INTRODUCTION

One of the main characteristics of malignant cell metabolism is that the tumor growth is never followed by an adequate vascularisation and these cells are faced with episodes of shorter or longer hypoxia and ischemia, with concomitant periods of reoxygenation. Permanent anoxia or ischemia lead to cell death and tumor necrosis. However, during the process of tumor evolution, cells are often exposed to shorter or longer periods of ischemia, followed by reoxygenation and recirculation. In accordance with this assumption is the fact that malignant cells are very resistant and well adapted to anoxia, remaining vital for a long time under anaerobic conditions (1,2).

Besides the well-known increase in glycolysis, one of the mechanisms of this adaptation is utilization of glutamine as a very important energy fuel for malignant cells and precursor of glutathione synthesis (2,3).

Numerous studies showed high rate of glutamine oxidation in tumor cells, but this oxidation is only partial and results in accumulation of glutamate and aspartate (4).

ALTERATIONS IN ENERGY METABOLISM INDUCED BY HYPOXIA AND REOXYGENATION MAY HAVE IMPORTANT INFLUENCE ON CELLULAR METABOLISM IN GENERAL AND FUNCTIONING OF ANTIOXIDATIVE SYSTEM, THAT IS FOLLOWED BY POSSIBILITY OF MULTILEVEL ADAPTATION OF MALIGNANT CELLS TO OXIDATIVE DAMAGE, REGENERATION OF THE ADENINE NUCLEOTIDE POOL (ADN) AND REGULATION OF CELLULAR ENERGY STATE (5).
The hypoxia of malignant cells followed by inhibition of DNA replication presents one of serious problems of cytostatic treatment and radiotherapy. The degradation of AdN pool during the course of anoxia is inevitable, while the reoxygenation will lead to regeneration of this pool if the changes are reversible (1).

The aims of our study was to elucidate the effects of transitory anoxia and reoxygenation on malignant cells and to study the role of supplementation of some metabolites, such as glutamine, glucose, N-acetylcysteine (NAC) and iodoacetate (IA) on GSH/GSSG system, which is a very important antioxidative mechanism in asctic carcinoma cells (13).

**MATERIALS AND METHODS**

Ehrlich ascites tumor cells (EAC) were propagated in the abdominal cavity of Swiss mice and harvested 7 days after transplantation. The same procedure was performed with cells of AS-30D hepatoma (ASH) in Sprague-Dawley rats. Cells of EAC and ASH were washed in Krebs-Ringer phosphate solution of the following composition: 145 mM NaCl, 5 mM KH2PO4, 1.5 mM MgSO4 and 6 mM KCl. The final pH was 7.4 and the temperature was 0°C. All experiments were performed in vitro. The cells were incubated at 30°C in the same physiological solution. In case when glucose was added, the solution contained 20 mM Tris-HCl and 20 mM MOPS, pH 7.4 in order to increase buffer capacity. Incubation of the cells was carried in an Eppendorf tubes (1.5 ml), full of cell suspension (approx. 120-130 mg wet wt. cells/ml), tightly closed in order to maintain the anaerobic conditions. Reoxygenation was performed under air in 25 ml Erlenmeyer flasks using Dubnoff metabolic shaker. The layer of the cell suspension was thin enough to avoid the lack of oxygen. After incubation, the reaction was terminated by the addition of sulfosalycilic acid (5% w/v) to the cell suspension. After centrifugation supernatant was decanted and used for the measurement of tGSH and GSSG. Concentration of these metabolites was determined by enzymic method (14). Cell weight was measured in 1.5 ml Eppendorf 3200 centrifuge tubes after centrifugation for 2.5 minutes and removal of supernatant. It was assumed that cell pellet contains 25% of incubation medium. One milligram of the pellet contains 0.1 mg of protein determined by the biuret method.

The measurements of the metabolites were done in triplicate, and means of three determinations were recorded. The crucial experiments were repeated two or three times and preliminary experiments were carried out in order to find proper experimental conditions (duration of anoxia and reoxygenation, concentration of supplemented metabolites). Viability of the cells was tested by trypan blue exclusion method and by measuring leakage of LDH into the incubation medium. The average viability of the cells was 95-96% of total cell number in thick suspension. All measurements are expressed per mg wet wt. of cells.

**RESULTS**

The influence of glutamine and glucose supplementation on GSH/GSSG system in EAC cells exposed to transitory anoxia

Anaerobic incubation for 60 minutes resulted in significant decrease of tGSH concentration (two times less than control) and may be the consequence of tGSH degradation. Accumulation of GSSG as a marker of oxidative stress and redox imbalance was also significant and it was followed by decrease of GSH concentration (only 20% in comparison to control aerobic group). Glutamine (10 mmol/l) and glucose (10 mmol/l) supplementation resulted in significant prevention of tGSH degradation and preservation of GSH/GSSG ratio. This ratio was 26.58 in aerobic control group of cells, in the presence of glutamine 5.1 and with glucose 3.65 in cells exposed to anaerobic conditions; without these substrates it was only 1.2.

The results of these experiments are presented in Table 1.

