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Implication of $\alpha_5\beta_1$ Integrin in Invasion of Drug-Resistant MCF-7/ADR Breast Carcinoma Cells: a Role for MMP-2 Collagenase

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Abstract—Expression of $\alpha_5\beta_1$ integrin in the drug-resistant MCF-7/ADR breast carcinoma cells was inhibited by treatment of these cells with α_5-specific siRNA. The decrease of $\alpha_5\beta_1$ expression resulted in a sharp decrease of expression of MMP-2 collagenase and inhibition of invasion activity of these cells *in vitro*. Similar decrease of invasion was also observed during inhibition of MMP-2 expression by treatment of these cells with MMP-2-specific siRNA. Inhibition of $\alpha_5\beta_1$ expression was also accompanied by significant decrease in cell content of active (phosphorylated) forms of signal protein kinases Akt and Erk1/2. Inhibition of activity of these kinases by treatment of cells with PI-3K/Akt-specific inhibitor LY294002 or Erk-specific inhibitor PD98059 resulted in inhibition of MMP-2 expression and the decrease of invasion *in vitro*. These data suggest that $\alpha_5\beta_1$ controls invasion ability of these cells by regulating expression of MMP-2, which involves PI-3K and Erk1/2 protein kinase signaling.

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Key words: integrins, signal protein kinases, matrix proteases, metastasis, invasion

Appearance of invasion ability and destruction of surrounding tissues are manifestations of malignant progression of tumor cells and the matrix-specific receptors of cell surface, integrins, play a key role in these processes [1, 2]. Integrins are the main messengers involved in cell interaction with extracellular matrix and transduction of the matrix-generated signals controlling such basic cell reactions as proliferation, motility, apoptotic death, etc. [3-5]. Malignant growth is accompanied by changes in each of these reactions.

Most integrins exhibit multiple ligand specificity; this results in ability of various receptors to interact with the same matrix protein and produce the same signals [6, 7]. This complicates analysis of properties of individual integrins. The only receptor exhibiting unique specificity is the fibronectin binding integrin $\alpha_5\beta_1$. Involvement of this receptor in malignant transformation and progression of tumor cells has been investigated in numerous studies, but the results are contradictory. There was both positive and negative correlation between $\alpha_5\beta_1$ expression and oncogenic properties and metastatic activity of various tumor cells [8-12]. The key mechanism by which integrins influence tumor progression consists of transduction of signals inducing expression of genes encoding matrix-specific metalloproteinases (MMP) [13, 14]. Their role in invasion and metastasis has been well documented in numerous studies [13, 15]. However, some elements of these signaling pathways, as well as identification of particular receptors controlling MMP, still require detailed investigation.

In this study, we have demonstrated for the first time that $\alpha_5\beta_1$ integrin controls invasion of breast carcinoma cells by regulating expression of MMP-2 collagenase, which employs PI-3K and Erk1/2 protein kinase signaling pathways.

MATERIALS AND METHODS

Cell lines and reagents. The MCF-7 cell line of human breast adenocarcinoma was purchased from

Abbreviations: MMP) matrix-specific metalloproteinases.

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ATCC (American Type Culture Collection, USA). A variant of this line, MCF-7/ADR obtained during selection of cells surviving in the presence of doxorubicin, was kindly supplied by T. N. Ignatova (University of Illinois, Chicago, USA). Cells were cultivated in DMEM medium containing 10% embryonic calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. MCF-7/ADR cells were cultivated in the presence of 1.7 μM doxorubicin. In all experiments, cells at the logarithmic phase of growth were used. Except specially stated cases, all the reagents were obtained from Sigma (USA). Polyclonal antibodies to α_5-subunit were purchased from Chemicon (USA). Short interfering RNA (siRNA) to α_5 integrin subunit (catalog No. sc-29372) and to MMP-2 collagenase (catalog No. sc-29398) and also reagents for transfection and antibody to β-actin were purchased from Santa Cruz Biotech (USA). Antibodies to Akt and Erk kinases were purchased from Cell Signaling Technology (USA). Reagents for reverse transcriptase polymerase chain reaction (RT-PCR) were purchased from Invitrogen (USA). The compounds LY294002 (inhibitor of protein kinase PI-3K) and PD98059 (inhibitor of Erk protein kinase) were purchased from Sigma and Calbiochem (USA).

