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A novel cancer therapy: combined liposomal hypoxia inducible factor 1 alpha antisense oligonucleotides and an anticancer drug

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Abstract

The combined influence of doxorubicin (DOX) and liposomal antisense oligonucleotides (ASOs) targeted to hypoxia-inducible factor 1 α (HIF1A) subunit on the apoptosis signaling pathways and cellular pump and nonpump resistance were investigated. Drug-sensitive A2780 and multidrug-resistant A2780/AD human ovarian carcinoma cells were used. Cells were incubated within 48 h in normoxic (21% O_2, 5% CO_2 and 74% N_2) or hypoxic (1% O_2, 5% CO_2 and 94% N_2) conditions, with or without DOX in the concentration corresponding to the IC_{50} dose, with or without liposomal ASO targeted to HIF1A mRNA. Apoptosis induction, lactic acid concentration, expression of genes and proteins involved in apoptosis signaling pathways, pump and nonpump cellular resistance were assessed. The results showed that overexpression of HIF1A protein induced by exposure to hypoxia and DOX activated both apoptotic cellular signal and cellular antiapoptotic defense. In addition, while hypoxia suppressed cellular pump resistance, due to multidrug resistance-associated protein family transporters, DOX activated pump resistance. A decrease in the expression of targeted protein (HIF1A) by liposomal HIF1A ASO effectively suppressed pump and nonpump cellular resistance and significantly enhanced apoptosis induction by hypoxia and DOX. Data obtained showed that ASO targeted to HIF1A mRNA that suppress cellular antihypoxic defense might be used as a powerful tool to improve the anticancer action of cytotoxic drug or even as an anticancer agent.

Keywords: Hypoxia-inducible factor 1 alpha; Antisense oligonucleotides; Liposomal delivery; Cancer; Doxorubicin; Hypoxia

1. Introduction

Hypoxia-limiting cellular oxidative metabolism and -activating anaerobic processes induces severe tissue damage by both apoptosis and necrosis [1,2]. However, rapid adaptation of cancer cells to endogenous tumor hypoxia leads to the increase in their resistance to exogenous oxygen deficiency and drug treatment. As a result of such adaptation, tumors grown in hypoxic conditions demonstrate a poor prognosis [3–5]. We hypothesize that suppression of such resistance in cancer cells might substantially increase the efficacy of other traditional treatments including chemotherapy.

Hypoxia-inducible factor-1 (HIF1) is a transcription factor found in mammalian cells cultured under reduced oxygen tension. It plays an essential role in cellular and systemic homeostatic responses to hypoxia [6–9]. HIF1 is a heterodimer composed of a 120-kDa HIF1-alpha subunit complexed with a 91–94-kDa HIF1-beta subunit. HIF1 plays a key role in cellular response to hypoxia, including the regulation of genes involved in energy metabolism, angiogenesis, and apoptosis. The alpha subunits of HIF are rapidly degraded by proteasomes under normal conditions but are stabilized by hypoxia. Upregulation of HIF1A induces the expression of various genes whose products play both adaptive and damaging roles in cellular response to hypoxia.

Abbreviations: ASO, antisense oligonucleotides; BCA, bicinchoninic acid; DOX, doxorubicin; FITC, fluorescein isothiocyanate; HIF, hypoxia-inducible factor; pNA, p-nitroanilide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling.

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Recently, we found that HIF1A protein plays a bimodal role in cellular hypoxic response in cancer cells inducing opposite effects of hypoxia: simultaneously promoting cell death and activating cellular antiapoptotic resistance [10]. A detailed analysis of the role of HIF1A in the development of the mentioned bimodal effects of hypoxia in cancer cells showed that the increase in cellular resistance prevails over the induction of cell death after overexpression of this protein during hypoxia. Moreover, we found that the downregulation of HIF1A promoted cell death induction and prevented activation of cellular defense by hypoxia. This suggested that HIF1A might be a potential candidate for anticancer therapeutic targeting.

In the present paper, we examine the influence of the suppression of HIF1A on the effects of hypoxia, the well-established anticancer drug – doxorubicin and their combination. The exogenous hypoxic exposure of cancer cells was used as an in vitro model of in vivo endogenous tumor hypoxia. The aim of this study is to suppress cellular-defensive mechanisms in order to enhance anticancer effects of endogenous tumor hypoxia, anticancer drug or their combination.

2. Materials and methods

2.1. Anticancer drug and cell lines

Doxorubicin (DOX) was purchased from Sigma Chemical Co. (St. Louis, MO). The human ovarian carcinoma cell lines, A2780 (sensitive) and A2780/AD (multidrug-resistant) were obtained from Dr. T.C. Hamilton (Fox Chase Cancer Center, PA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). All experiments were performed on cells in the exponential growth phase.

2.2. Hypoxia model

Cells were maintained at 37 °C in a humidified incubator containing 21% O_2, 5% CO_2 in air (referred to as normoxic conditions). Hypoxia was produced by placing cell culture plates in a modular incubator chamber (Billupus-Rottemberg, Inc., Del Mar, CA) and then flushed with a mixture of 1% O_2, 5% CO_2 and 94% N_2 at a flow rate of 3 L/min for 15 min [11]. The chamber was sealed and placed at 37 °C for 48 h. The flushing procedure was repeated every 12 h.

2.3. Liposomal delivery of antisense oligonucleotides targeted to HIF1A

The sequence of the antisense and sense HIF1A oligonucleotides was 5′-GCCGGCGCCCTCCAT-3′ and 5′-ATGGAGGGCGCCGGC-3′, respectively [12]. The DNA backbone of all bases in oligonucleotides was P-ethoxy modified to enhance nuclease resistance and increase incorporation efficacy into liposomes. Antisense oligonucleotides (ASOs) were synthesized by Oligos Etc. (Wilsonville, OR). Liposomes were used to deliver antisense or sense oligonucleotides in the cells. Liposomes were prepared using previously described lipid film rehydration method [2,10,13]. Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, evaporated to a thin film in a rotary evaporator, and rehydrated with citrate buffer. The lipid ratio for all formulations was 7:3:10 (egg phosphatidylcholine:1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine:cholesterol). Oligonucleotides were loaded into the liposomes by dissolving them in the rehydration buffer at concentrations of 0.5 mM. Free oligonucleotides were separated from those incorporated in the liposomes by gel-filtration prior to the experiments. The encapsulation efficacy ranged from 50 to 60% in various series of experiments. The mean liposome diameter was about 100 nm.

2.4. Intracellular localization of ASO and DOX

To analyze intracellular localization of ASO released from liposomes, a portion of oligonucleotides were labeled by fluorescein isothiocyanate (FITC) prior to the incorporation into the liposomes. The labeling was performed by Oligos Etc. (Wilsonville, OR). These labeled ASOs were used only in ASO release and localization experiments. In other experimental series ASOs were not labeled. The fluorescence of labels and DOX inside liposomes is quenched [14]. Therefore, the intracellular fluorescence of labeled ASO is attributed to oligonucleotides released from the liposomes. In fact, in preliminary experiments we did not registered measurable fluorescence of DOX and labeled ASO incorporated inside liposomes. In contrast, the destruction of the liposomal membranes, releasing DOX and labeled ASO restored fluorescence. Intracellular localization of ASO was studied by fluorescent microscopy. In these experiments, cell nuclei were additionally stained by Hoechst 33258 nuclear dye (Sigma, St. Louis, MO). The fluorescent substances were visualized by fluorescence microscopy (Zeiss Axiostar Plus fluorescence microscope) using the following filters: excitation 470/40 nm, emission 525/50 nm (FITC); excitation 546/12 nm, emission 590 and up nm (DOX); excitation 360/40 nm, emission 420 and up nm (Hoechst 33258).

