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A CHANGE IN THE SULFHYDRYL STATUS OF EHRLICH CARCINOMA CELLS BY EXPOSURE TO LIGHT IN THE PRESENCE OF CHLORIN-e_6 TRIMETHYL ESTER. THE EFFECT OF L-BUTHIONINE-[S, R]-SULFOXIMINE ON THE PHOTOSENSITIZED DAMAGE OF TUMOR CELLS

Only a complete understanding of the molecular mechanisms of a photosensitized destruction of tumor cells may contribute to a considerable increase in the effectiveness of laser photodynamic therapy (PDT). In particular, in the photodynamic influence (PDI) caused by the haematoporphyrin derivative (HpD) or chlorin-e_6, damage of the membrane structures of the target cell plays a significant role [1, 2, 3]. Thus, by photoirradiation of neoplasm cells in the presence of these photosensitizers (PS), the swelling of the former, an increase in the permeability of the cytoplasmic membrane and appearance of protrusions of different shape and number on its surface are observed [2]. At the same time, such damage of cells may lead to the rupture of their cytoplasmic membrane. Since by photoexcitation of the sensitizer the generation of active forms of oxygen takes place, then, in the opinion of some authors, the destruction of cellular membrane structures may result from the stimulated lipid peroxidation (LP), as well as the damage of sulfhydryl groups of the amino acids contained in the protein cellular membrane [1, 4]. In particular, on irradiation of neoplasm cells in the presence of HpD, Reyftmann et al. [5] observed the accumulation of fluorescent lipofuscin-like pigments considered to be the major product of LP.

As is generally known, in the protection of cells from the toxic effect of H_2O_2 as well as in the degradation of lipoperoxide compounds, the reduced glutathione (GSH) and some GSH-dependent enzymes play a key role. In mammalian cells at least two enzymes capable of reduction of organic hydroperoxides have been detected. The selenium-containing glutathione peroxidase (GP) consumes rapidly both H_2O_2 and organic hydroperoxides, including the lipoperoxides of fatty acids, acyl glycerides, steroids, and prostaglandins. Glutathione-S-transferase (GST), which is similar to nonselenium GP, reduces actively only organic hydroperoxides, but not H_2O_2 [6, 7].

A high content of GSH and the above GSH-dependent enzymes is often observed in tumors of different histogenesis, which exhibit resistance to some traditional chemotherapeutic agents, including alkylating ones, platinum preparations, and anthracyclinic antibiotics [8, 9, 10].

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The different roles of GSH, GP, and GST in the vital activity of a cell and its resistance to toxic effects made us undertake a study of their share in the resistance of tumor cells to photosensitized damage, using L-buthionine-[S, R]-sulfoximine (BSO), a low-toxity inhibitor of GSH biosynthesis [11, 12]. Chlorin-e_6 trimethyl ester (E6) was used as PS. This class of compounds, characterized by a low phototoxic effect on the skin, a rapid removal from the organism, and high spectral indices, may serve as an alternative to HpD PS, being promising for clinical use [13].

Materials and Methods

E6 was prepared by the method described by Lötjönen and Hynninen [14] on the basis of pheophytin extracted form nettle leaves. HpD was synthesized from haematoporphyrin IX dihydrochloride (Aldrich) according to the original method of Lipson et al. [15] modified by Kessel et al. [16].

Ehrlich ascites carcinoma (EAC) cells were weekly transplanted intraperitoneally on 25—30 g mongrel female mice, 3×10^7 cells per animal. Six days after inoculation of EAC the tumor-bearing animals were injected intraperitoneally with BSO (Sigma), 1 g per kg of body weight. The control group of EAC recipient animals was injected with an adequate volume of 0.9% NaCl. Eighteen hours later the tumor was withdrawn from the mouse abdominal cavity, 5 vols. of 0.9% NaCl were added and centrifuged at 4 °C for 6 min. The precipitated cells were resuspended in 0.9% NaCl and stored over melting ice.

