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Assessment of quantitative and qualitative changes of proteoglycans and glycosaminoglycans in normal breast tissue during the follicular and luteal phases of the menstrual cycle

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Summary

The effect of sex hormones on extracellular matrix compounds, such as proteoglycans (PGs) and glycosaminoglycans (GAGs), in mammary tissue remains poorly understood. The elucidation of extracellular matrix component functions could clarify pathophysiological conditions, such as cyclical mastalgia (breast pain). The authors examined the quantitative and qualitative changes of PGs and GAGs in normal breast tissue during the follicular and luteal phases of the menstrual cycle. Twenty-eight eumenorrheic patients with benign breast nodules were divided into groups: Group A included 15 follicular patients and Group B included 13 luteal phase patients. Breast tissue adjacent to the nodules was biochemically analyzed to evaluate the types and concentrations of PGS and GAGs. The distribution of proteoglycans during the menstrual cycle was analyzed with immunofluorescence. PG concentrations were elevated ($p < 0.01$) during the luteal phase compared with the follicular phase, whereas the concentrations of GAGs did not differ significantly. Immunofluorescence revealed that decorin was mainly found in the intralobular stroma. PG concentrations were elevated during the luteal phase, likely due to the influence of sex hormones on macromolecular synthesis. The PG decorin was observed in normal breast tissue in the intralobular stroma. Although the concentration of GAGs, including dermatan and heparan sulfate, varied cyclically, the differences were not significant.

Key words: Breast Tissue; Proteoglycans; Glycosaminoglycans; Menstrual Cycle.

Introduction

During the menstrual cycle, both the breast epithelium and stroma undergo morphologic, biochemical, and functional changes under the influence of sex hormones. Both tissues are more active during the luteal phase of the menstrual cycle [1-6]. Interlobular edema and ductal and acinar proliferation in this phase of the cycle are responsible for the increase in breast volume related to cyclical mastalgia (breast pain). The cyclical changes at the cellular level in the breast are associated with hormonal changes during the follicular and luteal phases of the menstrual cycle and are largely the result of changes in the extracellular matrix [7].

The extracellular matrix is a complex structure that surrounds and supports the cells to maintain an organized tissue structure. The extracellular matrix is closely related to cell growth, movement, and differentiation, and it controls the shape and function of tissues through receptors in cell surface [8]. Among several components of the extracellular matrix, proteoglycans (PGs), and glycosaminoglycans (GAGs) form a hydrophilic semi-fluid gel that allows the circulation of nutrients, hormones, and several chemical messengers [9]. Proteoglycans are macromolecules composed of a protein axis to which one or more GAG chains are covalently bound. Due to the presence of its sulfate and carboxyl groups, GAGs possess many negative charges that determine their functional properties, dictating the selective permeability of the basal membrane and the hydration of the extracellular matrix [10,11]. The objective of the study was to assess the PGs and GAGs in the extracellular matrix of breast tissue in different phases of the menstrual cycle.

Materials and Methods

Selection of patients, tissue samples, metabolic labeling of PGs with ^{35}S-sulfate, and extraction of PGs and GAGs

Thirty-one eumenorrheic women between the ages of 15 and 35 years were prospectively selected for the study. The participants had not used any hormonal contraceptives during the past six months and exhibited benign breast nodules confirmed by fine needle aspiration. Patients with endocrinopathies and those who were pregnant were excluded from the study.

During the surgical procedure to remove the nodules, fragments of adjacent breast tissue were removed for the study. The regularity of both the breast tissue and nodules were confirmed by histopathological testing. Two patients were excluded due to histopathological changes, and one patient was excluded due to difficulty in characterizing the menstrual cycle phase of the patient. Therefore, 28 patients were divided into two groups according their menstrual cycle phase. Dates of the previous period,

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subsequent period, and serum dosage of progesterone determined the cycle phase. Levels of serum progesterone equal or higher than three ng/ml defined the luteal phase [12]. Group A, which had 15 patients, represented samples collected during the follicular phase, and group B, with 13 patients, represented samples collected during the luteal phase.

Samples of regular breast tissue from follicular and luteal phases of the menstrual cycle obtained immediately after surgery were washed with five ml of PBS containing gentamicin (four mg/ml)and subsequently placed in culture bottles containing ten ml of F12 culture medium with no fetal bovine serum in the presence of 35S-sulfate (50 µCi/ml of medium), 100 µl of penicillin (10,000 U) and streptomycin (100 mg) antibiotic solution.The tissues were maintained in primary culture for 24 hours at 37°C in a CO_2 incubator. After this period, the medium was removed, and two volumes of methanol were added under agitation and maintained for 18 hours at -20ºC. The resulting precipitate was collected by centrifugation (1,300 X g, 15 minutes), dried, and resuspended in one ml of distilled water for further analysis. Proteoglycans and glycosaminoglycans were extracted from two pieces of removed tissue.

