

Estradiol and tamoxifen differently affects the inhibitory effects of vitamin A and their metabolites on the proliferation and expression of $\alpha_2\beta_1$ integrins in MCF-7 breast cancer cells

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ABSTRACT

Purpose: Retinoids are well known inhibitors of estrogen-dependent breast cancer cell growth and differentiation. $\alpha_2\beta_1$ integrins are involved in the normal growth and differentiation of breast cells, they also take part in many pathological processes including malignancies. The aim of the study was to evaluate the effect of estradiol and tamoxifen on the inhibitory action of retinoids on the proliferation of MCF-7 breast cancer cells and $\alpha_2\beta_1$ integrin expression.

Materials and methods: Evaluation was based on [³H]thymidine incorporation and the proliferative activity of PCNA- and Ki 67-positive cells. Expression of $\alpha_2\beta_1$ was assessed through immunocytochemical analysis.

Results: Treatment of cancer cells with the examined compounds and tamoxifen (10 μ M) revealed that only 13-*cis* retinoic acid (13-*cis* RA) and all-*trans* retinoic acid (ATRA) (10⁻⁵ M) decreased cells proliferation compared to the tamoxifen group (30.84%±3.32, p<0.01 and 31.05%±4.67, p<0.01, respectively). The lowest fraction of PCNA positive cells was also observed after the simultaneous addition ATRA (10⁻⁵ M) and tamoxifen (10 μ M) (30.75%±0.95, p<0.01, compared to the tamoxifen group). Our results showed that the decrease of $\alpha_2\beta_1$ integrin expression by 13-*cis* RA (10⁻⁵ M, 49.6±3.25%) and ATRA (10⁻⁹ M, 15.0%±5.0) was augmented by tamoxifen and to a lesser extent by estradiol, particularly in the case of ATRA at 10⁻⁷ or 10⁻⁹ M.

Conclusions: This data suggest that tamoxifen augments the inhibitory effect of retinoids on proliferation and $\alpha_2\beta_1$ integrin expression in MCF-7 cells.

Key words: retinol, carotenoids, retinoids, proliferation, $\alpha_2\beta_1$ integrins, MCF-7

INTRODUCTION

Most cells require adhesion to grow, differentiate and survive [1,2]. The major adhesion receptors in these processes are cell surface—integrins, that interact with ECM components. During tissue repair, immune response and malignant transformation, there are aberrations in the quality, quantity and function of integrins [2]. Up to now, there have been 18 α subunits and 8 β subunits [3] found, which are linked by noncovalent bonds to form over 24 $\alpha\beta$ heterodimers, mediating the adhesion between cells and between cells and the extracellular matrix [1,3,4]. The combination of α and β subunits also determines

ligand specificity. The integrins recognize several important proteins like fibronectin, laminin and collagen as their ligands [5,6], and the activation of integrin receptors triggers a cascade of signal transduction [1,7] via Ras, pp125^{FAK} and the protein kinase C (PKC) pathway [1,8]. Different cell types assemble and express different $\alpha\beta$ complexes. Normal human breast epithelial cells express β_1 , β_3 , β_4 , α_1 , α_2 , α_3 , α_6 , ν_8 integrins which dimerize to form $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_4$ and $\alpha_1\beta_3$ receptors [9-11]. Also, altered expression of the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ receptors is a common feature in breast tumors [9,12,13]. The expression of β_1 integrin in the adult breast is stable and its form of heterodimers with numerous α subunits suggests

that β_1 integrin is essential for the growth and differentiation of the mammary gland [13]. Also, expression of α_2 integrin is necessary for proper mammary gland development and is dependent on estrogen receptor expression [14]. The $\alpha_2\beta_1$ integrin was highly expressed on the epithelium of ducts and ductules of normal nonlactating breast tissue, and was expressed in benign lesions such as fibroadenoma or papilloma [15]. In contrast, a decrease or loss of the expression of the $\alpha_2\beta_1$ integrin was observed in many breast cancers [16,17].