The sensitivity of EAC cells to photosensitized injure of both the control group of animals and those treated with BSO was estimated *in vitro*. For this purpose the cell suspension was diluted with a physiological solution up to a concentration of 5×10^6 cells/ml, sodium phosphate buffer (pH 7.4) and glucose were added up to 10 mM and 5.55 mM, respectively. Immediately after adding the PS dissolved in 0.9% NaCl (for HpD) or in a minimal amount 5—10 μl of dimethyl sulfoxide (for E6), the cells were irradiated with light at 665 or 630 nm for E6 or HpD, respectively. The irradiation was carried out in quartz cuvettes (layer thickness 2 cm) by magnetic stirring at 20 °C, a 1 kW xenon lamp instrumented by a focusing optical system serving as the radiation source.

The proportion of damaged cells in the suspension was evaluated by staining them with 0.05% trypan blue (TB) in phosphate buffer (pH 7.2). The effectiveness of photosensitized damage was evaluated by the irradiation time during which 50% of the cells became stained ($t_{1/2}$).

The amount of non-protein (acid-soluble) sulfhydryl groups (NpSH) and proteins-associated sulfhydryl groups (PrSH) in EAC cells was determined by the method of Ellman [17]. In particular, 0.6 ml of 15% trichloroacetic acid (TCA) was added to $10—50\times10^6$ cells resuspended in 1.2 ml of 0.9% NaCl and incubated at 0—4 °C for 1 hour. Then the sample was centrifuged, the supernatant was separated and used to determine NpSH groups. For this purpose 0.9 ml of the extract was neutralized up to pH 7.2, adding 1.2 ml of 0.5 M sodium phosphate buffer (pH 6.8) and 0.3 ml of 1 M NaOH. Then, immediately prior to measurement, 0.3 ml of the 5,5′-dithiobis-2-nitrobenzoic acid (Sigma) 15 mg/ml solution in 10 mM sodium phosphate buffer (pH 6.8) was introduced. The optical density of the sample was recorded on a spectrophotometer at 412 nm and 0.9% NaCl was used instead of the NpSH extract in the control cuvette.

To determine the PrSH groups, the protein precipitate of EAC cells was washed twice with 10% TCA, dissolved in 2 ml of 0.125 mM TRIS-HCl buffer containing 8 M urea, and the pH was brought to 7.4. Then the sample was centrifuged at 4000 revs/min for 15 min. A transparent supernatant was used to determine the intracellular amount of PrSH groups. Subsequent analysis was carried out similarly to that described above for the NpSH groups.

The intensity of glucolysis was evaluated by glucose consumption and the amount of lactate formed. The carcinoma cells were incubated on a water bath at 37 °C in 0.9% NaCl containing 10 mM sodium-phosphate buffer (pH 7.4) and up to 0.2% of glucose. The density of the cellular suspension was 1×10^7 cells/ml. The lactate content was measured enzymatically, using a Sigma diagnostic kit, that of glucose colorimetrically by using standard *o*-toluidine reagent.

Results and Discussion

It was established that photoirradiation of EAC cells in the presence of E6 resulted in a considerable decrease in the intracellular content of both NpSH and PrSH groups (Fig. 1). At the same time, a practically complete staining of cells (with TB) was associated with an about 50% decrease in the content of NpSH and 30% decrease in the amount of PrSH groups (Fig. 1). What causes a decrease in the intracellular content of sulfhydryl groups? It may be suggested that this process is the result of their direct photosensitized oxidation. However, in the noncellular system, at an adequate light dose this PS did not induce

