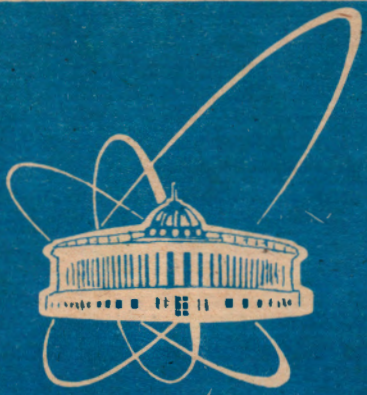

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N.L.Shmakova, V.I.Korogodin

ANAEROBIC GLYCOLYSIS AS A PROPERTY OF MALIGNANT CELLS AND ITS APPLICATION ASPECTS

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INTRODUCTION

The ability of malignant cells to brew glucose was discovered by Warburg (1) about seventy years ago. His follower Cori (2) found out that tumor cells feature this ability not only under hypoxia (the known "Pasteur effect") but also in the presence of oxygen. He called it the "inverse Pasteur effect". Since glucose fermentation may cause self-acidisation of tumor cells, he also came up with an idea that hyperglycemia can be used as an adjuvant in cancer treatment of some kinds.

In the late 1960s von Ardenne (3) decided to implement the idea and worked out a concept of "multistep therapy" for cancer. This concept was further developed in the Institute of Oncology and Radiology, Byelorussian Academy of Sciences (Minsk) (4). Von Ardenne's method was tested on 2500 patients with different malignant tumors. In some cases a few sessions of the hyperglycemia and radiation treatment revealed a noticeable advantage of the multistep therapy over traditional cancer treatments. It should be mentioned however that hyperglycemia, recommended for use in combination with other types of cancer therapy, is an extreme mode. In all cases system hyperglycemia three and more hours long was used. With radiation, local or general hyperthermia applied simultaneously or shortly after, this hyperglycemia is poorly tolerated, and the treatment results are often far from bright expectations. All this indicated that it is necessary to study cell mechanisms for hyperglycemia, optimize clinical hyperglycemia methods to increase its efficiency, and to find criteria for its individual application predictions.

We started this work about ten years ago. Experiments were carried out with Ehrlich ascites tumor cells, cells of various solid tumors and normal tissues of test animals.

MATERIALS AND METHODS

Cells of Ehrlich ascites tumor (EAT) or other solid tumors and normal tissues of rats were prepared by mechanical grinding and ferment treatment (for details see (5, 6)). Samples of these suspensions were subjected, in different combinations, to 1) incubation for different time at 37°C; 2) in a 0.8% solution of glucose; 3) incubation at different pH values produced in different ways; 4) determination of pH in suspensions of tumor

and normal cells; 5) irradiation with ^{137}Cs (γ-rays), 6) intravital staining of cells by trypan blue followed by counting the stained cells, 7) electron microscopy of cell samples, 8) determination of cell survival by ability of the cells to produce tumors. Methods for determination of EAT cell survival and for processing of results are described elsewhere (7).

RESULTS AND DISCUSSION

Combined effect of the glucose load and radiation. First of all we investigated the combined effect of the glucose load and radiation on EAT cells. The results of numerous experiments can be summed up as in Fig. 1 (see (6)). Cell incubation with glucose is seen to enhance the radiation effect. Yet, the efficiency of combined glucose-radiation treatment depends on conditions of EAT cell oxygenation under glucose loading. Only incubation under hypoxia, irrespective irradiation of cell conditions, showed a considerable increase in radiation damage. However the experiments failed to account for increasing radiation efficiency under glucose load. It is not clear whether radiosensibilization of EAT cells occurred or the glucose load effect and the radiation effect are independent.

In Fig. 2 there is an EAT cell survival curve for irradiation under hypoxia without glucose. The points, obtained in other experiments, are for irradiation of identical cells under hypoxia with glucose load. It is seen that the glucose load alone, without irradiation, decreases cell survival by two-four orders of magnitude depending on duration of incubation with glucose but does not affect either the size of the curve shoulder or the curve slope. This means that glucose load under hypoxia does not cause radiosensibilization of EAT cells but reduces their survival by the same value irrespective of whether or not they are irradiated.

