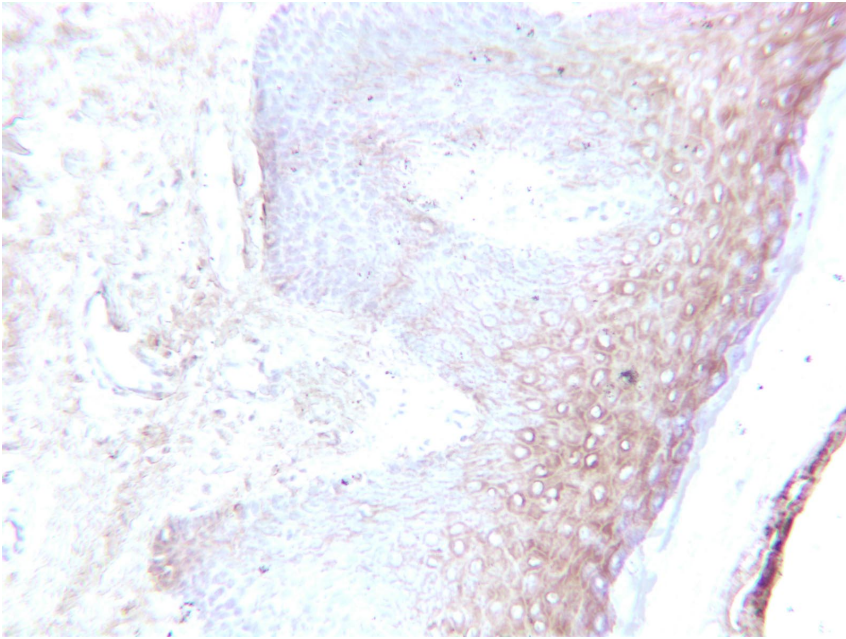
Liver- steroid sulfatase antisera



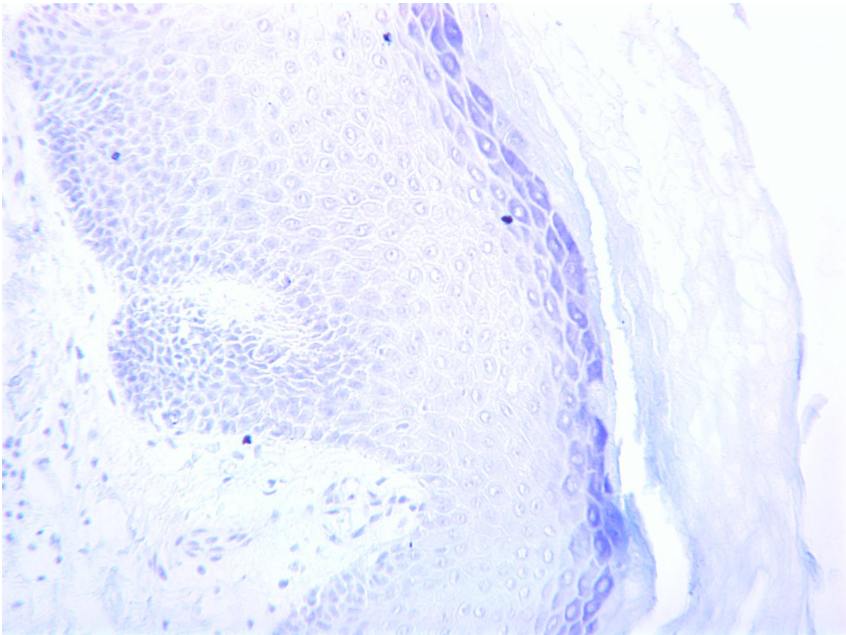
Liver- preimmune sera



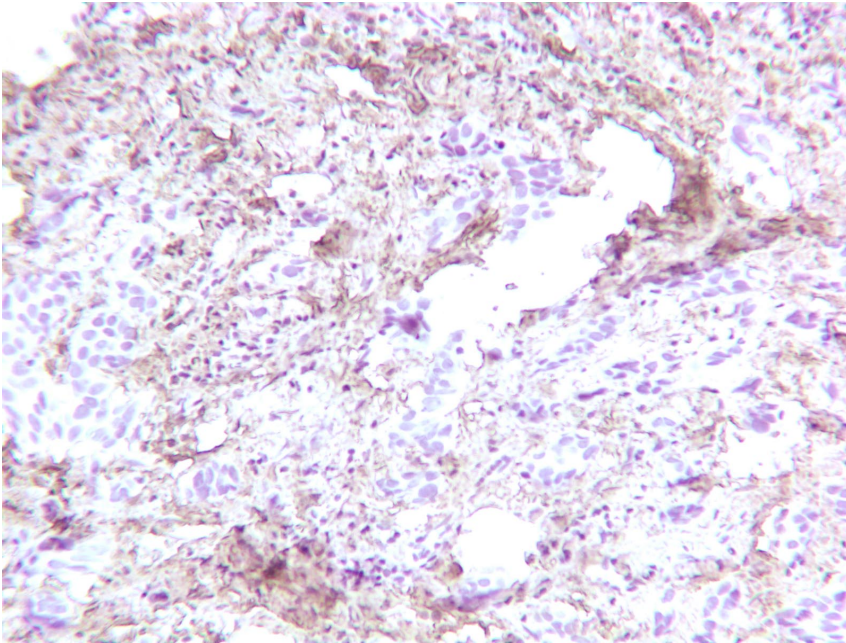
