ONCOLOGY

Development of Differential Sensitivity of CaOv Ovarian Adenocarcinoma Cells to Antitumor Agents under Conditions of Hypoxia

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We studied the role of VEGF signal pathway in autocrine regulation of tumor cell growth and survival under conditions of hypoxia. Hypoxia-resistant CaOv/H substrain with high level of VEGF-A secretion was obtained by long-term culturing of CaOv ovarian adenocarcinoma cells with $CoCl_2$ (hypoxia inductor). VEGF-A directly participates in autocrine regulation of CaOv cell growth, including the maintenance of cell growth under conditions of hypoxia or cytostatic treatment. On the other hand, CaOv/H cells retain high apoptotic potential and are characterized by high expression of $p27^{Kip1}$ (cyclin-dependent kinase inhibitor), which attests to possible involvement of this inhibitor into the regulation of apoptotic response of cells under conditions of hypoxia.

Key Words: *ovarian adenocarcinoma; hypoxia; vascular endothelium growth factor; proliferation*

Activation of hypoxia-inducible factor 1 (HIF-1) is an early manifestation of tumor cell reaction to hypoxia. HIF-1 regulates the expression of vascular endothelial growth factor (VEGF) and some other genes, whose activation leads to extra vascularization of the tumor and prevents the development of hypoxic syndrome in tumor tissue [9]. It was recently found that the functions of VEGF are not confined to stimulation of vascular growth in tumor tissue, but include autocrine regulation of tumor cell growth, if these cells carry specific VEGF receptors [3,7]. The mitogenic effect of VEGF is mediated through autophosphorylation of VEGF receptors (Flk-1/KDR and Flt-1) with subsequent activation of the RAS/MAP and PI3K/PKB signal pathways [8,10]. The scheme of signal pathways initiated by VEGF remains little studied in many aspects; the role of VEGF signal pathway in autocrine regulation of tumor cell growth and survival in hypoxia also remains unclear.

We previously showed that long-term culturing of CaOv ovarian adenocarcinoma cells under conditions of hypoxia leads to the formation of a hypoxia-resistant cell substrain characterized by constitutive activation of some mitogenic signal proteins (PI3K, STAT3) [2]. Here we studied the role of VEGF signal pathway in autocrine regulation of tumor cell growth and survival under conditions of hypoxia.

MATERIALS AND METHODS

Human ovarian adenocarcinoma CaOv cells were cultured in standard DMEM containing 10% FCS

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(Gibco) and gentamicin (50 U/ml) at 37°C and 5% CO_2. Hypoxia-resistant CaOv/H substrain was derived as a result of long-term (2 months) culturing of parental CaOv cell strain with 200 μl $CoCl_2$ and subsequent 4-month culturing in standard DMEM under conditions of normoxia.

Immunocytochemical evaluation of VEGFR2/KDR was carried out by the standard method with first antibodies to VEGFR2/KDR (clone A-3; Santa Cruz Biotech) and streptavidin-labeled second antibodies (LSAB®+kit; Dako) [4]. DAB^+ system (Dako) was used for visualization of immunocytochemical reaction. The reaction was carried out in darkness for 5-10 min. The sections were post-stained with Meyer hematoxylin and embedded in Canadian balm. The preparations were analyzed under a Leica light microscope. The number of positive cells and intensity of marker staining were evaluated (0: no staining; 1^+: weak staining; 2^+: medium staining; 3^+: intensive staining of cells). Cell staining index (SI) was calculated as follows: SI=1×% 1^+-positive cells+ 2×% 2^+-positive cells+3×% 3^+-positive cells.

For immunoblotting the cells at the stage of 80% confluence were removed from dishes in 1 ml phosphate buffer. Cells extracts were then isolated from these samples for application onto nitrocellulose filters (Amersham BS) as described previously [1]. Antibodies to $p27^{Kip1}$, $p21^{WAF1}$, and Akt/PKB (New England Biolabs Inc.) were used for immunoblotting. In order to prevent nonspecific adsorption, the filters were treated with 5% delipidated milk (Nestle) and then incubated with first antibodies for 3 h at ambient temperature. The filters were then washed, incubated for 1.5 h with peroxidase-conjugated (Amersham BS) second antibodies, and the resultant complexes were developed using chemiluminescent reagent (Amersham BS).

