Biochemistry (Moscow), Vol. 69, No. 3, 2004, pp. 322-330. Translated from Biokhimiya, Vol. 69, No. 3, 2004, pp. 399-409.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-146, February 29, 2004.

Role of Phosphatidylinositol-3 Kinase in Regulation of Differential Sensitivity of Melanoma Cells to Antitumor Agents. A Model for Hormone Resistance Development in Tumor Cells

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Received June 17, 2003
Revision received September 12, 2003

Abstract—Phosphatidylinositol-3 kinase (PI3K) belongs to one of the most important cellular proteins involved in the transmission of anti-apoptotic signal and regulation of survival pathways in tumor cells. Earlier we have found that prolonged treatment of melanoma cells with dexamethasone results in formation of a cell subline which was resistant to growth inhibitory dexamethasone action. We showed that constitutive activation of PI3K can be considered as one of the factors that regulate cell resistance to dexamethasone. Here we demonstrate that increased level of PI3K protein in dexamethasone-resistant cells correlates with partial decrease in expression of down-stream target of PI3K—protein kinase B (PKB). Study of the cell's sensitivity to various damaging agents showed that the cells after prolonged dexamethasone treatment are characterized by increased level of the resistance to both hormonal drugs and hypoxia, and at the same time with high sensitivity to ultraviolet (UV) radiation or anti-tumor agents such as adriamycin. As revealed, hypoxic conditions or short-term dexamethasone treatment of the resistant cells lead to a substantial increase in the PKB level, whereas neither UV radiation nor adriamycin affects the PKB level in these cells. We demonstrate that long-term dexamethasone treatment of melanoma cells results in the accumulation of the active form of mitogen-transducing signaling protein STAT3 (Signal Transducer and Activator of Transcription-3), which also contributes to inducing the melanoma cell's resistance to antiproliferative action of dexamethasone. We suggest that decreased level of PKB in combination with an activation of PI3K/STAT3 signaling in the melanoma cells after prolonged dexamethasone treatment may be one of the mechanisms of different sensitivity of these cells to hormonal drugs and damaging agents. The model of the progression of hormonal resistance of *in vitro* cultured tumor cells is presented.

Key words: melanoma, phosphatidylinositol-3 kinase, protein kinase B, STAT3, dexamethasone, resistance, apoptosis

One of major problems in the study of the mechanism of tumor growth is the identification of biochemical pathways providing the activation of protective mechanisms in tumor cells and determining hyperresistance of malignant tumors to damaging factors. Recent studies have shown that phosphatidylinositol-3 kinase (PI3K) and PI3K-dependent enzymatic systems controlling the major anti-apoptotic and mitogenic signaling pathways of cells take an active part in the regulation of tumor cell survival. The basis for the activation of PI3K is an interaction of p85 regulatory subunit of the enzyme with cellular tyrosine kinases, both non-receptor (p60-src) and receptors. Among the latter, the most important are the receptors for the mitogenic insulin-like and epidermal growth factors, as well as for the vascular endothelial growth factor (VEGF) activated in cells under hypoxia. Protein kinase B (PKB) controlling transmission of anti-apoptotic signal in cells is one of the main PI3K effectors.

Abbreviations: PI3K) phosphatidylinositol-3 kinase; PKB) protein kinase B; VEGF) vascular endothelial growth factor.

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Phosphorylated phosphatidylinositol derivatives, 3-OH phosphoinositides, whose production is catalyzed by PI3K, can interact with specific lipid-binding domains of PKB (so-called pleckstrin-homology (PH)-domains) resulting in conformational changes in the PKB molecule with its activation [1-4]. By now, the main PKB substrates involved in antiapoptotic and mitogenic signal transmission are identified, such as the signaling proteins PKC, p70 s6k, GSK-3, and Bad. Moreover, PI3K can participate in the mitogenic signal transmission independently of PKB via direct interaction with the signaling proteins of the RAS/RAF/ERK- and JAK/STAT-cascades [5-8].

In many cases, the adaptation of tumor cells to damaging factors is accompanied by the constitutive activation of the PI3K signaling pathway; however, the mechanism of these adaptive changes of PI3K is not yet clearly understood, and the same is true for the contribution of different PI3K effectors to the adaptation of tumor cells to damaging factors.

As we have demonstrated in our previous experiments, prolonged treatment of melanoma cells with dexamethasone leads to the constitutive activation of PI3K, whose hyperexpression largely determines a decrease in cell sensitivity to the anti-proliferation effect of the hormone. It was found that both transcription factors of the STAT family and JAK/STAT signaling pathway, which form with PI3K a joint autoregulatory loop, participate in maintenance of elevated level of PI3K [9].

In the present study, we have found for the first time that the activation of both PI3K and STAT3 (Signal Transducer and Activator of Transcription-3) caused by prolonged treatment of cells with dexamethasone is accompanied by partial suppression of the subjacent PI3K effector protein kinase B. This suppression is compensated under the short-term action of either hypoxidation factors or hormonal cytostatics, but remains when the cells are either treated with the antitumor drug adriamycin or irradiated, which can underlie the differential sensitivity of hormone-resistant cells to such factors.