It has been demonstrated that factors affecting the proliferation and differentiation of breast cancer cells can also influence integrin expression, e.g. estradiol and tamoxifen [18,19]. Some authors have also found that retinoids, which effectively inhibit the growth and differentiation of cancer cells (e.g. breast cancer cells), may also influence the migration and adhesion processes which have been observed in the culture of mesothelioma tumor cells [20] and in thymic stroma cells [21].

Retinoids constitute an important element of therapy in many branches of medicine like dermatology, hematology, rheumatology, chemotherapy and the chemoprevention of cancer. However, the application of retinoids in clinical practice requires careful dosage due to dose-related toxicity [22].

The purpose of the current study was to evaluate the effect of the vitamin A family on the growth, proliferation and expression of $\alpha_2\beta_1$ integrins in MCF-7 breast cancer cells as well as the possible role of estradiol and tamoxifen in these processes.

MATERIALS AND METHODS

Materials

Retinol (all-*trans* retinol), β -carotene, lycopene, 9-*cis* retinoic acid (9-*cis* Tretinoin), 13-*cis* retinoic acid (Isotretinoin), all-*trans* retinoic acid (Tretinoin), tamoxifen (Citrate Salt Tamoxifen), 17 β -estradiol (1,3,5 [10]-estratriene-3, 17 β -diol) were obtained from Sigma (St. Louis, MO, USA). The following antibodies: PCNA – Proliferating Cell Nuclear Antigen: monoclonal mouse antibody (clone PC 10), Ki 67: monoclonal mouse antibody (clone Ki 67) and integrin $\alpha_2\beta_1$: monoclonal mouse anti-human (clone P1E6) were obtained from Dako (Glostrup, Denmark).

Preparation of chemicals

Retinol, β -carotene and retinoids were diluted in ethyl alcohol and lycopene in tetrahydrofuran (THF) and then in the culture medium, to final concentrations of 10^{-9} - 10^{-5} M. Tamoxifen and 17 β -estradiol were added to the culture at a concentration of 10 μ M and 1 nM, respectively.

Culture of cell line MCF-7

The study was carried out on the hormone sensitive cell line (ER+) MCF-7 of human breast cancer (American Type Culture Collection, Rockville, MD) in DMEM medium

(Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Sigma, St. Louis, MO, USA), 50 μ g/ml streptomycin, and 100 U/ml penicillin in 75 cm² plastic flasks (Nunc, Roskilde, Denmark), at 37° C, in a humid incubator with 5% CO₂/95% air. The cell line was passaged once a week. The cells for the experiment were obtained from passages 3-7 and inoculated in 6-well plates (Nunc, Roskilde, Denmark) at 5×10^4 cells/well and grown to 85% confluence in Dulbecco's modified Eagle's medium (DME/F12, Sigma, St. Louis, MO, USA) supplemented as above. During the experiments, cells were detached with 0.05% trypsin/0.02% EDTA (Sigma, St. Louis, MO, USA).

Experiments were conducted in plates in DME/F12 Ham (Sigma, St. Louis, MO, USA), supplemented with a synthetic substitute of CPSR-1 serum (Sigma, St. Louis, MO, USA). Incubation of the MCF-7 cells with the examined substances was performed for 24 hours.