The results obtained suggested independent, or additive damaging effect of radiation and glucose load on EAT cells (6). Another team arrived at similar results and conclusions using another strain of tumor cells and another survival test method (8). Our data were also confirmed in experiments with animals by the method of morphometric measurement of tumor damage by combined treatment with radiation and hyperglycemia (9)

Laws of tumor cell damage under the effect of glucose load. Death of tumor cells affected by glucose under hypoxic conditions indicates that this effect is associated with utilization of glucose and that this utilization obeys the Pasteur law, i.e. drastic enhancement of glucose fermentation at a lack of oxygen. In other words, the glucose effect is the result of cell energy exchange changing from breathing to fermentation at a lack of oxygen, which leads to production of lactic acid. Then one can assume that the glucose load effect will be the stronger, the larger the degree of self-acidisation of cells is. The correlation between the glucose effect and pH due to accumulation of lactic acid is confirmed by the following simple experiment. Cell samples were incubated with glucose or in phosphate buffers of different pH. The observed cell survival variations are displayed in Table1 and Fig. 3. A decrease in survival with increasing acidity is seen to be the same irrespective of the nature of the cell acidising agent. The threshold pH value, below which EAT cells die, is 5.6 (6).

Mechanisms for death of tumor cells under glucose load. EAT cell damage dynamics under the effect of glucose load was determined by counting cells stained by trypan blue at different time after their incubation with glucose under hypoxia and by the electron microscopy method.

Typical of this dynamics is quickly vanishing viability of thus treated cells: over 99% of them die within 24 hours. The type of death is early pycnotic degeneration characteristic of the radiation effect only on differentiative cells. This type of death is not usually observed after irradiation of tumor cells, whose death normally results from division, just as they try to undergo mitosis.

Electron-microscopic investigations revealed that after an hour long glucose treatment under hypoxia cells show sharp destructive changes which indicate irreversible damage. This kind of damage usually stems from increasing permeability of plasma membranes, including those of lysosomes. This in turn leads to damage of all cellular nucleus material (5). It is clearity seen in Fig.4, 5. The damage pattern of glucose-treated tumor cells makes independence of the glucose and radiation effects understandable. Quick death and destruction of cells damaged at low pH is due to breaking of their lysosomes with proteolytic enzymes. The rest of the cells die of

chromosome aberrations after irradiation. This occurs later, at an attempt to undergo mitosis.

Comparison of glycolytic activity of normal tissue, benign tumor and malignant tumor cells. The above data arouse a question of whether the glycolysis effect under hypoxia is specific to malignant tumor cells or it can be observed in cells of other, including normal, tissues.

To compare anaerobic glycolysis properties of malignant and benign tumor cells as well as of normal tissue cells, we took them from rats in which tumors of different histogenesis were induced by single irradiation (10). A measure of glucose load efficiency was a decrease in pH. The results are given in Table 2. It is seen that after the same glucose treatment under hypoxia a significant (i.e. capable of killing part of the cells) decrease in pH was observed only in malignant tumor cells. This decrease in pH was never observed in samples of normal tissues or benign tumors.

With these data, it is believed that the damaging effect of the glucose load on tumor cells occurs under three conditions. First, there must be an increased concentration of glucose in the cell environment. Secondly, cell must suffer lack of oxygen. Thirdly, cells must have fermentation systems to provide take-up of glucose in an amount far exceeding their energy needs. Only such hypertrophied systems of anaerobic glycolysis may cause lactic acid overload of cells resulting in their mass death.

This is what one observes in malignant tumor cells. The adaptive meaning of the phenomenon is clear. Malignant growth is usually accompanied with increasing hypoxia in the tumor. Under these conditions tumor cells can survive only because they are capable of eagerly taking up glucose from the surrounding tissues (11). As a result, first, the tumor cells meet their energy needs and, secondly, the surrounding tissue cells die being "deprived" of vital breathing substrate. Of course, this favours the invasive tumor growth. On the other hand, with excess glucose in the environment, this ability of malignant tumor cells may result in their death because of self-acidisation.