Characterization of PGs and GAGs

The extracted PGs and GAGs (labeled or not with ^{35}S-sulfate) were characterized by a combination of agarose gel electrophoresis, polyacrylamide gel electrophoresis, immunoblotting, and degradation with specific mucopolysaccharides.

PGs and GAGs were analyzed by agarose gel electrophoresis in a 1,3-diaminopropane acetate buffer 0.05 M, pH 9.0 (PDA), developed by Jaques *et al.* [13] and modified by Dietrich and Dietrich [14].

Enzyme degradation with specific mucopolysaccharides (condroitinases AC, B) [15, 16] and heparitinase II [17] from *Flavobacterium heparinum*) were performed according to established methods [18, 19].

Quantification of 35S-sulfate labeled PGs and GAGs

Quantification of the radioactive compounds was performed by radioactivity count with scintillation cocktail. The electrophoresis gel bands in agarose or the radioactive chromatograms, identified by radioautogram, were removed, immersed in the scintillation cocktail, and radioactivity was quantified in the scintillation counter. Radioactivity in each sample was expressed as counts per minute (cpm).

Immunofluorescence

To understand tissue architectural changes, it is necessary to understand the location, distribution, and interaction with other extracellular matrix components. Thus, to analyze the distribution of PGs, the authors used immunofluorescence with anti-decorin and anti-versican monoclonal antibodies.

Statistical analysis

Student's t-test (software JMP 8 Statistical Discovery) was used to analyze the variables, with a level of significance of 5% ($p < 0.05$).

Results

Patients were between 16 and 35 years of age. The average age of patients in group A (first phase of the cycle) was 22.27 ± 1.35 years of age, and the average age in group B (second phase of the cycle) was 24.69 ± 1.63 years of age, demonstrating homogeneity between the groups. To evaluate PGs and GAGs in normal breast tissue, PGs and GAGs were extracted at greater than 90% yield, which was possible in only 12 cases due to the lack of material obtained for analysis without use of metabolic labeling. The quantification of PGs and GAGs was performed by slide densitometry. The average PG concentration in the follicular phase was 27.2 (± 3.6) µg/g of dry tissue and 43.6 (± 3.7) µg/g of dry tissue in the luteal phase. The average concentration of GAG in the first phase of the cycle was 1.2 (± 0.3) µg/g of dry tissue and 3.2 (± 0.9) µg/g of dry tissue in the second phase of the menstrual cycle. Student's t-test demonstrated that the differences observed between concentrations of PGs in normal breast tissue in follicular and luteal phases were statistically significant ($p < 0.01$). PG concentrations exhibited a mild initial increase during the proliferative phase, a decrease around the 14th day, a new increase during the luteal phase, and a decrease at the end of the menstrual cycle (Figure 1).

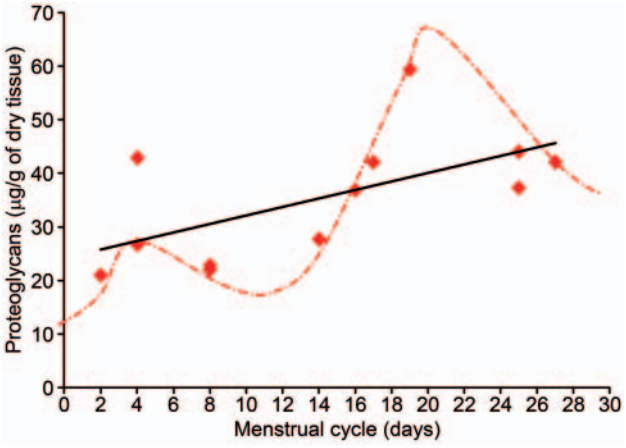

Figure 1. — Individual data of PG concentrations in regular breast tissue during the menstrual cycle.

The GAGs identified by agarose gel electrophoresis included dermatan sulfate and heparan sulfate. Immunoblotting identified the PG decorin. Figure 2 shows the immunolocation of decorin (green) and versican (red) in normal breast tissue during the follicular and luteal phases. The cells nuclei are marked in blue. Only decorin staining was positive in both phases of the menstrual cycle, with higher concentrations in the breast lobes. Specifically, concentrations were highest in the intralobular stroma, where there is higher concentration of cells, and concentrations were lowest in the interlobular stroma.