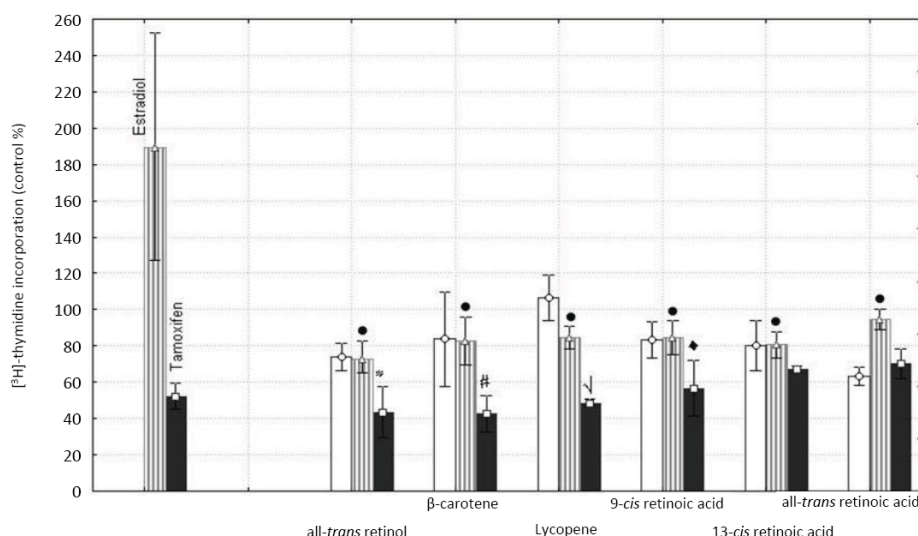
[³H]thymidine incorporation

Cell proliferation in the culture was assessed based on the incorporation of [³H]thymidine (Amersham, United Kingdom, specific activity 925 GBq/mmol), after incubation of the cell culture in the medium with or without the examined substances. Two hours prior to the termination of the experiment, [³H] thymidine was added to the culture at 18.8 KBq/well. After 2 - 3 washings of the culture with cold phosphate buffer, trypsinisation and precipitation (3 washings with 10% trichloroacetic acid), the precipitate was flooded with Instagel scintillation fluid (Packard, Groningen, The Netherlands). Radioactivity was expressed in dpm per well.

Immunocytochemical examinations

Immunocytochemical examinations were carried out in chambers for histochemical examinations (Lab-tek 4 well chamber slide, Nunc, Naperville, IL, USA). Cell material was fixed with cytofix (Cytofix, Merck, Darmstadt, Germany). A 2-step streptavidin-biotin LSAB kit + HRP kit (with horseradish peroxidase) was used for detection. Of primary antibodies: PCNA in dilution 1:100 and Ki 67 in dilution 1:25, incubation time 15 min. at room temperature and $\alpha_2\beta_1$ integrin antigen in dilution 1:50, incubation time 25 min. The incubation time with secondary antibodies was 15 min. at room temperature. The antigen-antibody reaction was visualized with the chromogen DAB (diaminobenzidine). The cells were counted with the use of Olympus MicroImage ImCD UDF morphometric program. The individual microscopic fields were photographed, and then the cells were counted on the monitor and the percentage of immunopositive cells in comparison to all cells were established. The intensity of reaction was expressed using the scale: low +, middle ++, high +++.

Figure 1. Influence of all-*trans* retinol, carotenoids and retinoids (concentrations 10^{-7} M) in combination with estradiol or with tamoxifen on [3 H]-thymidine incorporation (in %) into MCF-7 breast cancer cells. Exposure time 24 hrs. Data presented as mean values \pm SD (n = 4). Control = 100 %.



Symbols:

open bars: examined substances and no other additions

hatched bars: examined substances with estradiol (1 nM)

filled bars: examined substances with tamoxifen (10 μ M)

Compared:

- $p < 0.0001$ relative to the estradiol group
- * $p < 0.001$ relative to the retinol group
- # $p < 0.0001$ relative to the β -carotene group
- √ $p < 0.0001$ relative to the lycopene group
- ♦ $p < 0.01$ relative to the 9-*cis* retinoic acid group

Statistical analysis

In all the experiments, mean values \pm standard deviation (SD) for 4 measurements of each parameter were calculated. The U Mann-Whitney test was used to perform statistical analysis.

RESULTS

The effects of 24-hour exposure of breast cancer MCF-7 cells to retinol, carotenoids and retinoids on the incorporation of [3 H] thymidine.