MATERIALS AND METHODS

Cell culture. Human melanoma cells, the line FEMX, were grown in RPMI medium containing 10% of fetal serum (Gibco, USA) and 50 U/ml gentamicin at 37°C in an atmosphere of 5% CO_2. The hormone-resistant subline FEMX/D was selected from the parent line FEMX after prolonged (30 days) cultivation in medium containing 10^{-7} M dexamethasone. The following growth of FEMX/D cells was supported in medium without dexamethasone for 70-120 passages. In experiments on effect of UV radiation on cell survival, at the stage of 80% monolayer the FEMX or FEMX/D cells were irradiated with 16 J/m^2 UV and seeded into 4-well plates with following estimation of cell survival by the count of colonies formed 10-14 days later.

Transient transfection and determination of reporter gene activity. In experiments on transient transfection, we used a plasmid containing cDNA of the PI3K catalytic subunit p110 covalently linked to the iSH2 domain of the regulatory PI3K subunit [10]. As we reported previously, transient transfection with the p110-plasmid leads to significant growth of expression and activity of PI3K in cells [11]. The plasmid pcDNA3 was used for the control transfection. Transfection was performed using lipofectamine (Life Technologies-BRL, USA) for 4 h at 37°C. Mean transfection efficiency of this reagent was 28-30%. All of the following experiments with transfected cells were conducted not later than 72 h after the transfection was completed.

The transcription activity of STAT3 has been determined by means of co-transfection of cells with both a plasmid containing the luciferase reporter gene controlled by a STAT3-sensitive promoter and a plasmid encoding the STAT3-specific protein kinase JAK2 [12]. A parallel transfection of cells with a plasmid carrying the β-galactosidase gene was performed to control for the efficiency and possible toxicity of the transfection procedure. After 24 h, cells were removed from plates, lysed, and the activities of luciferase and β-galactosidase were determined according to standard protocols (Promega, USA). Luciferase activity in analyzed samples was calculated in relative units as a ratio of total luciferase activity to galactosidase activity.

Cell extract preparation and immunoblotting. Cells at the stage of 80% monolayer were removed from dishes in 1 ml of phosphate buffer, washed twice, and incubated for 15 min at 4°C in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% NP-40 (nonylphenoxypolyethoxyethanol), 150 mM NaCl, 1 mM EDTA, 1 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.1 mM sodium ortho-vanadate, and 1% aprotinin. Samples were centrifuged for 15 min at 15,000*g*, and supernatants were then used as total cell extracts. Protein was determined by the Lowry method. Electrophoresis of samples containing 100 μg of protein was performed in 10% polyacrylamide gel with subsequent electrotransfer of proteins onto nitrocellulose filters. Antibodies against PTEN, PKB, and phospho-PKB (Santa Cruz Biotechnology, USA) were used for immunoblotting. To prevent nonspecific sorption, the filters were treated with 3% BSA solution and then incubated with primary antibodies for 2 h at room temperature. Filters were washed, incubated for one hour with secondary antibodies conjugated with peroxidase, and the complexes formed were stained with ECL reagent (Amersham, UK).

VEGF-A in total cell extracts was quantitatively determined using a standard kit for ELISA (BioSource, USA).

Determination of MDR1 gene expression by RT-PCR. FEMX and FEMX/D cells were lysed in TRIzol (10^6 cells/ml) (from here on we used chemicals provided by Invitrogen, USA). Preparation of total RNA, reverse transcription, and PCR were performed according to [13] and manufacturer's recommendations. A mixture of random hexamers (1 μl, 50 ng) was added to the total RNA template (10 μl, 0.1 mg/ml), and after incubation for 10 min at 70°C the following ingredients were added to the sample up to the final concentrations: RT-buffer (Invitrogen); mixture of four dNTP, 0.5 mM; $MgCl_2$, 5 mM; DTT, 10 mM; and reverse transcriptase SuperScript^{RT}, 50 U. Samples were incubated for 50 min at 42°C and then 15 min at 70°C. The cDNA thus prepared was dissolved in water (1 : 3 v/v). The sample for PCR (25 μl) contained 3 μl cDNA; PCR-buffer (Invitrogen); mixture of four dNTP, 0.2 mM; 1.5 mM $MgCl_2$; 1 U Taq DNA-polymerase; and primers. The following primers were used: 1) β_2-microglobulin (forward: 5′-ACC CCC ACT GAA AAA GAT GA-3′; reverse: 5′-ATC TTC AAA CCT CCA TGA TG-3′; product length 120 bp); 2) *MDR1* (forward: 5′-CCC ATC ATT GCA ATA GCA GG-3′; reverse: 5′-GTT CAA ACT TCT GCT CCT GA-3′; product length 167 bp) [14]. Cycle duration: denaturation, 30 sec; annealing, 30 sec; and elongation, 1 min. Twenty seven cycles were used for the amplification of MDR1 cDNA, and 22 cycles for the amplification of β_2-microglobulin cDNA. The material was amplified on a Tertsik amplifier (DNA Technology, Russia). The products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and analyzed under UV light.

