Modulation of $\alpha_2\beta_1$ integrin changes during mammary gland development by $\beta$-oestradiol

Tessy Iype a, K. Jayasree b, P.R. Sudhakaran a, *

a Department of Biochemistry, University of Kerala, Kariavattom, Trivandrum 695581, India
b Department of Cytopathology, Regional Cancer Centre, Trivandrum 695011, India

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Abstract

In order to study the role of cell–matrix interactions in mammary gland function, temporal changes in $\alpha_2\beta_1$ integrin, the major receptor for collagen and the influence of $\beta$-oestradiol on its level and distribution in rat mammary gland at different stages of development were studied. The level of $\alpha_2\beta_1$ integrin determined by ELISA, was found to be high during different days of pregnancy, while in the lactating stage, it was significantly reduced. By immunocytochemical analysis, $\alpha_2\beta_1$ integrin was found to be localized towards the luminal side of acinar cells, both in the virgin and midpregnant stage, while it was not detected in the lactating stage. The possible role of hormones in modulating the level of integrin was examined in both in vitro and in vivo experiments using $\beta$-oestradiol. Supplementing $\beta$-oestradiol to isolated mammary epithelial cells from both virgin and lactating glands caused a concentration dependent increase in the incorporation of $\left[^{35}\text{S}\right]$methionine into $\alpha_2\beta_1$ integrin associated with the cells. Administration of $\beta$-oestradiol to virgin and lactating glands caused about 1.4–4-fold increase in the level of $\alpha_2$ integrin, indicating that upregulation of integrin during pregnancy may be due to oestrogen and as the oestrogen level falls during lactating phase, downregulation of $\alpha_2\beta_1$ integrin occurs. Treatment with $\beta$-oestradiol also resulted in the appearance of $\alpha_2\beta_1$ integrin in the acinar region of the lactating tissue, while in the untreated controls no staining for integrin was seen. These results indicate that oestrogen, apart from directly affecting the cellular activity, can influence mammary tissue function by affecting cell–ECM interactions through the modulation of integrin receptors for matrix proteins. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Interaction of epithelial cells with the extracellular matrix (ECM) is crucial in maintaining proper cellular morphology and tissue-specific gene expression [1–3]. Cells interact with the ECM through a variety of cell surface receptors such as the integrins. The integrins are a large family of transmembrane proteins which form heterodimers that mediate cell–ECM and cell–cell interactions [4,5]. In vitro studies have shown that consequent on ECM–integrin interaction a cascade of events occurs intracellularly, leading to tissue-specific gene expression [6,7].

Mammary gland undergoes proliferation, differentiation and regression during adult life. Functional differentiation in mammary epithelia requires specific hormones and local environmental signals. Various changes occur in the ECM, as well as in cell–ECM

* Corresponding author. Fax: +91-471-307158; E-mail: prsbn@md4.vsnl.net.in
interactions, during this ontogenic process. These changes may be either due to the action of matrix metalloproteinases (MMPs) which alters the cellular microenvironment [8–10] or due to alteration in cell–matrix interactions because of the altered expression of cell surface receptors such as the integrins. α2β1 Integrin has been identified as the major collagen receptor in the mammary gland and appears to play an important role in mammary gland morphogenesis and tissue-specific gene expression [11,12]. The different phases of mammary epithelial proliferation and differentiation are under stringent hormonal control [13,14]. Recent reports have suggested that hormones influence integrin expression [15–17] and matrix remodelling by upregulating MMPs involved in mammary gland involution [18].

The molecular mechanism of alteration in cell–matrix interactions was studied by examining the temporal pattern of expression of α2β1 integrin in rat mammary gland at different stages of development. The results presented here indicate that the changes in α2β1 integrin during mammary gland development were modulated by β-oestradiol.

2. Materials and methods

2.1. Materials

Acrylamide, bisacrylamide, β-oestradiol (water-soluble), Eagle’s minimal essential medium (MEM), collagenase, trypsin, foetal bovine serum, Hepes, DNase I, EGTA and α-dianisidine were from Sigma Chemical Co. (St. Louis, MO). [125I]NaI and [35S]methionine were products of BARC, Mumbai, India. ECL kit was from Boehringer Mannheim. Monoclonal mouse anti-integrin α2β1 antibody (P1E6, specific for α2), biotinylated anti-mouse IgG, streptavidin HRP and diaminobenzidine (DAB) were products of DAKO (Denmark). The anti-β1 antibody was a kind gift from R. Timpl, Max Plank Institute, Martinsreid, Munich. Collagen I–Sepharose (COL I–Sepharose) was prepared by the coupling of collagen I (COL I) to CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to the manufacturer’s instructions. Tissue culture plastics were from NUNC (Roskilde, Denmark).