It is perfectly clear why normal tissue cells do not have this ability (with some exceptions, see [11]). In terms of energy, breathing is an order of magnitude more effective than fermentation, which fully meets the needs of normal cells. As to benign tumors, it is their inability to ferment glucose that seems to prevent them from

malignancy. In other words, it is ability to ferment glucose, especially under hypoxia, that is thought to be one of the most important biochemical differences between two major types of tumors (i.e. malignant and benign ones).

All this allows the following hypothesis to be formulated. The fundamental difference between malignant and benign tumor cells is the ability to utilize glucose under anaerobic conditions. This hypothesis can be verified by comparing genetic structures of both cell types. Their genes responsible for malignancy are thought to be identical. As to epigenome systems responsible for fermentation, they must be different. Naturally, malignant and benign tumor cells of the same histogenesis must be compared. As far as we know, nobody has carried out investigations like this as yet.

Some application aspects. The relation established between the lethal effect of glucose load on tumor cells and their anaerobic glycolysis property, elucidation of mechanisms for this phenomenon allow some conclusions of importance for using artificial hyperglycemia in cancer therapy.

First, artificial hyperglycemia can be used in traditional types of treatment for it tends to "knock out" hypoxic pool cells. As is known, these cells are more resistant to radiation and chemical agents and are often responsible for tumor recorence after radiotherapy or chemotherapy course. The remaining oxygenated tumor cells, which show far less distinct decrease in pH and are not damaged under artificial hyperglycemia, can be "finished off" by radiation and chemical agents.

Secondly, from the aforesaid one can infer that the curing effect of artificial hyperglycemia must be much higher in treatment of large, often nonresectable tumors. It is of particular importance for clinical practice.

Thirdly, artificial hyperglycemia combined with local circular hypoxia is believed to enhance the glucose load effect owing to both more intense glycolysis and violated microcirculation of the tumor together with slowed-down outflow of lactic acid. Violation of blood circulation in the tumor is considered particularly important in cancer treatment (12, 13).

Fourthly, the relationship between a decrease in pH under glucose load and its effectiveness indicates that one should continuously monitor intratumour pH during artificial hyperglycemia sessions. In other words, a decrease in pH in the tumor should

be used as an indicator of individual tumor sensitivity to a given effect. Tumor pH monitoring can also be used to determine the effective duration of artificial hyperglycemia. As shown above, a decrease in pH below the threshold within an hour is enough to ensure mass death of tumor cells. Therefore the duration of artificial hyperglycemia can be substantially reduced for tumors with quite high glycolytic activity as compared with the present-day practice (infusion for not less than three hours).

All the above concepts that we worked out on the basis of in vitro investigations were fully confirmed by the research in the Belarus Institute of Oncology and Radiology with several strains of transplantated rat tumors (14, 15) and with a limited number of patients (16). Morphological investigations proved that artificial hyperglycemia causes selective interphase death of cell in hypoxic areas of the tumor (9). Effectiveness of artificial hyperglycemia increased with tumor growth (14). The degree to which pH reduced tumors of patients was shown to correlate with the curing effect of artificial hyperglycemia followed up for a month (16). It was also proved that pH in the tumor tissue can be further decreased by local circular hypoxia (by applying a tourniquet to the thigh), which increased the antitumour effectiveness of patients' irradiation by over 3-fold (16).

Thus, positively treating the clinical results of using hyperglycemia in combination with irradiation or hyperthermia (4, 16-20), one can also state that the currently employed mode of artificial hyperglycemia is not an optimum and can be substantially improved by following the above recommendations. The extreme effect of prolonged systemic artificial hyperglycemia on a patient can also be reduced by changing it for regional one when possible.

The only example of using regional intra-arterial artificial hyperglycemia is described in (21), where it was included in combined treatment of locally extended cancer of oral cavity and nasopharynx mucosa. Combination of irradiation and regional hyperglycemia was found to halve the number of relapses and to increase the number of cured patients by almost two-fold as compared with gamma-ray teletherapy alone.