siRNA transfection was carried out following the supplier's protocol (Santa Cruz Biotech). Briefly, $(1\text{-}2)\cdot10^5$ cells in 1 ml of DMEM medium lacking antibiotics but containing 10% embryonic serum were passed in 12-well plates up to 50% confluence. Cells were transfected with 50 nM (final concentration) of α_5-specific siRNA or control siRNA during 70 h using the reagent for transfection (Santa Cruz), and then cells were harvested by treatment with trypsin/EDTA and used for subsequent studies.

Invasion *in vitro* and MMP secretion were determined as described earlier [16] using Transwell clusters (12 μm pore membrane; Corning, The Netherlands). Secretion was evaluated by means of zymographic analysis of MMP activity in conditioned cell medium [16]. Medium (5-15 μl) was resolved in 7.5% SDS-PAGE with 1.0 mg/ml gelatin. The gel was washed three times with Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, 2.5% Triton X-100 and incubated in the same buffer containing 1% Triton X-100 for 5 h at 37°C. Gels were fixed, stained with Coomassie R-250, washed, and scanned.

RT-PCR. Isolation of total RNA and RT-PCR were carried out as described in [17]. The following primers were used in PCR: for α_5-integrin cDNA, 5′-catttccgagtctgggccaa-3′ (forward), 5′-tggaggcttgagctgagctt-3′ (reverse); for β-actin cDNA (internal standard), 5′-gtggggcgccccaggcacca-3′ (forward), 5′-ctccttaatgtcacgcacgatttc-3′ (reverse); for MMP-2 cDNA, 5′-tggcagtgcaatacctgaac-3′ (forward), 5′-caaggtccatagctcatcgtc-3′ (reverse). Amplification conditions for α_5 and MMP-2 were the following: 30 cycles, denaturation – 94°C for 60 sec, annealing – 58°C for 60 sec, elongation – 72°C for 60 sec; for β-actin: 30 cycles, denaturation – 94°C for 30 sec, annealing – 60°C for 30 sec, elongation – 72°C for 30 sec. Amplification products were separated by electrophoresis in 1.5% agarose gel, scanned, and analyzed using ImageJ NIH (USA) software.

