Role of Phosphatidylinositol Signaling Pathway in the Development of Cross-Resistance of Tumor Cells to Hormonal Cytostatics and Hypoxia

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Long-term *in vitro* culturing of CaOv ovarian adenocarcinoma cells in the presence of a synthetic analogue of glucocorticoid hormone dexamethasone allowed us to obtain a subline of CaOv/D cells resistant to the antiproliferative effect of dexamethasone and characterized by high resistance to hypoxia. It was found that CaOv/D cells are characterized by constitutive increase in phosphatidylinositol 3-kinase expression and hypersecretion of vascular endothelial growth factor VEGF-A. Culturing of cells under hypoxic conditions was accompanied by a significant increase in phosphatidylinositol 3-kinase expression and VEGF-A synthesis. Experiments with cell transfection with phosphatidylinositol 3-kinase catalytic subunit proved its participation in the regulation of VEGF-A synthesis and maintenance of cell growth under condition of hypoxia. Our results indicate that coordinated activation of phosphatidylinositol 3-kinase and VEGF-A can be a factor determining the development of cross-resistance of tumor cells to hormonal cytostatics and hypoxia.

Key Words: *ovarian adenocarcinoma; dexamethasone; hypoxia; phosphatidylinositol 3-kinase; proliferation*

Signal pathways regulated by phosphatidylinositol 3-kinase (PI3K, an enzyme phospharylating inositol at the 3-hydroxyl of the inositol ring), play a key role in the regulation of protective mechanisms in tumor cells. 3-OH phosphorylated inositides interact with specific lipid-binding sequences of proteins (PH domains, pleckstrin homology) and activate the corresponding target proteins [1,4], *e.g.* PKB/Akt, PKC, mTOR, p70 S6 kinase, GSK-3, Bad, *etc*. [3,4,6]. PI3K plays an important role in the regulation of growth and survival of tumor cells by determining their resistance to adverse factors [4,8,14].

The role of PI3K in adaptation of tumor cells to hypoxia is of particular interest. The early reactions of tumor cells to hypoxia include activation of hypoxia-induced factor 1 (HIF-1), which regulates expression of angiogenic peptide vascular endothelial growth factor (VEGF) [9,10,13]. Adaptation of cells to hypoxia *in vitro* can be associated not only with intensive angiogenesis in the tumor tissue, but also with activation of some intracellular signaling proteins maintaining tumor growth under hypoxic conditions. Activation of one of these proteins, PI3K, often accompanies cell response to hypoxia [12,15]. However, the role of PI3K in adaptation of tumor cells to hypoxia remains unclear.

The development of acquired resistance to hormonal cytostatics is accompanied by constitutive activation of PI3K. PI3K plays a role in the maintenance of hormone-independent phenotype of cells [2,5]. Here we studied the role of PI3K in the regulation of cell response to hypoxia and the contri-

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bution of coordinated activation of PI3K and VEGF to the development of cross-resistance of tumor cells to hormonal cytostatics and hypoxia.

MATERIALS AND METHODS

Experiments were performed with CaOv human ovarian adenocarcinoma cells sensitive to the growth-inhibitory effect of glucocorticoid hormones. Hormone-resistant subline CaOv/D was obtained after cell culturing in the presence of synthetic analogue of glucocorticoid hormone dexamethasone (10^{-7} M). The cells were then transferred to dexamethasone-free medium and cultured for not less than 3 months.

CaOv human ovarian adenocarcinoma cells were cultured in standard DMEM containing 10% fetal bovine serum (Gibco) and gentamicin (50 U/ml) at 37°C and 5% CO_2. Growth rate was estimated in the MTT test. This method is based on accumulation of fluorescent MTT reagent in living cells [5].

Experiments with transitory transfection were performed on a plasmid containing cDNA of PI3K catalytic subunit p110 covalently bound to the iSH2 domain from the regulatory subunit of PI3K [7]. Control transfection involved the pcDNA3 plasmid. Transfection was performed using Lipofectamine reagent (Life Technologies-BRL) at 37°C for 4 h. Further experiments with transfected cells were performed not later than 72 h after transfection.