RESULTS

Role of PI3K in regulation of UV-resistance in FEMX and FEMX/D melanoma cells. The experiments were performed on both dexamethasone-sensitive FEMX melanoma cells and hormone-resistant FEMX/D subline, which was selected from FEMX cells by long-term hormone treatment and characterized by increased level of PI3K expression [9]. Cell survival after UV irradiation (16 J/m^2) was estimated from their colony-forming efficiency after placing onto 4-well plates. As seen from Fig. 1a, the transfection of parental FEMX cells with a plasmid carrying cDNA for the catalytic p110 subunit of PI3K results in decrease in cell sensitivity to UV irradiation, which correlates with previously described decrease in sensitivity of p110-transfectants to the cytostatic effect of dexamethasone [9, 11]. However, parallel experiments on UV-sensitivity of hormone-resistant FEMX/D cells characterized by high level of PI3K have not shown the expected increase in their UV resistance (Fig. 1b). We

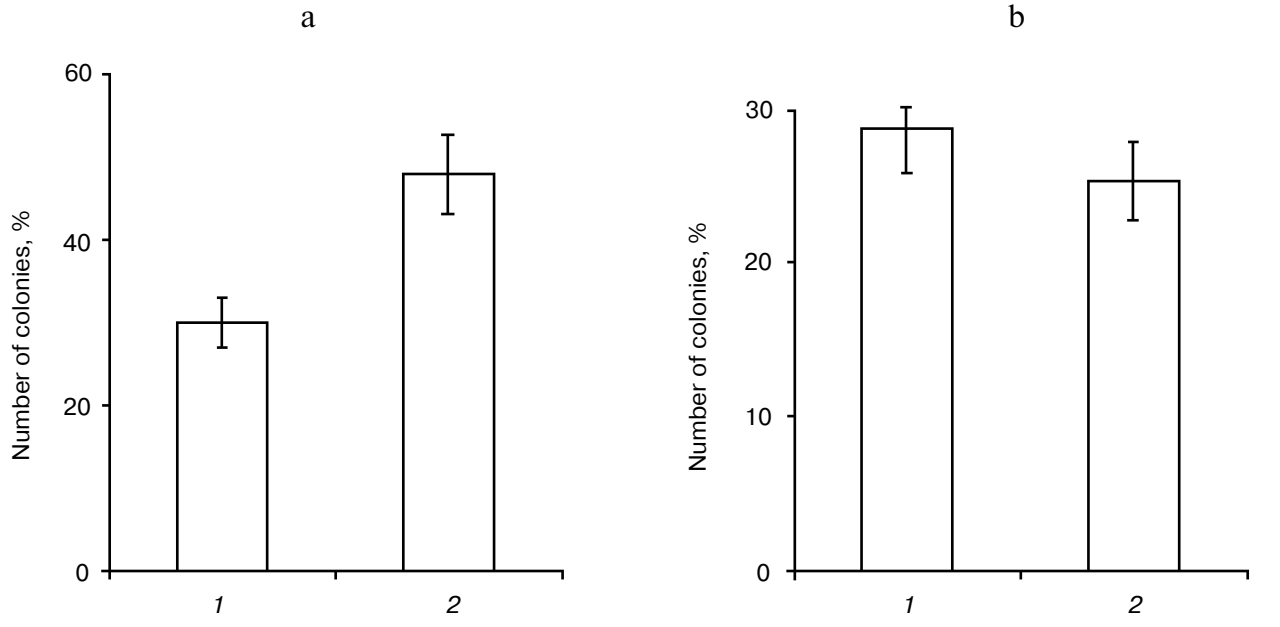

Fig. 1. Sensitivity of melanoma cells to UV irradiation. a) Effect of PI3K on the UV sensitivity of melanoma cells. FEMX cells were transfected with either the control vector pcDNA3 (*1*) or a p110-carrying plasmid (*2*) as described in "Materials and Methods". Twenty-four hours after the transfection a part of the cells was irradiated with UV (16 J/m^2) and the cells were seeded onto 4-well plates; the number of colonies formed was counted after 10 days of growth. The diagram shows the number of colonies after the irradiation in percent; the number of colonies formed by non-irradiated cells is taken as 100%. b) UV sensitivities of the FEMX (*1*) and FEMX/D (*2*) cells. Cells at the stage of 80% monolayer were irradiated with UV and seeded with following colony counting as described above. Mean ± SD values of three independent experiments are presented.

presume these data to be indicative of specific metabolic changes in hormone-resistant cells, which lead to a selective suppression of antiapoptotic, but not mitogenic, effect of PI3K.

Sensitivity to adriamycin and level of MDR1 expression in FEMX and FEMX/D cells. To test a supposition on possible disturbances in antiapoptotic signaling pathways of hormone-resistant cells, we have compared the sensitivity of FEMX and FEMX/D cells to apoptosis-inducing chemical dugs such as adriamycin. Analysis of cell survival after adriamycin treatment has demonstrated a small but significant decrease in survival of hormone-resistant FEMX/D cells compared with the parental ones (Fig. 2a).