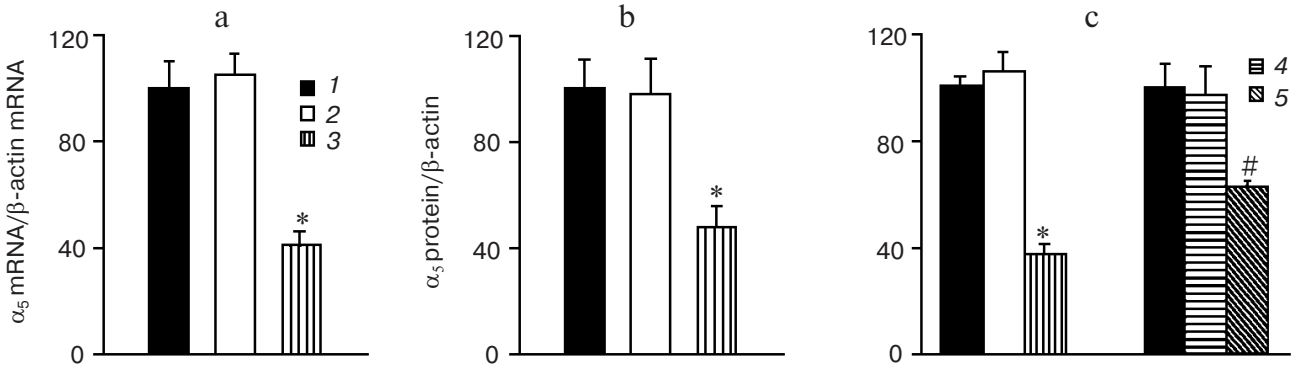

Fig. 1. Effect of α_5 integrin siRNA on expression of $\alpha_5\beta_1$ integrin in MCF-7/ADR cells and their invasive activity *in vitro*. a) RT-PCR analysis. Total RNA was isolated from cells not treated with siRNA (*1*) and cells treated with control siRNA (*2*) or α_5-siRNA (*3*) (the same designations are used in panels (b) and (c)), and mRNA content was determined by RT-PCR as described in "Materials and Methods". The ratio of α_5 mRNA to β-actin mRNA in untreated cells was defined as 100%. b) Immunoblotting of proteins: 30-40 μg of cell lysate proteins prepared from cells treated or not treated with siRNA (as indicated in (a)) were fractionated by PAGE, transferred onto a nitrocellulose membrane, and identified using specific antibodies. Relative content of α_5-subunit and β-actin was determined by processing of scanning data using the program ImageJ NIH. The ratio α_5/β-actin in untreated cells was defined as 100%. c) Analysis of invasive activity *in vitro*: $2.5\cdot10^5$ cells not treated (*1*) or treated with siRNA (*2*, *3*) or peptides RGES (*4*) or GRGDS (*5*), each in 300 μl of DMEM medium containing 0.5% embryonic serum, were placed onto 300 μg of matrigel formed in the upper chamber of a two-chamber cell. Peptides (500 μg/ml) were added to a cell suspension before application to matrigel. Cells were incubated at 37°C for 24 h, and then cells that migrated to the lower chamber were counted. The ordinate shows the ratio of number of migrated siRNA-treated cells to the number of migrated untreated cells (as percent). In all series data represent mean ± SEM for three experiments; * $p < 0.01$ (versus untreated cells), # $p < 0.05$ (versus untreated cells).

