

Estrogen receptor β participate in the regulation of metabolism of extracellular matrix in estrogen α negative breast cancer

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Abstract: The biology of breast cancer is closely related to sex steroid hormones. Estrogen receptor β is overexpressed in around 70% breast cancer cases, referred to as "ER positive". Estrogens bind to estrogen receptor and stimulate the transcription of genes involved in control of cell proliferation. Moreover, estrogens may induce growth factors and components of extracellular matrix and interact with them in a complex manner. Extracellular matrix and integrins play an important role in cell functions and their aberrant expressions are implicated in breast cancer development, invasion and metastasis. ER β is certainly associated with more differentiated tumors, while evidence of role of ER β is controversial. The highly invasive breast cancer ER β negative cell line MDA-MB 231 can be the model of exam the role of ER β in breast cancer. The aim of this study was to examine the role of activation of ER β on the metabolism of the extracellular matrix and the expression of β -1 integrin in the breast cancer cell line MDA-MB 231. The cells were exposed on the estradiol, tamoxifen, raloxifen and genisteina in dose dependent concentrations. To determine the relative rate of collagen synthesis we measured the time-dependent reduction of collagen-bound radioactivity after pulse-chase labeling with [³H] proline by Peterkofsky methods. The expression of β -1 integrin was determined by Western blot analysis. The activity of MMP2 and 9 were measured using gelatin zymography with an image analysis system. Our data suggest on the role of estrogen receptor β on the metabolism of extracellular matrix in the breast cancer line MDA – MB 231. Estradiol and SERMs regulate the expression of ECM proteins: collagen, integrins and enhance activity of metalloproteinases 2 and 9.

Key words: breast cancer, estrogen receptor β , extracellular matrix, estradiol, tamoxifen, raloxifen, genisteina

Introduction

Estrogen are well known to contribute immensely to the development of breast cancer [1,2]. Estrogen effects are exerted through the binding of estrogen to two types of specific ligand – activated receptors α and β . ERS are the members of steroid hormone receptor superfamily [3,4]. Although α and β receptor share homology at DNA and ligand binding domains available data provide evidence that they have both distinct functions as well as overlapping and interactive or cooperative functions. Following ligand binding

receptors undergoes conformational changes, dimerization, nuclear translocation complex formation with various transcriptional co – activators or co – repressors and subsequent bind to specific DNA motif called estrogen – response – elements (ERE). EREs is located frequently near the promoter regions of estrogen – responsive genes. ERs can also influence gene expression by interacting with general transcriptional factors, for example AP-1, NF κ B, Sp1 [4-6]. Over the last few years it has been confirmed the presence of membrane and possibly mitochondrial – localized estrogen receptor exerted non – genomic effects. There is evidence that estrogen receptor expression is profoundly altered during breast tumorigenesis. In normal mammary gland there is very low expression of ER α and predominate ER β [7,8]. The opposite situation is

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observed in 70% of breast cancer where ER α is over-expressed. 30% of breast cancer doesn't expressed protein of estrogen receptor α . In mammary carcinogenesis even though the mitogenic effect of estrogens is well demonstrated the presence of ER α is associated with more differentiated and less invasive tumors and more favorable prognosis ER α is a well – established marker of breast cancer hormone sensitivity ER α negative breast cancer is more aggressive in its clinical course, metastasizes more frequently and the prognosis is definitely much more unfavourable [9-15].

The second receptor ER β has likewise been detected in human breast cancer but their role in breast cancer isn't well known. It is suggested that this receptor partakes in cells differentiation, regulating the function of mitochondria and synthesis of intracellular matrix [16-23].

Clinical significance of presence of estrogen receptor β in breast cancer published so far are full of contradictions [24-26]. According to some authors, the presence of the receptor has a favourable prognostic influence, according to others it has an unfavourable prognostic impact. Understanding the role of estrogen receptor β in the biology of neoplastic breast cell is clinically important and might potentially create new forms of hormonal treatment of cancers at present recognized as hormone-independent cancers [14,27-30].

Human cell lines constitute powerful model for analysis of cancer. Human cell lines MDA-MB 231 is the prototype for the study of hormone independent, highly aggressive breast cancer. The MDA – MB 231 breast cancer cell line was obtained from a pleural effusion of a 51 year – old patient. It was shown that the MDA-MB 231 cell lines had a lack of expression of estrogen receptor α but estrogen receptor β is expressed.