In parallel, the level of *MDR1* gene mRNA was examined by PCR in FEMX and FEMX/D cells; this gene encodes P-glycoprotein, a transporter protein that

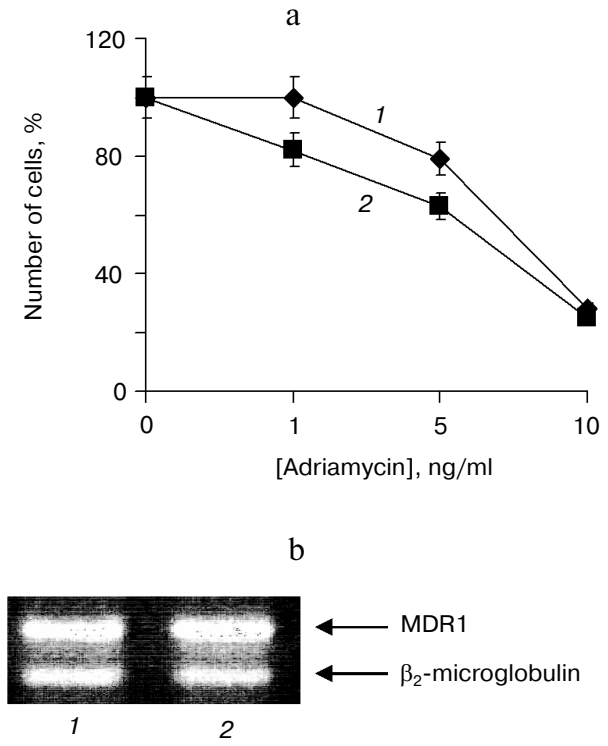

Fig. 2. Effect of adriamycin on the growth of FEMX (*1*) and FEMX/D (*2*) melanoma cells. a) Adriamycin-sensitivity of FEMX and FEMX/D cells. The cells were seeded onto 24-well plates and 24 h later adriamycin was added at concentration of 0-10 ng/ml. After three days of growth the number of cells was counted in a Goryaev chamber using staining with Trypan blue to exclude dead cells. The number of surviving cells was calculated in percent, the number of cells grown without adriamycin was taken as 100%. Mean ± SD values of three independent experiments are presented. b) MDR1 levels in FEMX (*1*) and FEMX/D (*2*) cells. Expression of *MDR1* gene was determined by RT-PCR of total RNA isolated from lysed cells (see "Materials and Methods"). Arrows indicate the locations of products specific for MDR1 and β_2-microglobulin (internal PCR control).

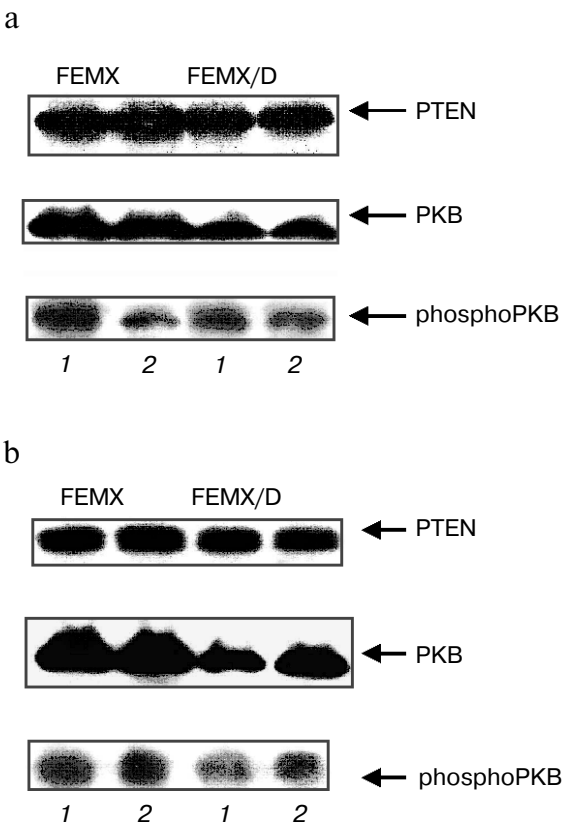

Fig. 3. Expression of PTEN and PKB in FEMX and FEMX/D cells. a) Effect of UV irradiation on the PTEN and PKB levels in melanoma cells. The cells were plated on plastic dishes, and one day later some part of the cells was irradiated with UV (exposure dose 16 J/m^2) with following growth of control (*1*) and UV-irradiated (*2*) cells for 6 h. The cells were then removed from dishes, lysed, and PTEN, PKB, and phospho-PKB contents were determined by immunoblotting by a hybridization of cellular proteins immobilized on nitrocellulose membrane with corresponding antibodies. b) Effect of dexamethasone on the levels of PTEN and PKB. Cells at the stage of 80% monolayer were grown either without (*1*) or in the presence of 10^{-7} M dexamethasone (*2*) for 24 h; PTEN, PKB, and phospho-PKB were then determined by immunoblotting.

mediates resistance of cells to xenobiotics including adriamycin. The levels of *MDR1* mRNA are virtually equal in FEMX and FEMX/D cells (Fig. 2b) suggesting a P-glycoprotein-independent mechanism of increased sensitivity to adriamycin in hormone-resistant cells.

Levels of PTEN and PKB in FEMX and FEMX/D cells. Western-blotting analysis of the physiologically active antagonist of PI3K, a phosphatase encoded by the tumor growth suppressor gene *PTEN* (Protein Tyrosine Phosphatase with Homology to Tensin), has shown no significant difference in its level in FEMX and FEMX/D cells (Fig. 3a).