2.5. Lactic acid and protein concentration

To confirm the existence of cellular hypoxia, the concentration of lactic acid in cell lysates was measured by enzymatic assay kit 755-10 (Sigma, St. Louis, MO) and was expressed per gram of protein determined using the BCA protein assay kit (Pierce, Rockford, IL).

2.6. Gene expression

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used for the analysis of gene expression as previously described [15–17]. Total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) with 1 μg of total cellular RNA (from 1×10^7 cells) and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences, Piscataway, NJ). After synthesis, the reaction mixture was immediately subjected to PCR, which was carried out using GenAmp PCR System 2400 (Perkin Elmer, Shelton, CT). The pairs of primers used to amplify each type of cDNA are shown in Table 1. β_2-Microglobulin (β_2-m) was used as an internal standard. PCR regimen was: 94 °C/4 min, 55 °C/1 min, 72 °C/1 min for 1 cycle; 94 °C/1 min, 55 °C/50 s, 72 °C/1 min for 28 cycles, 60 °C for 10 min. PCR products were separated in 4% NuSieve 3:1 Reliant® agarose gels (BMA, Rockland, ME) in 1× TBE buffer (0.089 M Tris/borate, 0.002 M EDTA, pH 8.3; Research Organics, Inc., Cleveland, OH) by submarine electrophoresis. The gels were stained with ethidium bromide, digitally photographed and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA). Gene expression was calculated as the ratio of mean band density of analyzed RT-PCR product to that of the internal standard (β_2-m).

2.7. Protein expression

To confirm RT-PCR data, the expression of HIF1A protein and caspase 9 was measured. The identification of the above proteins was determined by Western immunoblotting analysis and processed using scanning densitometry to quantify the expressed protein. To this end, harvested cells were lysed in Ripa buffer (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using a needle and syringe. Following incubation on ice for 45 min, the cells were centrifuged at 10,000 × *g* for 10 min. Protein content in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) and 50 μg of protein was run on a 15% sodium dodecyl sulphate (SDS) polyacrylamide gel immersed in Tris/glycine/SDS buffer (BioRad, Hercules, CA) for 90 min at 70 V. Proteins were transferred to an Immobilon-P nitrocellulose membrane (Millipore, Bedford, MA) in a Tris/glycine buffer (BioRad, Hercules, CA) for 90 min at 100 V. The membrane was blocked in non-fat milk for 2 h at room temperature on a rotating shaker to prevent non-specific binding, washed and incubated overnight with anti-HIF1A mouse primary antibody (1:100 dilution, Lab Vision Corp., Fremont, CA) and anti-caspase 9 rabbit primary antibody (1:2000 dilution, Stress Gen Biotechnologies, Victoria State, BC, Canada) at 4 °C. Following further washing, the membrane was immersed in goat anti-rabbit and goat anti-mouse IgG biotinylated antibody (1:3000 dilution and 1:1000 dilution, respectively, BioRad, Hercules, CA) at room temperature for 1.5 h on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (BioRad, Hercules, CA). The bands were digitally photographed and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA).

Table 1
List of primers used in RT-PCR

Genes	Primers (5′–3′)	
	Sense primer	Antisense primer
HIF1A	cacagaaatggccttgtgaa	ccaagcaggtcataggtggt
VHL	ggtcacctttggctcttcag	tgacgatgtccagtctcctg
MDR1	cccatcattgcaatagcagg	gttcaaacttctgctcctga
MRP1	atgtcacgtggaataccagc	gaagactgaactcccttcct
MRP2	tggagtctacggagctctggg	tgctggtgctcaaaggcacgg
TOP2A	gtagcaataatctaaacctct	ggttgtagaattaagaatagc
P53	gaagacccaggtccagatga	ggtaggttttctgggaaggg
BCL2	ggattgtggccttctttgag	ccaaactgagcagagtcttc
BAX	tttgcttcagggtttcatcc	gccactcggaaaaagacctc
CASP3	tggaattgatgcgtgatgtt	ggcaggcctgaataatgaaa
CASP9	tgactgccaagaaaatggtg	cagctggtcccattgaagat
β_2-m (internal standard)	acccccactgaaaaagatga	atcttcaaacctccatgatg

2.8. Caspase activity

The direct measurements of caspase 3 activity were made using a colorimetric protease assay kit (MBL International, Watertown, MA) as previously described [13]. The assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the substrates X-pNA, where X stands for amino acid sequence recognized by the specific caspase (DEVD for caspases 3). The increase in the caspase activity was determined by comparing these results with the level of the untreated control incubated with saline.

2.9. Apoptosis

Two approaches were used to assess apoptosis induction. The first approach was based on measuring the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in the cell cytoplasm using anti-histone and anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as previously described [18,19]. The second approach was based on the detection of single- and double-stranded DNA breaks (nicks) by an in situ cell death detection kit (Roche, Nutley, NJ) using terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described [18,19]. Briefly, cells were fixed, permeabilized and incubated with the TUNEL reaction mixture. The label incorporated at the damaged sites of the DNA was visualized by a fluorescence microscope.

2.10. Statistical analysis

Data obtained were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA) and presented as mean value ± S.D. from 4 to 8 independent measurements. The difference between variants was considered significant if $P < 0.05$.

3. Results

3.1. Intracellular release and localization of liposomal HIF1A ASO

Analysis of intracellular localization of FITC-labeled ASO targeted to HIF1A by fluorescent microscopy showed that oligonucleotides released from liposomes accumulated in cell nuclei (Fig. 1). A superimposition of light and fluorescent pictures obtained using excitation wavelengths corresponding to Hoechst 33258 (nuclear stain) and FITC on the same cells clearly support such a conclusion. The fluorescence of labels inside micelles or liposomes in an aqueous solution is known to be strongly quenched [14]. Therefore, the absence of visible fluorescence in the cytosol suggests that fluorescent molecules associated with ASO are transferred in liposomes within a pinocytic-endosomic vesicle to the nucleus. The analysis of intracellular localization by fluorescent microscopy showed that ASOs were released from the liposomal drug delivery system in perinuclear region and accumulated in nuclei (Fig. 1). We did not observe visible fluorescence in the cytosol. This suggests that free ASO did not accumulate in the cytoplasm and that transfer of these substances was achieved within endosome vesicles. The liposomal drug delivery system significantly increased the transfection efficacy of ASO. Significant ASO fluorescence was observed in 75–90% of cells after 48 h of incubation. In contrast free fluorescent ASO do not enter cells even when they were used in the highest attainable concentration (∼1 mg/ml, data not shown). Similar results were obtained in sensitive and multidrug-resistant ovarian carcinoma cells both under normoxia and hypoxia (data not shown).