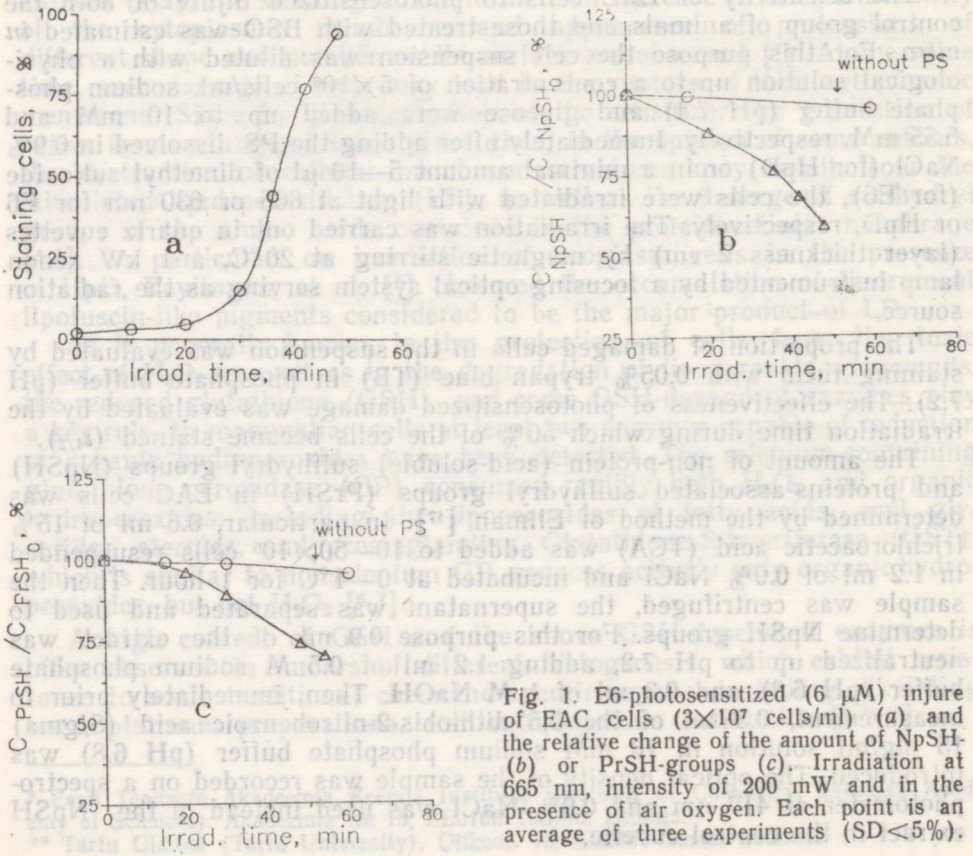

Fig. 1. E6-photosensitized (6 μM) injure of EAC cells (3×10^7 cells/ml) (*a*) and the relative change of the amount of NpSH- (*b*) or PrSH-groups (*c*). Irradiation at 665 nm, intensity of 200 mW and in the presence of air oxygen. Each point is an average of three experiments (SD<5%).

the photodestruction of SH-groups of L-cysteine and GSH. As the content of NpSH groups of mammalian cells in GSH is 90%, then, most obviously, a decrease in the number of NpSH groups results from an enzymatic consumption of GSH in the reduction reactions of H_2O_2 or the lipoperoxide compounds formed:

$$HOOH \text{ or } ROOH + 2GSH \rightarrow H_2O \text{ or } ROH + GSSG + H_2O.$$

As the intracellular amount of NpSH groups decreases about 1.5 times as fast as that of PrSH ones, it may be supposed that by the photosensitized damage of tumor cells GSH somewhat protects PrSH groups from oxidative dectruction. This suggestion is supported by a strong positive relationship between the contents of PrSH and NpSH groups in both intact EAC cells (Fig. 2) and those treated with BSO (Fig. 3), i. e. the higher the number of NpSH groups (GSH) in the cell, the higher the amount of PrSH groups detected in it. Moreover, we have established that the GSH administered exogenously increased slowly (about 15—20%) the resistance of EAC cells to the E6-photosensitized damage [2]. The low effectiveness of exogenous GSH may be caused by a low permeability of the cytoplasmic cell membrane with respect to the said peptide [11].