Discussion

Several studies in literature consistently describe parallel changes in the menstrual cycle and breast lobular kinetics, reporting relative inactivity during the follicular phase and more intense cell proliferation during the luteal

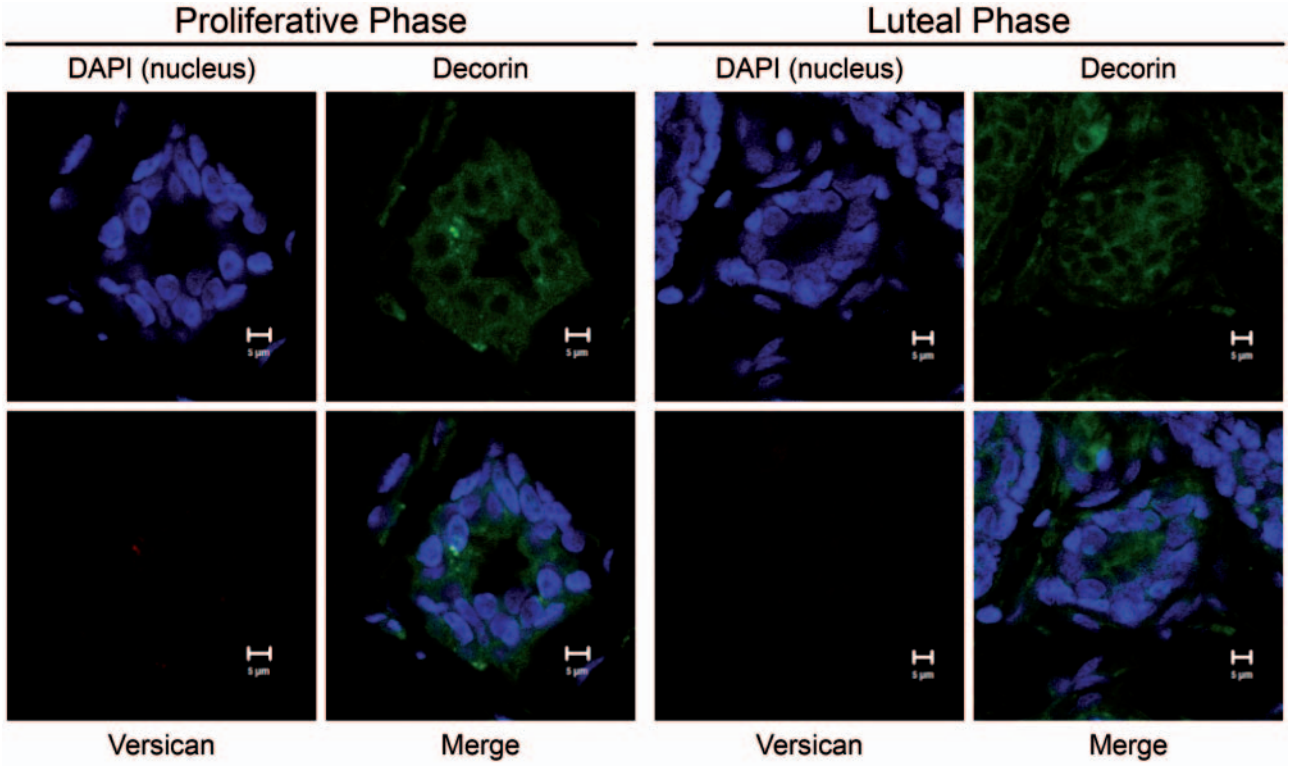

Figure 2. — Immunofluorescence microscopy of the PGs decorin and versican from normal breast tissue in the proliferative and luteal phases of the menstrual cycle. (A) DAPI (nucleus); (B) decorin; (C) versican; (D) image superposition. Scale bar: five μm.

