Chronic Stress Induces the Expression of the Proliferation Marker Ki-67 in Female Rat Breast

NAGWA E. EL-NEFIAWY, M.D.; AHMED DESOUKY, M.D.; SAMY A. ABUSIKKIEN, M.D. and HESHAM I. ABDALLAH, M.D.

The Department of Anatomy & Embryology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Abstract

Background: It is thought that the dynamic ability of breast cells to be continuously influenced and remodeled under the influence of hormones and growth factors makes them susceptible to carcinogenesis. Few studies investigated the effect of estrous cycle hormones on mammary gland proliferation. However, the relationship between stress and mammary gland proliferation remains to be elucidated.

Aim of the Work: To define the impact of stress exposure (acute and chronic) on proliferation, apoptosis and steroid receptor expression in adult female rat breast.

Material and Methods: Vaginal smears were performed first to define rats at same phase of estrous cycle. 18 Adult female albino rats were divided into three equal groups; control, acute stress, and chronic stress groups. Rats were subjected to restraint stress for either 5h once (acute stress) or 5h daily for 2 weeks in chronic stress. At the end of experiment fixed pairs of breast were collected, fixed in 10% formalin and processed into paraffin blocks. Cut sections were stained with H & E and immunohistochemically stained for steroid receptors estrogen (ER) and progesterone (PR), proliferation marker (Ki-67), and P53 antibody.

Results: Histologically evident proliferation of the epithelial compartment was detected in chronic stress group. Acute stress group revealed massive inflammatory criteria like blood congestion and cell infiltrates in breast parenchyma. Immunohistochemistry showed negative immune staining for ER and trivial positive immune staining for PR with stromal localization in the three groups. Proliferation marker (Ki-67) as well as P53 revealed marked intense immune staining in chronic stress group contrary to other groups.

Conclusion: Chronic but not acute stress induces proliferation of breast parenchyma regardless of steroid receptors expression as shown by the expression of Ki-67 and P53 markers. The novel observations presented here might have implications for understanding breast biology together with the pathogenesis of breast cancer.


Introduction

THE mammary gland is both a source of food and a site of disease. In the rat, mammary glands develop from ectodermal thickening called the milk line that extends on both sides of the body from the neck to the inguinal region. Number one gland is located in the neck region, adjacent to the salivary glands. Number 2 and 3 mammary glands are situated on the chest wall, separated by a thin layer of pectoralis muscle. Number 4 gland is located on the abdominal wall. Lastly, number 5 gland is located in the inguinal region [1].

The mammary gland is composed of epithelial parenchyma and mesenchymal stroma [2]. The functional portions of rat mammary gland are termed lobulo-alveolar units or terminal end buds. These structures are hormone sensitive, dynamically active through estrous cycle, regress with menopause, and are the site of milk production. They are also the site of origin of most mammary cancers [3].

In most mammals, the normal breast epithelium expresses variety of receptors with different functions. Of these receptors are the steroid receptors, the cell cycle-associated antigen Ki-67, and the tumor suppressor protein P53 [4,5].

Estrogen receptors are a group of proteins found inside cells and are activated by the hormone estrogen. Estrogen receptor once activated is able to bind to DNA and regulate the activity of many different genes. Progesterone receptor is a protein found inside cells and is activated by the hormone progesterone [6].

The cell cycle-associated antigen Ki-67 is a marker of proliferation. The expression of the Ki-67 protein is strictly associated with cell proliferation. The fact that the Ki-67 protein is present
during all active phases of the cell cycle (G (1), S, G (2), and mitosis), but is absent from resting cells (G (0)), makes it an excellent marker for determining the growth fraction of a given cell population [7].

P53 may be regarded as dispensable for normal development, but it plays an important role in regulating cell fate in response to various stresses. P53 exerts its anti-proliferative action by inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis. It may also enhance DNA repair and inhibit angiogenesis. It has been suggested that P53 could have evolved in higher organisms specifically to prevent tumor development. Indeed, loss of P53 activity disrupts apoptosis and accelerates the appearance of tumors in transgenic mice [5].