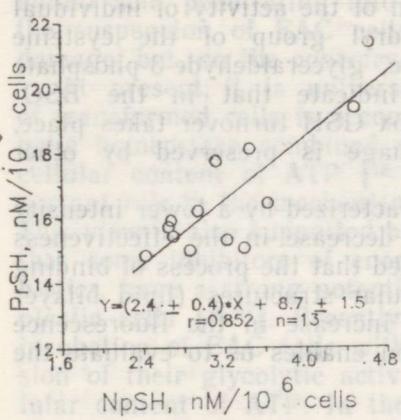

Fig. 2. The relationship between the amount of NpSH- and PrSH-groups in the intact EAC cells.

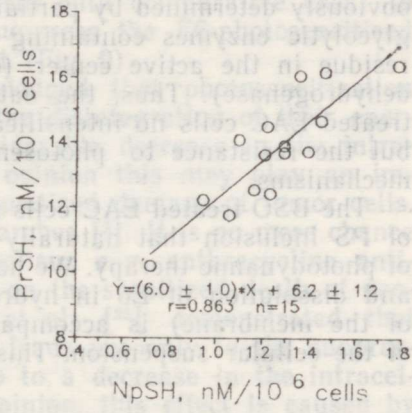

Fig. 3. The relationship between the amount of NpSH- and PrSH-groups in the EAC cells pretreated with BSO.

Thus, the problem is whether the intracellular GSH determines the resistance of neoplastic cells to photosensitized damage. It turned out (Table) that the BSO-induced (the inhibitor of γ-glutamylcysteine synthetase) 2.5-fold decrease in the content of GSH in EAC cells did not increase (as expected) their sensitivity to photodynamic therapy, but, on the contrary, an almost 30% reliable increase in their resistance could be observed.

By such a considerable suppression of GSH biosynthesis, the protection of cell from the toxic effect of H_2O_2, an effective degradation of the lipoperoxidation compounds formed and, consequently, the maintenance of resistance of tumor cells to PDI are possible only by a considerable activation of the pentose phosphate pathway of glucose turnover. As is known, such activation of the dehydrogenation reaction of glucose-6-phosphate provides the supply of NADPH which is necessary for reducing the oxidized glutathione by glutathione reductase.

The effect of L-buthionine-[S, R]-sulfoximine on the intracellular amount of SH groups intensity of glycolysis, binding of photosensitizer, and the resistance of EAC cells to photodynamic effect of chlorin e_6 trimethyl ester (mean ±S. D.)

EAC cells	$t_{1/2}$, min	Rate of glucose utilization, nM/min/10^6 cells	Intensity of anaerobic glycolysis, nM lactat/ /min/10^6 cells	NpSH-groups content, nM/10^6 cells	PrSH-groups content, nM/10^6 cells	Binding of E6 with EAC cells, min^{-1}
Intact	21.9±1.3 $n=13$	3.31±0.16 $n=16$	5.45±0.18 $n=16$	3.14±0.14 $n=20$	17.05±0.49 $n=19$	0.0487±0.0012 $n=4$
Pretreated with BSO	27.2±1.6 $n=13$	2.87±0.18 $n=16$	4.95±0.24 $n=16$	1.31±0.05 $n=21$	13.8±0.4 $n=21$	0.0516±0.0015 $n=3$

$t_{1/2}$ — time of irradiation during which 50% of the cells become stained.

In fact (Fig. 4), there is a positive correlation between the intensity of glycolysis and the level of GSH in EAC cells. However, the BSO-treated neoplastic cells are characterized not only by decreased glucose consumption, but also by intensive anaerobic glycolysis (Table). This is obviously determined by partial suppression of the activity of individual glycolytic enzymes containing the sulfhydryl group of the cysteine residue in the active center (e. g. aldolase, glyceraldehyde-3-phosphate dehydrogenase). Thus, the data obtained indicate that in the BSO-treated EAC cells no intensification of redox GSH turnover takes place, but the resistance to photosensitized damage is preserved by other mechanisms.