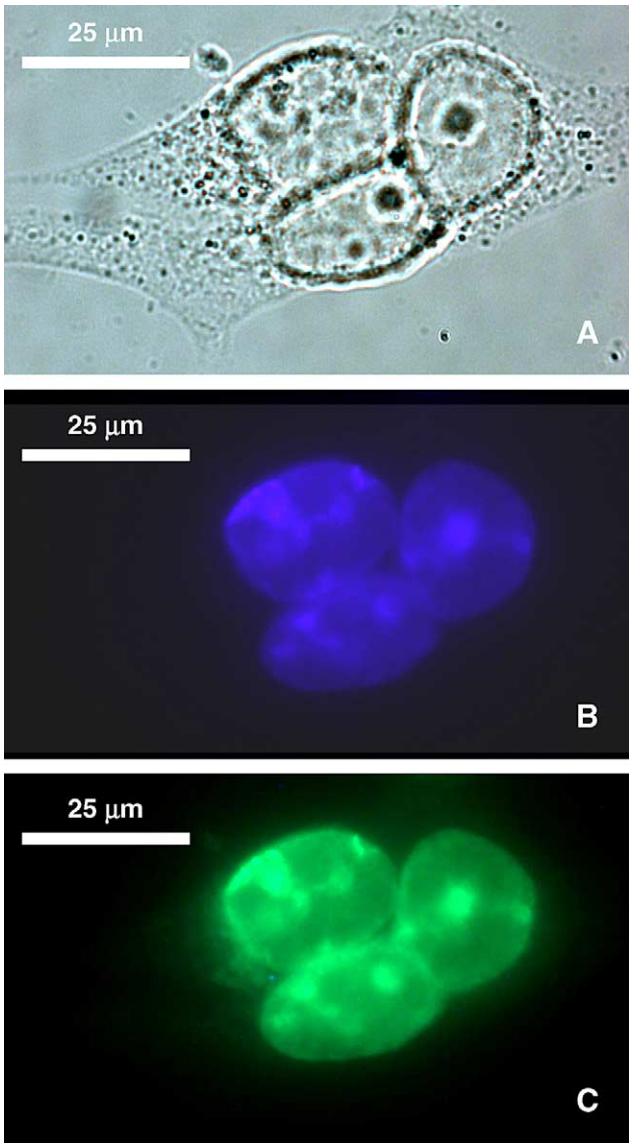

Fig. 1. Intracellular localization of FITC-labeled antisense oligonucleotides targeted to HIF1A mRNA in A2780/AD multidrug-resistant human ovarian carcinoma cells: (A) light microscopy; (B) fluorescent microscopy (Hoechst 33258 nuclear stain); (C) fluorescent microscopy (FITC – labeled ASO).

3.2. Liposomal ASO targeted to HIF1A mRNA decreased the overexpression of the HIF1A gene and protein induced by hypoxia and DOX

Experimental data show, as expected, that hypoxia induced the overexpression of HIF1A mRNA (Fig. 2, bar 5) and the protein itself (Fig. 3, bar 3) both in sensitive and resistant cells. We also found that DOX under normoxia increased the expression of HIF1A mRNA (Fig. 2, bar 7), and protein (Fig. 3, bar 5). In addition, DOX and hypoxia demonstrated a synergetic effect leading to the considerable overexpression of HIF1A mRNA (Fig. 2, bar 9) and protein (Fig. 3, bar 7). In these experiments, the concentrations of DOX corresponded to the IC_{50} doses (the concentration of the drug that induces the death of 50% of whole cell population) for A2780 sensitive and A2780 multidrug-resistant cells equal to 0.2 and 3 μM, respectively. It is interesting that in such conditions these effects were more pronounced in multidrug-resistant cells when compared with their drug-sensitive variant. In fact, overexpression of the studied protein under the same hypoxic conditions was more evident in A2780/AD human ovarian carcinoma cells (compare bar 3 in left and right panels of Fig. 3). Incubation under hypoxic conditions of sensitive and multidrug-resistant cells with DOX in the same effective concentration (IC_{50} dose) induced more pronounced overexpression of HIF1A protein (compare bar 7 in left and right panels of Fig. 3). While empty liposomes and liposomes with sense oligonucleotides (controls) did not

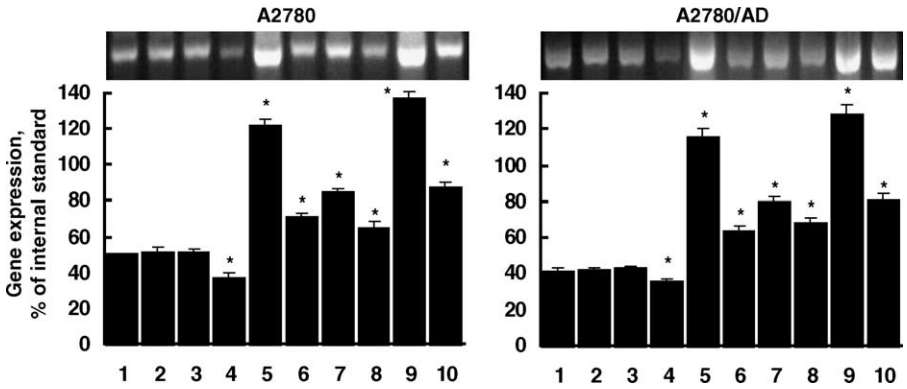

Fig. 2. Typical images of gel electrophoresis of RT-PCR products and expression of HIF1A mRNA (percentage of internal standard, β_2-microglobulin) in A2780 and A2780/AD human ovarian carcinoma cells. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, empty liposomes; 3 – normoxia, liposomal HIF1A sense oligonucleotides; 4 – normoxia, liposomal HIF1A antisense oligonucleotides; 5 – hypoxia, no treatment; 6 – hypoxia, liposomal HIF1A antisense oligonucleotides; 7 – normoxia, DOX; 8 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 9 – hypoxia, DOX; 10 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides. *$P < 0.05$ when compared with control (normoxia, no treatment).

influence the expression of HIF1A mRNA (Fig. 2, bars 2 and 3), the application of liposomal HIF1A ASO decreased the expression level under normoxia (Fig. 2, bar 4; Fig. 3, bar 2) and limited the increase in the expression under hypoxia or DOX exposure and after combined action of hypoxia and DOX (Fig. 2, compare bar 5 with bars 6, 8, 10 and Fig. 3, compare bar 3 with bars 4, 6, 8). These data suggest that selected liposomal ASO substantially decreased the expression of targeted HIF1A gene.

The analysis of the expression of HIF counterpart – the von Hippel–Lindau tumor suppressor gene product (VHL) – showed that liposomal ASO targeted *HIF1A* gene decreased the expression of the *VHL* gene in normoxia (Fig. 4, bar 2). Hypoxia alone significantly downregulated the expression of this gene (Fig. 4, bar 3). Liposomal HIF1A ASO substantially limit this effect of hypoxia (Fig. 4, bar 4). Therefore after the action of HIF1A ASO under the hypoxic conditions the expression of the *VHL* gene was substantially higher when compared with hypoxia alone (Fig. 4, compare bars 3 and 4). Analysis of an influence of DOX on the expression of *VHL* gene showed that the exposure of cells to DOX led to the overexpression of this gene both in normoxia and hypoxia (Fig. 4, bars 5 and 7). Liposomal HIF1A ASO diminished this effect of DOX decreasing the expression of *VHL* gene both under normoxia and hypoxia (Fig. 4, bar 8). Therefore, despite the opposite effects of hypoxia and DOX on the expression of VHL gene, liposomal ASO targeted to HIF1A decreased both of those effects.