Stress is an organic response of the hypothalamic-pituitary-adrenal axis to chemical, physical and emotional stimuli. Researchers categorize stress into several phases that can lead to either fast reactions with no major consequences or the collapse of vital organ systems [8]. An important distinguishing characteristic of stress is its duration. Acute stress is defined as an abrupt, short-lasting (seconds to hours timescale) and isolated perturbation, whereas chronic stress is recurring, persisting for several hours a day for weeks, months or longer [8]. Studies using restraint stress models in rats are well-documented and were performed to provide important information about different disease pathogenesis and progression [9].

Observational studies have established that stressful life events increase the risks of mental disorders, acute infections, and total and cause-specific mortality [9]. Thus, studies on the effect of stress on breast tissue are demanding. The present study was undertaken first to study the effect of stress on the histological structure and the expression of the proliferation marker Ki-67 in correlation with steroid and anti-proliferative (P53) receptors in the female rat mammary gland in vivo using immunohistochemical technique. The second aim of this study was to determine which has more influence on breast tissue acute or chronic stress.

Material and Methods

Experimental design:

Previous investigators emphasized that in the evaluation of the rat mammary gland, a systematic approach to study design, sampling, and processing for histology is critical. The morphologic characteristics of the rat mammary gland are both site and age-specific. Therefore it is important to use rats that are sexually mature at study start and to collect the same mammary gland pair [3].

The effect of estrous cycle hormones on the breast tissue is a controversial issue. Some researchers state that in rats mammary epithelium responds to cyclic changes in ovarian hormone levels with slight changes in the glandular morphology [10].

Other investigators emphasize upon the dramatic cyclic changes of rat breast tissue similar to human [11,12].

Thus in the present work, following criteria were considered:

1- The female rats used in the present study were adult and mature.
2- The same pair (4 th & 5 th) of mammary glands was obtained in all groups.
3- Vaginal smears were performed to obtain rats at the same phase of the estrous cycle.

Animals:

The present study was carried out on 18 adult female Albino rats with average 200gm B.W. Animals were purchased and kept in the Experimental Unit-Medical Research Center and Bilharzial Researches, Ain Shams University. Rats were maintained under pathogen free conditions with free access to food and water in a room maintained at a temperature of 23±3°C and 12h dark: 12h light cycle. The experimental design and procedures were approved by the Animal Care and Use Committee of Faculty of Medicine, Ain Shams University.

Vaginal smears were performed using pipette smear technique to estimate the day of estrous cycle for all rats of the study. A drop of distilled water was introduced into the vagina with the help of a dropper. Water was collected back and placed on a clean slide. The prepared smear was examined microscopically under low power for different types of cells [11].

Animal grouping:

Rats were randomly divided into 3 groups (6 rats/group) as follows:

Group I (control group): Animals were not subjected to any manipulations.

Group II (chronic stress group): Animals were subjected to chronic restraint stress.
Group III (acute stress group): Animals were subjected to acute restraint stress.

Animals were sacrificed at the end of the experiment by over dose of ether inhalation.

Restraint stress model:
Rats react stressfully to immobilization in tight quarters, perhaps due to the ever-present dangers of entrapment in their natural burrowing environment [13].

Re-usable restraint devices were constructed of flexible stainless steel wire mesh with latex borders and used to individually and painlessly enclose the rat, enabling it to breathe freely with minimal movement possible. The rat was introduced into the space without undue force and maintained in this immobilized position for varying periods according to protocol. For acute stress protocol, rats were introduced once into the restraint devices for a period of 5 hours. For rats in the chronic stress protocol, restraint stress was applied for 5 hours and repeated daily for 2 weeks [9].

Histological techniques:
Immediately after animal sacrifice, breast specimens were extracted and fixed in 10% neutral formalin in water, dehydrated in ascending grades of ethanol and cleared in xylol. Paraffin blocks were prepared and 5-µm thick transverse sections were cut and stained with haematoxylin and eosin (H & E) then examined by Vanox light microscope (Olympus, Tokyo, Japan) [14].