2.2. Experimental animals

Mammary tissues (from inguinal and abdominal glands) at various stages of ontogeny were isolated from female Sprague–Dawley rats. Virgin tissue from animals at the pro-oestrous stage was used.

2.3. Hormone treatment

Both virgin and lactating rats were injected with β-oestradiol (0.6 mg/kg body weight) subcutaneously to the inguinal mammary gland. Saline injected animals served as control.

2.4. Preparation of the plasma membrane extract from rat mammary gland tissue

Mammary gland tissue was washed with phosphate-buffered saline (PBS) and homogenized in 20 volumes of hypotonic buffer (1 mM NaHCO3, 0.5 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride (PMSF)) under ice-cold conditions. It was filtered through cheesecloth and centrifuged at 800 × g. The pellet was repeatedly extracted with the buffer and finally washed with 0.025 M Tris–HCl buffer (pH 7.4) containing 1 mM PMSF. Pure plasma membrane was prepared by ultracentrifugation of the resuspended pellet over sucrose gradient [19]. The pellet was extracted with detergent buffer (0.025 M Tris–HCl, 0.15 M NaCl, 1 mM PMSF, 0.5% deoxycholate, 0.5% NP40) for 12 h, centrifuged at 13000 rpm for 15 min, and the supernatant was used for affinity chromatography.

2.5. Isolation of collagen I binding proteins

The plasma membrane extract prepared from the midpregnant rat mammary gland was subjected to affinity chromatography on COL I–Sepharose column, and the collagen I binding proteins were eluted with 20 mM EDTA and radiiodinated by chloramine T method [20]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was done according to the procedure of Laemmli [21] and was visualized by silver staining [22], and was further confirmed by immunoblotting using antibodies against integrins. Binding to collagen I and IV...
was also tested using radiolabelled protein by dot blot assay.

2.6. Enzyme-linked immunosorbent assay (ELISA) for the quantitation of integrin

The amount of protein present in the plasma membrane extracts from the mammary gland tissues was estimated according to the method of Lowry et al.[23]. Membrane extract equivalent to 20 μg of protein from each set was used for coating the multiwells. After incubation at 37°C for 3 h, blocking was done for another 1 h in 0.2% casein/0.05% Tween-20 in PBS. The primary antibody was added and incubated again for 1 h, followed by washing in Tween-PBS and incubation with secondary antibody. Streptavidin-HRP was added and kept for 1 h at room temp. One ml of substrate-chromogen mixture was added to the wells (to 60 ml of 0.1 M citrate-phosphate buffer was added 12 μl of 30% H₂O₂ followed by 500 μl of o-dianisidine (10 mg/ml)) and the reaction was stopped after 5 min with 50 μl of 5 N HCl. Yellow-orange colour was developed and the absorbance was measured at 400 nm.

2.7. Immunocytochemical analysis

Immunocytochemical analysis was carried out with mammary tissue sections from different developmental stages. Virgin and lactating glands after oestradiol treatment were also taken. For these studies, tissues from the inguinal glands were used. Cryostat sections were fixed in cold acetone for 10 min and blocked with 0.3% H₂O₂ in methanol for 30 min to reduce endogenous peroxidase activity. The sections were rinsed with distilled water and incubated with 3% BSA to reduce non-specific antibody binding. The primary antibody, monoclonal anti-integrin α₂ was added and kept overnight at 4°C. washed in PBS and incubated further for 30 min each at room temperature with biotinylated antimouse IgG followed by peroxidase-conjugated streptavidin. After each step the sections were washed in PBS and the reaction was developed by the application of dianinobenzidine, and were lightly counterstained with haematoxylin. In control experiments, the primary antibody was omitted [18].