3.3. Liposomal ASO targeted to HIF1A enhanced cellular lactate accumulation induced by exogenous oxygen deficiency and DOX

To analyze the degree of cellular hypoxia in cancer cells, we measured the accumulation of final product of anaerobic metabolism – lactic acid in cell lysates (Fig. 5). We found that empty liposomes and liposomal sense oligonucleotides did not influence lactate accumulation in sensitive and multidrug-resistant cells when compared with the

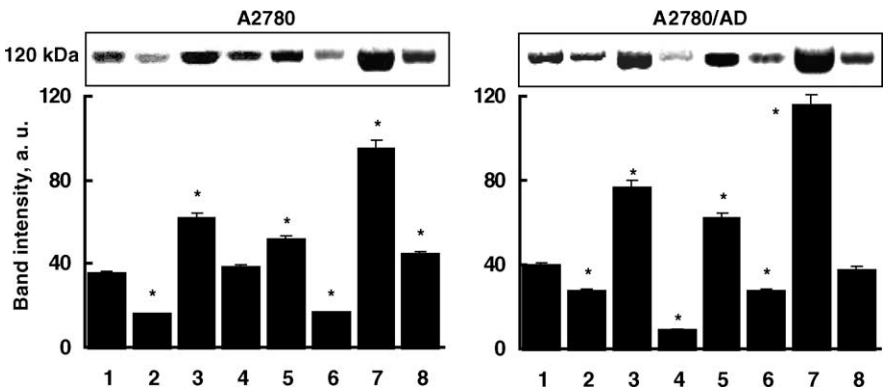

Fig. 3. Typical images of Western blots of HIF1A protein and densitometric analysis of bands in A2780 and A2780/AD human ovarian carcinoma cells. Band intensities are shown in arbitrary units. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, liposomal HIF1A antisense oligonucleotides; 3 – hypoxia, no treatment; 4 – hypoxia, liposomal HIF1A antisense oligonucleotides; 5 – normoxia, DOX; 6 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 7 – hypoxia, DOX; 8 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides. *$P <$ 0.05 when compared with control (normoxia, no treatment).

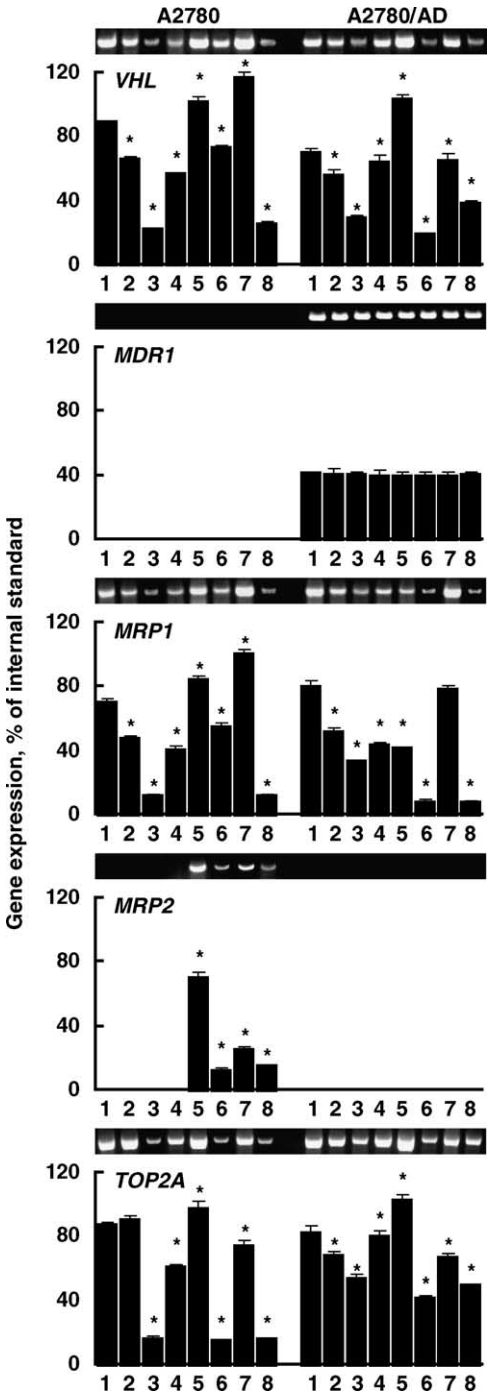

Fig. 4. Typical images of gel electrophoresis of RT-PCR products and expression of *VHL*, *MDR1*, *MRP1*, *MRP2* and *TOP2A* genes (percentage of internal standard, β_2-microglobulin) in A2780 and A2780/AD human ovarian carcinoma cells. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, liposomal HIF1A antisense oligonucleotides; 3 – hypoxia, no treatment; 4 – hypoxia, liposomal HIF1A antisense oligonucleotides; 5 – normoxia, DOX; 6 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 7 – hypoxia, DOX; 8 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides. $^{*}P < 0.05$ when compared with control (normoxia, no treatment).

control (Fig. 5, compare bars 2 and 3 with bar 1). As expected, exogenous hypoxia led to the development of cellular hypoxia in both sensitive and multidrug-resistant cancer cells (Fig. 5, bar 7). Similarly, DOX treatment induced lactate accumulation (Fig. 5, bar 5). Liposomal HIF1A ASO enhanced lactate accumulation in the presence of hypoxia and in both types of cells (Fig. 5).

3.4. Influence of liposomal ASO targeted to HIF1A on pump, nonpump cellular resistance and cell death signaling pathways

We analyzed by RT-PCR the influence of liposomal ASO targeted to HIF1A in the expression of mRNA encoding proteins involved in pump (MDR1, MRP1 and MRP2) and nonpump (Topoisomerase IIα and BCL2) cellular resistance and apoptosis induction pathways (BAX, P53, caspases 9 and 3).

The *MDR1* gene encoding P-glycoprotein – the main protein involved in drug efflux in multidrug-resistant A2780/AD human ovarian carcinoma cells was constituently expressed in resistant cells and was not expressed in drug-sensitive cells (Fig. 4, compare bars 1–8 in A2780 and A2780/AD cells). Neither hypoxia nor DOX influenced the expression of this gene in both sensitive and multidrug-resistant cells. The second type of proteins involved in pump resistance – multidrug resistance associated proteins, encoded by *MRP1* and *MRP2* genes – exhibited different behaviors when compared with the *MDR1* gene. While MRP1 mRNA was expressed in both sensitive and multidrug-resistant cells, its expression in normoxia without treatment was more pronounced in multidrug-resistant cells (Fig. 4, bar 1). It was found that hypoxia downregulates the *MRP1* gene in sensitive and multidrug-resistant cells (Fig. 4, bar 3). In contrast, under normoxia and hypoxia in sensitive cells and under hypoxia in resistant cells, DOX increased the expression of this gene (Fig. 4, bars 5 and 7). The highest level of the MRP1 expression was found after DOX treatment under hypoxia in both sensitive and multidrug-resistant cells (Fig. 4, bar 7). Liposomal HIF1A ASO downregulated the expression of MRP1 mRNA under normoxic and hypoxic conditions (Fig. 4, bars 2 and 4). HIF1A ASO delivered by liposomes under the action of DOX significantly limited the expression of the *MRP1* gene preventing its overexpression by DOX (Fig. 4, compare bars 5 and 6) and combination of DOX and hypoxia (Fig. 4, compare bars 7 and 8). In contrast to MRP1, MRP2 mRNA was not expressed in sensitive and multidrug-resistant cells in normoxia and hypoxic conditions (Fig. 4). DOX exposure led to the overexpression of the *MRP2* gene in sensitive cells (Fig. 4, bars 5 and 7), while HIF1A ASO suppressed this overexpression (Fig. 4, bars 6 and 8).