Of particular interest is the only case we know of treating a cancer patient with artificial hyperglycemia alone. According to Prof. A.F.Tsyb, director of the Institute of Medical Radiology, Russian Academy of Medical Sciences, in the clinic of this institute

they also used regional hyperglycemia alone, without irradiation, to treat a patient with a nonresectable tongue tumor. After a period of treatment, the tumor got soon inoculated without signs of intoxication, and no relapse was observed.

CONCLUSION

We have investigated a phenomenon of glucose fermentation by tumor cells, discovered by Warburg. It was confirmed that the phenomenon obeys the Pasteur law (i.e. drastic enhancement of glucose fermentation under hypoxic conditions) and shown that it is only typical of malignant tumor cells and is not observed in cells of benign tumors and normal tissues. A phenomenon of fast damage and death of tumor cells under hyperglycemia and hypoxia was also investigated. The main laws and mechanisms for antitumour effect of glucose load arising from self-acidisation of tumor cells by glycolysis products were explored. A new clinical application of artificial hyperglycemia was proposed for malignant tumor treatment.

The phenomenon can be further investigated in terms of fundamental and applied knowledge.

Fundamental aspects of the antitumour effect of glucose load are directly related to the problem of distinguishing between benign and malignant tumors. We put forward a hypothesis that tumors of both types contain identical genes responsible for uncontrolled multiplication of cells. Besides, malignant tumor cells have hypertrophied systems of anaerobic glycolysis, which makes them malignant in the end. The available methods of molecular biology and gene engineering allow experimental verification of this assumption.

Applied aspects are related to the use of artificial hyperglycemia for treatment of malignant tumors. This method can be first of all applied to large nonresectable tumors admitting of regional hyperglycemia and local hypoxia with simultaneous monitoring of tumor pH variation.

Table 1. The influence of medium pH on the viability of EAT cells measured in vivo

pH	Medium	Oxigen conditions	Cell survival (fraction)[a]	Average value for given pH[b]
7.0	buffer	N_2	2.9	2.9
5.6	buffer	N_2	1.9	
		O_2	6.8	2,6
	glucose load	N_2	1.9	
		O_2	1.8	
5.3	buffer	N_2	0.017	0.017
5.0	buffer	N_2	0.0010	
		O_2	0.0046	0.0021
	glucose load	N_2	0.0020	
4.5	buffer	N_2	0.0008	0.0008

[a] Cell survival is related to standard conditions.

[b] Average values calculated from average log values of cell survival.

Table 1. EAT cells were treated by 0.1 M phosphate buffer in medium at different pH under the condition of well oxygenation or in anoxia and by glucose load at pH 5.6 in the presence of oxygen or at pH 5.0 in hypoxia (these values are usually achieved by glycolisis). The influence of pH on cell's survival was independent on oxygen tension and on the way by which pH was achived.

Table 2. Results of determining glycolytic activity of normal tissue, benign and malignant tumor samples taken from rats

Tissue	No Sample	pH of suspension		
		Original	After incubation with glucose	Difference
Normal tissues				
Lung	1	7.00	6.60	0.40
	2	7.05	7.05	0.00
Kidney	1	7.30	6.95	0.35
	2	7.30	7.10	0.20
Spleen	1	6.75	6.45	0.30
	2	6.80	6.45	0.35
Marrow	1	6.55	6.25	0.30
Benign tumors				
Mammary gland	1	7.40	7.00	0.40
	2	6.90	6.60	0.30
	3	7.30	7.00	0.30
	4	7.00	6.70	0.30
	5	6.70	6.60	0.10
	6	7.10	7.00	0.10
	7	7.05	6.70	0.35
	8	7.3	6.60	0.70
	9	6.90	6,05	0.85
Malignant tumors				
Mammary gland	1	6.70	6.70	0.00
	2	6.80	5.95	0.85
	3	7.00	6.10	0.90
Thymus	1	6.45	5.90	0.55
	2	6.40	5.00	1.40
	3	6.05	4.65	1.40
Lung	1	6.90	6.00	0.90
	2	6.50	5.75	0.75
	3	6.90	6.00	0.90
	4	6.85	5.95	0.90

Table 2. The cell suspensions ($2 \cdot 10^6$ cells/ml in medium with 1 mM Hepes and 0.8% glucose) from normal, bening and malignant tissues were incubated under the anoxia during 1 hour. The difference between original and incubated with glucose pH was registered.