2.8. Isolation of cells and metabolic labelling

Mammary glands were excised from virgin and lactating rats and the epithelial cells were isolated by the method of Emerman and Bissell [24]. The mammary tissue was dissociated by treating with a medium consisted of 0.1% collagenase, 0.15% trypsin, 1% FCS, 0.12% NaHCO₃, 0.15% Hepes in MEM, for 1 h at 37°C and centrifuged at 20×g for 1 min. The epithelial and fibroblast cells were separated by repeated centrifugation and final pellet consisting of epithelial cells was digested with DNase I (1 ml, 0.4% at 37°C for 15 min) to give a greater number of single cells. Viability of the cells was checked using 0.1% trypan blue. The cells were then seeded onto 35-mm plastic petri dishes at a density of 3–4×10⁶ cells per plate for 30 min at 37°C in MEM, and then methionine-free medium containing 20 μCi/ml of [³⁵S]methionine was added to the cells. Different concentrations (10⁻⁴ M, 10⁻⁵ M, 10⁻⁶ M) of oestradiol was supplemented in the medium. After 12 h, the medium and cell layer were separated. The cell layer was extracted with 50 mM Tris–HCl buffer (pH 7.5) containing deoxycholate and NP40. The extract was then passed through a COL I-Sepharose column, and the integrin was eluted with 20 mM EGTA. The fractions were subjected to SDS-PAGE analysis, visualized by silver staining, and the bands corresponding to α₂β₁ integrin were cut and dissolved in H₂O₂ and the radioactivity was measured in a LKB Rack beta liquid scintillation counter.

3. Results

3.1. Isolation and characterization of α₂β₁ integrin from rat mammary gland

In order to confirm the presence of α₂β₁ integrin in mammary gland plasma membrane extract of the midpregnant rat mammary gland was subjected to affinity chromatography on COL I-Sepharose and the bound proteins were eluted using 20 mM EDTA. The eluted proteins were characterized by electrophoresis. Two bands of molecular size 160 000 and 130 000 appeared on electrophoresis under reducing conditions. Western blotting followed
by detection with specific antibodies further confirmed the presence of \( \alpha_2\beta_1 \) integrin (Fig. 1). Their binding to COL I and COL IV was confirmed by dot blot assay using radio iodinated \( \alpha_2\beta_1 \) integrin.

3.2. Changes in \( \alpha_2\beta_1 \) integrin expression in the mammary gland at different stages of development

Mammary gland undergoes developmental changes during adult life and therefore the changes in the level and/or distribution of the \( \alpha_2\beta_1 \) integrin in the gland during different stages of ontogeny was studied. Mammary glands from varying stages of development, viz. virgin, midpregnant, lactating and involuting stages, were extracted and analysed by ELISA using antibodies against \( \alpha_2 \) integrin. The virgin tissue was found to express a considerable amount of \( \alpha_2\beta_1 \) integrin which increased significantly in the midpregnant stage. The level of the integrin during pregnancy was found to remain high up to the later stages. A significant decrease in \( \alpha_2 \) integrin level was found in the lactating stage when compared with midpregnant or virgin. As involution sets in, the integrin reappears gradually attaining a level similar to that of the virgin tissue (Fig. 2). Thus there is an upregulation of the \( \alpha_2\beta_1 \) integrin in pregnancy while it is significantly downregulated in the lactating phase.

3.3. Localization of the \( \alpha_2\beta_1 \) integrin by immunocytochemical analysis

The changes, if any, in the distribution pattern of \( \alpha_2\beta_1 \) integrin, as the mammary gland undergoes developmental changes, were also studied by immunocytochemical analysis. Tissue sections from mammary gland at different stages were stained with antibody against \( \alpha_2 \) integrin. Positive reaction was indicated by brown staining. \( \alpha_2 \) Integrin was found to be expressed by virgin, midpregnant and involuting mammary glands, while the lactating gland did not show positive reaction (Fig. 3). For the virgin tissue, expression was moderate and the distribution was found to be mainly towards the luminal area of epithelial cells, along with the ducts and ductules of the gland. In the midpregnant stage also, a similar expression pattern was seen. Although due to the
packing of the acinar structures, the intensity of staining was apparently moderate in the acinar region like the virgin tissue, because of an increase in the number of acini during pregnancy, the total amount of $\alpha_2\beta_1$ integrin in the gland is increased. It was followed by a downregulation of the integrin in the lactating stage. But as involution sets in, the integrin reappeared and the intensity of staining was mild. No staining was seen in control sections untreated with primary antibody, indicating that the reaction observed was not non-specific.