The BSO-treated EAC cells may be characterized by a lower intensity of PS inclusion that naturally leads to a decrease in the effectiveness of photodynamic therapy. We have established that the process of binding and dissolution of E6 in hydrophobic cellular structures (lipid bilayer of the membrane) is accompanied by an increase in the fluorescence of the cellular suspension. This phenomenon enables us to evaluate the

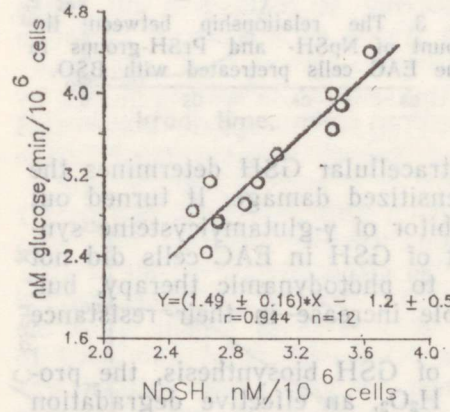

Fig. 4. The relationship between the intensity of aerobic glucose utilization and the amount of NpSH-groups (GSH) in the intact EAC cells.

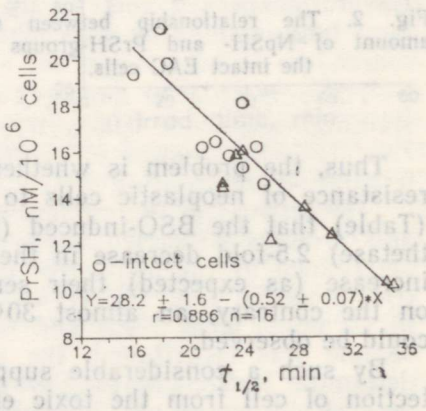

Fig. 5. The relationship between the content of PrSH-groups in intact or pretreated with BSO EAC cells and the resistance to PDT in the presence of E6 (2μM). Irradiation at 665 nm; intensity 200 mW; $t_{1/2}$ — time of irradiation during which 50% of the cells become stained.

accumulation of PS in EAC cells. It appeared that a typical kinetic dependence reflecting the binding of E6 to the neoplastic cells is described by a first-order equation. The calculations made indicate that the constant of binding of E6 (Table) is practically similar for both the intact and carcinoma cells pretreated *in vivo* with BSO. In addition, a considerable decrease in the intracellular content of GSH caused by BSO did not provide an increase in the photosensitivity of EAC cells also by using HpD as PS.

It should be pointed out that the inhibition of GSH biosynthesis in neoplastic cells is associated with a negligible 20—30% decrease in the amount of PrSH groups (Table). On the basis of the data obtained (Fig. 5) it was established that there is an inverse correlative dependence between the content of PrSH groups and the resistance of EAC cells to photosensitized damage, i.e. the smaller the amount of PrSH groups in EAC cells, the less considerable the intensity of their photosensitized injure.

Thus, irrespective of the fact that GSH has a considerable protective effect upon the PrSH groups (Fig. 2), the EAC cells, characterized by a low intracellular content of PrSH (Fig. 5) and NpSH groups (Figs. 6 and 7) and a low glycolytic activity (Fig. 8), have a higher resistance to photosensitized inactivation. At the same time, this is not dependent on the nature of the PS used. Moreover, the addition of L-cysteine (the amino acid penetrating into the cytoplasm with comparative ease) to the suspension of EAC cells did not suppress the E6-photosensitized damage, but, on the contrary, promoted it (Fig. 9).

