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ORIGINAL CONTRIBUTIONS

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CHARACTERISTICS OF HOMOCYSTEINE-INDUCED MULTIDRUG RESISTANCE OF HUMAN MCF-7 BREAST CANCER CELLS AND HUMAN A2780 OVARIAN CANCER CELLS

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Aim: **To study the influence of homocysteine on the mechanisms of drug resistance formation.** ***Methods:*** **In current study human MCF-7 breast cancer cells and A2780 ovarian cancer cells sensitive to anticancer drugs were used. To access the viability of cells, we applied 3-[4,5-dimethylthiazol-2–1]-2,5-diphenyltetrazolium bromide colorimetric assay (MTT-test). Expression of Bcl-2, p-glycoprotein (P-gp), glutathione S-transferase (GST) and E-cadherin was studied by immunocytochemistry.** ***Results:*** **A2780 and MCF-7 cells were treated by homocysteine. It was shown that every next treatment with homocysteine (up to 5th) decreased the sensitivity of A2780 and MCF-7 cells to cytotoxic drugs. Immunocytochemical study of molecular profile of A2780 and MCF-7 cells after long-term cultivation with homocysteine has been carried out and has revealed that such treatment resulted in the induction of Bcl-2, P-gp, GST and E-cadherin expression. This indicates that incubation of studied cells with homocysteine leads to simultaneous induction of expression of drug resistance markers to cisplatin and doxorubicin.** ***Conclusion:*** **Cultivation of MCF-7 and A2780 cells with homocysteine leads to simultaneous development of resistance to doxorubicine and cisplatin. The development of drug resistance is diverse for different drugs and varies among cell lines.**

Key Words: **MCF-7 cells, A2780 cells, cisplatin, doxorubicin, drug resistance, homocysteine, immunocytochemistry.**

Drug resistance is a complex and comprehensive problem of modern oncology that determines a number of failures during therapy of cancer patients. The study of mechanisms of the development of resistance to cancer preparations is important for understanding the way of correction of tumor cell phenotype toward elevation of its sensitivity to chemotherapy.

Due to the development of resistance, cells acquire new properties that are reflected at morphological level as well as on the change of their molecular, phenotypic and biochemical patterns [1–8]. That's why comparative study of biological patterns of sensitive cell and its resistant analog will help to identify the mechanisms of drug resistance development.

Cultivated lines of resistant cells of different histogenesis may serve as a convenient model for such research. The method of formation of drug resistance, a main method of experimental oncology, is grounded on prolonged cultivation of tumor cells in the medium with gradually increasing concentrations of anticancer preparations [9]. However, the method is associated with certain difficulties. Firstly, this process requires significant time period; for example, achievement of resistance to doxorubicin in human MCF-7 breast cancer cell line requires at least 4–5 months (resistance level 16), to cisplatin — 7–8 months (resistance level 4), while the development of resistance to doxorubicin in human A2780 ovarian cancer cell line occupies 11–12 months (resistance level 6), and to cisplatin — 6.5–8 months (resistance level 8). Secondly, drug resistance in human tumor is often characterized by cross-resistance to two and more anticancer preparations, while the mentioned method allows achieve the resistance only to one anticancer preparation at the time. Thirdly, further cultivation of resistant cells without addition of anticancer preparations leads to significant decrease of resistance level [9].

It is known that in blood plasma of patients with tumors of different localization elevated level of homocysteine could be detected, and in some cases it may be explained by the development of drug resistance. Homocysteine is a sulfur-containing amino acid that is not present in natural proteins but is an intermediate product of exchange between amino acids methionine and cysteine [10–14]. According to the data of numerous studies [15–22], elevated homocysteine content is directly linked to deficiency in methyl groups what in turn leads to hypomethylation. It is known that disturbance in DNA methylation map plays a role in regulation of expression of genes [20–25], the protein products of which determine different mechanisms of drug resistance formation: functioning of transport systems (genes *mdr1* [24–28], *mrp1* [27–29], *lrp* [28, 29]) that results in decreased intracellular accumulation of cytostatic preparations; altered proteins — apoptosis regulators (genes *p53* [29], *bcl-2* [30, 31]); elevated activity of detoxification systems (genes *GSTπ* [31], *MT* [31–33]), system of DNA-adducts reparation (gene *MGMT* [33]) that appear during interaction of a number of anticancer preparations with DNA molecule, etc. So, the study of influence of elevated concentrations of homocysteine on the mechanisms of formation of drug resistance allows reveal the initial chains involved in the development of this process.