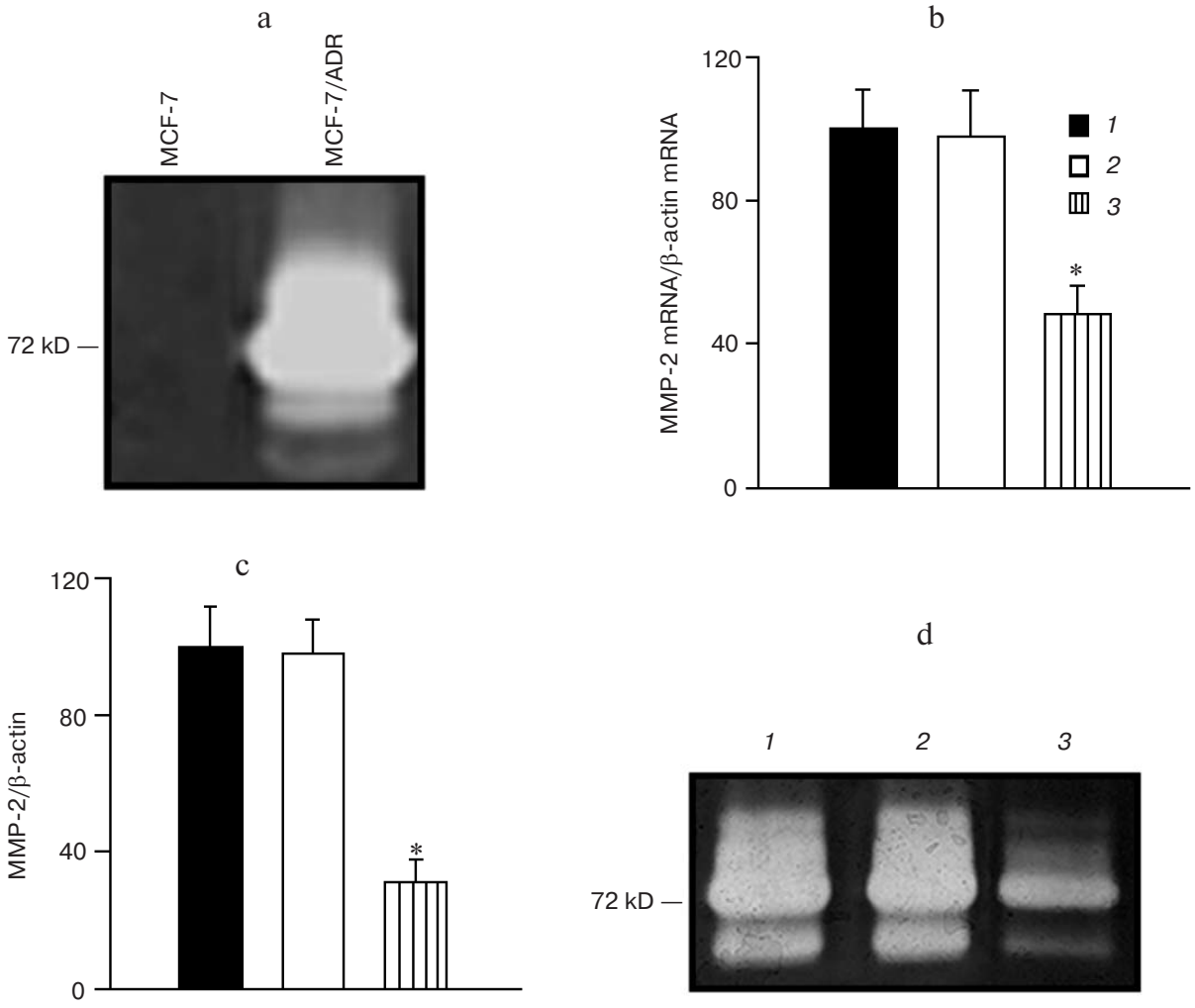

Fig. 2. Effect of α_5-integrin siRNA on expression and activity of MMP-2 collagenase in MCF-7/ADR cells. a) Zymographic analysis of collagenase activity in the conditioned medium of MCF-7 and MCF-7/ADR. The medium was obtained after cell growth for 24 h. Numbers on the left indicate molecular mass of inactive form of MMP-2. b) RT-PCR. The content of MMP-2 and β-actin mRNA was determined in MCF-7/ADR cells not treated with siRNA (*1*) and cells treated with control siRNA (*2*) or α_5-siRNA (*3*) (the same designations are used in panels (c) and (d)) as indicated in "Materials and Methods". The ratio of MMP-2 mRNA to β-actin mRNA in untreated cells was defined as 100%. c) Immunoblotting of proteins: 30-40 μg of cell lysate protein was fractionated by PAGE, followed by subsequent electrotransfer onto a nitrocellulose membrane and identification by specific antibodies. Relative content of MMP-2 and β-actin was determined as indicated in the legend to Fig. 1b. The ratio MMP-2/β-actin in untreated cells was defined as 100%. d) Zymographic analysis of MMP-2 activity in the conditioned medium of MCF-7/ADR treated or not treated with siRNA. The medium was obtained after cell growth for 24 h. Panels (a) and (d) show results of a typical experiment. Data of panels (b) and (c) represent mean ± SEM of three experiments; * $p < 0.01$ (versus untreated).

Immunoblotting. Cell lysate proteins (15-30 μg) separated in 7.5% SDS-PAGE were electroblotted onto a nitrocellulose membrane. After incubation with primary antibodies for 1 h at room temperature, the membrane was incubated with HRP-conjugated secondary antibodies (Amersham, Great Britain) (dilution 1 : 5000), and developed using an Enhanced Chemiluminescence (ECL) detection system (Amersham) followed by scanning and quantifying with ImageJ NIH software.

Statistical treatment. Differences between groups were evaluated by means of Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Inhibition of $\alpha_5\beta_1$ integrin expression suppresses invasive activity of MCF-7/ADR cells *in vitro*. We earlier demonstrated that the MCF-7/ADR cell line differs from the parent MCF-7 by much higher invasion activity *in vitro* [18]. We have revealed significant differences between these strains in the expression of some integrins at both gene transcription and protein biosynthesis levels. MCF-7 cells are highly active in expression of $\alpha_2\beta_1$ integrin, whereas MCF-7/ADR cells do not express this receptor. On the contrary, MCF-7/ADR cells are highly