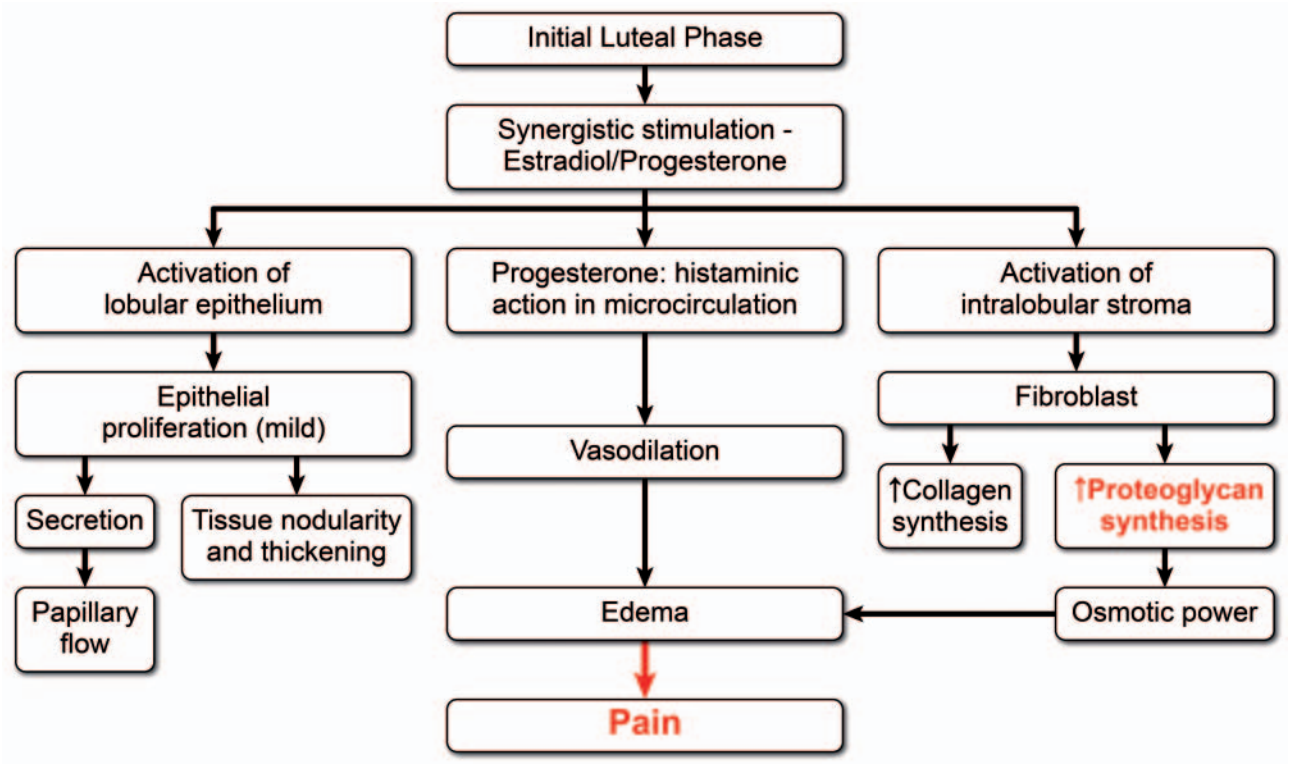

Figure 3. — Proposed explanatory model for the origination of cyclical mastalgia (breast pain).

phase. Proliferative activity reaches its peak around the 25th day of the cycle, simultaneous with the progesterone peak and the second increase in estrogen [1-4]. Thus, changes in the proliferative activity of the breast lobe correspond perfectly to the hormonal curve of estradiol and progesterone.

Few studies in the literature describe the effect of sex hormones on the extracellular matrix of female breast tissue, and the understanding and importance of extracellular matrix changes has only recently been appreciated.

Some animal studies report a possible synergistic effect between sex hormones and GAG synthesis [20]. Further, the breast stroma rich in extracellular matrix expresses hormonal receptors. These findings have motivated the present authors to study the effect of female sex hormones on the synthesis of PGs and GAGs in female breast tissue. Moreover, the pathophysiology of mastalgia, a frequent complaint in the clinical practice, is likely affected by its relationship with hormonal changes during the menstrual cycle, although the relationship remains to be fully elucidated.

Data obtained from the present study demonstrate a significant increase in the concentration of the PG decorin in the intralobular stroma during the luteal phase. The GAGs dermatan and heparan sulfate were also observed and tended to be elevated during the second phase of the cycle. Thus, sex hormones may influence PGs and GAGs, given that their concentrations and rate of synthesis change during the menstrual cycle.

The GAGs present in PGs exhibit a large anionic charge due to their sulfate and carboxyl groups. Therefore, these molecules attract a cloud of cations, mainly sodium. By osmosis, water is brought into the extracellular matrix, forming a gel. This mechanism could explain the common breast edema in the pre-menstrual phase and the consequent breast pain during this period.

The prevalence of breast pain is variable, according to different studies, and affects approximately 70% of women [21]. In less severe cases, the initial clinical approach involves verbal instructions to differentiate this pain from that of breast cancer, and the problem is resolved in approximately 85% of cases [22].

Drug treatment is only used in severe and incapacitating cases of breast pain. The literature reports a large number of drug treatment options, and placebo treatment itself exhibits a response rate of 19% in clinical trials [23]. Tamoxifen and danazol are highly effective, but their numerous side effects lead to low treatment compliance.

The present study proposes an explanatory model for cyclical mastalgia pathophysiology, as shown in the algorithm in Figure 3. Furthermore, these results may initiate a line of research to identify effective drugs to treat mastalgia, including dinoprostone (prostaglandin E2) and misoprostol (synthetic prostaglandin E1 analog), which exhibit proven activity on the extracellular matrix of other sites, such as the cervix [24-30].

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