Similar to pump resistance, an opposite action of hypoxia and DOX on topoisomerase IIα expression was observed (Fig. 4). While hypoxia downregulated the expression of gene-encoding topoisomerase IIα (*TOP2A*, Fig. 4, compare bars 1 and 3), DOX exposure led to the overexpression of this gene (Fig. 4, bars 1 and 5). Once again it was found that HIF1A ASO decreased effects of

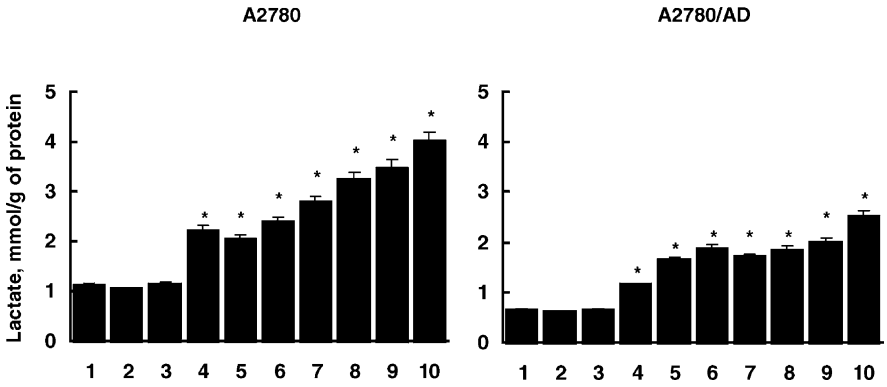

Fig. 5. Lactate concentration in A2780 and A2780/AD human ovarian carcinoma cells. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, empty liposomes; 3 – normoxia, liposomal HIF1A sense oligonucleotides; 4 – normoxia, liposomal HIF1A antisense oligonucleotides; 5 – normoxia, DOX; 6 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 7 – hypoxia; 8 – hypoxia, liposomal HIF1A antisense oligonucleotides; 9 – hypoxia, DOX; 10 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides. *$P < 0.05$ when compared with control (normoxia, no treatment).

hypoxia and DOX almost completely eliminating overexpression of gene-encoding topoisomerase IIα caused by DOX (Fig. 4, compare bars 5, 7 and 6, 8).

Liposomal ASO targeted to HIF1A mRNA downregulated the expression of the *BCL2* gene under normoxia (Fig. 6, compare bars 1 and 2). Both hypoxia and DOX led to the overexpression of the main player in cellular antiapoptotic defense – the *BCL2* gene (Fig. 6, bars 3, 5, 7). HIF1A ASO prevented this overexpression (Fig. 6, compare bars 3 with 4, 5 with 6, 7 with 8). Analysis of the main proapoptotic member of BCL2 protein family – BAX – showed that DOX and especially hypoxia upregulated the expression of the BAX mRNA (Fig. 6, bars 3, 5, 7). While HIF1A ASO diminished the effects of hypoxia on the expression of the *BAX* gene (Fig. 6, bar 4), they substantially promoted the overexpression of the *BAX* mRNA under the combined action of hypoxia and DOX (Fig. 6, bar 8). Both hypoxia and DOX increased the expression of P53 mRNA when compared with control (Fig. 6, compare bars 3, 5, 7 with bar 1). ASO targeted to HIF1A delivered by liposomes also upregulated P53 mRNA under normoxia, hypoxia (Fig. 6, compare bars 2, 4 with bar 1) and in combination with DOX (Fig. 6, bars 6 and 8). Such effect was seen both in sensitive and multidrug-resistant cells. Similar effect of HIF1A ASO was observed on the expression of genes encoding caspases 9 (*CASP9*) and 3 (*CASP3*). It was found that liposomal delivery of HIF1A ASO led to the overexpression of caspases 9 and 3 under normoxic and hypoxic conditions (Fig. 6, compare bars 2 and 4 with bar 1). While DOX alone activated caspases 3 and 9, HIF1A ASO enhanced this activation (Fig. 6, compare bar 6 with 5 and bar 8 with 7).

3.5. Liposomal ASO against HIF1A enhanced the ability of hypoxia and DOX to induce apoptosis by caspase-dependent pathway

Results obtained during the analysis of gene expression by RT-PCR are supported by the measurement of the caspase 9 expression by Western immunoblotting (Fig. 7). The experimental data show that hypoxia and DOX not only increase the expression of procaspase 9, but also induced the cleavage of the procaspase and increasing of its active form, responsible for the initiation of cell death by apoptosis (Fig. 7, bars 3, 5, 7). HIF1A ASO was found to enhance such effect of hypoxia, DOX and their combination (Fig. 7, bars 4, 6, 8). Active caspase 9 cleaved the main executer of apoptosis – caspase 3 – and increased its apoptosis-inducing activity. Direct measurement of caspase 3 activity (Fig. 8) showed that after the exposure to hypoxia, DOX and their combination, the activity of caspase 3 increased reaching plateau after 24 h. While similar results were obtained in sensitive and multidrug-resistant cells, the magnitude of the maximal activity induced by DOX was lower in multidrug-resistant cells despite the fact that both cell types were incubated with similar equivalent concentrations of the drug (the IC_{50} dose for sensitive and multidrug-resistant cells, respectively). Liposomal HIF1A ASO alone activated caspase 3 and enhanced the ability of hypoxia and DOX to trigger caspase-dependent pathway of apoptosis. The maximum activity of caspase 3 was observed in both sensitive and multidrug-resistant cells after the combined action of hypoxia, DOX and liposomal ASO targeted to HIF1A mRNA (Fig. 8, line 8).