Our previous results on the antiproliferative activity of retinol, carotenoids and retinoids in a 24-hour culture of cancer cells revealed that the retinol and β -carotene concentrations used in the experiment did not inhibit [3 H] thymidine incorporation at IC_{50} . The lowest values were obtained using these compounds at a concentration of 10^{-7} M, while retinoids at a concentration 10^{-5} M. We found that within the range of the concentrations used (10^{-9} - 10^{-5} M), lycopene exhibited a stimulatory effect, which was the lowest at 10^{-7} M. Therefore, these concentrations were used for further experiments [unpublished data].

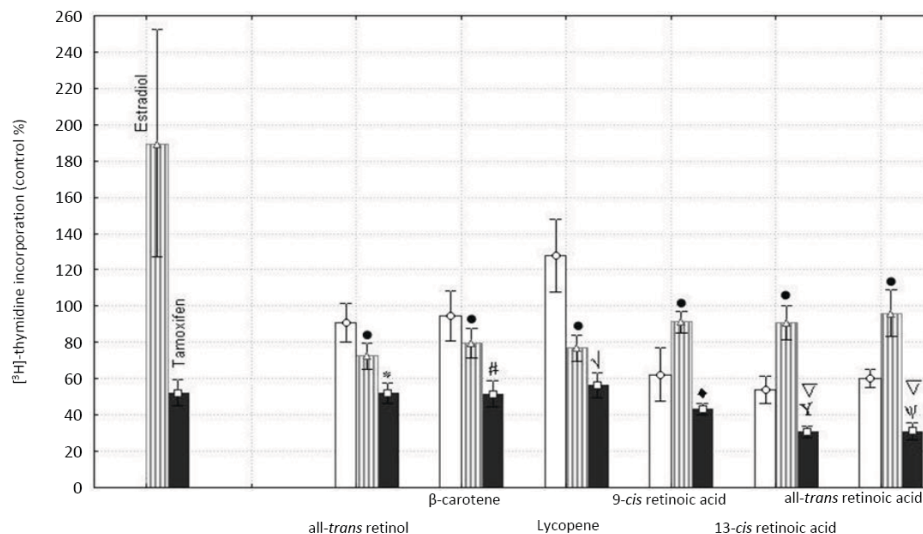
The effect of 24-hour exposure of breast cancer MCF-7 cells to retinol, carotenoids and retinoids combined with 17 β -estradiol or with tamoxifen on the incorporation of [3 H] thymidine.

A comparison of the antiproliferative activity of retinol, carotenoids and retinoids at the concentrations of 10^{-7} and 10^{-5} M has demonstrated that all these compounds when combined with estradiol reduce its stimulatory activity by approximately 50% irrespective of the concentration used (compared to the estradiol group, 189.25%, $p < 0.0001$) (Fig. 1,2). However, for combination with tamoxifen, statistically significant values were obtained using retinol, β -carotene, lycopene and 9-*cis* retinoic acid (9-*cis* RA) at a concentration of 10^{-7} M and all the substances examined at 10^{-5} M (compared to the examined substances alone groups). A statistically significant reduction in the percentage of the proliferating cells was also obtained for 13-*cis* RA and ATRA at 10^{-5} M (30.84% and 31.05%, $p < 0.01$, respectively), compared to the tamoxifen group (52.3%) (Fig. 1,2).

Evaluation of the immunocytochemical reaction of MCF-7 cells to PCNA and Ki 67 antigens

A 24-hour culture of cancer cells in the presence of retinol, β -carotene and retinoids alone and in combination with tamoxifen showed an evident and significant decrease in the

Figure 2. Influence of all-*trans* retinol, carotenoids and retinoids (concentrations 10^{-5} M) in combination with estradiol or with tamoxifen on [3 H]-thymidine incorporation (in %) into MCF-7 breast cancer cells. Exposure time 24 hrs. Data presented as mean values \pm SD (n = 4). Control = 100 %.