For immunoblotting, the monolayer of cells (80%) was taken from plates and placed in 1 ml phosphate buffered saline. Extracted samples were put on nitrocellulose filters (Amersham BS) [2,5]. Electrophoresis of samples with 100 μg protein was performed in 10% polyacrylamide gel. Proteins were electrotransferred onto nitrocellulose filters. The filters were treated with 3% bovine serum albumin to prevent nonspecific sorption, and then hybridized with primary antibodies against the p85 subunit of PI3k (Sigma) at room temperature for 2 h, washed, and hybridized with peroxidase-conjugated secondary antibodies for 1 h. The complexes were developed with the ECL reagent (Amersham).

Isolation of total RNA and reverse transcription PCR (RT-PCR) were performed as described elsewhere [2] according to manufacturer recommendations. PCR was conducted with specific primers for VEGFR2 (direct, 5'-TATGTCTATGTTCAAGAT TAC-3'; indirect, 5-AAGTTTCTTATGCTGAT GCT-3') and β_2-microglobulin (control) on a Tertsik device (DNK-Tekhnologiya). Reaction products were separated by electrophoresis in 2% agarose, stained with ethidium bromide, and studied in UV light.

The results were analyzed using Statistica 6.0 and Origin 6.0 softwares.

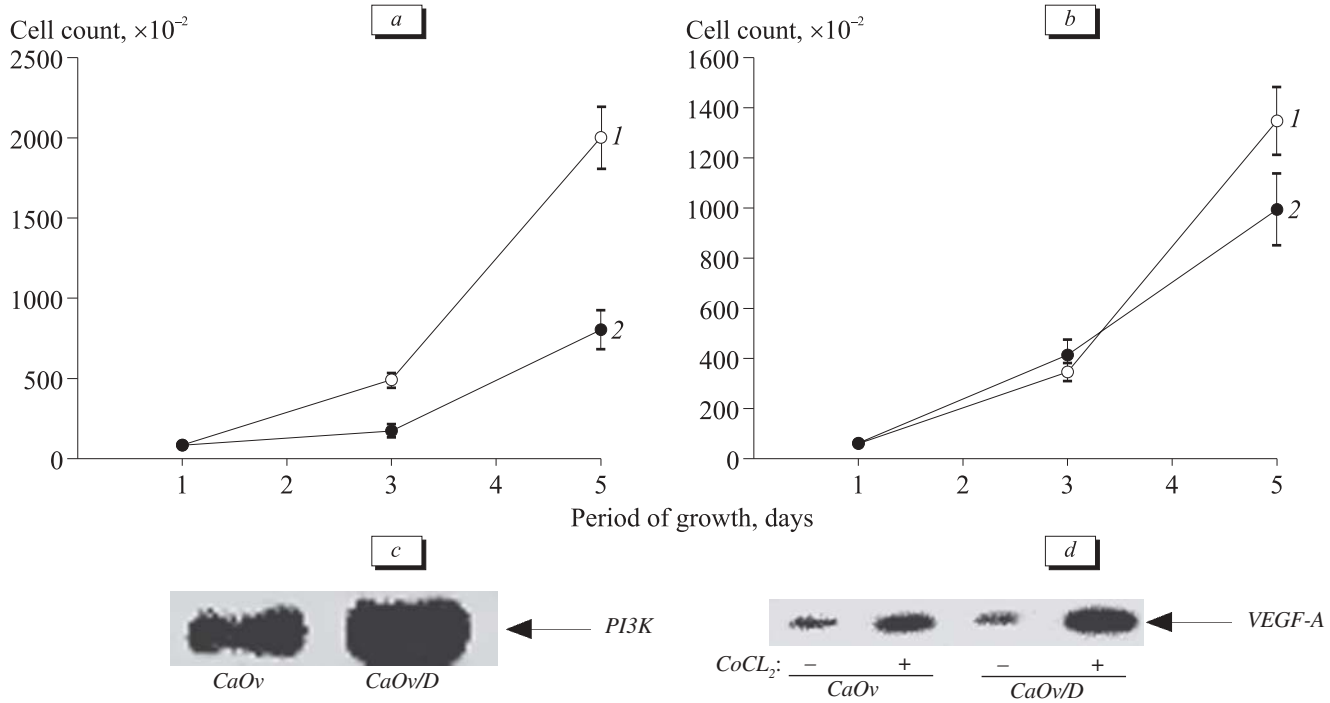

Fig. 1. Immunoblotting assay of CaOv (*a*) and CaOv/D cells (*b*) for sensitivity to dexamethasone and expression of PI3K (*c*) and VEGF-A (*d*). Culturing without dexamethasone (*1*); culturing with 10^{-7} M dexamethasone for 3 days (*2*).

RESULTS

Hormone-resistant CaOv/D cells were characterized by high resistance to the antiproliferative effect of dexamethasone, which was observed after cells culturing in a hormone-free medium (Fig. 1, *a*, *b*).

Study of PI3K expression showed that PI3K activity in dexamethasone-resistant CaOv/D cells is much higher than in parent cells (Fig. 1, *c*).