**Table 1.** Comparison of protective effect of glutamine and glucose on tGSH, GSSG and GSH concentration in EAC cells exposed to transitory anoxia (60 minutes) and reoxygenation (up to 120 minutes)

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>tGSH</th>
<th>GSSG</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>1.331</td>
<td>1.372</td>
<td>0.136</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>6.861</td>
<td>1.068</td>
<td>0.251</td>
</tr>
<tr>
<td>Anaerobic + Gln</td>
<td>1.051</td>
<td>1.198</td>
<td>0.153</td>
</tr>
<tr>
<td>Anaerobic+Gluc</td>
<td>6.959</td>
<td>1.189</td>
<td>0.124</td>
</tr>
</tbody>
</table>

Effects of N-acetylcysteine (NAC; 1 mmol/l) and iodoacetate (IA; 1 mmol/l) on GSH/GSSG system in EAC and ASH cells in transitory anoxia

The results of supplementation of EAC cells, exposed to anaerobic conditions during 60 minutes, with N-acetylcysteine are shown in Figures 1a, 1b and 1c.
The presence of NAC in medium resulted in less pronounced degradation of tGSH and disturbance of GSH/GSSG ratio. During reoxygenation, GSH/GSSG ratio increased from 4.8 to 5.7.

We incubated ASH cells under anaerobic conditions during 60 minutes in the presence of iodoacetate (1 mmol/l), which is the inhibitor of Embden-Meyerhoff’s glycolysis pathway. Figures 2a, 2b and 2c show that inhibition of glycolysis results in significant decrease of tGSH concentration (more than five times), and in significant disturbance of GSH/GSSG ratio (from 27.4 in control cell suspension to 1.25 after transitory anoxia and 2.17 after reoxygenation) during 60 minutes of anaerobiosis and reoxygenation (up to 120 minutes).
DISCUSSION

Many authors showed that anaerobiosis causes significant metabolic disturbances that induce increased sensitivity of cells to the effects of reoxygenation (2,6,7,8,15). The absence of expected further oxidative damage in EAC and ASH cells induced by reoxygenation, which is found in normal cells and tissues, may indicate some specificities of malignant cells phenotype, first of all the absence of enzyme xantine oxidase that is responsible for generation of superoxide during reoxygenation (4).

Reoxygenation is considered by many authors as a primary phase for generation of oxygen radicals. Jaescke et al. (10), in their studies of perfusates of rat liver and muscle (12), show that in hypoxic-anoxic phase, which precedes reperfusion and reoxygenation, GSSG as a marker of oxidative damage is intensively generated. This is followed by decrease of ATP concentration and leakage of LDH, which is a marker of cell membrane damage, while reoxygenation results in partial regeneration of glutathione, and this is in accordance with our results obtained with EAC cells. Supplementation of glutamine, which presents a major metabolic energy fuel and essential component for transformation to glutamate and precursor for glutathione biosynthesis, was performed in order to stimulate resynthesis and to prevent degradation of glutathione during anoxia and reoxygenation. Incubation of cells with metabolic substrates such as glutamine and glucose, which stimulate pentose-phosphate pathway and production of reductive equivalents, resulted in prevention of tGSH depletion and in partial preservation of GSH/GSSG ratio.

Experimental results of Kovačević et al. (1,16) show the increase of the total adenylate pool under aerobic conditions in the presence of glutamine and inosine as a result of AMP resynthesis in adenylosuccinate synthetase reaction. Anaerobic conditions induce depletion of one part of the pool, but the other part of the pool is stable. Frequent episodes of anoxia and reoxygenation result in significant utilization of aspartate that accumulates as a product of partial oxidation of glutamine. Generally, malignant cells and especially rapidly proliferating tumors, exist between aerobicosis and anaerobiosis. Adaptation to these conditions may be very important not only for proliferation of malignant cells, but also as a stimulus for tumor progression (15).

Our results showed different sensitivity of the glutathione system in EAC and ASH cells in conditions of oxidative stress induced by anaerobic incubation and reoxygenation. In EAC cells exposed to anoxia and reoxygenation, we observed the preservation of tGSH levels due to supplementation with N-acetylcysteine as intracellular scavenger of free radicals (17,18) and as a reducing thiol reagent. Hepatoma AS-30D cells, with a store of glycogen, showed relatively strong resistance to anaerobic incubation, followed by preservation of glutathione level, and this is significantly different from EAC cells' response to transitory anoxia. Addition of iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase in Embden-Meyerhoff's glycolysis pathway, to the medium was performed in order to inhibit glycolysis that preserves ATP level and energetically supports essential cellular functions. This may indicate the presence of the glycolysis in ASH cells without addition of glucose to medium. These results support our presumption that stimulation of pentose-phosphate pathway during oxidative stress is the factor that may influence protection of malignant cells from oxidative damage.

CONCLUSION

Our results indicate that in malignant cells, exposed to transitory anoxia and reoxygenation, persist constant cooperation between energy metabolism and antioxidative system of glutathione. Malignant cells showed some specificities in sensitivity and different response to transitory anoxia and reoxygenation, probably due to specificities of energy metabolism and enzyme function.

REFERENCES