Materials and methods

Culture cells MDA MB 231 were maintained in 75 cm² tissue culture flask (Sarsted, USA), under standard culture conditions; at 37°C, without CO₂ humidified incubator in Leibovitz medium (Sigma-Aldrich), were renewal every 3-4 days. Culture media contained 15% of fetal bovine serum, and enriched with 50 U/ml antibiotic, antimycotic solution (penicillin, streptomycin, amphotericin B (Sigma-Aldrich Poland). When cells reached 80% confluence cells were rinse with 0.05% trypsin-0.53 mM EDTA solution to remove all traces of serum. After that culture were incubated with 3 ml of trypsin-EDTA solution. Cells layer were observed under an inverted microscope and cells dispersed usually within 5 to 15 minutes of incubation. Cells were aspirate by pipeting with complete growth medium (6 to 8 ml). Appropriate medium were added for cell suspension in new culture vessels. Subcultivation ratio was 1:2. For experiment cells were collected and seeded in 6 well culture plates (Sarsted, USA). The cells were incubated for 24 h prior to treatment and allowed to proliferate to 70% – 80% confluence before commencement of each experiment, without serum and phenol red in DMEM / F-12 medium. The cells were exposed on the estradiol, tamoxifen, raloxifen and genisteina for 24 h in dose dependent concentrations (2 nM, 5 nM, 10 nM estradiol, 1 nM, 10 nM, 100 nM, 1 μ M tamoxifen, raloxifen, genistein).

Western immunoblot analysis. After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2 m pore-sized nitrocellulose at 100 mA for 90 minutes using a BioRad electrophoresis unit according to the method described in the manual accompanying the unit. The membrane was blocked with 5% dried milk in TBS-T for 1 hour in room temperature, slowly shaking. Then the nitrocellulose was incubated with monoclonal antibody against b₁-integrin at concentration 1:5,000 or polyclonal antibodies against β -actin at concentration 1:1,000 overnight. After the incubation, nitrocellulose was washed with TBS-T (1 \times 15 min and 2 \times 10 min) intensively shaking. In order to analyze the proteins second alkaline phosphatase labeled antibodies against mouse Fc IgG or against rabbit's IgG were added at concentration 1:2,500 in TBS-T and incubated for 1 hour slowly shaking. Then the membrane was extensively washed with TBS-T (5 \times 10 min) and submitted to BCIP/NBT western blotting detection system.

Collagen production. Incorporation of radioactive precursor into extracellular matrix components was measured after labeling confluent cells in serum-free medium for 24 h with the 5-[³H] proline (5 m Ci/ml, 28 Ci/mmol). Incorporation of label into collagen was determined by digesting proteins with purified *C. histolyticum* collagenase according to the method of Peterkofsky *et al.* [16]. Results are shown as combined values for cell plus medium fractions.

Determination of prolidase activity. The activity of prolidase was determined according to the method of Myara [14] which is based on measurement of proline by Chinard's reagent [5]. Briefly, the monolayer was washed three times with 0.15 M of NaCl. Cells were harvested by scraping and suspended in 0.15 M NaCl, centrifuged at low speed (200 \times g) and the supernatant was discarded. The cell pellet was suspended in 0.3 ml of 0.05 M Tris-HCl, pH 7.8, and sonicated 3 times for 10 seconds at 0°C. Samples were then centrifuged (16,000 \times g, 30 min.) at 4°C. Supernatant was used for protein determination and then prolidase activity assay. Activation of prolidase required incubation with manganese; 100 ml of supernatant incubated with 100 ml of 0.05 M Tris-HCl, pH 7.8 containing 2 mM MnCl (II) for 2 hours at 37°C. The prolidase reaction was initiated by adding 100 ml of the incubated mixture to 100 ml of 94 mM gly-cyl-proline (Gly-Pro) for a final concentration of 47 mM Gly-Pro. After additional incubation for 1 hour at 37°C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes reaction was terminated at time "zero" (without incubation). The released proline was determined by adding of 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min. at 90°C. The amount of proline released was determined colorimetrically by absorbance at 515 nm and calculated by using proline standards. Protein concentration measured by the method of Lowry. Enzyme activity was reported as nanomoles per minute per milligram of supernatant protein.