Analysis of both total PKB, the major antiapoptotic effector of PI3K, and activated (phosphorylated) PKB

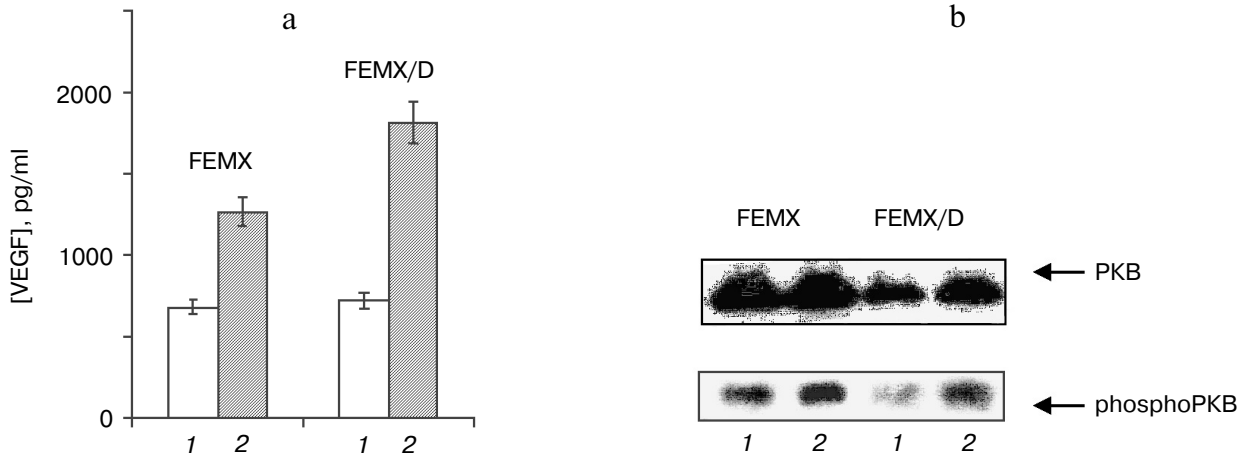

Fig. 4. Level of VEGF and expression of PKB in FEMX and FEMX/D cells. a) Effect of hypoxia inductor $CoCl_2$ on VEGF level. Cells were grown either in absence (*1*) or presence of 200 μM $CoCl_2$ (*2*) for three days, then removed from dishes and lysed with subsequent determination of intracellular VEGF using a standard ELISA kit (BioSource, USA). Mean ± SD values of three independent experiments are presented. b) Effect of $CoCl_2$ on the expression of PKB and phospho-PKB. Cells were grown for 24 h either in the medium without supplements (*1*) or in the presence of 200 μM $CoCl_2$ (*2*) with subsequent determination of PKB and phospho-PKB by immunoblotting.

has revealed the prominent decrease in PKB expression and activation in hormone-resistant FEMX/D cells despite the elevated level of PI3K in these cells; the PKB level also remained decreased after irradiation (Fig. 3a). A relatively low level of PKB was also observed in FEMX/D cells pretreated with adriamycin (data not shown) suggesting the decreased level of PKB to be one of the factors determining high sensitivity of hormone-resistant melanoma cells to apoptotic stimuli.

At the same time, we have found that short-term (for one day) treatment of FEMX and FEMX/D cells with dexamethasone results in increase in PKB activity with no distinct changes in the level of PTEN (Fig. 3b).

Effect of hypoxia inductor $CoCl_2$ on levels of VEGF-A and PKB. Sensitivity of FEMX and FEMX/D cells to hypoxia. Growing of FEMX cells under hypoxic conditions (caused by 200 μM $CoCl_2$ added to the culture medium) leads to a drastic increase in the intracellular level of VEGF-A (vascular endothelial growth factor), one of the positive regulators of the PI3K/PKB signaling pathway. Comparative analysis of VEGF-A level in parental and hormone-resistant FEMX cells grown under hypoxic conditions has revealed higher content of VEGF-A in the hormone-resistant cell line (Fig. 4a). Analysis of PKB has shown that increase in VEGF-A level under hypoxia correlates with the proportional

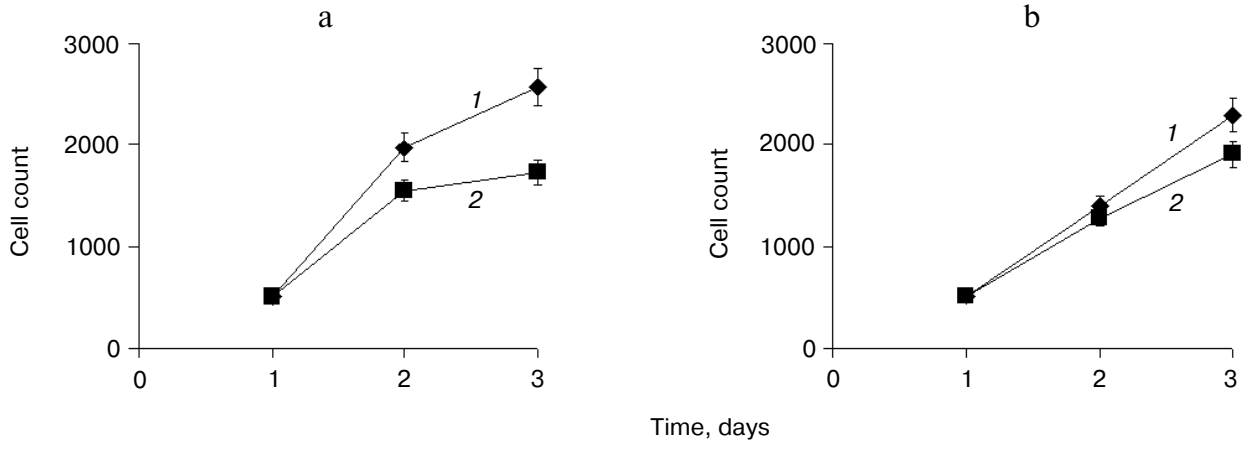

Fig. 5. Effect of hypoxia inductor $CoCl_2$ on rate of growth of FEMX (a) and FEMX/D (b) cells. Cells were grown either in the absence (*1*) or presence of 200 μM $CoCl_2$ (*2*) for three days; cells were counted in a Goryaev chamber. Mean ± SD values of four independent experiments are presented.