Total RNA was obtained and reverse transcription with polymerase chain reaction (RT-PCR) was carried out as described previously [1] and in accordance with manufacturer's instruction. PCR was carried out with specific primers to VEGF-A (direct: 5'-AGTGGTGAAGTTCATGGATGTC-3', reverse: 5'-TGGTCTATCTTTCTTTGGTCTG-3'), VEGFR2 (direct: 5'-TATGTCTATGTTCAAGATTAC-3', reverse: 5'-AAGTTTCTTATGCTGATGCT-3'), and β_2-microglobulin for control [5] on a Tertsik device (DNA Tekhnologiya). The reaction products were separated by electrophoresis in 2% agarose, stained with ethidium bromide, and analyzed in UV light.

RESULTS

The experiments were carried out on CaOv human ovarian adenocarcinoma cells and CaOv/H cell substrain derived as a result of long-term culturing of the parent strain with hypoxia inductor $CoCl_2$. Morphological analysis of cells under conditions of short-term cell culture was carried out by light microscopy. Control tumor cells (CaOv) were compared with experimental substrain (CaOv/H) cells. The structure and capacity of cells to monolayer formation were evaluated. Experimental substrain cells were morphologically better preserved and their capacity to form a monolayer was significantly higher in comparison with the parental CaOv cells (Fig. 1).

Secretion of VEGF-A stimulating angiogenesis in adjacent tissue plays an important role in the development of resistance to hypoxia. Comparative analysis of VEGF-A level in CaOv and CaOv/H cells by RT-PCR showed a significant increase in VEGF synthesis in CaOv/H cells (Fig. 2, *a*). Analysis of cell growth rate in the presence of VEGF inhibitor (recombinant low-molecular-weight fragment of VEGF type 1 receptor, sFlt-1/Fc-chimera)

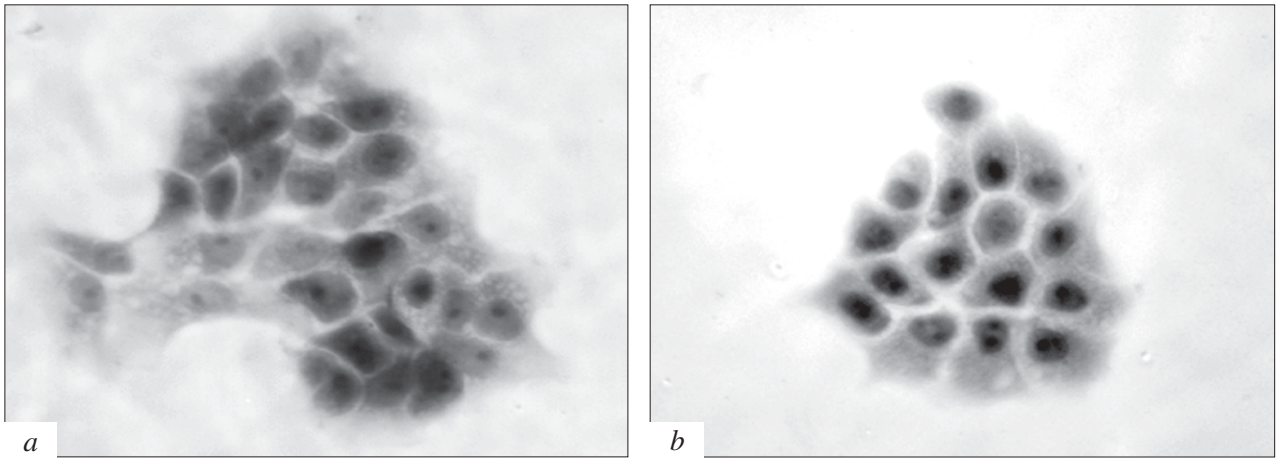

Fig. 1. CaOv ovarian adenocarcinoma cells (*a*) and hypoxia-resistant CaOv/H substrain (*b*). Hematoxylin and eosin staining, ×450.