For evaluation of possible correlation between PI3K activity and VEGF-A synthesis we compared VEGF-A content in CaOv and CaOv/D cells cultured for 5 days under normoxic conditions in the presence of $CoCl_2$, a compound modulating the effect of hypoxia *in vitro* [11]. Basal VEGF-A level was practically similar in cells of both lines. However, hormone-resistant CaOv/D cells were characterized by more pronounced induction of VEGF-A in the presence of 200 μM $CoCl_2$ compared to CaOv cells (Fig. 1, *d*).

Evaluation of the dynamics of CaOv and CaOv/D cell growth in the presence of $CoCl_2$ showed that dexamethasone-resistant CaOv/D cells exhibited increased resistance to $CoCl_2$ (Fig. 2, *a*, *b*). RT-PCR revealed expression of for VEGFR2/KDR mRNA (specific VEGF-A receptor) in both CaOv and CaOv/D cells (Fig. 2, *c*), which attested to possible involvement of VEGF-A in the autocrine regulation of cell growth. Thus, hormone-resistant CaOv/D cells retain high expression of VEGFR2/KDR and are characterized by greater induction of VEGF-A under hypoxic conditions, which correlates with increased resistance of these cells to growth-inhibitory effect of hypoxic factors.

For better understanding of the role of the PI3K signaling pathway in the regulation of VEGF-A expression we transfected parent CaOv cells with a plasmid containing the gene encoding PI3K catalytic subunit p110. Intracellular concentration of VEGF-A significantly increased after transfection of cells with p110 plasmid (Fig. 3, *a*). Analysis of the growth dynamics of transfected cells in the presence of $CoCl_2$ showed that transfection of the p110 plasmid significantly increases cell resistance to $CoCl_2$ (Fig. 3, *b*, *c*).

The observed correlation between PI3K activity and cell resistance to hypoxia suggests that activation of the PI3K-dependent signaling pathway can serve as a component of the protective response of tumor cells to hypoxia. Culturing in the presence of $CoCl_2$ was accompanied by PI3K accumulation in CaOv cells and more so in hormone-resistant CaOv/D cells with high resistance to hypoxia (Fig. 3, *d*).