increase in the level of PKB, both total and phosphorylated (Fig 4b). Hormone-resistant cells under hypoxia were found to possess higher survival compared to the parental cell line. The data presented in Fig. 5a show that the incubation of FEMX cells in the presence of $CoCl_2$ for 3 days results in a marked (more than 35%) decrease in the number of viable cells in comparison with the FEMX cells grown under normal conditions. Alternatively, hormone-resistant FEMX/D cells growing under hypoxia are characterized by significantly less (within 16%) decrease in the number of viable cells (Fig. 5b).

Overall, the data indicate that the hormone-resistant FEMX/D cells are characterized by decreased PKB level, which remains decreased under either the action of adriamycin or UV irradiation but is compensated in hypoxia; this phenomenon possibly underlies the differential sensitivity to these factors that we have found in hormone-resistant cells.

Effect of dexamethasone on transcription activity of STAT3. As we have previously demonstrated, the hormone-resistant FEMX/D cells are characterized by increased level of not only PI3K, but also of the transcription factor STAT3, which is a basal mitogen-transmission signaling protein and is in functional cooperation with the PI3K-signaling pathway [9]. In the experiments presented here, we studied the effect of short-term and prolonged hormone treatment on the transcription activity of STAT3 in FEMX cells. To do this, 24 h before harvesting of the cells growing with dexamethasone, we transfected them with both a plasmid carrying the luciferase reporter gene controlled by the STAT3-specific promoter and a plasmid encoding protein kinase JAK2 responsible for STAT3 phosphorylation. Yet, the relatively short-term (for three days) growth of cells with dexamethasone was found to result in increase in the transcription activity of STAT3, which became more pronounced with further prolongation of cell growth in the presence of dexamethasone up to 30 days (Fig. 6). One can hypothesize considering the known role of STAT3 in the mitogenic signal transmission that the significant elevation of STAT3 activity during prolonged cell growth in the presence of the hormone is one of the factors counteracting the cytostatic effect of dexamethasone and determining low sensitivity of the FEMX/D cells to the hormone.

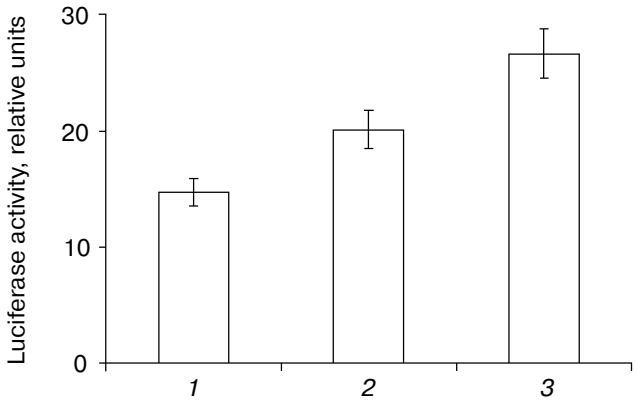

Fig. 6. Effect of dexamethasone on the transcription activity of STAT3. FEMX cells growing either in the absence (*1*) or presence of dexamethasone for 3 (*2*) and 30 days (*3*) were transfected with both a plasmid carrying the luciferase reporter gene controlled by a STAT3-sensitive promoter and a plasmid encoding JAK2. To determine the transfection efficiency, cells were simultaneously transfected with a plasmid carrying the β-galactosidase gene. The cells were harvested 20 h after the transfection, and luciferase and β-galactosidase activities were determined as described in the "Materials and Methods". Mean ± SD values of three independent experiments are presented.

DISCUSSION

Phosphatidylinositol-3 kinase, an enzyme directly involved in mitogenic and antiapoptotic signal transmission in cells, is one of the most important intracellular proteins controlling the resistance of tumor cells to damaging factors. The main objective of this study was to examine PI3K and PI3K-dependent protein activities under the action of hormonal cytostatics on tumor cells, as well as to investigate the role of PI3K-dependent protective system in regulation of sensitivity of cells to hormonal drugs or damaging agents of non-hormonal nature.

We have shown previously that a prolonged treatment of the FEMX melanoma cells with dexamethasone leads to decreased sensitivity of the cells to the anti-proliferative effect of the hormone with no expressed changes in the level of glucocorticoid-binding receptors. In hormone-resistant cells, a significant decrease in the PI3K level was revealed, which was maintained for a long time in hormone-free medium [9].

One could expect considering the ability of PI3K to block the apoptosis that the constitutive activation of PI3K must also lead to decreased sensitivity of cells to proapoptotic factors, such as irradiation and chemical inducers of apoptosis. However, the studies on the sensitivity of the hormone-resistant FEMX/D cells, which are characterized by increased expression of PI3K, to UV radiation and adriamycin revealed no increase in resistance of cells to those factors, and in the case of adriamycin the hormone-resistant cells became yet more sensitive. As it occurred, the level of P-glycoprotein mRNA (this protein transports xenobiotics out of the cell) remained unchanged in hormone-resistant cells compared to the parental cell line. These data are indicative of possible decrease in activity of signaling pathways responsible for the transmission of antiapoptotic signal from PI3K in hormone-resistant cells.