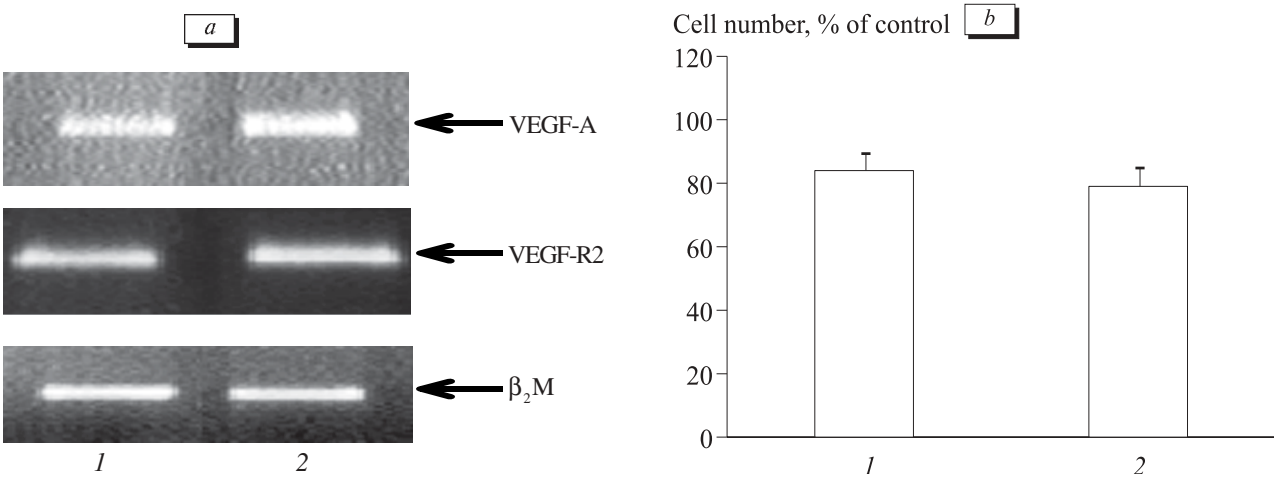

Fig. 2. The role of VEGF-A in the regulation of CaOv (*1*) and CaOv/H cells (*2*) proliferation. *a*) RT-PCR with specific primers to VEGF-A, VEGF-R2, and β_2-microglobulin ($\times\beta_2$M); *b*) effect of VEGF inhibitor (sFlt-1/Fc) on cell growth.

was carried out for evaluating the role of VEGF-A in cell growth regulation. Addition of VEGF inhibitor led to inhibition of CaOv and CaOv/H cells (Fig. 2, *b*).

The effect of VEGF in target cells is realized through the formation of complexes with membrane-bound receptors; their presence in the cells is an obligatory condition for the realization of bio-