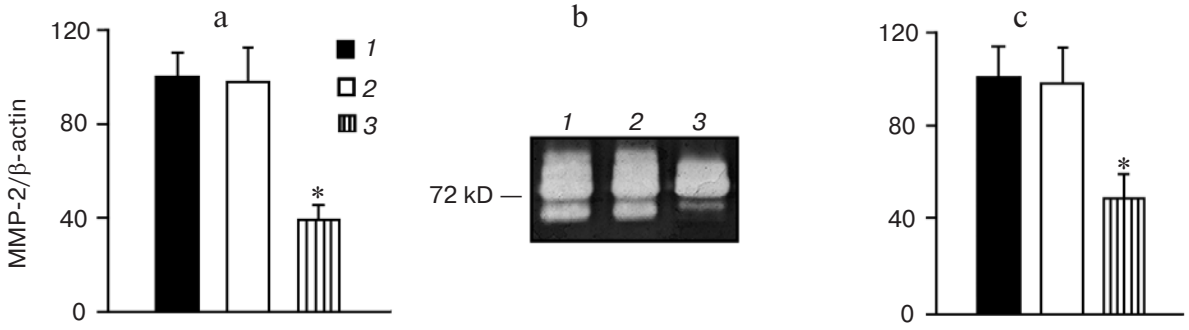

Fig. 3. Effect of MMP-2 specific siRNA on expression and secretion of MMP-2 and invasiveness of MCF-7/ADR cells *in vitro*. a) Immunoblotting of proteins: 30-40 μg of cell lysate protein obtained from untreated cells (*1*) or cells treated with control siRNA (*2*) or MMP-2-specific siRNA (*3*) (the same designations are used in panels (b) and (c)) was fractionated and identified as indicated in the legend to Fig. 1b. The ratio of MMP-2 to β-actin in untreated cells was defined as 100%. b) Zymographic analysis of MMP-2 activity in the conditioned medium. The medium was obtained after cell growth for 24 h. c) Analysis of invasiveness *in vitro*. Conditions are described in the legend to Fig. 1c. The ordinate shows number of migrated cells treated with siRNA as % of migrated untreated cells. Data of panels (a) and (c) represent mean ± SEM of three experiments; * $p < 0.01$ (versus untreated cells).

active in expression of $\alpha_5\beta_1$, whereas the parent cells do not express it.

Cytofluorimetric analysis showed that $\alpha_2\beta_1$ is the most abundant receptor in MCF-7, whereas $\alpha_5\beta_1$ is the most abundant receptor in MCF-7/ADR cells. It has also been shown that MCF-7/ADR cells do not express integrins containing α_v subunit (data not shown).

For elucidation whether high invasive activity of MCF-7/ADR is associated with increased expression of $\alpha_5\beta_1$ integrin, we have investigated the effect of down-regulation of this receptor, which was accomplished by cell transfection with the α_5-specific siRNA, on the invasive activity. Figure 1 (a and b) shows that transfection with α_5-specific siRNA causes significant reduction of corresponding mRNA and protein. Blockade of expression of this integrin was accompanied by a potent decrease in invasion (60%) of MCF-7/ADR cells in matrigel (Fig. 1c). The RGD-containing antagonist of $\alpha_5\beta_1$ receptor and of α_v-containing integrins [7] exhibited a similar effect on invasive activity. Since MCF-7/ADR cells do not express α_v-integrins, the effect of RGD-peptide can be attributed to its effect only on $\alpha_5\beta_1$ receptor.

Effect of $\alpha_5\beta_1$ integrin on invasive activity is mediated by MMP-2. One of the key mechanisms of tumor invasion involves increased production of MMP [13, 15].

Figure 2a shows that the MCF-7/ADR cell line with high invasive potential is characterized by high level of gelatinase activity of MMP-2 in the conditioned medium, whereas parent MCF-7 cells exhibiting low invasiveness do not secrete this enzyme into conditioned medium. Neither cell line expressed MMP-9 collagenase (data not shown). It was reasonable to suggest that inhibition of invasive activity of MCF-7/ADR cells by α_5-specific siRNA would be attributed to inhibition of expression and/or activity of MMP-2. Indeed, data of Fig. 2 (b-d) show that treatment of MCF-7/ADR cells with α_5-specific siRNA caused significant inhibition of MMP-2 expression at the stages of gene transcription, protein synthesis, and secretion of gelatinase activity.