Symbols:

open bars: examined substances and no other additions
hatched bars: examined substances with estradiol (1 nM)
filled bars: examined substances with tamoxifen (10 μ M)

Compared:

- $p < 0.0001$ relative to the estradiol group
- * $p < 0.001$ relative to the retinol group
- # $p < 0.001$ relative to the β -carotene group
- √ $p < 0.0001$ relative to the lycopene group
- ♦ $p < 0.01$ relative to the 9-*cis* retinoic acid group
- Y $p < 0.01$ relative to the 13-*cis* retinoic acid group
- ψ $p < 0.01$ relative to the all-*trans* retinoic acid group
- ∇ $p < 0.01$ relative to the tamoxifen group

percentage of PCNA and Ki 67 positive cells compared to the control.

In comparison to the tamoxifen group, only the culture of cancer cells after a simultaneous supplementation with ATRA (10^{-5} M) and tamoxifen (10 μ M) demonstrated a statistically significant drop in the percentage of PCNA-positive cells (Tab. 1).

Expression of $\alpha_2\beta_1$ integrin on MCF-7 breast carcinoma cells

We found no statistically significant differences in the expression of $\alpha_2\beta_1$ integrins using retinol, carotenoids and retinoids at concentrations of 10^{-7} and 10^{-5} M. Thus, the concentrations of 10^{-7} M for retinol, β -carotene and lycopene, and 10^{-5} M for retinoids were chosen for further experiments.

Retinol, β -carotene and lycopene did not statistically significantly alter the expression of $\alpha_2\beta_1$ integrins in the culture, compared to the values obtained in the control (73.3%) and estradiol (92.5%) groups. Only lycopene in combination with tamoxifen caused a statistically significant reduction in the expression of $\alpha_2\beta_1$ integrins (43.5% as compared to the control, tamoxifen and lycopene group; 73.3%, $p < 0.01$, 65.72%, $p < 0.01$ and 75.5%, $p < 0.001$, respectively) (Fig. 3).

Of the retinoids examined, only 13-*cis* RA statistically significantly altered the expression of $\alpha_2\beta_1$ integrins in the MCF-7 cell line culture as compared to the control (49.6% vs. control 73.3%, $p < 0.01$). As there was no effect of ATRA at a concentration of 10^{-5} M on the level of the integrins, we used its lower concentrations; but even at 10^{-7} M, ATRA had no effect. However, when used in combination with estradiol or tamoxifen, the acid was found to statistically significantly reduce the integrin expression. Moreover, even the lowest concentration of retinoid used (10^{-9} M) caused a statistically significant drop in the integrin expression to the approximate level of 15%, with the expression after retinoid application with estradiol and tamoxifen similar to that observed at 10^{-7} M (Fig. 4).

DISCUSSION

The present study demonstrates that vitamin A and its metabolites exhibit antiproliferative activity in MCF-7 breast cancer cells, and some of these compounds affect $\alpha_2\beta_1$ integrin expression.

The retinoids in our experiment were more potent inhibitors than carotenoids. The most active were 13-*cis* RA and ATRA.

Table 1. Percentage of PCNA and Ki67 positive MCF-7 breast carcinoma cells.