Two methods were used to assess apoptosis induction in cancer cells under the action of liposomal ASO, hypoxia, DOX and their combination. The first method, the measurement of the enrichment of cell cytoplasm and media by ELISA, showed that HIF1A ASO alone induced apoptosis in both types of studied cells under normoxia and hypoxia (Fig. 9, bar 3). Exposure of cancer cells to hypoxia and DOX also led to the apoptosis induction (Fig. 9, bar 4), which was enhanced by ASO targeted to HIF1A in both sensitive and multidrug-resistant cancer cells (Fig. 9, bar 5). Analysis of apoptosis by the second method – TUNEL (Fig. 10) – supports the data obtained by the first method (Fig. 9). Similar to ELISA data, the maximal fluorescence

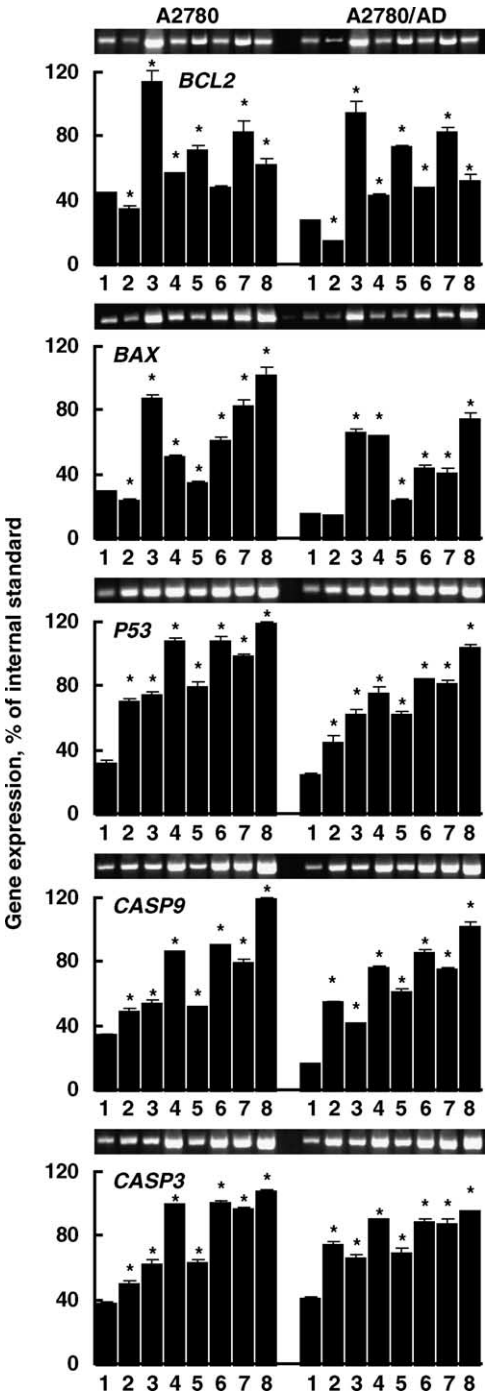

Fig. 6. Typical images of gel electrophoresis of RT-PCR products and expression of *BCL2*, *BAX*, *P53*, *CASP9* and *CASP3* genes (percentage of internal standard, β_2-microglobulin) in A2780 and A2780/AD human ovarian carcinoma cells. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, liposomal HIF1A antisense oligonucleotides; 3 – hypoxia, no treatment; 4 – hypoxia, liposomal HIF1A antisense oligonucleotides; 5 – normoxia, DOX; 6 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 7 – hypoxia, DOX; 8 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides. $^{*}P < 0.05$ when compared with control (normoxia, no treatment).

of labeled DNA brakes was seen when the combination of DOX and liposomal ASO was applied under hypoxic conditions.

4. Discussion

The above data help to clarify several issues in cancer therapy of solid tumors: (1) similarities and differences in mechanisms of cell death induction by applied exogenous DOX and endogenous hypoxia; (2) synergism of hypoxia and DOX in apoptosis induction; (3) the role of HIF1A in apoptosis induction during hypoxia and DOX exposure.

DOX and hypoxia exhibited certain similarities in their mechanisms of apoptosis induction. Both hypoxia and DOX exposure led to the overexpression of the HIF1 protein. It is remarkable that the overexpression of this protein under equivalent conditions of hypoxia and DOX application was more pronounced in multidrug-resistant cells than sensitive cells. Taking into account the involvement of this protein in the activation of cellular defense, one can suggest that this protein might be responsible, at least in part, for the development of resistance in multidrug-resistant human ovarian cells. Both hypoxia and DOX activated cellular nonpump resistance by increasing the expression of the *BCL2* gene which plays a central role in cellular antiapoptotic defense. However, these stimuli simultaneously activated cell death pathway by upregulation of the *BAX* and *P53* genes and initiated caspase-dependent pathway of apoptosis. While both activation of apoptosis and increase in cellular antiapoptotic defense depend on the expression of HIF1A protein, the application of liposomal ASO targeted to HIF1A during hypoxia and DOX exposure significantly enhanced apoptosis induction by these factors. Therefore, we conclude that overexpression of HIF1A protein activated cellular antiapoptotic defense more significantly when compared with the apoptosis induction.

Another studied damaging factor – anaerobic glycolysis and lactate accumulation – also was activated by both DOX and exogenous hypoxia. We found that hypoxia, DOX and liposomal ASO targeted to HIF1A mRNA alone or in combination shifted cellular metabolism toward anaerobic glycolysis and increased cellular lactate accumulation. It is interesting that lactate accumulation in multidrug-resistant cells were less pronounced when compared with the sensitive cells under equivalent degree of hypoxia and DOX impact. This may suggest that the cancer cells resistant to chemotherapy are also resistant to other exogenous damaging impacts including hypoxia. The decreased lactate accumulation in multidrug-resistant cells was not related to the higher activity of efflux pumps because the lactate accumulation in cellular media was also less pronounced in multidrug-resistant cells when compared with drug-sensitive cells (data not shown). Therefore, this finding may suggest that similar mechanisms might be responsible for the resistance to chemotherapy and hypoxia. Furthermore, the experimental data discussed above suggest that the mechanisms of resistance to hypoxia may not be related to efflux pumps. It is highly probable that antiapoptotic cellular resistance to the

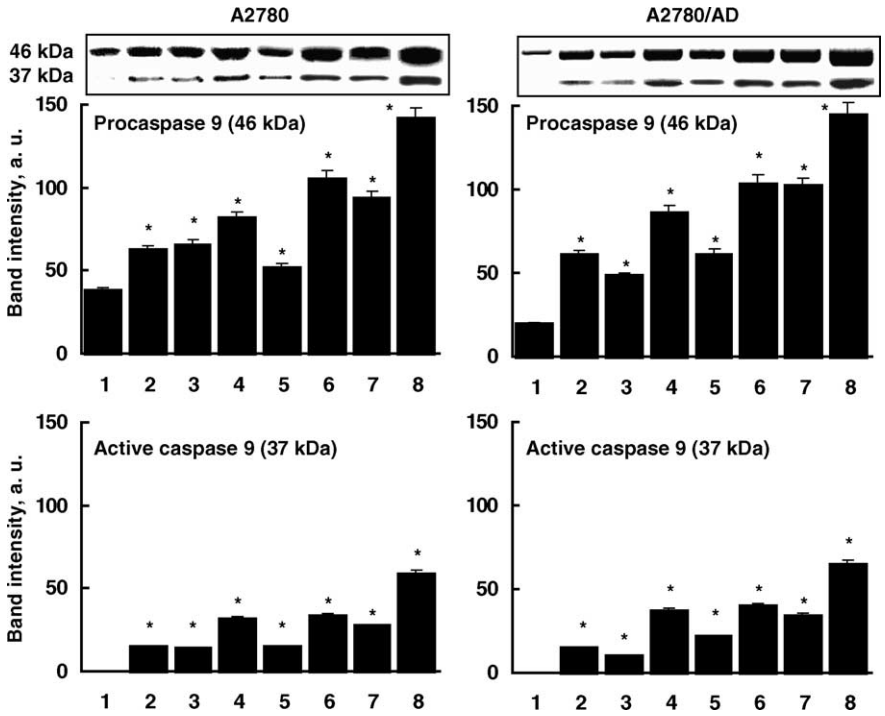

Fig. 7. Typical images of Western blots of caspase 9 and densitometric analysis of bands in A2780 and A2780/AD human ovarian carcinoma cells. Band intensities are shown in arbitrary units. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, liposomal HIF1A antisense oligonucleotides; 3 – hypoxia, no treatment; 4 – hypoxia, liposomal HIF1A antisense oligonucleotides; 5 – normoxia, DOX; 6 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 7 – hypoxia, DOX; 8 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides. *$P < 0.05$ when compared with control (normoxia, no treatment).

hypoxic conditions and exposure to an anticancer drug have related mechanisms at root. Analysis of experimental data also clearly shows the involvement of HIF1A protein in the development of cellular response to hypoxia during hypoxic conditions and DOX exposure. In fact, the suppression of this protein by liposomal HIF1A ASO led to the substantial enhancement of the degree of hypoxia and lactate accumulation in normoxia, hypoxia, DOX and their combined presence.