At present, it is universally acknowledged that photosensitization of transformed cells is accompanied by the disintegration of their energetic homeostase combined with a considerable decrease in the intracellular content of ATP [18, 19]. In our opinion this may play an important role in the mechanism of photosensitized damage of tumor cells. This view is also supported by other researches [2]. It is no mere chance that some inhibitors of energetic metabolism, e. g. anthracycline antibiotics, have a strong potentiating effect on the sensitized death of neoplastic cells [20, 21]. Recently Guminska et al. [22] demonstrated that incubation of EAC cells with L-cysteine leads not only to the suppression of their glycolytic activity, but also to a decrease in the intracellular content of ATP. In the authors' opinion, this effect is caused by the suppression of the activity of the pyruvate-kinase isoform sensitive

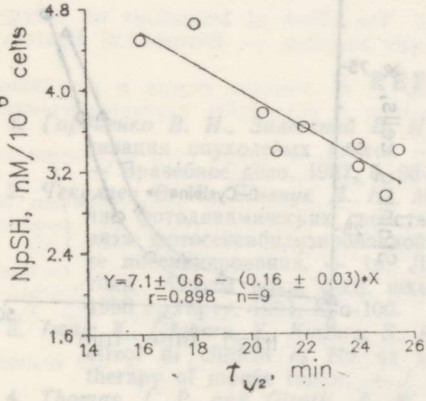

Fig. 6. The relationship between the content of NpSH-groups (glutathione) in intact EAC cells and its resistance to PDT during photoirradiation. See Fig. 5 for irradiation conditions.

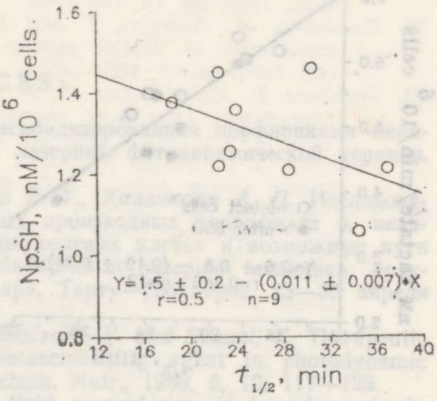

Fig. 7. The relationship between the content of NpSH-groups (glutathione) in EAC cells (pretreated with BSO) and its resistance to PDT during photoirradiation. See Fig. 5 for irradiation conditions.

to L-cysteine. The potentiating effect of cysteine on the photosensitized destruction of EAC cells is therefore easy to explain.

A graph of the dynamics of photodamage of EAC cells vs. decrease in their content of PrSH and NrSH groups (Fig. 1) shows that the avalanche-like staining of neoplastic cells is preceded by a relatively negligible decrease (about 25%) in the overall content of SH groups. We are of the opinion that such a negligible change in the intracellular content of SH groups cannot be responsible for subsequent events leading to an irreversible damage of carcinoma cells, in particular, their membrane structures.

Thus, the results obtained indicate that the resistance of EAC cells to E6- and HpD-induced PDI is determined neither by GSH nor the group of enzymes dependent on GSH. On the contrary, when studying the role of GSH and selenium-dependent glutathione peroxidase in the mechanism of photokilling of cells. Thomas and Girotti [4] observed that the L1210 cells which either grow in a Se-free medium or are subjected to BSO-treatment, are characterized not only by an intensive LP, but also by a noticeable decrease in survival by their HpD-induced photosensitization. Such disagreement between our conclusion and those of other researchers about the role of GSH in the resistance of neoplastic cells to photodynamic therapy is obviously due to differences in the histological type of the experimental models used. The latter may considerably differ in antioxidant activity which is of importance in the suppression of LP. It was recently shown that the antioxidative activity of selenium cannot be caused only by the functioning of the selenium-dependent glutathione peroxidase mechanisms of hydroperoxide consumption [23]. Besides, catalase and such protein as ceruloplasmin may take part in the suppression of LP whose expression has been recorded in tumors of nonliver origin [24].