	PCNA		Ki67	
Control	85.75 ± 8.09 ++/+++		96.25 ± 4.78 +++	
Tamoxifen	52.5 ± 1.7 +++		53.0 ± 2.21 +++	
all-trans retinol	54.5 ± 4.20 ++/+++	$p < 0.004$	55.5 ± 4.2 +++	$p < 0.0001$
all-trans retinol + Tamoxifen	59.75 ± 1.70 +++	$p < 0.004$	51.25 ± 2.98 +++	$p < 0.0001$
β- carotene	44.25 ± 3.77 ++	$p < 0.0001$	65.75 ± 2.21 +++	$p < 0.0003$
β- carotene + Tamoxifen	58.5 ± 2.64 ++	$p < 0.004$	61.0 ± 7.07 +++	$p < 0.0001$
Lycopene	87.75 ± 2.21 ++		95.75 ± 4.34 ++/+++	
Lycopene + Tamoxifen	85.75 ± 4.34		91.25 ± 1.70 +/++	
9-cis retinoic acid	65.0 ± 4.08 +++	$p < 0.01$	47.5 ± 5.06 ++	$p < 0.0001$
9-cis retinoic acid + Tamoxifen	54.25 ± 4.34 +++	$p < 0.004$	55.25 ± 5.19 ++/+++	$p < 0.0002$
13-cis retinoic acid	56.25 ± 4.78 +++	$p < 0.0004$	55.75 ± 4.42 +++	$p < 0.0002$
13-cis retinoic acid + Tamoxifen	44.75 ± 4.57 +++	$p < 0.0001$	59.75 ± 1.70 +++	$p < 0.0001$
all-trans retinoic acid	45.0 ± 4.08 +++	$p < 0.0001$	52.0 ± 0.82 +++	$p < 0.0001$
all-trans retinoic acid + Tamoxifen	30.75 ± 0.95 ++/+++	$p < 0.0001$ *	64.75 ± 5.37 +++	$p < 0.0003$

Exposure time 24 hrs. Data presented as mean values ± SD (n = 4).

Control = percentage of PCNA and Ki67 positive MCF-7 cells.

Concentrations: tamoxifen – 10 μM, retinol, β-carotene and lycopene – 10⁻⁷ M, 9-cis retinoic acid, 13-cis retinoic acid, all-trans retinoic acid – 10⁻⁵ M

Statistically significant differences relative to the control group.

* p < 0.01 relative to the tamoxifen group.

Intensity of the reaction: low +, middle ++, high +++.

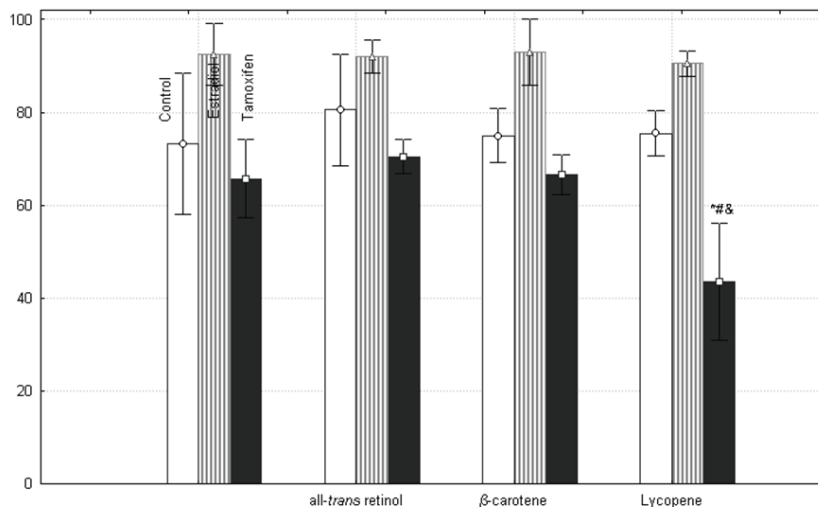
In the case of ATRA, the differences were not statistically significantly concentration-dependent.

In our study, the compounds examined can decrease the stimulatory activity of estrogens, the more so as the normal retinoid signaling pathway has been found to be altered in breast carcinoma cells [23]. Leede et al. demonstrated that the addition of 10⁻⁹ M estradiol to the culture of MCF-7 line resulted in a significant increase in the level of RARα protein, and in these conditions the growth of cancer cells was evidently inhibited by retinoic acid [24]. On the other hand, Muller et al. revealed that the transcriptional factor (Hairy and Enhancer of Split homologue-1) is a mediator of the antiproliferative effect of ATRA in ERα breast cancer cell line (MCF-7) [25].