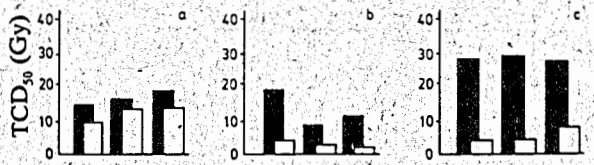


Fig. 1. The dependence of TCD_{50}(Gy) on the conditions of irradiation and treatment of EAT cells by glucose: the treatment by glucose in oxygen (a) and in anoxia (b,c), irradiation in oxygen (a,b) and in anoxia (c). Shaded and open areas correspond to irradiation only and irradiation with glucose load, respectively. Nine experiments. TCD is the radiation dose (Gy) of tumor cells to reduce tumor inoculability from 100% to 50%.

Treatment of EAT cells in vitro in medium containing 0.8% glucose. Viability was tasted in vivo by ability of cells to produce tumors. Anaxia was achieved by metabolic method.

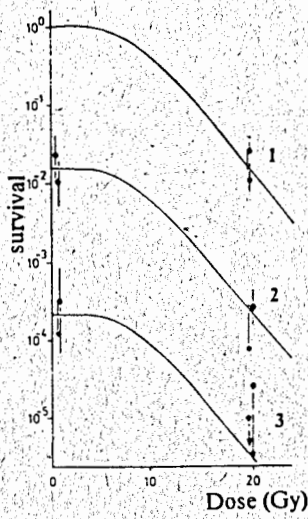


Fig. 2. Survival curve of EAT cells after irradiation in anoxia as compared with experimental points taken from in vivo tested viability of EAT cells: after irradiation by a dose of 20 Gy (1) and after combined treatment by irradiation and glucose for 15 (2) or 60 (3) minutes.

Survival curve EAT cells after irradiation in anaxia tested in vitro . Experimental poits - treatment of EAT cells performed in vitro in medium containing 0.8% glucose; viability tested in vivo by ability of cells to produce tumors.

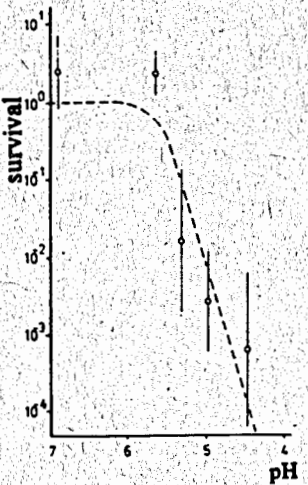
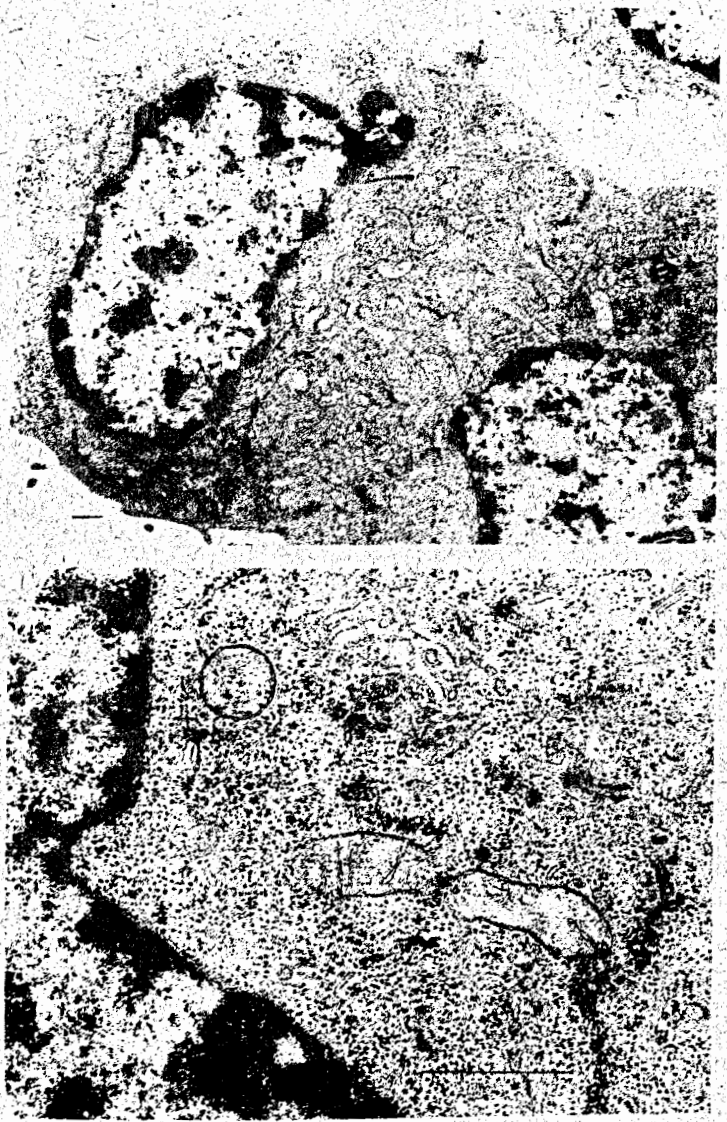