There are two main differences in the responses of cancer cells to hypoxia and DOX exposure. While hypoxia significantly suppressed the expression of *TOP2A* gene,

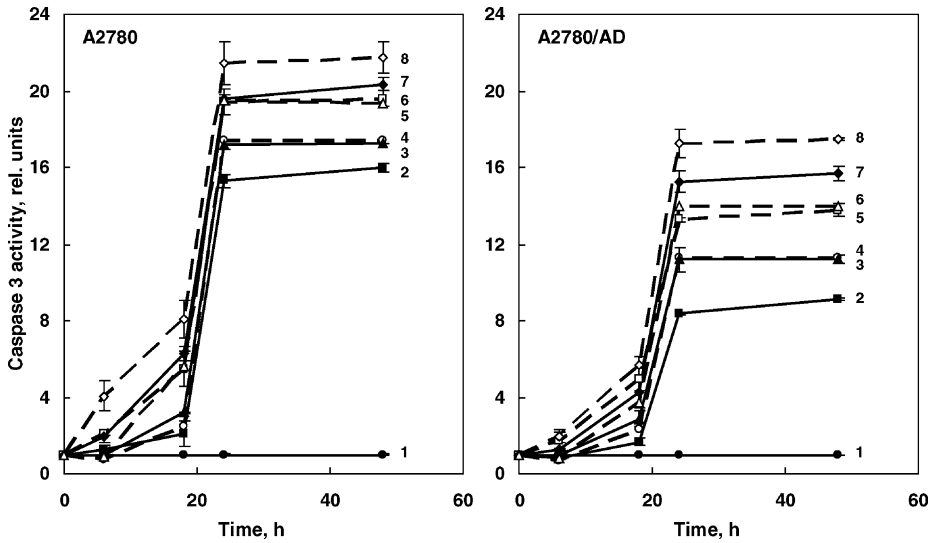

Fig. 8. Time-dependent activity of caspase 3 in A2780 and A2780/AD human ovarian carcinoma cells. Activities in the control (normoxia, no treatment) were set to a relative unit of 1. Means ± S.D. from four independent measurements are shown: 1 – normoxia, no treatment (control); 2 – normoxia, liposomal HIF1A antisense oligonucleotides; 3 – hypoxia, no treatment; 4 – hypoxia, liposomal HIF1A antisense oligonucleotides; 5 – normoxia, DOX; 6 – normoxia, DOX, liposomal HIF1A antisense oligonucleotides; 7 – hypoxia, DOX; 8 – hypoxia, DOX, liposomal HIF1A antisense oligonucleotides.

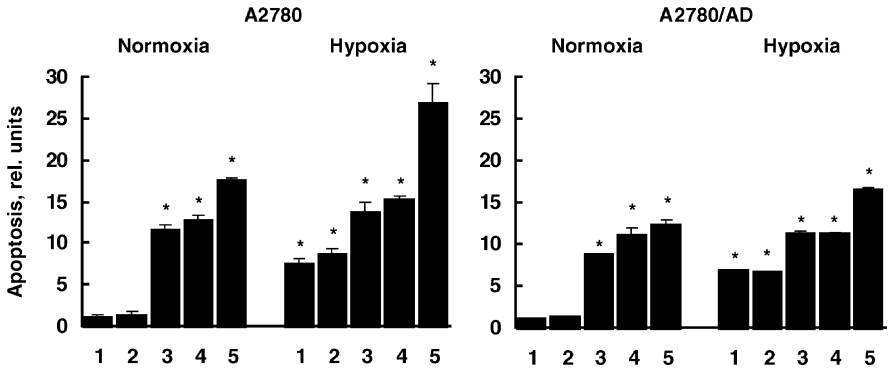

Fig. 9. Apoptosis induction in A2780 and A2780/AD human ovarian carcinoma cells. The enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in control (normoxia, no treatment) cells was set to 1 unit, and the degree of apoptosis was expressed in relative units. Means ± S.D. from four independent measurements are shown: 1 – no treatment (control); 2 – empty liposomes; 3 – liposomal HIF1A antisense oligonucleotides; 4 – DOX; 5 – DOX, liposomal HIF1A antisense oligonucleotides. $*P < 0.05$ when compared with control (normoxia, no treatment).

DOX substantially upregulated this expression. Both effects evidently were dependent on the HIF protein, because the suppression of its expression by liposomal ASO targeted to HIF1A mRNA significantly diminished them. The expression of the TOP2A gene under hypoxia increased and under the DOX exposure decreased. After the combined action of hypoxia, DOX and HIF1A ASO a net decrease in the expression of TOP2A mRNA is observed. Therefore such combination was effective in terms of the preventing of the activation of DNA repair mechanisms associated with topoisomerase IIα – the main target of DOX.

The second difference is related to the different influence of hypoxia and DOX on pump resistance. As expected, the *MDR1* which encodes in human the main drug efflux pump – P-glycoprotein – was expressed only in multidrug-resistant variant of human ovarian carcinoma cells. We found that the expression of this gene was unchanged after the exposure to hypoxia, DOX and their combination with or without liposomal ASO targeted to HIF1A mRNA. The expression of the drug efflux pump, encoded by members of the MRP family (*MRP1*) was observed in both sensitive and multidrug-resistant cells. It was also observed in sensitive cells after DOX exposure. Hypoxia decreased the expression of *MRP1* gene and did not influence on the *MRP2* gene in the absence of DOX. In contrast, DOX upregulated the expression of both *MRP1* and *MRP2* genes. Therefore, hypoxia suppressed cellular pump resistance while DOX activated pump resistance. It is interesting that ASO targeted to HIF1A mRNA significantly suppressed DOX-induced activation of MRP-related drug efflux pumps and did not interfere with the effects of hypoxia. As a result, the combination of DOX and hypoxia with ASO targeted to HIF1A successfully suppressed this type of multidrug resistance. Therefore, the combination of endogenous hypoxia and treatment with DOX and liposomal ASO targeted to HIF1A might be very effective in suppressing multidrug resistance in resistant cancers which predominantly overexpress drug efflux transporters from the MRP cluster, such as certain types of lung cancer [20–22]. Experimental data showed that DOX, hypoxia and liposomal ASO applied alone or used in the combination did not significantly influence on P-glycoprotein family of drug efflux pumps in MDR1-expressing multidrug-resistant ovarian carcinoma cells. Previously we found that prolonged incubation of sensitive human ovarian carcinoma cells with low concentrations of DOX progressively increased their resistance and finally led to the development of stable P-glycoprotein-dependent multidrug resistance [23]. A significant increase in cellular resistance to DOX was seen long before the overexpression of the *MDR1* gene encoding P-glycoprotein efflux pump. Taking into account the present data about the induction of MRP-dependent multidrug resistance by DOX, we can suggest that the increase in the resistance of sensitive cells in the early phase of adaptation to drug was caused by the overexpression of other multidrug-resistant transporters which are related to the MRP protein family. Our previous data which showed that the suppression of this early rapid phase of multidrug resistance prevented the development of later stable MDR1-gene-dependent resistance [23], lead to the conclusion that liposomal ASO targeted to HIF1A might be used to suppress the resistance in drug-sensitive cancer cells and prevent the development of stable multidrug resistance. Once again we would like to state that the suppression of MRP-type of drug resistance might prevent the development of P-glycoprotein type of multidrug resistance that is observed in most tumors during chemotherapy. More importantly, the present investigations show that the DOX-HIF1A ASO combination will be effective in cancers that express P-glycoprotein, not only in cancers that overexpress MRP proteins. This hypothesis, if confirmed by future experiments, may result in a completely new approach in preventing the development of multidrug resistance during cancer chemotherapy.