MATERIALS AND METHODS

Parental human MCF-7 breast cancer cells and A2780 ovarian cancer cells sensitive to anticancer

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Abbreviations used: GST – glutathione S-transferase; MAbs – monoclonal antibodies; MTT – 3-[4,5-dimethylthiazol-2–1]-2,5-diphenyltetrazolium bromide; P-gp – p-glycoprotein.

drugs (cisplatin and doxorubicin) were studied. Cisplatin and doxorubicin were from Ebewe, Austria. The cells of both lines were cultured in modified Dulbecco ISCOV medium (Sigma, Germany) supplemented with 10% of fetal calf serum (Sangva, Ukraine) at 37 °C in humidified 5% CO_2. Cells were cultured for 24 h, and then homocysteine (Sigma, Germany) was added to culture medium at the concentrations of 50 or 100 mM. Incubation with homocysteine lasted for 72 h, then the cells were passaged, the cycle of abovementioned treatment was repeated 10 times, and then the cells were cultured for 3 months without homocysteine. The control cells of both lines were cultured without addition of homocysteine to culture medium.

Cytotoxicity test was performed after 1, 3, 5, and 10 cycles of homocysteine treatment and after 3-months period of homocysteine-free cultivation using 3-[4,5-dimethylthiazol-2–1]-2,5-diphenyltetrazolium bromide (Sigma, Germany) in standard MTT-test [34].

Expression of surface and intracellular protein (apoptosis regulator Bcl-2), proteins associated with drug resistance (glycoprotein (P-gp) and glutathione S-transferase (GST)), molecules of intercellular adhesion (E-cadherins) was studied by immunocytochemical peroxydase-antiperoxydase (PAP) method using murine monoclonal antibodies (MAbs), secondary rabbit antibodies against mice immunoglobulins, and complex of MAbs against peroxydase with raddish peroxidase, and also EnVision visualization system (DakoCytomation, Denmark) [2, 35].

RESULTS AND DISCUSSION

It has been shown that 72 h incubation of ovarian cancer cells of A2780 line with homocysteine resulted in the decrease of their sensitivity to the action of cisplatin by 2.5-fold, and to doxorubicin — by 2-fold, compared to the control cells, while 3rd treatment with homocysteine — by 2.7-fold and 2.2-fold decrease, and after 5-th — 4.5-fold and 2.7 decrease, respectively. It's necessary to note that further cultivation of the cells with homocysteine didn't lead to elevation of their drug resistance level.

An acquirement of drug resistant phenotype in A2780 cells treated for long time with elevating concentrations of cisplatin occurs via antiapoptotic program [2] and is accompanied by an appearance of expression of Bcl-2 oncoprotein and GST — the protein responsible for intracellular detoxification (Fig. 1) [2]. Also, development of resistance to doxorubicin in these cells occurs via induction of P-gp and E-cadherin expression (see Fig. 1) [2]. So, development of resistance to cisplatin and doxorubicin in cells of this histogenesis occurs via different mechanisms. That's why immunocytochemical study of molecular profile of A2780 cells after long-term cultivation with homocysteine has been carried out, and has revealed that such treatment resulted in the induction of Bcl-2, P-gp, GST and E-cadherin expression (Fig. 2). This indicates that incubation of A2780 cells with homocysteine leads to simultaneous induction of expression of drug resistance markers which are common in cells resistant to cisplatin and doxorubicin, and intensity of immunocytochemical reaction (i. e. higher level of markers expression) elevates along with increasing number of passages.