Skin- steroid sulfatase antisera



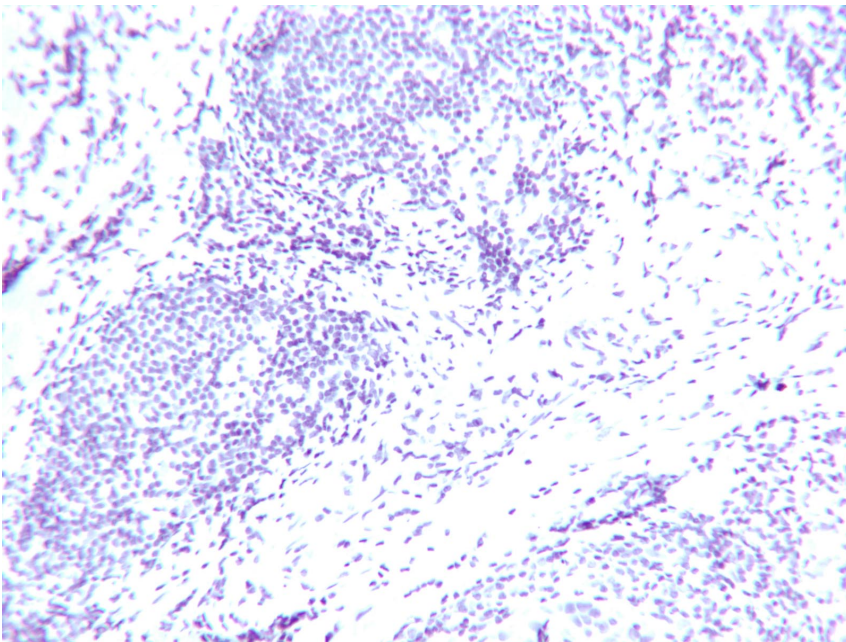
Skin- preimmune sera



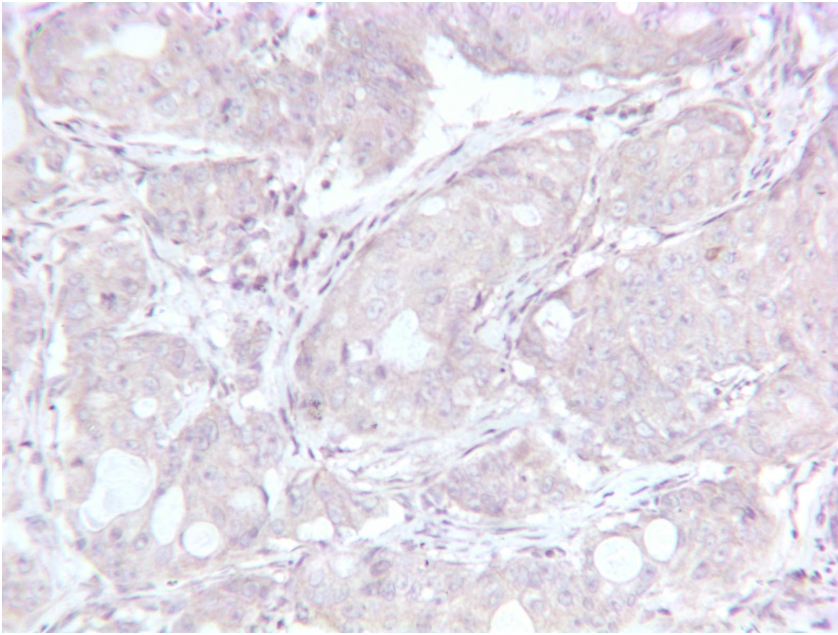
Breast carcinoma (ER/PR-+/+)- steroid sulfatase antisera