Zymography. Gelatinolytic activity was determined according to the method of Unemori and Werb [23]. Cell culture medium was concentrated 10 times using 3 kDa cut off "centricons", mixed with Laemmli sample buffer, Laemmli [11] containing 2,5% SDS (without reducing agent). An equal amounts (about 20 mg) of protein was electrophoresed under non-reducing conditions on 10% polyacrylamide gels impregnated with 1 mg/ml gelatin. After electrophoresis, the gels were incubated in 2% Triton X-100 for 30 min at 37°C to remove SDS and incubated for 18-24 h at 37°C in substrate buffer (50 mM Tris-HCl buffer, pH 8.0, containing 5 mM CaCl₂). After staining with Coomassie brilliant blue R250, gelatin-degrading enzymes present in tissue extract were identified as clear zones in a blue background.

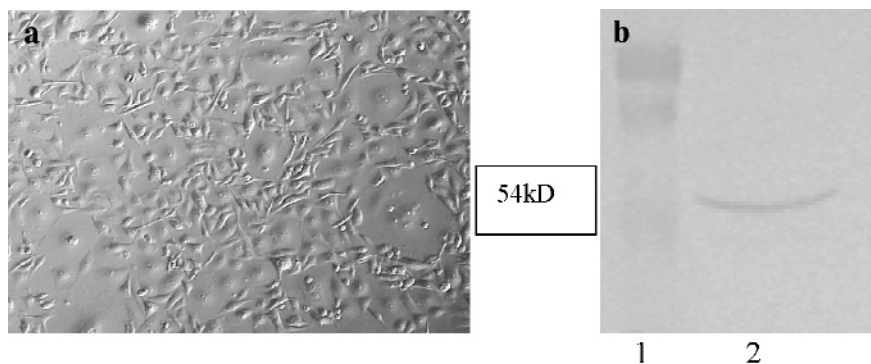


Fig. 1. a. Cell culture of MDA – MB231. **b.** Estrogen receptor β expression in MDA-MB 231 (1 – standard weight, 2 – ER β receptor protein)

Statistical analysis. In all experiments, the mean values for six independent experiments \pm standard deviation were calculated. The results were submitted to statistical analysis using Student's t-test, accepting $P < 0.05$ as significant.

Results

Western blotting method revealed the presence of estrogen receptor β protein in the cells of MDA-MB 231 line (Fig. 1).

After 24-hour incubation of neoplastic cells with estradiol, a threefold increase in synthesis of collagen was observed even at the lowest doses of estradiol. Increase in the dose to 10 nM resulted in a sixfold increase in the synthesis of collagen (Table 1).

After administration of raloxifen, the increase was proportional to the dose (Table 2).

After administration of tamoxifen, maximum increase in the synthesis of collagen was observed regardless of administered dose (Table 3).

After administration of genisteine in concentrations of 1.10 and 100 nM, a twofold increase in the synthesis of collagen was observed, a sixfold increase occurred after the concentration of 1 μ M (Table 4).

The exposure of MDA MB 231 cell line to the examined substances also resulted in changes of increase in the prolidase activity depending on concentrations of administered substances (Fig. 2).

Conducted experiment indicates that after the exposure of neoplastic cells of MDA-MB 231 line there occurs an increase in the β -1 integrin receptors expression even at the lowest doses of SERM estradiol compared to the expression observed in standard conditions. The increase in the concentration of investigated substances results in increased expression of β -1 integrin receptor (Fig. 3)

Examined cells show the presence of collagenolysis with relative molecular masses of 72kD and 92kD which are consistent with metalloproteinases 9 and 2. The cells of the examined line revealed higher activity of metalloproteinase 9 than that of metalloproteinase 2. Activity of both metalloproteinases was highest after administration of genisteine. Zymo-

Table 1. Biosynthesis of collagen in the cells of MDA-MB 231 line after 24-hour exposure to estradiol in concentrations of 2 nM, 5 nM, 10 nM

Controls	2 nM Estradiol	5 nM Estradiol	10 nM Estradiol
4.02 \pm 0.1	12.13 \pm 2.5 $p < 0.01$	11.58 \pm 2.7 $p < 0.01$	25.34 \pm 2.5 $p < 0.01$

Table 2. Biosynthesis of collagen in the cells of MDA-MB 231 line after 24-hour exposure to raloxifen in concentrations of 1nM, 10 nM, 100 nM 1 μ M.