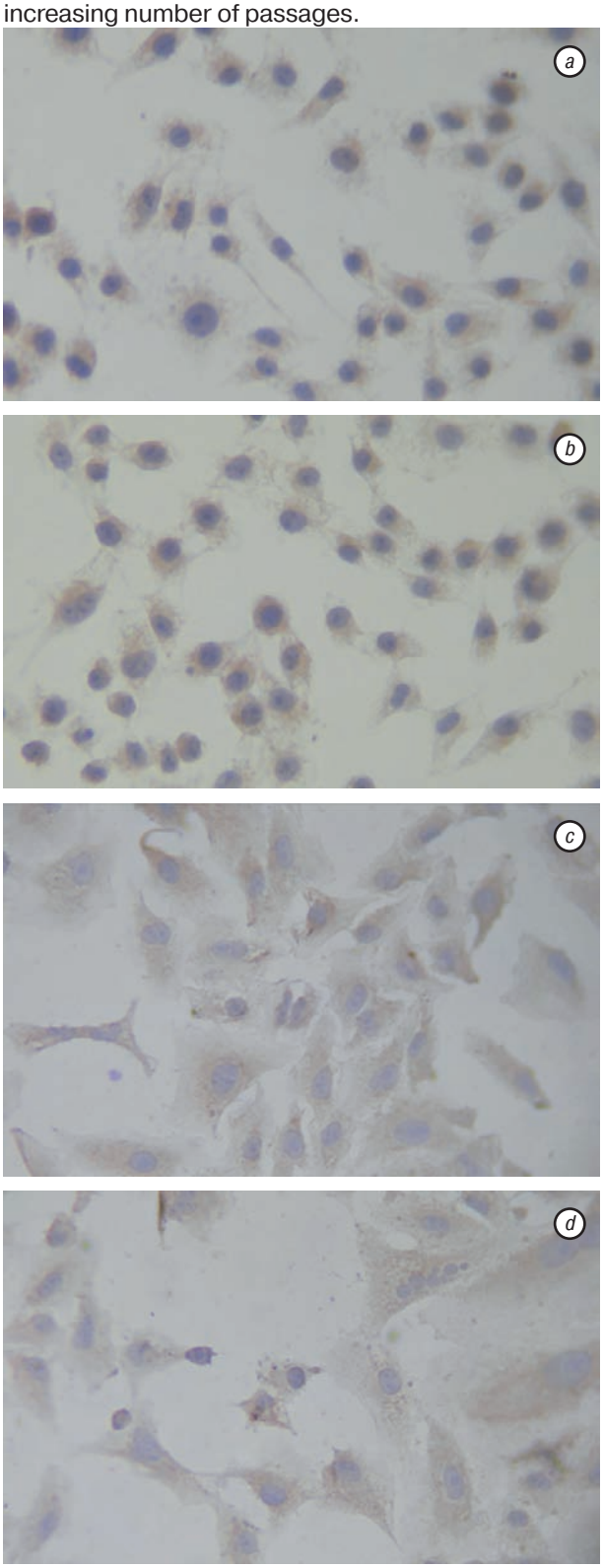

Fig. 1. Bcl-2 (*a*) and GST (*b*) expression in A2780/DDP cells, P-gp (*c*) and E-cadherin (*d*) expression in A2780/Dox cells

Incubation of MCF-7 cells with homocysteine for 72 h resulted in the decrease of cells sensitivity to doxorubicin by 2.5-fold and to cisplatin — by 1.5-fold, compared to the control, while after third introduction of homocysteine into the culture medium the degree of MCF-cells resistance was equal to 3, and to cisplatin —

1.7, and after the fifth treatment these values were equal to 5.0 and 2.5 respectively. Further cultivation of MCF-7 cells with homocysteine had no additional impact on their resistance to mentioned drugs.