It should be pointed out that the role of LP in the mechanism of photosensitized damage of neoplastic cells has not yet been thoroughly studied. Thus, Chernitsky and Bolodon [25] observed that pretreatment of human erythrocytes with N-ethylmaleimide, despite a considerable decrease in the amount of GSH (to 20—30% of the initial) and the resulting stimulation of LP, did not lead to any increase in the rate of their photosensitized hemolysis. The authors reached a conclusion that

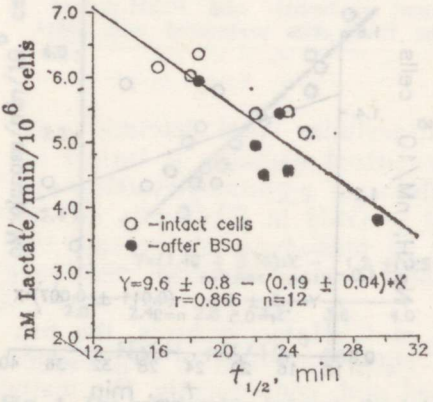

Fig. 8. The relationship between anaerobic glycolysis (lactat production) of intact or pretreated with BSO EAC cells and the resistance to PDT during photoirradiation. See Fig. 5 for irradiation conditions.

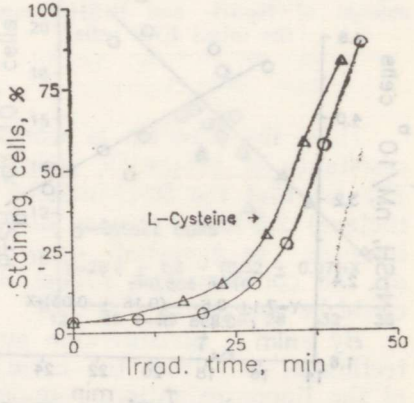

Fig. 9. The intensity of E6-photosensitized (2μM) injure of EAC cells (5×10^6 cells/ml, pH 7.4, 5.55 mM glucose) without or in the presence of 1 mM L-cysteine. Irradiation at 665 nm and intensity of 125 mW.

the intensification of LP observed by photohemolysis of erythrocytes is the result rather than the reason of erythrocytes lysis. It is known that such strong inhibitors of SH groups as *cis*-platinum preparations widely used in the chemotherapy of malignant neoplasm of humans may contribute considerably to LP [26]. However, as shown by Nahabedian et al. [21], their combination with HpD-sensitized PDI did not lead to a noticeable potentiating effect.

Why do the EAC cells pretreated with BSO and characterized by a decreased intracellular content of PrSH groups exhibit a raised resistance to E6-sensitized treatment (Table, Fig. 5)? In our opinion, this may be caused by a joint effect of different mechanisms. First, by the suppression of the mitotic activity of neoplastic cells and, second, by partial inhibition of the functional activity of some ionic pumps.

In fact, the neoplastic cells which are in the *S*-phase of a mitotic cycle, have a higher sensitivity to PDI [1]. However, a considerable decrease in the content of reduced GSH induced by BSO or N-ethylmaleimide may lead to a decrease in the proliferative activity of transformed cells [27, 28]. This may be accounted for by an important role of GSH in the regulation of microtubular proteins assemble in the spindle system [28].

It is known that up to 60% of the energy generated is consumed by the cell for the functioning of its ionic pumps. This consumption is especially high in tumor cells. In this connection, K^+, Na^+-ATP-ase, whose activity is greatly determined by the SH group, is of special interest. Also, cysteine and the reduced GSH not only inhibit the inactivation of this ATP-ase with thiol toxins, but also act as its activators. Thus, the BSO-induced decrease in the content of GSH, obviously, releases the above mechanisms that leads to a noticeable reduction in energy expenditure and, as a result, to a decrease in the photosensitivity of neoplastic cells.