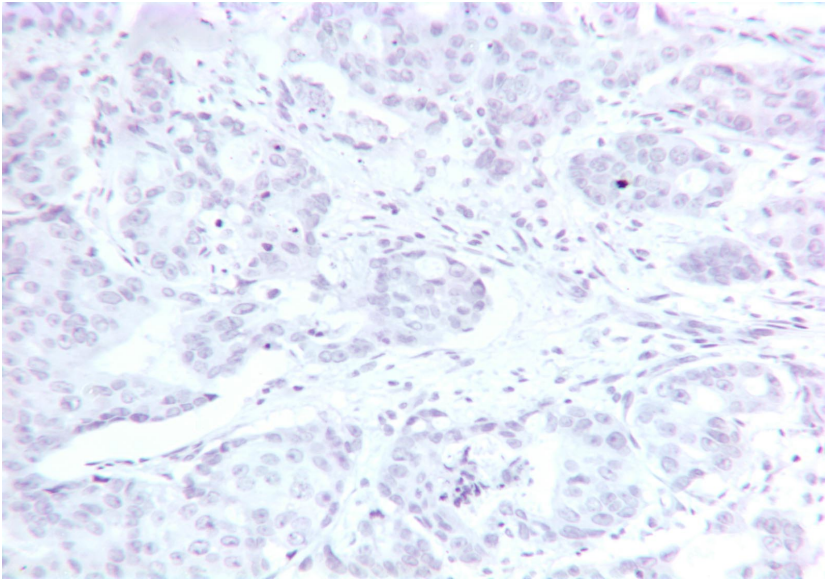
Breast carcinoma (ER/PR-+/+)- preimmune antisera



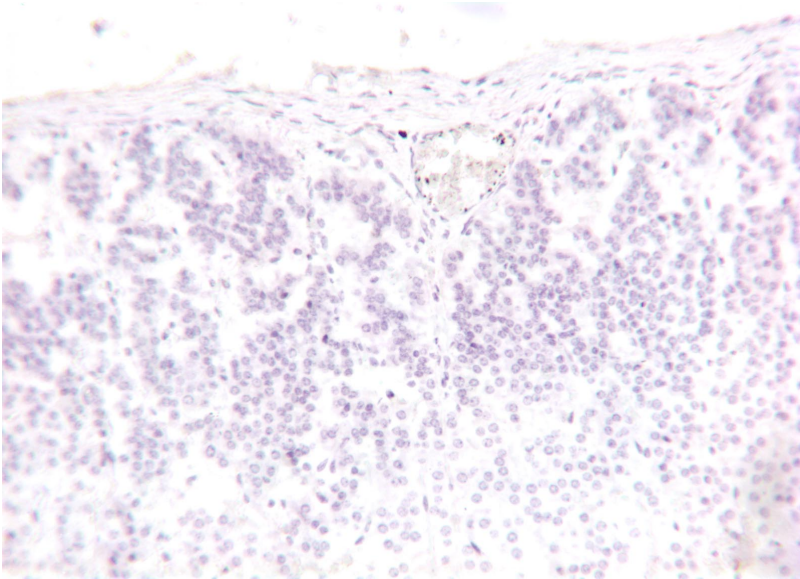
Breast carcinoma (ER/PR -/-)- steroid sulfatase antisera