For direct validation of our suggestion, we investigated the effect of transfection of MMP-2 specific siRNA on invasiveness of MCF-7/ADR cells. Figure 3 shows that the transfection resulted in significant inhibition of MMP-2 expression and decrease of its gelatinase activity in the conditioned medium as well as a sharp decrease of the invasiveness of the transfected cells.

Blocking effect of α_5-siRNA on expression of MMP-2 and invasive activity *in vitro* is mediated by PI-3K/Akt and Erk1/2 signaling pathways. Several studies have shown that integrin effect on cell behavior is mainly mediated by two protein kinase signaling pathways: PI-3K/Akt-dependent and Erk-dependent ones [2, 19, 20]. In this study we investigated: i) whether blockade of $\alpha_5\beta_1$-generated signals influences Akt and Erk protein kinases; ii) whether decrease of MMP-2 and invasiveness of MCF-7/ADR cells treated with α_5-siRNA are related to changes in expression and/or activity of these enzymes.

Figure 4 (a and b) shows that the decrease of expression of the $\alpha_5\beta_1$ receptor in these cells did not influence total protein content of Akt and Erk1/2 assayed in cell lysate, but caused significant decrease in phosphorylated (active) forms of these protein kinases.

The role of signal kinases in inhibition of invasion activity caused by blockade of $\alpha_5\beta_1$ expression was then confirmed in subsequent study of the effects of inhibitors of these enzymes on MCF-7/ADR cells. Treatment of these cells with LY294002, blocking PI-3K/Akt-mediated signaling, or PD98059, the inhibitor of Erk-mediated signaling, resulted in both significant decrease of MMP-2 expression (Fig. 4c) and significant inhibition of invasiveness of cells (Fig. 4d).