Controls	1 nM Raloksifen	10 nM Raloksifen	100 nM Raloksifen	1 μ M Raloksifen
4.02 \pm 1.9	8.02 \pm 4.2 $p < 0.01$	14.68 \pm 2.2 $p < 0.01$	25.40 \pm 4.17 $p < 0.01$	37.03 \pm 3.88 $p < 0.01$

Table 3. Biosynthesis of collagen in the cells of MDA-MB 231 line after 24-hour exposure to tamoxifen in concentrations of 1nM, 10nM, 100nM 1 μ M.

Controls	1 nM Tamoksifen	10 nM Tamoksifen	100 nM Tamoksifen	1 μ M Tamoksifen
4.02 \pm 1.9	11.78 \pm 4.3 $p < 0.01$	14.12 \pm 5.61 $p < 0.01$	11.10 \pm 5.66 $p < 0.01$	14.02 \pm 3.02 $p < 0.01$

Table 4. Biosynthesis of collagen in the cells of MDA-MB 231 line after 24-hour exposure to genisteine in concentrations of 1nM, 10 nM, 100 nM 1 μ M.

Controls	1 nM Genisteine	10 nM Genisteine	100 nM Genisteine	1 μ M Genisteine
4.02 \pm 0.9	9.21 \pm 2.82 $p < 0.01$	7.01 \pm 2.0 $p < 0.01$	9.49 \pm 4.25 $p < 0.01$	23.8 \pm 2.31 $p < 0.01$

gram shows the collagenolytic activity of cells cultured in standard conditions (control) and after 24-hour exposure to estradiol in concentrations of 2nM (Fig. 4).

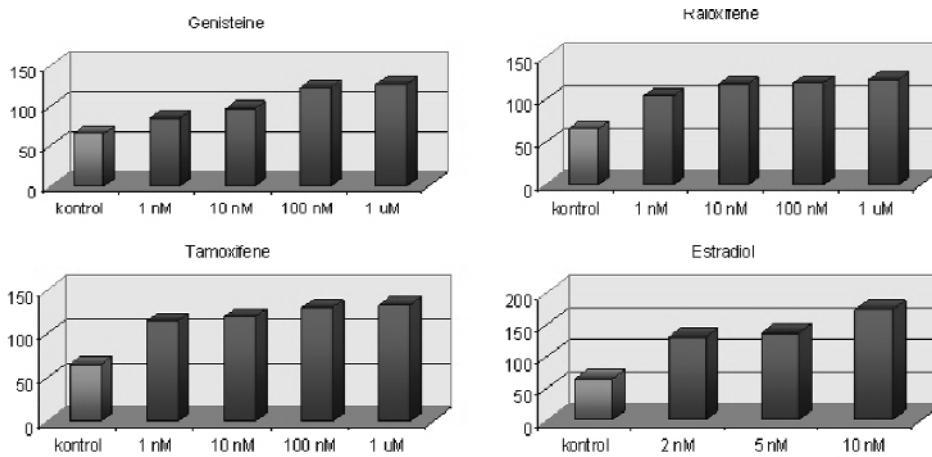


Fig. 2. Activity of prolidase

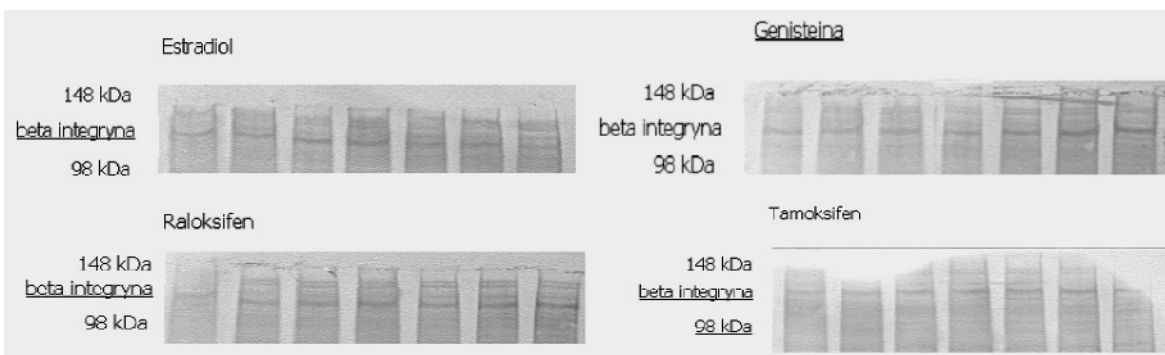
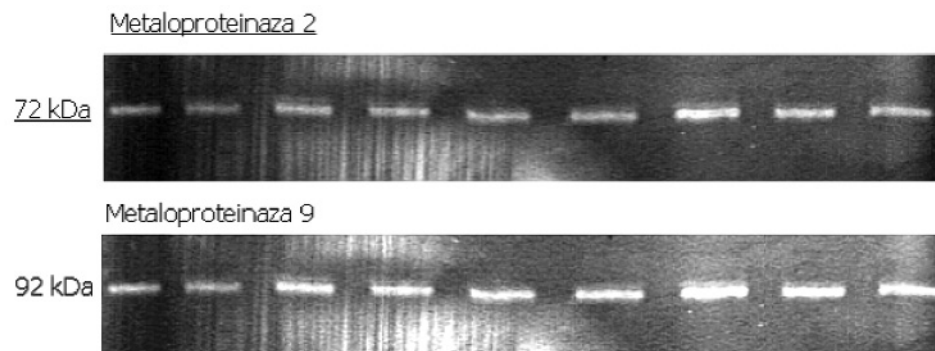
Fig. 3. Expression of β -1 integrin receptor after 24-hour exposure to estradiol, raloxifen, tamoxifen and genisteine.