However despite certain differences in their mechanisms of action, hypoxia, DOX and liposomal ASO targeted to HIF1A mRNA showed synergism in terms of cell death induction. Experimental data showed that apoptosis was more pronounced after the combined action of hypoxia and

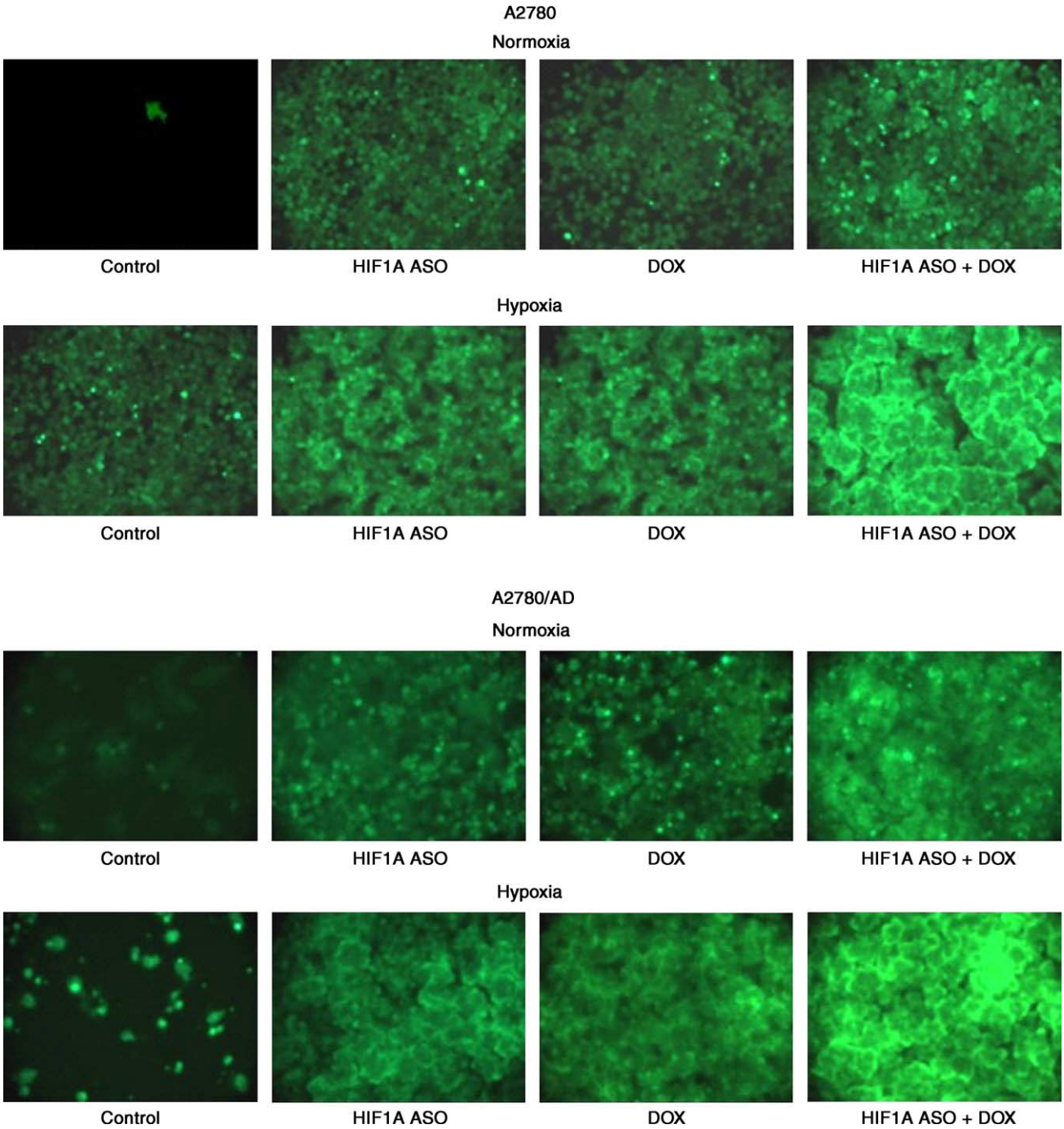

Fig. 10. Typical fluorescence microscopy images of TUNEL-labeled A2780 and A2780/AD human ovarian carcinoma cells.

DOX. HIF1A ASO in all cases enhanced the apoptosis induction be hypoxia, DOX and their combination and had the ability to induce apoptosis even under normoxia.

The present paper demonstrates a bimodal role of HIF1A protein during hypoxia. The overexpression of this protein induced by hypoxic conditions triggers apoptosis in cancer cells. On the other hand, this protein plays a critical role in the activation of cellular antiapoptotic defense under hypoxia. Similar to hypoxia, HIF1A protein plays two opposite roles during DOX exposure, initiating apoptosis and simultaneously activating cellular pump and nonpump resistance. Data obtained after the application of liposomal ASO targeted to HIF1A mRNA showed that activation of antiapoptotic cellular defense overwhelms the apoptosis induction. Hypoxia suppresses the antiapoptotic overexpression in the presence of DOX and thus more significantly inhibits cellular defense. Apoptosis induction mechanisms not related to the expression of HIF1A remain intact under these conditions and therefore lead to the enhancement of apoptosis induction. This confirms the possibility of using HIF1A protein as target for cancer chemotherapy.

In conclusion, the results of present experimental work further support the proposal that liposomal ASO targeted to HIF1A mRNA can potentially be used to enhance the efficacy of cancer chemotherapy. This effect of ASO is based on their ability to suppress existing pump and non-pump resistance and prevent their activation after treatment with an anticancer drug. Moreover, ASO targeted to HIF1A mRNA that suppress cellular defense might be used as a powerful tool to enhance the anticancer action of cytotoxic drug or even as an anticancer agent especially on the background of endogenous tumor hypoxia in solid tumors.

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