Immunohistochemistry:
A- Primary antibodies:
1- Anti-Estrogen Receptor (nuclear epitope) polyclonal antibody with species reactivity to rat, human, and ovine and was used at dilution of 1-2µg/ml (Pierce Biotechnology, Meridian Road Rockford, USA).

2- Anti-progesteron Receptor (nuclear epitope, MA1-411) mouse anti-rat monoclonal antibody (Pierce Biotechnology, Meridian Road Rockford, USA, Catalog Number; MA1-411).

3- Ki-67 (nuclear protein): Rabbit polyclonal anti-Ki 67 antibody with species reactivity to human and rat (Pierce Biotechnology, Meridian Road Rockford, USA, Catalog Number; MA1-12648).

B- Staining technique:
Five-micrometer sections were cut from the formalin-fixed, paraffin-embedded blocks. Sections were mounted on positively charged glass slides. Sections were first subjected to heat mediated antigen retrieval in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) prior to blocking in 5% normal goat serum + 1% BSA for 2 hours at 37°C. The streptavidin biotin peroxidase method was used for Immune-staining. Breast sections were deparaffinized in xylene, and placed in absolute ethanol. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 20 minutes. To minimize non-specific reaction, sections were initially incubated with fetal calf serum 1.5 for 30min. at 37°C. Sections were then incubated with the primary antibody overnight at 4°C and rinsed in phosphate buffer saline (PBS). A biotinylated secondary antibody, was applied for 30min. at 37°C. After another rinse in PBS, sections were incubated with PAP reagent 1:200 for 30min. at 37°C. Finally sections were treated with diaminobenzidine- H2O2 mixture and counterstained with hematoxylin. Negative control staining was performed after omitting the primary antibody [15].

Image photography:
Light microscopic digital images were captured then transformed into 32-bit color images using a digital camera (FUJIX HC-2000; Fuji Photo Film, Tokyo, Japan) attached to a light microscope (VANOX AHBS3; Olympus, Tokyo, Japan).

Results
In the present study breast tissue was collected for the experiment in the late metaestrous early diestrous phase of the cycle for all groups. Vaginal smear at this stage of the estrous cycle was characterized by presence of nearly equal number of both large epithelial cells and leucocytes with some cornified cells. Some tightly packed clumps of cells were also seen (Fig. 1).

Light microscopy:
I- Control group (group I):
H & E stained breast sections of the control group revealed the classic appearance of breast tissue. Scattered lobes composed of ducts and acini were seen immersed among obviously wide area of connective tissue stroma containing fat cells. Acini were lined by single layer of low cuboidal
cells and outer basal layer of myoepithelium. Small ductules were lined by inner layer of columnar or cuboidal epithelium. While, large ducts were lined with pseudostratified columnar or double layer cuboidal epithelium (Fig. 2-A,B).

II- Chronic stress group (Group II):

In this group there was obvious increase in the lobulo-alveolar epithelial component on the expense of the connective tissue stroma and fat cells of the breast tissue. Blood vessels were noticed in the connective tissue (Fig. 3).

III- Acute stress group (Group III):

Breast tissue revealed dispersed epithelial component of ducts and acini among plenty of connective tissue stroma. Numerous dilated congested blood vessels were a very prominent feature. Extensive infiltration of the stroma with inflammatory cell infiltrate was very striking. Inflammatory reaction was more pronounced in the peri-glandular and peri-vascular regions (Fig. 4-A,B).

Immunohistochemistry:

A- ER: Negative immune reaction was obtained in breast sections of the three studied groups (Fig. 5-A,B,C).

B- PR: In the three studied groups, few sporadic positive nuclear staining of cells scattered in the connective tissue stroma, especially at the periductal and peri-vascular areas. The epithelial component of the breast at the ducts and acini revealed negative immune staining (Fig. 6-A,B). Illustrates PR immune stain distribution.

C- Ki-67: In control group; few sporadic positive epithelial cells scattered across the epithelial compartment of the breast revealed light brown nuclear staining (Fig. 7-A).