Breast carcinoma (ER/PR -/-)- preimmune sera



Adrenal gland- steroid sulfatase antisera



Adrenal gland- preimmune sera

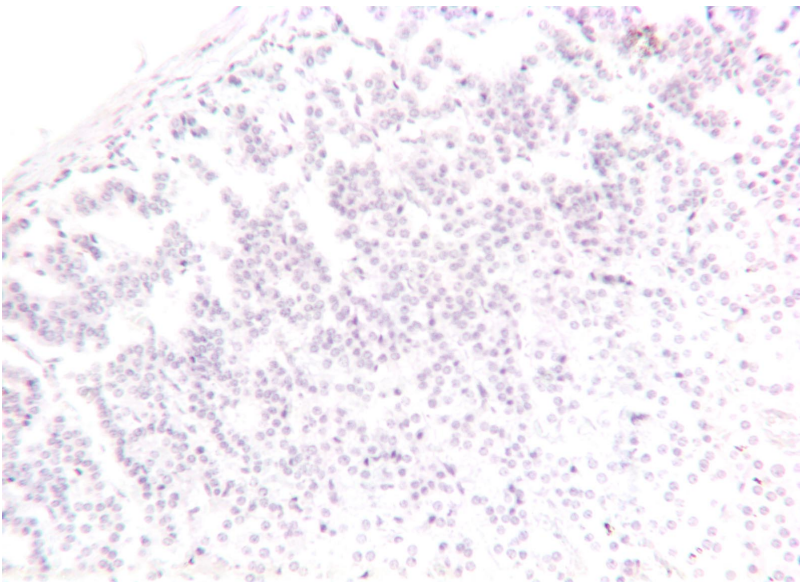


Figure 7: Slides showing immunohistochemical analysis for steroid sulfatase of various human tissues. See methods for the exact procedure. For each slide, one side was treated with purified steroid sulfatase antiserum and the other side was treated with purified preimmune serum. Slides were counterstained with hematoxylin

Discussion

Steroid sulfatase has been suggested as a prognostic indicator for breast cancer (Suzuki et al., 2003; Miyoshi et al., 2003), based mainly on studies of steroid sulfatase mRNA. Immunohistochemistry may also be useful to determine if steroid sulfatase is indeed of value as a marker. I sought to employ immunohistochemistry to screen various human tissues for steroid sulfatase status. Tissues were obtained from Spring Bioscience (Fremont, CA).

Not much information is available on the tissues that were obtained from Spring Bioscience. They only mention the site from where the tissue was obtained and that it was meant for immunohistochemical studies. Hence, more studies need to be carried out in future with tissues that provide with more information about the source.

The steroid sulfatase antibody used by other laboratories was prepared by purifying the steroid sulfatase protein to some extent and then injecting it in rabbit or mouse to get the antibody (Miki et al., 2002). However, our steroid sulfatase antibody was prepared by a different approach. Steroid sulfatase cDNA sequences of human, rat and mouse were aligned and a conserved region was selected. A peptide was synthesized using this sequence and injected in rabbits to get the antisera. By Western blotting of microsomal preparations of placenta, MCF-7 and MDA-MB-231 cells, this antiserum crossreacted with 66kDa protein that is the reported size of steroid sulfatase (Selcer et al., 2001). This band was eliminated when the Western was carried out after the antiserum was exposed to the peptide fragment used to generate the antiserum. The antisera have also been shown by Western blotting to bind to microsomal preparations of rat liver, rat granulosa cells, porcine ovaries and monkey ovaries. The preimmune and the immune

sera were purified by protein A chromatography, checked by ELISA and used for immunohistochemistry.

Miki et al., 2002, similarly screened various human adult and fetal tissues looking for steroid sulfatase immunoreactivity and steroid sulfatase mRNA. This group found steroid sulfatase immunoreactivity only in adult placenta, and mRNA expression was undetectable or present in very low levels in most of the adult and fetal tissues screened. My findings show high steroid sulfatase immunoreactivity in placenta, liver, normal breast, breast carcinoma (ER+/ PR+) and skeletal muscle. Skin, lung, brain and uterine carcinoma showed moderate steroid sulfatase immunoreactivity, whereas, adrenal gland, breast carcinoma (ER-/ PR-), ovarian carcinoma and prostate had very low steroid sulfatase immunoreactivity.

Placenta has high steroid sulfatase immunoreactivity and that was confirmed by my findings (Shankaran et al., 1991). Defect in the enzyme steroid sulfatase causes X-linked ichthyosis that affects males with generalized scaling of the skin (DiGiovanna and Robinson-Bostom, 2003). This supports the finding of moderate immunoreactivity of steroid sulfatase in the skin. Steroid sulfatase is present in liver and brain (Shankaran et al., 1991). Moreover, steroid sulfatase levels have been found to increase in maternal brain after delivery and during lactation (Mortaud et al., 1996). *In vitro* studies have also shown that the steroid sulfatase pathway is active in producing free steroids in carcinoma cells of vaginal, ovarian and endometrial uterine carcinomas (Milewich and Porter, 1987).