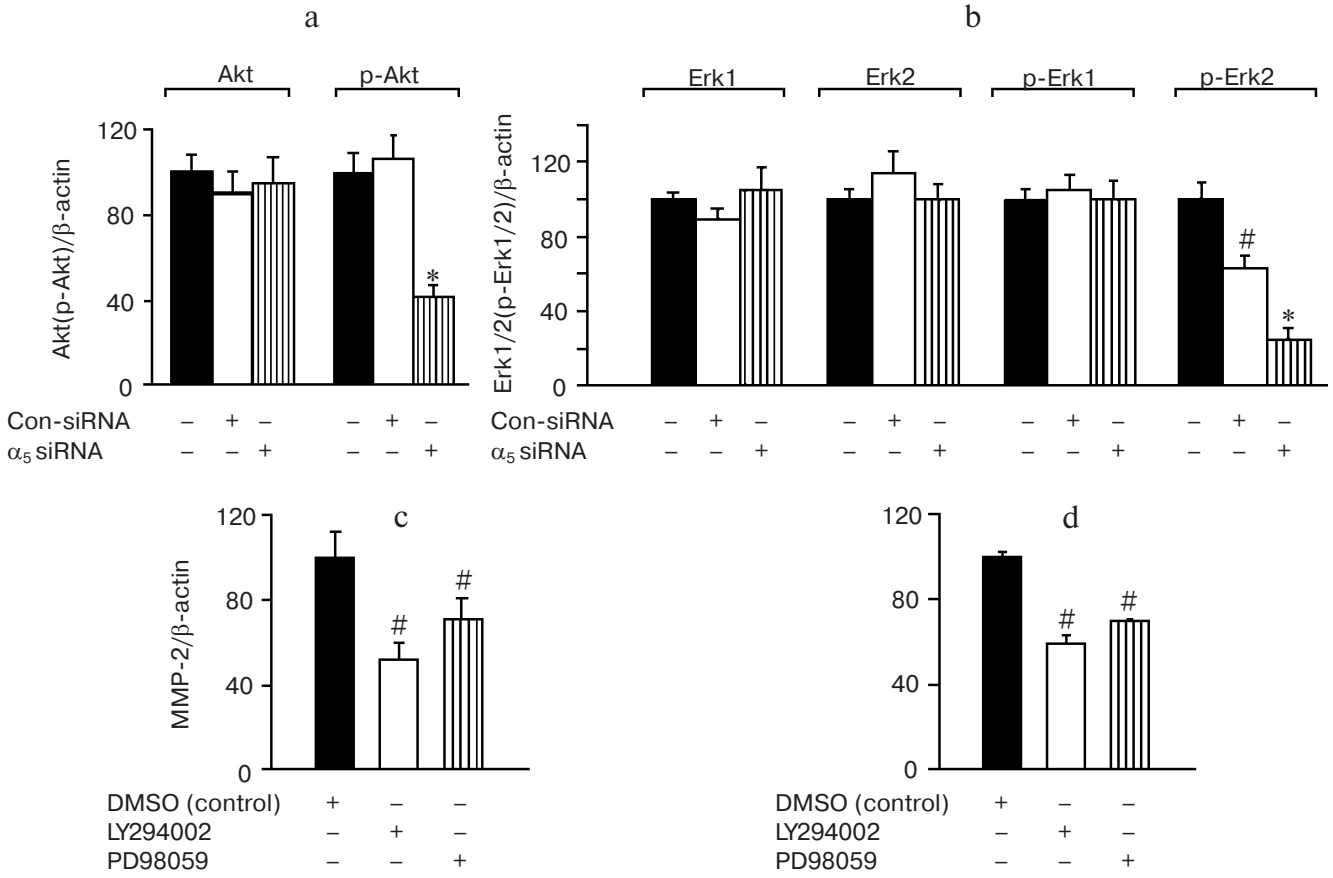

Fig. 4. a) Effect of α_5-integrin siRNA on expression and activity of Akt protein kinase of MCF-7/ADR cells (immunoblotting of proteins). Fractionation of cell lysate (40-50 μg protein) obtained from untreated cells or cells treated with control (Con-siRNA) or α_5-specific siRNA and analysis of content of Akt (total protein), pAkt (phosphorylated Akt), and β-actin were carried out as indicated in the legend to Fig. 1b. The ratio of Akt/β-actin or pAkt/β-actin for untreated cells was defined as 100%. b) Effect of α_5-integrin siRNA on expression and activity of protein kinase Erk1/2 of MCF-7/ADR cells (immunoblotting of proteins). The content of Erk1 and Erk2 total protein, phosphorylated forms (pErk1 and pErk2), and β-actin was analyzed as in the case of Akt. The ratio of Erk1/2 or pErk1/2 to β-actin for untreated cells was defined as 100%. c) Effect of kinase inhibitors on MMP-2 expression in MCF-7/ADR cells. Cells ($5 \cdot 10^5$) in DMEM medium containing 10% embryonic serum were treated with 50 μM LY294002 or PD98059 (final concentration) dissolved in DMSO (final DMSO concentration was 0.5%) for 24 h at 37°C. Cells were then washed three times and lysed. Immunoblotting of cell lysate proteins, identification, and expression of MMP-2 were carried out as indicated in the legend to Fig. 1b. The ratio MMP-2/β-actin in cells incubated only in the presence of 0.5% DMSO was defined as 100%. d) Effect of kinase inhibitors on invasive activity of MCF-7/ADR cells *in vitro*. Cells ($2.5 \cdot 10^5$) in DMEM medium containing 0.5% embryonic serum were treated with LY294002 or PD98059 as described above and applied to the upper chamber onto matrigel, and then invasiveness was detected as described in the legend to Fig. 1c. The ordinate shows number of treated and migrated cells as percent to migrated cells pretreated with 0.5% DMSO only. In all series data represent mean ± SEM for three experiments; * $p < 0.01$ (versus untreated cells), # $p < 0.05$ (versus untreated cells).

DISCUSSION

We have already indicated that data on the role of $\alpha_5\beta_1$ integrin in growth and malignant progression of tumor cells are contradictory. For example, *de novo* expression of $\alpha_5\beta_1$ decreased oncogenic potential and inhibited proliferation of colon carcinoma cells [8]. Histochemical analysis of human carcinomas growing in various tissues (stomach, colon, breast, liver) showed that the level of $\alpha_5\beta_1$ was lower in tumors than in non-tumor sites [9, 10]. This result is inconsistent with observations that $\alpha_5\beta_1$ prevents apoptotic death of Chinese hamster cells [11] and that this receptor is required for metastasis of mouse melanoma cells [12].