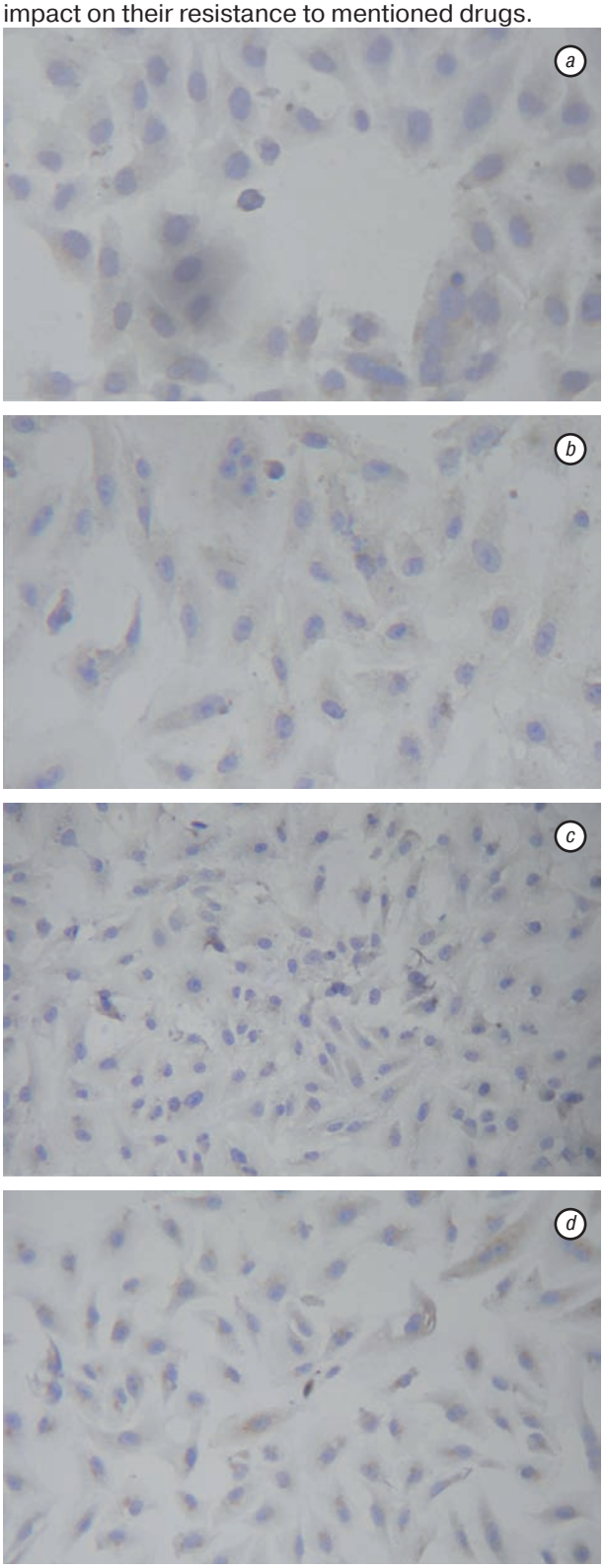

Fig. 2. Bcl-2 (*a*), P-gp (*b*), GST (*c*) and E-cadherin (*d*) expression in A2780 cells with homocysteine-induced resistance

It is known that the development of drug resistance in MCF-7 cells upon action of elevating concentrations of cisplatin and doxorubicin is accompanied by the increase of cell adhesion (Fig. 3) [36]. Development of resistance to cisplatin in MCF-7 cells occurs via antiapoptotic mechanisms (decreased Bcl-2 expression) and the system of intracellular detoxification (GST) [36], while doxorubicin-resistant MCF-7 cells acquire such properties via MDR-dependent mechanisms (elevated P-gp expression) (see Fig. 3).