Analysis of the expression level of the physiological PI3K antagonist, the phosphatase encoded by the tumor

growth suppressor gene *PTEN*, has not revealed apparent changes in hormone-resistant cells. Nonetheless, analysis of the main antiapoptotic PI3K effector, PKB, has shown an apparent decrease in both expression and activation of PKB in hormone-resistant cells, in spite of the greatly increased PI3K level in these cells. It is worth noting that the decrease in the PKB activity caused by the hyperexpression of PI3K is a fact that has also been reported by others [15]. A standard approach for those studies is cell transfection with the gene encoding the catalytic subunit of PI3K followed by selection of stable clones and determination of PKB level in the transfected cells [15]. In the present study, we have shown that the decrease in PI3K level also occurs in tumor cells under prolonged action of dexamethasone that leads to the constitutive PI3K activation. Reduced level of PKB was also retained after the action of apoptotic agents, such as irradiation and adriamycin, allowing the reduced level of PKB to be considered as one of the factors determining the elevated sensitivity of hormone-resistant cells to apoptotic factors.

A different pattern was observed in studies on FEMX and FEMX/D cell survival under additional stimulation of PKB. The growth of FEMX and FEMX/D under hypoxic conditions results in drastic increase in the synthesis of cellular VEGF-A, which is one of the positive PKB regulators [16, 17], the increase being more profoundly expressed in hormone-resistant cells. Further experiments on cell survival have demonstrated that in hypoxia, but not under the action of irradiation or adriamycin, the hormone-resistant FEMX/D cells possess higher survival compared to the parental cells.

The same regularity was found in studies on the effect of dexamethasone on the PKB level. The short-term treatment of FEMX cells with dexamethasone appears to result in increased expression of PI3K and activation of PKB, which are more expressed in hormone-resistant cells. The data indicate that the decrease in the PKB level, which is characteristic of hormone-resistant cells, compensated in hypoxia or under the short-term exposure to dexamethasone, but remained decreased under irradiation or treatment with adriamycin, might underlie the differential sensitivity of hormone-resistant cells to such factors.

The most important question is what is the nature of the alterations in cell metabolism that determine the increase in cell resistance to the cytostatic effect of dexamethasone? Taking into consideration that the cells after prolonged hormone treatment are resistant to the cytostatic effect of dexamethasone, but remain highly sensitive to apoptotic agents, we have suggested that the development of resistance to dexamethasone might be associated with activation of PI3K effectors possessing mitogenic activity.

One of the PI3K-dependent pathways involved in growth regulation is the JAK/STAT signaling pathway. STAT proteins belong to the family of transcription factors involved in the transmission of extracellular signals in cytokine- and growth factor-treated cells. The proteins of the STAT family are classified as double-activity proteins, which act in cells not only as transcription factors, but also as true signaling messengers. STAT phosphorylation occurs on complex formation with tyrosine kinases of the JAK family or with some other serine-threonine kinases, such as the ones belonging to the MAP-kinase cascade [18-21]. Additional activation of STAT3 occurs via the complex formation between regulatory PI3K subunit and STAT3 protein [8].

Previous experiments performed in our laboratory have demonstrated that the transfection of FEMX melanoma cells with plasmids carrying DNAs encoding either p110 subunit of PI3K or STAT3 results in a decrease in cell sensitivity to the cytostatic effect of dexamethasone. Studies of the hormone-resistant subline selected after prolonged treatment of FEMX cells with dexamethasone have revealed the coordinated activation of PI3K and STAT3, which form a joint regulatory loop in resistant cells [9]. We have suggested that in the course of dexamethasone treatment of FEMX cells, the intracellular level of activated STAT3 and/or PI3K gradually increases allowing the cell, due to the positive feedback between PI3K and STAT3, to maintain high activities of these signaling proteins after removal of the hormone from the culture medium. To test this hypothesis, in the present study we have performed a comparative analysis of the transcription activities of STAT3 in melanoma cells cultured with dexamethasone for different times. It was actually found that the activity of STAT3 in cells significantly increases with prolongation of growth with dexamethasone to 30 days. The same tendency was found in studies on the effect of dexamethasone on the level of PI3K (data not shown). Overall, the data suggest that the activation of PI3K/STAT3 signaling pathway caused by the long-term exposure of cells to the hormone can serve as one of the factors determining the development of cell resistance to the cytostatic effect of the hormone.

A model for development of hormone resistance in cells. Taken together, previously obtained data and the data of the present study prompted us to develop a model for the formation of cell resistance to antiproliferative action of glucocorticoids hormones. This model is based on the ability of glucocorticoids to increase the activities of some mitogen-transmitting signaling proteins, such as PI3K and STAT3, simultaneously with repression of cell division *in vitro*. Our data indicate that PI3K and STAT3 can be considered as components of one and the same regulatory chain, which are coupled by positive feedback and activated by glucocorticoids [9]. It is worth noting that, as judged from the latest literature, the activation of positive feedback between signaling proteins can be of the utmost importance in regulation of metabolism and maintenance of high proliferation activity of tumor cell, including a cell in response to a mitogenic stimulus [22-24].