3.4. Effect of oestradiol on the production of $\alpha_2\beta_1$ integrin by isolated mammary epithelial cells in culture

In order to study whether the altered expression of $\alpha_2\beta_1$ integrin in the mammary gland was due to hor-
monal control, the effect of oestradiol on the $\alpha_2\beta_1$ integrin production by the mammary epithelial cells was studied. Mammary epithelial cells from virgin and lactating tissues in culture were treated with different concentrations of the hormone ($10^{-6}$–$10^{-4}$ M) in $[^{35}S]$methionine (20 $\mu$Ci/ml) containing medium. The medium and the cell layer were collected after 12 h. The detergent extract of the cell layer was subjected to affinity chromatography on COL I-Sepharose and determined the amount of radioactivity incorporated into $\alpha_2\beta_1$ integrin. There was an increase in the amount of $\alpha_2\beta_1$ integrin synthesized both by the virgin tissue and the lactating tissue with increase in the concentration of $\beta$-oestradiol, and the maximum effect was observed at a concentration of $10^{-4}$ M of the hormone (Fig. 4). The amount of $\alpha_2\beta_1$ integrin in the cells from virgin stage tissue increased with increase in the amount of hormone. Though the integrin level of the lactating stage was very low compared with the virgin stage, after oestradiol treatment of cells from lactating tissue, there was an increase in the level of integrin.

3.5. Effect of oestradiol treatment in vivo on $\alpha_2\beta_1$ integrin in mammary gland

In order to study the effect of oestradiol in vivo, virgin and lactating rats were administered oestradiol and the plasma membrane extracts were prepared and ELISA was done for the quantitation of $\alpha_2\beta_1$ integrin using specific antibody against the integrin. In both cases, after oestradiol administration the level of the integrin was elevated. Though the level of the integrin in the normal lactating gland was very low, after oestradiol treatment it was elevated by about four times the control levels (Fig. 5).

3.6. Immunocytochemical analysis of oestradiol treated tissues

The expression of $\alpha_2\beta_1$ integrin on treatment with $\beta$-oestradiol was also tested by immunocytochemical analysis. Sections of both virgin and lactating tissues after oestradiol administration were subjected to immunocytochemical analysis. The amount of $\alpha_2\beta_1$ integrin in the virgin stage was found to be increased after oestradiol administration. The luminal side of cells in each acini, the ducts and ductules of the gland showed positive staining (Fig. 6). The normal
lactating gland did not show significant reaction, but after oestradiol treatment the staining was found to be intense. Immune positivity was found to be towards the luminal side of cells in acini; the ducts and myoepithelial cells also showed positive staining. While L-oestradiol administration caused only a slight increase in intensity of staining in virgin tissues, it resulted in intense positive reaction in lactating tissues.

4. Discussion

In vitro studies showed that ECM influence the expression of tissue-specific functions in mammary epithelial cells. In these studies the crucial role of the extracellular matrix has been demonstrated by showing that mammary cells acquire a glandular morphology, synthesize an organized basement membrane and maintain mammary-specific gene expression when cultured on EHS tumour matrix [25–27].

Evidence in support of a role for ECM in the control of tissue-specific functions in vivo is also accumulating. Alterations in the basement membrane components and their degradation in the mammary gland during development may influence cell functions in vivo [28]. The remodelling of the basement membrane by the degradation of ECM components through the coordinated action of matrix degrading metalloproteinases (MMPs) have also been demonstrated [29,30]. The coordinated expression of MMPs and their endogenous regulators, and expression of tissue-specific function such as casein production in mammary gland suggested that the interaction of mammary cells with the basement membrane components in vivo is critically important [31].