The investigations carried out indicate that the decrease in the content of SH groups observed by irradiation of EAC cells in the presence of E6 is the result rather than the reason of their photosensitized damage. A comparison of the results obtained with those of other researchers shows that the effectiveness of combining PDI with the use of the preparations decreasing the intracellular amount of reduced glutathione is obviously predetermined by the histological type of target cells and specificities of their antioxidant systems.

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EHRLICHI ASTSIITSE KARTSINOOMI RAKKUDE SULFHÜDRIILSE TASEME MUUTUS VALGUSE EKSPOSITSIOONIL JA KLORIIN-e_6 TRIMETÜÜLESTRI MANULUSEL. L-BUTIONIIN-[S, R]-SULFOKSIMIINI MÕJU KASVAJARAKKUDE FOTOSENSIBILISEERITUD VIGASTAMISE EFEKTIIVSUSELE

On leitud, et sulfhüdriilsete (SH) rühmade hulga langus Ehrlichi astsiitse kartsinoomi rakkudes (EAK) kloriin-e_6 ja valguse toimel on eelkõige tagajärg, mitte aga rakkude fotosensibiliseeritud vigastuste põhjus. On täheldatud glutatiooni biosünteesi 2,5-kordset ja valkassotsieerunud SH-rühmade hulga 30-protsendilist langust siis, kui EAK eeltöödeldi glutatiooni biosünteesi inhibiitoriga — L-butioniin-[S, R]-sulfoksimiiniga (BSO). BSO-ga indutseeritud sulfhüdriilsete rühmade hulga langus ei suurendanud EAK tundlikkust fotodünaamilisele mõjustusele (FDM), vaid vastupidi, selle resistentsus isegi kasvas 1,5 korda. Saadud tulemuste analüüs ja nende vastandamine teiste autorite andmetega näitavad, et FDM-i ja rakusisest redutseeritud glutatiooni taset vähendavate preparaatide kombinatsiooni efektiivsus sõltub tõenäoliselt märkrakkude histoloogilisest tüübist ja antioksüdeerimissüsteemi iseärasustest.

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ИЗМЕНЕНИЕ СУЛЬФГИДРИЛЬНОГО СТАТУСА КЛЕТОК АСЦИТНОЙ КАРЦИНОМЫ ЭРЛИХА ПРИ СВЕТОВОЙ ЭКСПОЗИЦИИ В ПРИСУТСТВИИ ТРИМЕТИЛОВОГО ЭФИРА ХЛОРИНА e_6. ВЛИЯНИЕ L-БУТИОНИН-[S, R]-СУЛЬФОКСИМИНА НА ЭФФЕКТИВНОСТЬ ФОТОСЕНСИБИЛИЗИРОВАННОГО ПОВРЕЖДЕНИЯ ОПУХОЛЕВЫХ КЛЕТОК

Показано, что снижение содержания сульфгидрильных групп, наблюдаемое при облучении клеток асцитной карциномы Эрлиха (АКЭ) в присутствии хлорина e_6, является скорее всего не причиной, а следствием их фотосенсибилизированного повреждения. Действительно, предобработка клеток АКЭ L-бутионин-[S, R]-сульфоксимином (БСО) — ингибитором биосинтеза глутатиона — снижала до 2,5 раза содержание глутатиона и приблизительно на 30% уровень белоккоциированных SH-групп. Однако эти индуцированные БСО изменения сульфгидрильного статуса опухолевых клеток не повышали их чувствительность к фотодинамическому воздействию (ФДВ). Более того, их резистентность возрастала почти в полтора раза. Анализ полученных результатов, а также их сопоставление с данными других исследователей показывают, что эффективность комбинации ФДВ с препаратами, понижающими внутриклеточный уровень восстановленного глутатиона, предопределяется, по-видимому, гистологическим типом клеток-мишеней и особенностями их антиоксидантных систем.