Miyoshi et al., 2003, showed that steroid sulfatase mRNA levels are elevated in ER-positive breast cancer but not in ER-negative breast cancers (Miyoshi et al., 2003).

My findings support that. I found that human breast carcinoma (ER+/ PR+) had high steroid sulfatase immunoreactivity whereas human breast carcinoma (ER-/ PR-) had minimal immunoreactivity for steroid sulfatase. Suzuki et al. (2003) studied the steroid sulfatase immunoreactivity in human breast cancer and found that steroid sulfatase expression was present only in carcinoma cells. They also found that steroid sulfatase immunoreactivity was associated with increased recurrence and poor prognosis. These data suggest that steroid sulfatase could be used as an independent prognostic marker for breast cancer.

Here, I screened various different tissues for steroid sulfatase status and established a protocol. I found a significant difference between estrogen-positive breast cancer and estrogen-negative breast cancer. The next logical step would be to screen breast cancer tissues for steroid sulfatase status and evaluate its role with other prognostic markers like tumor size, lymph node involvement, distant metastasis and estrogen-positivity. Additionally, one could examine the effect of Tamoxifen, a nonsteroidal competitive inhibitor of estrogens, a drug used in treatment of estrogen-positive breast cancer, on steroid sulfatase status (Dhingra, 2001). The eventual goal would be to evaluate steroid sulfatase as a prognostic marker for relapse and to further refine the diagnosis and treatment of breast cancer.

References

- Adams, J., Garcia, M., Rochefort, H., 1981. Estrogenic effects of physiological concentrations of 5-androstene-3 beta, 17 beta-diol and its metabolism in MCF7 human breast cancer cells. *Cancer Res.* 41: 4720-4726.
- Adams, J.B., 1998. Adrenal androgens and human breast cancer: a new appraisal. *Breast Cancer Res Treat.* 51:183-188.
- Ahmed, S., Owen, C.P., James, K., Sampson, L., Patel, C.K., 2002. Review of estrone sulfatase and its inhibitors--an important new target against hormone dependent breast cancer. *Curr Med Chem.* 9: 263-273.
- American Cancer Society, 2002. www.cancer.org
- American Type Culture Collection, 2003. www.atcc.org
- Bates, S.E., Valverius, E.M., Ennis, B.W., Bronzert, D.A., Sheridan, J.P., Stampfer, M.R., Mendelsohn, J., Lippman, M.E., Dickson, R.B., 1990. Expression of the transforming growth factor-alpha/epidermal growth factor receptor pathway in normal human breast epithelial cells. *Endocrinology.* 126:596-607.
- Braverman, C.B., Gurbide, E., 1986. In vitro effects of human prolactin and oxytocin on sulfatase activity in isolated human decidual cells. *J. of Clin. Endocrin. Metab.* 63:725-729.
- Brodie, A., 2002. Aromatase inhibitors in breast cancer. *Trends Endocrinol Metab.* 13: 61-65.
- Cailleau, R., Young, R., Olive, M., Reeves, W.J. Jr., 1974. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst.* 53:661-674.

- Chang, P.L., Varey, P.A., Rosa, N.E., Ameen, M., Davidson, R.G., 1986. Association of steroid sulfatase with one of the arylsulfatase C isozymes in human fibroblasts. *J Biol Chem.* 261:14443-14447.
- Chetrite, G., Paris, J., Boetlla, J., Pasqualini, J.R., 1996. Effect of norgestrel acetate on estrone sulfatase and 17 β -hydroxysteroid dehydrogenase activities in human breast cancer cells. *J. Steroid Biochem. Molec. Biol.* 58: 525-531.
- Chetrite, G., Kloosterboer, H.J., Pasqualini, J.R., 1997. Effect of tibolone (Org OD14) and its metabolites on estrone sulfatase activity in MCF-7 and T-47D mammary cancer cells. *Anticancer Res.* 17: 135-140.
- Chetrite, G., Ebert, C., Wright, F., Philippe, A.C., Pasqualini, J.R., 1999. Control of sulfatase and sulfotransferase activities by medrogestone in the hormone-dependent MCF-7 and T-47D human breast cancer cell lines. *J. Steroid Biochem. Molec. Biol.* 70: 39-45.
- Clemens, J.W., Kabler, H.L., Sarap, J.L., Beyer, A.R., Li, P.K., Selcer, K.W., 2000. Steroid sulfatase activity in the rat ovary, cultured granulosa cells, and a granulosa cell line. *J Steroid Biochem Mol Biol.* 75:245-252.
- Dhingra, K., 2001. Selective estrogen receptor modulation: the search for an ideal hormonal therapy for breast cancer. *Cancer Invest.* 19: 649-659.
- DiGiovanna, J.J., Robinson-Bostom, L., 2003. Ichthyosis: etiology, diagnosis, and management. *Am J Clin Dermatol.* 4:81-95.
- Duke, J.C., Wright, A., Gottlicher, M., Okret, S., Gustafsson, J.A., 1995. Molecular Mechanisms of Hormone Action: Regulation of Target Cell Function by the Steroid Hormone Receptor Supergene Family. In: *Endocrinology and*