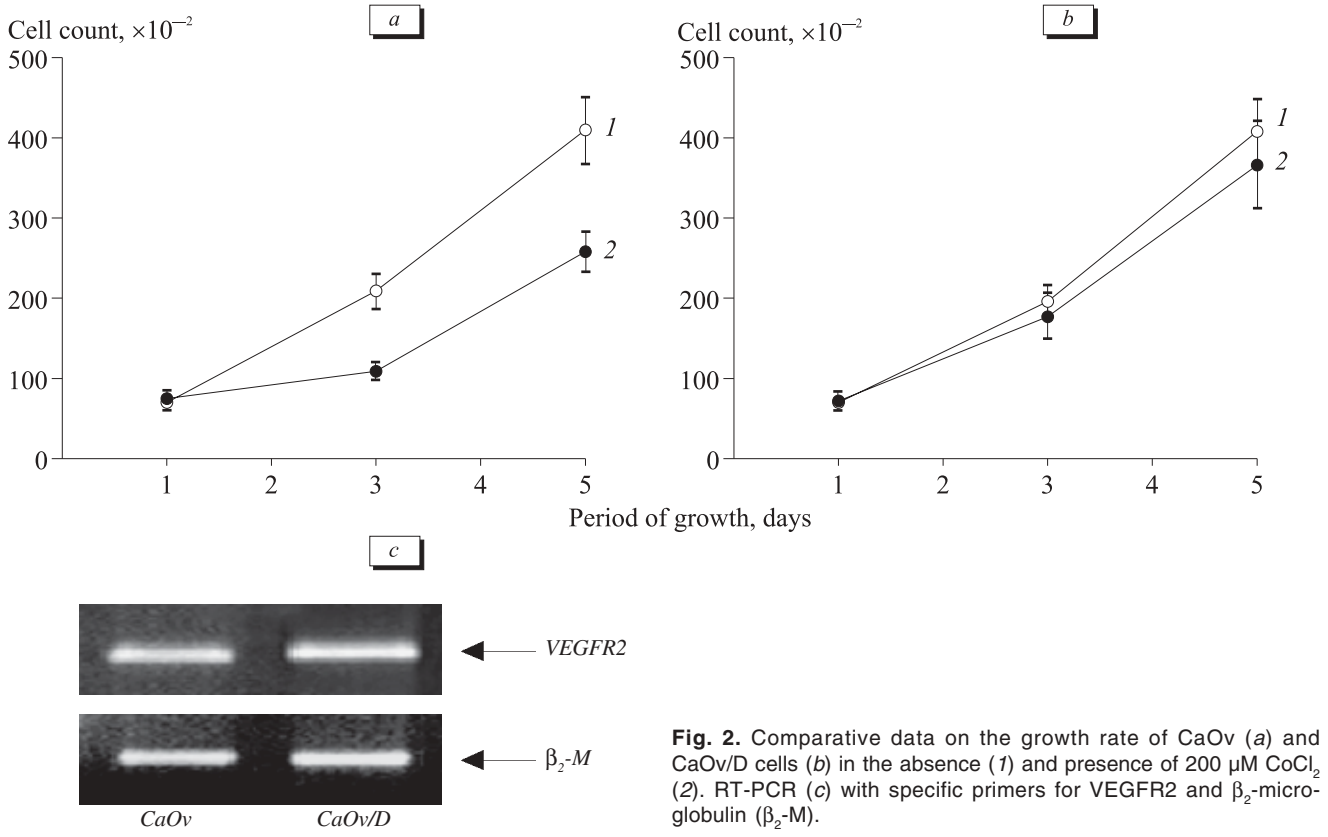

Fig. 2. Comparative data on the growth rate of CaOv (*a*) and CaOv/D cells (*b*) in the absence (*1*) and presence of 200 μM $CoCl_2$ (*2*). RT-PCR (*c*) with specific primers for VEGFR2 and β_2-microglobulin (β_2-M).