The results presented here give further evidence that the molecular mechanisms involved in cell–matrix interactions in intact tissue is altered as the mammary gland undergoes developmental changes. This is evidenced by the changes in α2β1 integrin which is a receptor for COL I and COL IV. α2β1 integrin is
the major collagen receptor of the mammary gland and was isolated by affinity chromatography on COL I-Sepharose which appeared as 160,000/130,000 bands in SDS-PAGE analysis. In the virgin mammary tissue, the $\alpha_2\beta_1$ integrin was seen towards the luminal side of the epithelial cells in the acini, and also in the duct system. During gestation stage, when proliferation of epithelial cells occurs, there is a significant increase in $\alpha_2\beta_1$ integrin level, which appeared at the cell–matrix contact sites. This may cause alterations in cell–matrix interaction and contribute to tissue remodelling during mammary gland development. Keely et al. [12] have shown that the $\alpha_2\beta_1$ integrin was present on the basal, lateral and apical surfaces of the mammary epithelium throughout post natal development and pregnancy. A high level of expression of the $\alpha_2\beta_1$ integrin is associated with orderly and regulated proliferation of epithelial cells, including the ducts and ductules of normal breast [11]. Our results show that in the lactating tissue, where epithelia showed a structural change and tissue-specific gene expression such as milk production is maintained, $\alpha_2$ integrin level is very low. It appears that there is a down regulation of $\alpha_2$ integrin, during lactating stage.

Earlier reports have suggested that $\alpha_2\beta_1$ integrin might mediate some mammary cell response to collagen [11]. A decrease in $\alpha_2\beta_1$ integrin levels disrupted the ability of mammary cells to organize in three-dimensional collagen gels, indicating that $\alpha_2\beta_1$ integrin plays a critical role in collagen induced morphogenesis [32]. From our results, it is evident that a high level of $\alpha_2\beta_1$ integrin is maintained during gestation, when epithelial proliferation and glandular structure formation occur in the mammary gland. The downregulation of the integrin in the lactating stage may be a regulatory mechanism to arrest the morphogenetic event. The normal development and ductal morphogenesis of the mammary gland depend on functional $\beta_1$ integrins, which permit contacts with the ECM and with laminin in particular [33]. It is also suggested that cellular growth, survival and morphogenesis of acinar structures by normal cells are integrin dependent and loss of proper integrin mediated cell–ECM interactions may be critical to breast tumour formation [34]. The results presented here provide further evidence in support of a critical role for the interaction of cells with ECM in vivo in mammary epithelial function. Changes in the level of $\alpha_2\beta_1$ integrin not only affect epithelial-basement membrane interactions, but also may influence intracellular events.

The increase in $\alpha_2\beta_1$ integrin during pregnancy may be proliferation associated, but during lactation there is a down regulation of the integrin, though the epithelial mass remains the same. This may also be due to the action of hormones, since the development of the mammary epithelium and milk production during the later stages of pregnancy and in lactation are under hormonal control. The normal development of the mammary gland was found to be partly under control of interaction between gonadotropic hormone and oestrogen [14]. Results on the production and distribution of $\alpha_2\beta_1$ integrin presented here suggest that hormones influence cell–matrix interactions as well. It appears that the production and distribution of $\alpha_2\beta_1$ integrin is modulated by oestrogen. This conclusion is based on the following observations. (a) In mammary glands from midpregnant rats, which is under oestrogen stress, the level of the $\alpha_2\beta_1$ integrin is elevated. (b) In lactating tissue, which is relieved from oestrogen stress, there is decrease in $\alpha_2\beta_1$ integrin level. (c) Administration of $\beta$-oestradiol caused an increase in the level of the integrin. Both in the virgin and in the lactating stage, the level of the integrin was significantly enhanced after oestradiol treatment. The distribution pattern of the $\alpha_2\beta_1$ integrin in lactating glands treated with oestradiol was also similar to that of virgin and pregnant stages. (d) In vitro experiments by supplementing oestradiol to primary cultures of mammary epithelial cells also demonstrated that the $\alpha_2\beta_1$ integrin production by cells from both the virgin and lactating tissues is increased in the presence of oestradiol. Although these experiments do not indicate how oestrogen affects the production and distribution of $\alpha_2\beta_1$ integrin in mammary gland, its enhanced production by primary cultures of mammary epithelial cells on treatment with oestradiol indicates that oestradiol effect is not a systemic effect. The influence of hormones on the cell surface receptors and changes in tissue-specific functions have been reported in different systems [15–17]. The addition of oestradiol and progesterone to cultured stromal cells of the human endometrium in the early proliferative phase increased the expression of $\beta_1$ integrins in vitro.
References