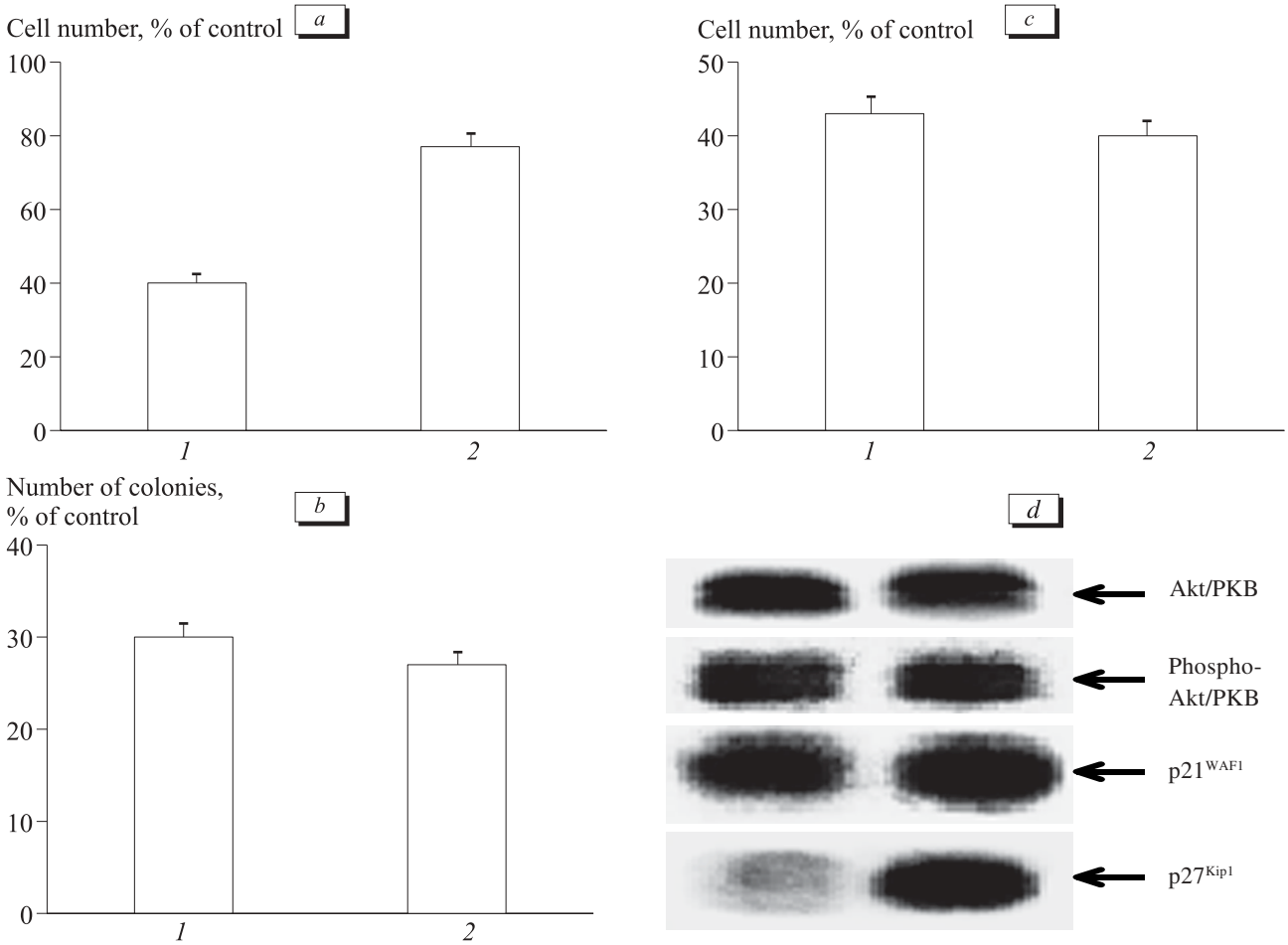

Fig. 3. Comparative analysis of CaOv (*1*) and CaOv/H cells (*2*) sensitivity to dexamethasone (*a*), UV irradiation (*b*), and adriamycin (*c*). *a-c*) control (untreated samples); *d*) expression of Akt/PKB, phospho-Akt/PKB, $p21^{WAF1}$, and $p27^{Kip1}$ in CaOv and CaOv/H cells.

logical activity of VEGF [6]. RT-PCR analysis of VEGFR2/KDR mRNA content revealed the presence of receptor mRNA in both variants of CaOv cells (Fig. 2, *a*). The presence of VEGFR2 in CaOv and CaOv/H cell membranes was confirmed by immunocytochemical analysis with monoclonal antibodies to VEGFR2. The index of staining for Flk-1 receptor was 80±16 for CaOv and 100±24% for CaOv/H. These data indicate direct participation of the VEGF signaling pathway in autocrine regulation of CaOv cell growth, including the maintenance of cell growth during adaptation to hypoxia.

Stable growth of CaOv/H cells under conditions of hypoxia suggests that these cells are highly resistant to other growth-inhibitory factors. In our experiments we used dexamethasone (synthetic glucocorticoid) as a cytostatic. Evaluation of cell sensitivity to the growth-inhibitory effect of dexamethasone showed high resistance of CaOv/H cells to dexamethasone in comparison with the parent strain (Fig. 3, *a*). However, the study of cell sensitivity to proapoptotic influences, such as adriamycin or UV irradiation, showed no appreciable changes in CaOv/H cell survival in comparison with CaOv cells (Fig. 3, *b*, *c*). For further evaluation of the apoptotic potential of CaOv and CaOv/H cells we compared the expression of some key intracellular proteins, antiapoptotic (Akt/PKB) and responsible for proliferation suppression and transmission of the apoptotic signal ($p27^{Kip1}$, $p21^{WAF1}$). The expression of Akt/PKB, phospho-Akt/PKB (active form of the enzyme), and $p21^{WAF1}$ was virtually the same in CaOv and CaOv/H cells, while the level of $p27^{Kip1}$ was significantly higher in CaOv/H cells in comparison with the parental strain (Fig. 3, *d*).

These data indicate adaptation of *in vitro* cultured tumor cells to hypoxia, developing in the presence of intense production of VEGF by the cells. Though VEGF is a specific angiogenic peptide, hypersecretion of this factor can play an important role in autocrine regulation of tumor cell proliferation, maintaining their growth under conditions of hypoxia. On the other hand, cell adaptation to hypoxia is not paralleled by an increase in cell resistance to apoptotic factors and develops in the presence of constitutive increase in the expression of $p27^{Kip1}$ (cyclin-dependent kinase inhibitor), one of the proapoptotic proteins. We hope that further studies in this direction will clear out the role of $p27^{Kip1}$ in the regulation of apoptotic response in CaOv/H cells and characterize the signal pathways responsible for the maintenance of high apoptotic potential of cells during adaptation to hypoxia.

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