- Metabolism. Felig, P., Baxter, J.D., Frohman, L.A., Third Edition. McGRAW-HILL INC., New York. 169-200.
- Duncan, L., Purohit, A., Howarth, N.M., Potter, B.V., Reed, M.J., 1993. Inhibition of estrone sulfatase activity by estrone-3- methylthiophosphonate: a potential therapeutic agent in breast cancer. *Cancer Res.* 53: 298-303.
- Elger, W., Schwarz, S., Hedden, A., Reddersen, G., Schneider, B., 1995. Sulfamates of various estrogens are prodrugs with increased systemic and reduced hepatic estrogenicity at oral application. *J Steroid Biochem Mol Biol.* 55: 395-403.
- Frohman, L.A., 1995. Diseases of the Anterior Pituitary. In: *Endocrinology and Metabolism.* Felig, P., Baxter, J.D., Frohman, L.A., Third Edition. McGRAW-HILL INC., New York. 289-384.
- Gill, G.N., 1995. Biosynthesis, Secretion and Metabolism of hormones. In: *Endocrinology and Metabolism.* Felig, P., Baxter, J.D., Frohman, L.A., Third Edition. McGRAW-HILL INC., New York. 69-90.
- James, V. H., Reed, M.H., Purohit, A., 1992. Inhibition of oestrogen synthesis in postmenopausal women with breast cancer. *J. Steroid Biochem. Mol. Biol.* 43: 149-53.
- Jordan, V. C., 1997. Tamoxifen treatment for breast cancer: concept to gold standard. *Oncology (Huntingt).* 11: 7-13.
- Kolli, A., Chu, G.H., Rhodes, M.E., Inoue, K., Selcer, K.W., Li, P.K., 1999. Development of (p-O-sulfamoyl)-N-alkanoyl-phenylalkyl amines as non-steroidal estrone sulfatase inhibitors. *J Steroid Biochem Mol Biol.* 68:31-40.

- Li, P.K., Chu, G.H., Guo, J.P., Peters, A., Selcer, K.W., 1998. Development of potent non-estrogenic estrone sulfatase inhibitors. *Steroids*. 63: 425-432.
- MacIndoe, J.H., 1988. The hydrolysis of estrone sulfate and dehydroepiandrosterone sulfate by MCF-7 human breast cancer cells. *Endocrinology*. 123:1281-1287.
- Miki, Y., Nakata, T., Suzuki, T., Darnel, A.D., Moriya, T., Kaneko, C., Hidaka, K., Shiotsu, Y., Kusaka, H., Sasano, H., 2002. Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues. *J Clin Endocrinol Metab*. 87: 5760-5768.
- Milewich, L., Porter, J.C., 1987. In situ steroid sulfatase activity in human epithelial carcinoma cells of vaginal, ovarian, and endometrial origin. *J Clin Endocrinol Metab*. 65: 164-169.
- Mitchell, B.F., Cross, J., Hobkirk, R., Challis, J.R., 1984. Formation of unconjugated estrogens from estrone sulfate by dispersed cells from human fetal membranes and decidua. *J Clin Endocrinol Metab*. 58: 845-849.
- Miyoshi, Y., Ando, A., Hasegawa, S., Ishitobi, M., Taguchi, T., Tamaki, Y., Noguchi, S., 2003. High Expression of Steroid Sulfatase mRNA Predicts Poor Prognosis in Patients with Estrogen Receptor-positive Breast Cancer. *Clin Cancer Res*. 9: 2288-2293.
- Morris, C., 2002. The role of EGFR-directed therapy in the treatment of breast cancer. *Breast Cancer Res Treat*. 75 Suppl 1:S51-5.
- Mortaud, S., Donsez-Darcel, E., Roubertoux, P.L., Degrelle, H., 1996. Murine steroid sulfatase gene expression in the brain during postnatal development and adulthood. *Neurosci Lett*. 215: 145-148.