In chronic stress group; remarkable number of epithelial cells showed strong positive immune staining in this group compared with the control group. Positive staining involved large proportion of the breast lobules. The positive immune staining revealed a spatial localization that involved some breast lobules rather than others and some cells in the same lobule rather than others. The stroma revealed negative immune staining (Fig. 7-B).

In acute stress group; sporadic cells revealed light brown immune staining were recognized that were nearly comparable to the control group (Fig. 7-A,B,C). Illustrates Ki-67 immune staining results.

Fig. (1): Light microscopic image of vaginal smear of adult female albino rat used in the present study showing many leucocytes (T) and squamous epithelial cells ( ) denoting late metaestrous/early diestrous phase of the cycle. (x200).

Fig. (2): Light microscopic images of control adult female rat breast. (a) Definite lobules containing acini & ducts and surrounded by plenty of connective tissue stroma is seen. (b) ( ) Refers to myoepithelial cell surrounding acinus, ( T) point to ducts lined by columnar or pseudostratified columnar cells. D=Ducts, A=Acini, V=Vessels. (H & E a x200 & b x400).
Fig (3): Light microscopic image of adult female rat breast subjected to chronic stress. Breast lobules showed increased number of lobulo-alveolar components compared with the control. V=Blood vessels. (H & E a x200).

Fig. (4): Light microscopic images of adult female rat breast subjected to acute stress. (a) Scattered units of acini and ducts among plenty of connective tissue stroma can be visualized. (b) Numerous dilated congested blood vessels (V) are seen also excessive number of leucocyte infiltration is evident. D=Duct, A=Acinus. (H & E a x200 & b x400).

Fig. (5): Light microscopic images of adult female rat breast subjected to immune-histochemistry with streptavidin biotin peroxidase method for ER receptor showed negative immune staining for the three groups (a) control, (b) chronic stress, and (c) acute stress group. (T)=Acini, (*)=Connective tissue stroma. (x200).
D- P53: In control group; few cells revealed positive light brown immune staining at the epithelial compartment of the breast (Fig. 8-A).

In chronic stress group; dark positive immune staining was visualized at large scale of epithelial cells compared with the control. Positive immune staining did not involve all epithelial cells but showed differential localization (Fig. 8-B).

In acute stress group; Light sporadic foci of positive immune staining at epithelial cells of ducts & acini was frequently encountered (Fig. 8-C).

Results of the immune staining with the four markers were qualitatively assessed and illustrated in Table (1).

<table>
<thead>
<tr>
<th>Marker</th>
<th>ER</th>
<th>P53</th>
<th>Ki-67</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−ve</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chronic stress</td>
<td>−ve</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Acute stress</td>
<td>−ve</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Discussion

Published reports on the immune-histochemical expression of hormone and proliferation receptors in the mammary tissue were centered primarily on normal or malignant mammary tissue with limited references on the implications of stress on these receptors expression. The present study declared that chronic and not acute stress accentuated the expression of the proliferation marker Ki-67 and the anti-proliferation protein P53 in the epithelial compartment of the sexually mature adult female rat breast in vivo using immunohistochemical technique.

In the present study breast tissues of all groups were collected in the late metaestrous/early diestrous phase of the cycle which corresponded to luteal phase of menstrual cycle in human. Estrogen hormone level at this time of the cycle is minimal [11].

Immune staining for the ER demonstrated negative results for all groups which coincided well with the vaginal smear cytology performed. Our results are in agreement with the previous reports which stated that the expression of ER in human breast was strongly correlated with the hormones of the menstrual cycle in a way that it is prominent in the follicular phase and weak or negative in the luteal phase [16].

The ovarian steroid hormone estrogen plays an important role in the development of the mammary gland [10]. Estrogen stimulates both proliferation and progesterone synthesis in the epithelial component of normal human breast tissue implanted into athymic nude mice [4]. In addition, estradiol plays an important role in the progression of breast cancer, and a majority of the human breast cancers start out as estrogen dependent and express the estrogen receptor (ER) [17].