In the present study, the use of the most common antiestrogen of the SERM group – tamoxifen caused a reduction in the percentage of the proliferating cells; however, it was dependent on the concentrations of retinol, carotenoid pigments and retinoids used for the experiment.

Estrogen-induced proliferation in the normal mammary gland requires undefined stromal signaling, but these signals are greatly altered during tumorigenesis [26,27]. Antiestrogens, like estrogens, can control the adhesive behavior of breast tumor cells as well. Estradiol enhanced and tamoxifen induced a reduction in the adhesion of MCF-7 cells [18,19]. De Pasquale suggests that estradiol may promote breast cancer cell migratory activity by estrogen receptor-dependent and –independent pathways [28]. Our results seem to indicate that retinol and carotenoids do not alter the expression of α₂β₁ integrins, when added to the culture simultaneously with estradiol. Of the compounds used in the current study, only 13-cis RA and ATRA used alone at a very low concentration (10⁻⁹ M) and in combination with estradiol and tamoxifen (ATRA 10⁻⁹ and 10⁻⁷ M), as well as lycopene in combination with tamoxifen reduced α₂β₁ integrin expression. Therefore, the all-trans retinol and 9-cis RA as well as β-carotene have no direct influence on integrin receptor α₂β₁. According to our results, such activity is observed in the case of β-carotene

Figure 3. Expression of $\alpha_2\beta_1$ integrin on MCF-7 breast cancer cells. Exposure time 24 hrs. Data presented as mean values \pm SD (n = 4). Control = percentage of positive $\alpha_2\beta_1$ MCF-7 cells.



Symbols:

open bars: examined substances and no other additions (concentrations of all-*trans* retinol, b-carotene and lycopene 10^{-7} M)

hatched bars: examined substances with estradiol (1 nM)

filled bars: examined substances with tamoxifen (10 μ M)

Compared:

* $p < 0.01$ relative to the control group

& $p < 0.001$ relative to the lycopene group

$p < 0.01$ relative to the tamoxifen group

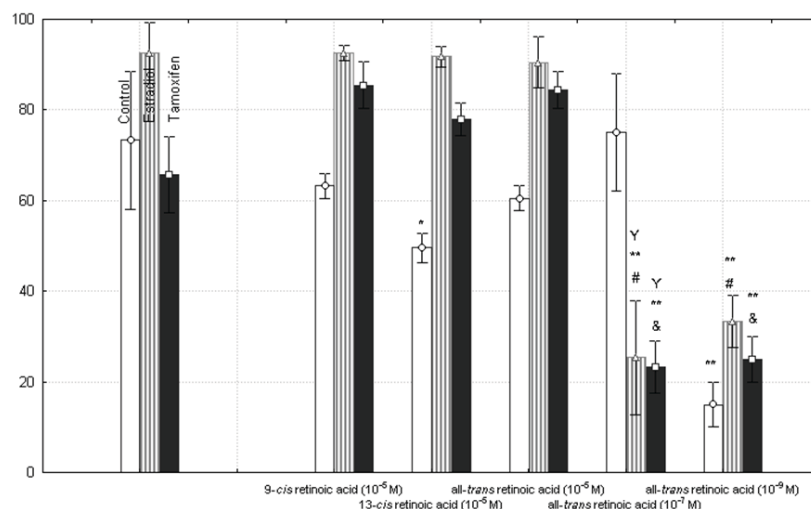
metabolites (ATRA, 13-*cis* RA). The mechanism of lycopene effect on cancer cells still remains an open question. Probably, it depends on the concentration and time of exposure. Our results showed that this carotenoid needs some interaction with antiestrogens. The molecular functions and actions of carotenoids are also determined by the physical and chemical properties of the molecules [29]. Maybe some lycopene derivatives (like in the case of β -carotene and retinoic acid) may interact with nuclear receptors [30]. It is also difficult to explain the effect of various concentrations of ATRA used alone (10^{-9} , 10^{-7} and 10^{-5} M) and in combination with estradiol and tamoxifen on the expression of $\alpha_2\beta_1$ integrins in our experiment.