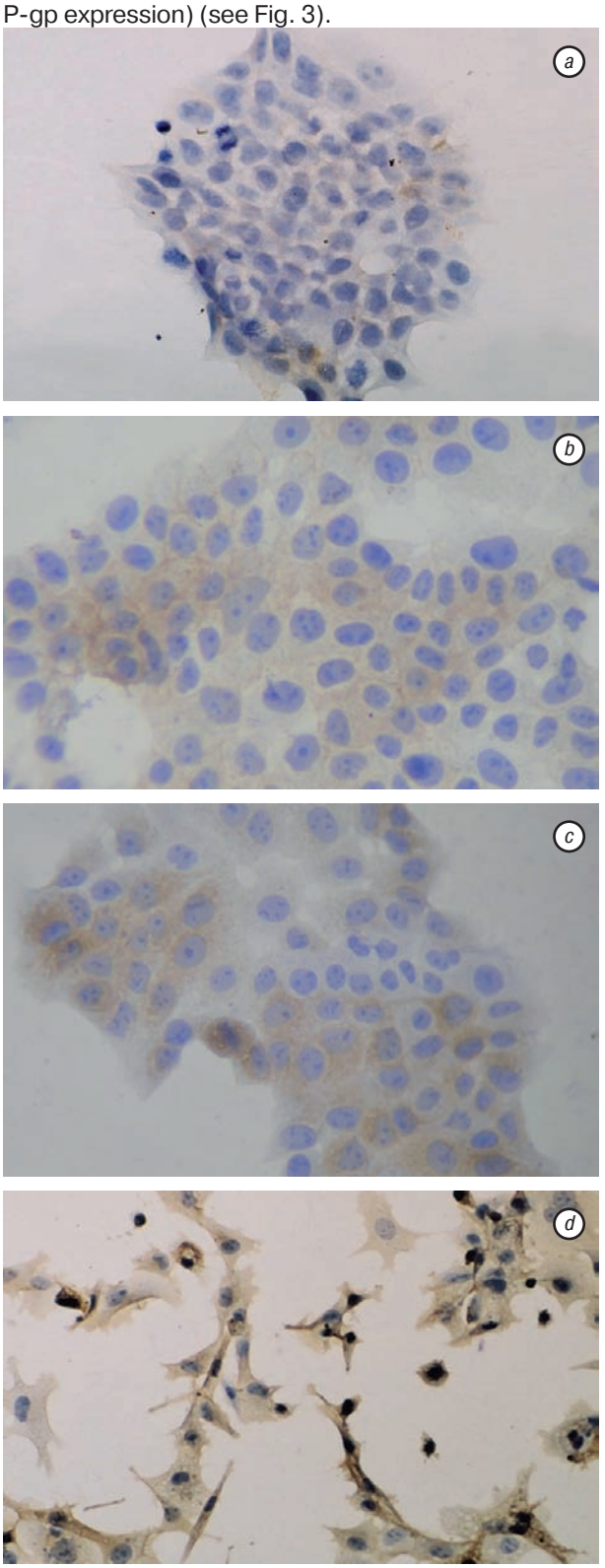

Fig. 3. Bcl-2 (*a*), GST (*b*) and E-cadherin (*c*) expression in MCF-7/DDP cells, P-gp (*d*) expression in MCF-7/Dox cells

Immunocytochemical study has demonstrated that incubation of MCF-7 cells with homocysteine resulted in the induction of P-gp, GST and E-cadherin expression and significant decrease of Bcl-2 (Fig. 4). So, our

data have shown that alteration of molecular profile of MCF-7 cells upon the action of homocysteine reflects an involvement of both mechanisms responsible for the development of resistance to cisplatin and doxorubicin.