- Munroe, D.G., Chang, P.L., 1987. Tissue-specific expression of human arylsulfatase-C isozymes and steroid sulfatase. *Am J Hum Genet.* 40:102-114.
- Newman, S. P., Purohit, A., Ghilchik, M.W., Potter, B.V., Reed, M.J., 2000. Regulation of steroid sulphatase expression and activity in breast cancer. *J. Steroid Biochem. Mol. Biol.* 75: 259-264.
- Ng, J. H., Nesaretnam, K., Reimann, K., Lai, L.C., 2000. Effect of retinoic acid and palm oil carotenoids on oestrone sulphatase and oestradiol-17beta hydroxysteroid dehydrogenase activities in MCF-7 and MDA-MB-231 breast cancer cell lines. *Int. J. Cancer.* 88: 135-138.
- O'Malley, B.W., Strott, C.A., 1991. Steroid Hormones: Metabolism and Mechanism of Action. In: *Reproductive Endocrinology*. Yen, S. S. C. and Jaffe, R.B., Third Edition. W.B. Saunders Company. Philadelphia. 156-180.
- Pasqualini, J.R., Chetrite, G., Nestour, E.L., 1996. Control and expression of estrone sulfatase activities in human breast cancer. *J. Endocrinol.* 150:99-105.
- Pasqualini, J.R., Chetrite, G., 2001. Paradoxical effect of estradiol: it can block its own bioformation in human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 78: 21-24.
- Poulin, R. and Labrie, F., 1986. Stimulation of cell proliferation and estrogenic response by adrenal C19-delta 5-steroids in the ZR-75-1 human breast cancer cell line. *Cancer Res.* 46: 4933-4937.
- Pratt, S.E., Pollak, M.N., 1993. Estrogen and antiestrogen modulation of MCF7 human breast cancer cell proliferation is associated with specific alterations in

- accumulation of insulin-like growth factor-binding proteins in conditioned media. *Cancer Res.* 53:5193-5198.
- Purohit, A., Dauvois, S., Parker, M.G., Potter, B.V., Williams, G.J., Reed, M.J., 1994. The hydrolysis of oestrone sulphate and dehydroepiandrosterone sulphate by human steroid sulphatase expressed in transfected COS-1 cells. *J. Steroid Biochem. Mol. Biol.* 50: 101-104.
- Purohit, A., Woo, L.W., Singh, A., Winterborn, C.J., Potter, B.V., Reed, M.J., 1996. In vivo activity of 4-methylcoumarin-7-O-sulfamate, a nonsteroidal, nonestrogenic steroid sulfatase inhibitor. *Cancer Res.* 56: 4950-4955.
- Purohit, A., Woo, L. W., Potter, B.V., Reed, M.J., 2000. In vivo inhibition of estrone sulfatase activity and growth of nitrosomethylurea-induced mammary tumors by 667 COUMATE. *Cancer Res.* 60: 3394-3396.
- Pusztai, L., Esteva, F.J., Cristofanilli, M., Hung, M.C., Hortobagyi, G.N., 1999. Chemo-signal therapy, an emerging new approach to modify drug resistance in breast cancer. *Cancer Treat Rev.* 25(5):271-7
- Reed, M.J., Purohit, A., 1993. Sulfatase inhibitors: the rationale for the development of a new endocrine therapy. *Rev. Endocr. Relat. Cancer.* 45:51-62.
- Reed, M.J., Purohit, A., Duncan, L.J., Singh, A., Roberts, C.J., Williams, G.J., Potter, B.V., 1995. The role of cytokines and sulphatase inhibitors in regulating oestrogen synthesis in breast tumours. *J. Steroid Biochem. Mol. Biol.* 53: 413-420.
- Reed, M.J., Purohit, A., 1997. Breast cancer and the role of cytokines in regulating estrogen synthesis: an emerging hypothesis. *Endocr. Rev.* 18:701-715.