Currently, it is difficult to indicate a direct relationship between the action of carotenoids and retinoids on the expression of integrins and the resulting implications for breast cancer cells. Our results demonstrate that among vitamin A metabolites, ATRA is the most active compound, and in low concentrations has the strongest action on $\alpha_2\beta_1$ integrin expression. The most stable system is the one observed in benign mammary epithelium where integrins are expressed at very high levels and no invasion occurs [31]. The increase in $\alpha_2\beta_1$ integrin expression has been described in most malignant tumors [32-34]. The altered expression of the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ receptors is a common occurrence in breast tumors [5], but Alford et al. described a decrease in the levels of expression of the $\alpha_2\beta_1$ integrin in

many breast cancer cells [16]. Maemura et al. revealed that the in vitro tested breast carcinoma cells (ER+ and ER-) and human mammary epithelial cells (HMEC) expressed α_2 and β_1 subunits on their surface. The expression levels on HMEC and ER(+) MCF-7 cell line were comparable [6]. The integrins control mammary gland development through co-operation with growth factors and hormone receptors, e.g. estrogen receptor expression [17,35]. Although the cross-talk between hormone receptors and integrins has been less investigated, several studies suggest that cell-ECM interactions have a significant impact on the hormonal control of this process [36]. Lanzafame et al. suggests that changes in $\alpha_2\beta_1$ expression correlate with the histological type and hormonal receptor status in breast carcinomas. A decrease of $\alpha_2\beta_1$ expression together with a loss of ER expression were found on poorly differentiated breast adenocarcinoma cells [17]. A reduction in breast cancer cell contacts is also crucial for the initiation of metastatic invasion. The $\alpha_2\beta_1$ integrins are also known to be involved in angiogenesis – a process that also refers to proliferation and invasion of cancer cells. Retinoids can also affect the angiogenic process in humans [37] through an effect on growth factors. Since integrins can affect or in turn be influenced by other factors e.g. cytokines, growth factors, this may be the common mechanism of the action of carotenoids, retinoids and integrins.

Retinoids have been described to modulate extracellular matrix synthesis as well as cell adhesion and migration [21,38].

Figure 4. Expression of $\alpha_2\beta_1$ integrin on MCF-7 breast cancer cells. Exposure time 24 hrs. Data presented as mean values \pm SD (n = 4). Control = percentage of positive $\alpha_2\beta_1$ MCF-7 cells.



Symbols:

open bars: examined substances and no other additions (concentrations of 9-*cis* retinoic acid, 13-*cis* retinoic acid 10^{-5} M and all-*trans* retinoic acid 10^{-5} , 10^{-7} , 10^{-9} M)

hatched bars: examined substances with estradiol (1 nM)

filled bars: examined substances with tamoxifen (10 μ M)

Compared:

* $p < 0.01$ relative to the control group

** $p < 0.0001$ relative to the control group

$p < 0.0001$ relative to the estradiol group

& $p < 0.0001$ relative to the tamoxifen group

Y $p < 0.001$ relative to the all-*trans* retinoic acid group

Retinoic acid could favorably modulate the diffusive and invasive behavior of the mesothelioma tumor, thus affecting migration [20] through the reduced synthesis of fibronectin and laminin. The latter ligand is also characteristic for $\alpha_2\beta_1$ integrin in MCF-7 cell line [6,39].

In physiological conditions, integrins take part in the control of growth and function of the epithelial breast cells. The expression of integrins becomes affected in carcinogenesis. In view at the presented studies it seems that retinol, carotenoids or retinoids may play an important role.

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