The model given below for the development of hormone resistance is applied to a monoclonal population of tumor cells, in which the cells have identical biochemical parameters, and possible cell selection during the long-term influence of hormone is minimal. The model includes the following main stages in the development of hormone resistance in cells.

1. In the absence of hormone the cell maintains a certain basal level A_{bas} of the activity of mitogen-transmitting signaling proteins, P_1-P_n, which comprise a joint regulatory loop:

$$P_1 \rightarrow P_2 \rightarrow \text{ ------ } \rightarrow P_n \rightarrow P_1 .$$

2. When a hormone is added, additional stimulation of one or more components of this loop occurs resulting in increment in activity of each component to a definite value A_{stim}.

3. Positive back-couplings are activated between individual components of the regulatory loop resulting in further increase in protein activities. The value A_{stim} gradually increases with duration of hormone action and tends toward an allowed maximum (Fig. 7, curve *1*).

4. After withdrawal of hormone, the activity of regulatory loop components begins to decrease. The level of protein activities after withdrawal of hormone A_{rest} is to be in direct dependence on the level A_{stim} achieved at the moment of hormone removal and in inverse dependence on the time t elapsed from the termination of hormone action on condition that other cellular parameters remain constant:

$$A_{rest} = A_{bas} + A_{\Delta},$$

where $A_{\Delta} = f(A_{stim}, t)$.

The value A_{Δ} is a temporal function, whose decrease rate dA_{Δ}/dt inversely depends on the value A_{stim}.

Further behavior of the system is determined by the extent of the activation of regulatory loop components at the time of hormone withdrawal and by the elapsed time from termination of the influence of hormone. Figure 7 shows schematically possible levels of the regulatory loop activation and degree of cellular resistance to the antiproliferative effect of hormone. On short-term exposure of cells to hormone, the level A_{stim} is not significant, and the level of signaling proteins quickly returns back to the basal level and is insufficient to overcome the cytostatic effect of the hormone, so that the cells retain virtually unchanged the former level of hormone sensitivity (Fig. 7, curve *2a*). Under a prolonged influence of hormone, high A_{stim} values prevent an abrupt decrease in activity of signaling proteins so that the autoregulatory loop is functioning in an excited state for a long time. So, after withdrawal of hormone, cells keep up high level of signaling protein activity, which is sufficient for overcoming the antiproliferative effect of the hormone (Fig. 7, curve *2b*).

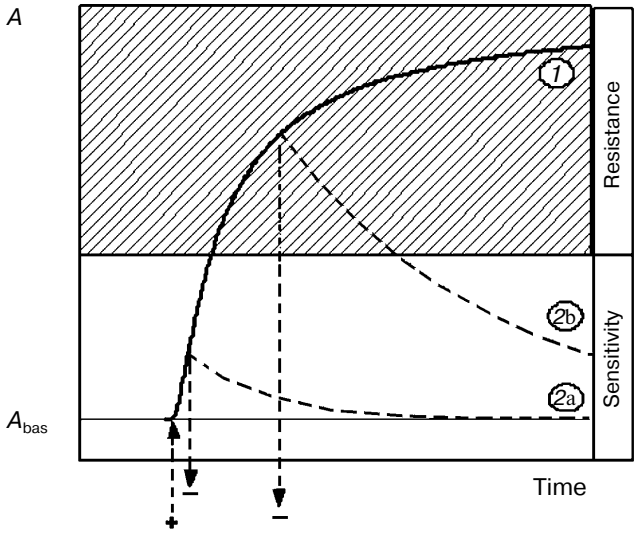

Fig. 7. A scheme for the development of hormone resistance in cells. *1*) Activation of the regulatory loop in a presence of hormone; *2*) decrease in activity of the regulatory loop after withdrawal of short-term (a) or prolonged (b) influence of the hormone. Arrows indicate the start (+) and finish (–) of hormone action. See the text for other explanations. Plots were drawn using the software Origin 7 (OriginLab Corporation, USA).

When all other factors are the same, the duration for which the regulatory loop is in the excited state and cells demonstrate high-level resistance to hormone is determined by the A_{stim} and t values.

Systems with positive feedbacks including the model we have proposed are described by complex kinematic parameters. Some nonlinear disturbances can appear in these systems, such as undulations, additional maxima and minima, and modulated damping [22, 24]. We expect further studies on kinetic parameters for the main components of the regulatory loop, as well as search for additional regulatory factors and their study, to allow certainly the refining of the functional dependence determining duration for which the regulatory loop we have described is in an excited state.

It must be emphasized that the given model does not encompass all variants of hormone resistance development including those connected with either loss or inactivation of specific hormone-binding receptors, mutations in hormone-sensitive sequences of target genes, etc. This model is intended mainly for the variants of resistance development in monoclonal tumor cells, in which the system of hormonal signal transmission remains intact, but a response of the cells to the hormonal antiproliferative stimulus is dramatically depressed or absent. Further exploration in this field will allow a significant extension of our knowledge on the nature of steroid hormone-activated metabolic regulatory loops and determination of their role in development of various hormone-resistance types in tumor cells.

This study was supported by the Russian Foundation for Basic Research, grant No. 01-04-49816.

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