In the present study, evident proliferation in the epithelial component (namely acini) of female rat breasts was noticed histologically in the group subjected to chronic stress yet negative immune staining for estrogen receptor was obtained. Previous investigators correlated the level of ER and cell proliferation in normal human breast tissue.
They found that the degree of down-regulation of ER is directly related to the proliferative rate of the breast tissue [16].

As regards the effects of the estrous cycle on mammary gland proliferation in rats, few studies are available and conflicting results have been obtained. Two studies reported high proliferation during proestrous and diestrous [18,19]. Whereas, other two studies reported high proliferation during metestrous and early diestrous [11,12].

Our present work was performed in metestrous phase and demonstrated obvious proliferation of rat mammary gland particularly in response to chronic stress exposure histologically and immunohistochemically compared with control group.

The relation between breast cell proliferation and the development of breast cancer is well established. Cells stimulated into DNA synthesis showed increased susceptibility to chemical carcinogens. Also it had been shown that the risk of breast cancer was reduced if breast proliferation was reduced following ovarian ablation [20]. In the present work, low expression of ER and dense staining for the proliferation marker were manifested after exposure to chronic stress implying that stress could be regarded on a histological basis as an obvious risk factor inducing breast cell proliferation that may lead to breast cancer. The present results go in line with observational studies which established that stressful life events contribute to various diseases including cancer [20].

In the present study, immune staining for progesterone receptor (PR) demonstrated few sporadic positive stained cells in the three study groups. The interesting finding is that these positive cells were exclusively localized in the stroma particularly in perivascular and periductal regions with negative staining of epithelial compartment. In the metestrous phase the level of progesterone will soon decline to a minimum [11]. This explains that in our work, the number of positive immune-stained cells for progesterone hormone was minimal. However, the localization of these cells at the stromal compartment still needs an explanation. Though both steroid hormone receptors revealed either negative or very low expression, yet proliferation marker demonstrated extensive positive immune staining in chronic stress group. These results imply that chronic stress might have mediated this proliferative effect via other mechanisms rather than steroid hormones.

The exact events that trigger proliferation of mammary epithelial cells in vivo are still unclear. However, it has been speculated that estradiol and progesterone control proliferation of epithelial cells through paracrine mechanisms [22,23]. In fact, previous studies have demonstrated that estrogen, progesterone and growth hormone are able to stimulate the production of growth factors through paracrine epithelial-stromal interactions, which subsequently stimulate proliferation of mammary epithelial cells [11,24,25].

In the present study, positive immune staining for P53 and proliferation marker (Ki-67) showed preferential localization. These results could be explained in view of previous investigations which concluded that the content of ER and PR in the normal mammary tissue varies with the degree of lobular development, in parallel with cell proliferation. They added that the expression of hormone receptors occurs in cells other than the proliferating cells, speculating that breast epithelial compartment represent at least two separate cell populations [21].

Other investigators hypothesized that either ER is down-regulated in breast epithelial cells passing through the cell cycle or that ER-positive and proliferating cells are separate populations [11].

P53 plays an important role in regulating cell fate in response to various stresses. P53 exerts its anti-proliferative action by inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis [8]. Indeed, loss of P53 activity disrupts apoptosis and accelerates the appearance of tumours in transgenic mice [26]. In normal cells not exposed to stress, the level and activity of P53 are very low. Upon stress, P53 is activated through a series of post-translational modifications and becomes able to bind to specific DNA sequences [8]. In the present study, breast tissue in female rats exposed to stress revealed intense positive immune staining for the P53 marker in contrast to the control rats. Our study results are in agreement with previous reports.

In the present study, we attempted to define the relationship between stress exposure and proliferation in normal female rat breast. Chronic stress accentuated the expression of the proliferation marker Ki-67 as demonstrated by the immunohistochemical staining. In the control and acute stress specimens very low degree of expression of the proliferation marker was noticed in the epithelial cells of the breast tissue. The exact mechanism through which chronic stress mediated these results was not elucidated in the present study and future research is needed to explore them.
References