- Robertson, G.L., 1995. Posterior Pituitary. In: *Endocrinology and Metabolism*. Felig, P., Baxter, J.D., Frohman, L.A., Third Edition. McGRAW-HILL INC., New York. 385-434.
- Santner, S. J., Feil, P.D., Santen, R.J., 1984. In situ estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway. *J. Clin. Endocrinol. Metab.* 59: 29-33.
- Santner, S.J., Leszczynski, D., Wright, C., Manni, A., Feil, P.D., Santen, R.J., 1986. Estrone sulfate: a potential source of estradiol in human breast cancer tissues. *Breast Cancer Res. Treat.* 7:35-44.
- Selcer, K.W., Jagannathan, S., Rhodes, M.E., Li, P.K., 1996. Inhibition of placental estrone sulfatase activity and MCF-7 breast cancer cell proliferation by estrone-3-amino derivatives. *J Steroid Biochem Mol Biol.* 59:83-91.
- Selcer, K. W., Hegde, P.V., Li, P.K., 1997. Inhibition of estrone sulfatase and proliferation of human breast cancer cells by nonsteroidal (p-O-sulfamoyl)-N-alkanoyl tyramines. *Cancer Res.* 57: 702-707.
- Selcer, K.W., Beyer, A.R., Parker, S., Li, P.K., Clemens, J.W., 2001. Development and characterization of a steryl sulfatase antibody. *Biol Reprod.* 64(Supp 1):248.
- Selcer, K.W., Kabler, H., Sarap, J., Xiao, Z., Li, P.K., 2002. Inhibition of steryl sulfatase activity in LNCaP human prostate cancer cells. *Steroids.* 67:821-826.
- Shankaran, R., Ameen, M., Daniel, W.L., Davidson, R.G., Chang, P.L., 1991. Characterization of arylsulfatase C isozymes from human liver and placenta. *Biochim Biophys Acta.* 1078:251-257.

- Soule, H.D., Vazquez, J., Long, A., Albert, S., Brennan, M., 1973. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst.* 51:1409-1416.
- Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladino, M.A. Jr., Shepard, H.M. 1985. Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. *Science.* 230:943-945.
- Suzuki, T., Nakata, T., Miki, Y., Kaneko, C., Moriya, T., Ishida, T., Akinaga, S., Hirakawa, H., Kimura, M., Sasano, H., 2003. Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. *Cancer Res.* 63: 2762-2770.
- Toniolo, P., Bruning, P.F., Akhmedkhanov, A., Bonfrer, J.M., Koenig, K.L., Lukanova, A., Shore, R.E., Zeleniuch-Jacquotte, A., 2000. Serum insulin-like growth factor-I and breast cancer. *Int J Cancer.* 88: 828-832.
- Utsumi, T., Yoshimura, N., Takeuchi, S., Maruta, M., Maeda, K., Harada, N., 2000. Elevated steroid sulfatase expression in breast cancers. *J. Steroid Biochem. Mol. Biol.* 73: 141-145.
- Utsumi, T., Yoshimura, N., Maruta, M., Takeuchi, S., Ando, J., Maeda, K., Harada, N., 1999. Significance of steroid sulfatase expression in human breast cancer. *Breast Cancer.* 6: 298-300.
- van Doorn, L. G., Poortman, J., Thijssen, J.H., Schwarz, F., 1981. Actions and interactions of delta 5-androstene-3 beta, 17 beta-diol and 17 beta-estradiol in the immature rat uterus. *Endocrinology.* 108: 1587-1593.

- Woo, L. W., Howarth, N.M., Purohit, A., Hejaz, H.A., Reed, M.J., Potter, B.V., 1998. Steroidal and nonsteroidal sulfamates as potent inhibitors of steroid sulfatase. *J. Med. Chem.* 41: 1068-1083.
- Woo, L. W., Purohit, A., Malini, B., Reed, M.J., Potter, B.V., 2000. Potent active site-directed inhibition of steroid sulphatase by tricyclic coumarin-based sulphamates. *Chem. Biol.* 7: 773-791.
- Yen, P.H., Allen, E., Marsh, B., Mohandas, T., Wang, N., Taggart, R.T., Shapiro, L.J., 1987. Cloning and expression of steroid sulfatase cDNA and the frequent occurrence of deletions in STS deficiency: implications for X-Y interchange. *Cell.* 49:443-454.