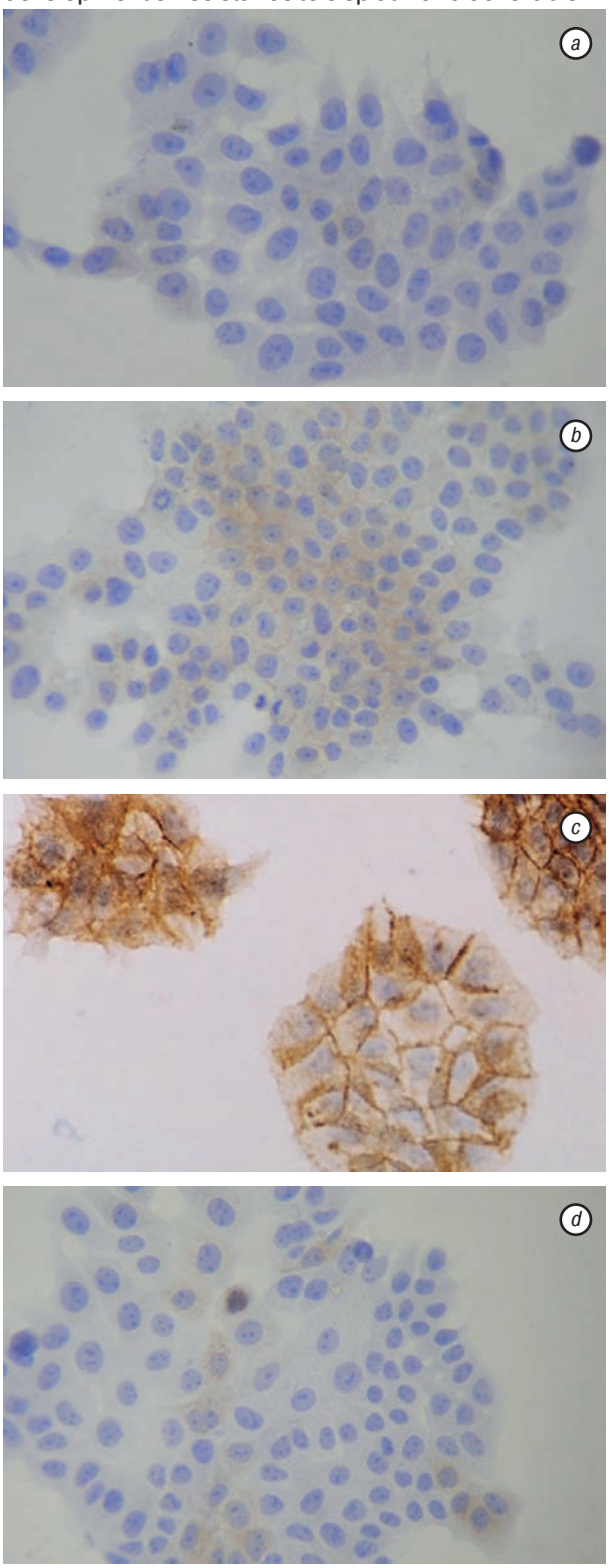

Fig. 4. Bcl-2 (*a*), GST (*b*), E-cadherin (*c*) and P-gp (*d*) expression in MCF-7 cells with homocysteine-induced resistance

It has been shown that further three months long cultivation of drug resistant cells in the medium without homocysteine doesn't lead to decrease of resistance level.

So, cultivation of MCF-7 and A2780 cells with homocysteine leads to simultaneous development of resistance to doxorubicin and cisplatin. Increase of homocysteine concentration in culture medium over certain critical level causes maximal tension of systems of its utilization that can't release the cell from its excess, what results in total genome demethylation upon conditions of deficiency of methyl groups donors, and consequently leads to elevation of activity of the majority of genes thus creating the grounds of tumor cell genetic instability.

Summarizing abovementioned, one should note that the creation of *in vitro* hyperhomocysteinemia model allows in 2–3 weeks receive cell strains with cross-resistance to doxorubicin and cisplatin. The recent studies have shown that the development of multidrug resistance phenotype involves a number of mechanisms and activation of respective genes; altered P-gp, GST and E-cadherin expression are directly linked to DNA methylation [13, 14, 37, 38]. From other hand, hypomethylation of DNA may be caused by disturbed metabolism of folic acids that leads to hyperhomocysteinemia.

Along with this, the mechanisms of formation of drug resistance upon elevated homocysteine level are far from being clear yet. Such process is associated with altered DNA methylation map (its hypomethylation) due to deficiency of methyl groups that appears as a result of compensatory elevation of activity of the systems of homocysteine utilization in response to its increased concentration in intracellular medium, and, possibly, due to inhibition of functions of DNA-methyltransferase-1 via deficiency of its substrate. Further studies of homocysteine-induced multidrug resistance in MCF-7 and A2780 cells will be required for understanding the ways to overcome natural and acquired drug resistance.

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