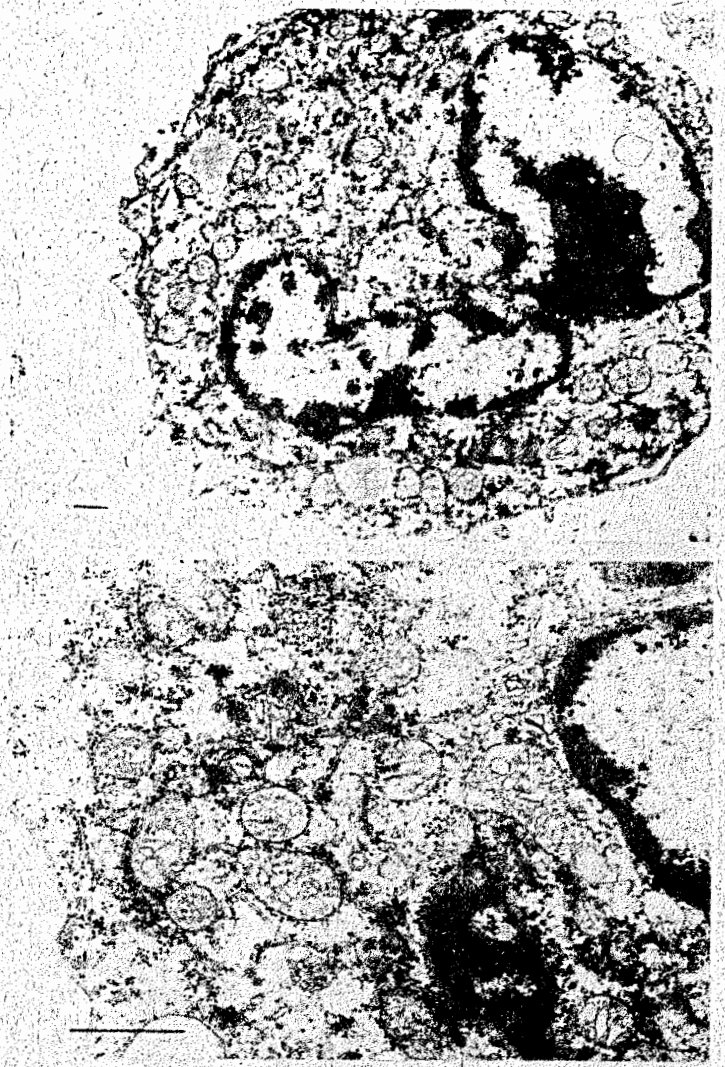
Fig. 3. The dependence of EAT cell survival after incubation at various pH on the value of pH. The curve has a threshold at pH 5.5 (see table 1).



(a)

(b)

Fig. 4. Morphology (a) and ultrastructure (b) of intact EAT cells.



(a)

(b)

Fig. 5. Morphology (a) and ultrastructure (b) of EAT cells after glucose load in vitro for 1 hour.

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