Mechanisms responsible for "bi-directed" functions of integrins (including $\alpha_5\beta_1$) obviously depend on activity of numerous signaling pathways stimulating or blocking individual "behavioral" reactions of cells. The pattern of these pathways can be specific for certain cell type or cell line. Recent studies have shown that complexing of $\alpha_6\beta_4$ integrin and epidermal growth factor receptor erbB2 is essential for proliferation and invasion (invasive growth) of breast carcinoma cells. The signaling cascade initiated by this complex includes oncoprotein RAS and transcription factors STAT3 and c-Jun [21]. However, in colon carcinoma cells, $\alpha_6\beta_4$ activates proapoptotic protein p53 and induces cell cycle arrest followed by subsequent apoptosis. This effect is not observed in cells with inactivated p53 [22].

These differences in the effect of $\alpha_5\beta_1$ integrin are also determined by particular signaling pathways in certain tumor cell lines. In hepatoma cells, this receptor forms a complex with urokinase receptor uPAR, FAK kinase, and receptor of epidermal growth factor (EGFR) and initiates potent Ras-Erk mediated mitogenic effect [23]. However, in colon cancer cells with initially low level of $\alpha_5\beta_1$ expression, re-expression of this receptor inhibited mitogenesis [24]. It has been found that the receptor of human epidermal growth factor HER-2 expressed by these cells and playing a key role in their proliferative and tumorigenic activity forms a complex with $\alpha_5\beta_1$ and becomes a target for lysosomal cleavage. This results in sharp decrease in its expression and inhibition of cell growth.

In this study, we have demonstrated for the first time that inhibition of tumor cell invasion induced by blockade of $\alpha_5\beta_1$ receptor expression occurs via inhibition of expression and secretion of MMP-2 collagenase. This also involves signaling phosphokinases PI-3K/Akt and Erk as intermediates of the regulatory pathways. Involvement of PI-3K/Akt and Erk in $\alpha_5\beta_1$-mediated signaling, which controls progression of tumor cells, is documented in several studies [19, 20, 25].

Several studies have shown that in tumor cells signals mediated by $\alpha_5\beta_1$ integrin can control expression of other matrix-specific MMPs. For example, fibronectin stimulation of $\alpha_5\beta_1$ receptor of breast carcinoma cells and lung cancer cells results in the increase of MMP-1 collagenase expression, which is accompanied by increased invasive activity *in vitro* [14]. Synovial fibroblasts stimulated by antibodies to $\alpha_5\beta_1$, were characterized by increased expression of MMP-3 collagenase [26].

There is significant problem in elucidation of mechanisms that are employed by integrin-mediated signals for control of expression and/or activity of matrix proteinases. Recent study has shown [20] that in lung carcinoma cells sequential signal transduction from $\alpha_5\beta_1$ integrin to PI-3K and Erk kinases results in activation of c-Fos proto-oncoprotein, which is a subunit of the transcription factor AP-1. This reaction stimulated AP-1 binding to a regulatory sequence in the promoter of the gene encoding MMP-9 and its transcription as well.

The other putative pathway might be the formation of a complex between integrin and proteinase, which results in enzyme positioning on the cell surface and its activation followed by subsequent destruction of the matrix. This suggestion is supported by data on $\alpha_2\beta_1$ integrin interaction with MMP-1 collagenase; these associates are formed due to interaction of I-domain of α_2-subunit with the hemopexin domain of MMP-1 [27]. Since α_5-subunit lacks I-domain, direct interaction between $\alpha_5\beta_1$ and MMP-2 is questionable. However, the interaction of integrin with MMP-2 may be mediated by collagen followed by formation of ternary complex MMP-2–collagen–$\alpha_2\beta_1$ integrin [28].

Our preliminary data suggest that in MCF-7/ADR cells $\alpha_5\beta_1$ receptor forms associates with MMP-2 collagenase. The nature of these complexes is now being investigated.

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