Fig. 4. Zymographic assessment of collagenolytic activity in mammary cancer cells of MDA-MB 231 line after 24-hour exposure to estradiol, raloxifen, tamoxifen and genisteine.

Discussion

It has been known for a long time now that estrogens in mammary cancer cells stimulate proliferation. Surazyński and Wolczyński demonstrated that estrogens in hormone-dependent lines influence the composition and metabolism of extracellular matrix [19]. The question remains open whether estrogens may also influence the course of hormone-independent breast cancer [2,13]. Our own research has shown the lack of α estrogen receptor and the presence of β estrogen receptor in the MDA MB 231 cell line. This

is further supported by observations performed by Vladusic, Gustafsson, Murphy [30]. We have demonstrated that the exposure of cells to estrogens did not have any effect upon the proliferation speed in the culture. However, we have confirmed that β estrogen receptor activation by estradiol, tamoxifen, raloxifen and genisteine influences the metabolism of extracellular matrix. So it may be assumed that this process happens through beta estrogen receptor. It is known that extracellular matrix is not a static compartment and that very intensive transformations occur in it

[4,9]. The elements of intercellular matrix modulate the cell phenotype through integrin receptors. It is known that the changes in interactions of integrins with the matrix determine the malignant phenotype of the *in vivo* culture. Stimulated integrin mediation in the adhesion of extracellular matrix determines the survival rate of cells and their invasiveness [22]. The development of breast cancer carries with it significant changes in the composition of extracellular matrix.

In the presence of estradiol, genisteine and raloxifen, biosynthesis of collagen increased depending on the dose, whereas the presence of tamoxifen resulted in increased collagen biosynthesis, but increasing doses of tamoxifen did not result in further increase in collagen biosynthesis.

Examined substances also increased prolidase activity – the enzyme participating in the extracellular metabolism of collagen and supplier of proline – product for collagen resynthesis. The exposure of MDA MB 231 cell line to the examined substances also resulted in changes of β -1 integrin expression. Observed changes prove that, contrary to common belief, estrogens influence the tumorigenesis in tumors without alpha estrogen receptor, but containing β estrogen receptor. This might explain the observations of some authors who associate the presence of β estrogen receptor with poor prognosis.

In primary tumor, the exposure to estrogens might increase the cell adhesion. However, it is known that such tumors are characterized by the presence of a large pool of metastatic cells. Outside the primary tumor, estrogens may promote the creation and development of metastases. It is known that inhibition of cellular matrix metabolism decreases, to a large extent, the tumor progression, its invasiveness and creation of metastases.

Conclusions

1. Functional estrogen receptor b occurs in MDA-MB 231 breast cancer cell line.
2. Activation of estrogen receptor b in MDA-MB 231 breast cancer cell line influences the metabolism of the main component of intercellular matrix – collagen and adhesive properties of cells.
3. Estradiol and SERMs stimulate biosynthesis of collagen, enhance the activity of prolidase and of metalloproteinases 2 and 9.
4. Estradiol and SERMs in MDA-MB 231 breast-cancer cell line regulate the expression of beta-1 integrin.
5. Substances operating via estrogen receptor b may influence the biological properties of neoplastic cells in MDA-MB 231 breast cancer cell line.

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