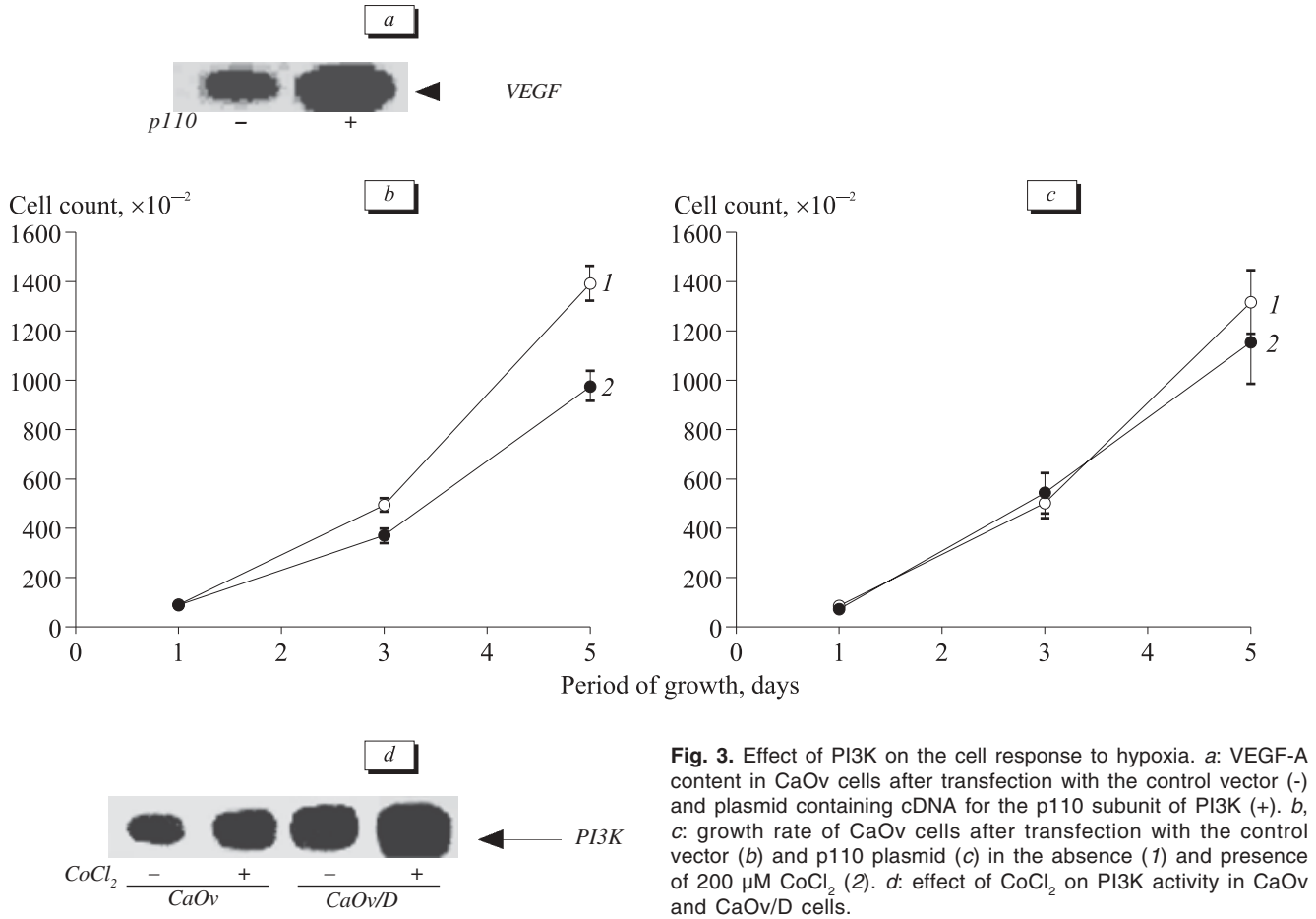

Fig. 3. Effect of PI3K on the cell response to hypoxia. *a*: VEGF-A content in CaOv cells after transfection with the control vector (-) and plasmid containing cDNA for the p110 subunit of PI3K (+). *b*, *c*: growth rate of CaOv cells after transfection with the control vector (*b*) and p110 plasmid (*c*) in the absence (*1*) and presence of 200 μM $CoCl_2$ (*2*). *d*: effect of $CoCl_2$ on PI3K activity in CaOv and CaOv/D cells.

Our results show that long-term culturing of ovarian carcinoma cells with dexamethasone induced cell resistance to the antiproliferative effect of this hormone and increased their resistance to hypoxia. Resistant cells are characterized by constitutive increase in PI3K expression and hypersecretion of VEGF-A under hypoxic conditions. These data provide direct evidence that the PI3K signaling pathway plays a role in the regulation of VEGF-A synthesis and maintenance of cell growth during hypoxia. Our study indicates that coordinated activation of PI3K and VEGF-A can serve as a factor determining cross-resistance of tumor cells to hormonal cytostatics and hypoxia.

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