The Role of Glycolysis in the Growth of Tumor Cells

II. THE EFFECT OF OXAMIC ACID ON THE GROWTH OF HELA CELLS IN TISSUE CULTURE*

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The finding that potassium oxamate inhibits glycolysis by ascites tumor cells in vitro, without inhibiting oxidative metabolism (1), indicated that oxamate might be applied to the study of the role of glycolysis in tumor growth in vivo. If, as Warburg (2, 3) states, tumor cells derive much of their energy for survival and growth from glycolysis, then the inhibition of glycolysis in tumor-bearing animals should result in the regression of the tumors. At the same time, if normal cells do not depend on glycolysis for energy, they should not be affected by specific inhibitors of glycolysis. It therefore seemed of interest to use oxamate as a glycolytic inhibitor in ascites tumor-bearing mice.

However, preliminary experiments showed that although potassium oxamate is a nontoxic substance in doses up to 3 g per kg, it is excreted so very rapidly that it cannot be used conveniently as an inhibitor of glycolysis in animals.

It was decided, therefore, to study its effects on actively growing malignant cells in tissue culture, where the concentration of inhibitor can be readily controlled. Inasmuch as the ascites cells used previously could not be grown readily in culture, the experiments reported here were done with HeLa cells. It is the purpose of these experiments to test directly whether these cells are dependent on glycolysis for growth. A preliminary report of this work has appeared (4).

EXPERIMENTAL PROCEDURE

Cells—Two strains of HeLa cells were used, the HeLa 22a strain, donated to us by Dr. Michael Bender of the Oak Ridge National Laboratories, and the HeLa S-3 strain, supplied to us by Dr. Harold Nitowsky of Sinai Hospital, Baltimore, Maryland.

Although we have used the HeLa 22a strain successfully in several early experiments, it was found that, in our hands, the HeLa S-3 was a more vigorously growing strain. The HeLa S-3 cells were therefore used in the remaining experiments.

Media—The cells were grown in a medium described by Puck et al. (5, 6). The complete medium was made up before use by mixing the following three component solutions, in the designated proportions.

Oxamate—Potassium oxamate was prepared by partial ammonolysis of diethyl oxalate

Formulae—The sodium ion concentration in the media with and without sodium oxamate was kept constant by replacing the sodium chloride of Eagle's balanced salt solution (8, 9), and 20% human serum.

For stock cultures, the complete medium consisted of 40% nutrient solution (glucose, amino acids, vitamins, and growth factors in a balanced saline solution), 40% Hank's balanced salt solution (7), and 20% human serum. The medium used for experimental purposes consisted of 40% nutrient solution, 40% Eagle's balanced salt solution (8, 9), and 20% human serum. The sodium ion concentration in the medium with and without sodium oxamate was kept constant by replacing the sodium chloride of Eagle's balanced salt solution with equivalent amounts of sodium oxamate. The final media varied only in their oxamate ion and chloride ion concentration. The final media also contained penicillin and streptomycin to reduce the possibility of contamination. Final sterilization of the complete medium was achieved by passage through a Selas porcelain bacteriological filter with a maximal pore diameter of 0.02 μ. The human serum was obtained from Microbiological Associates, and from the Philadelphia Serum Exchange.

Conditions for Cell Growth—Stock cultures were grown on the flat glass surface of 100-ml milk dilution bottles containing 5 ml of the complete medium described above. The cultures were incubated at 37° in a constantly gassed (5% CO₂-95% air), humidified incubator. Stock cultures were trypsinized by addition of 5 ml of 0.05% trypsin-saline A (5) for 15 minutes at 37°, followed by the addition of an equal volume of complete medium which contained enough human serum to stop the action of trypsin. The cells were dispersed by rapid and repeated pipetting of the suspension. The dispersed cells were then transferred in 0.1 ml aliquots, into screw-cap tubes (5 in ¥ ½ in) which contained 1.5 ml of complete medium. The cultures were placed horizontally in racks in the gassed incubator at 37° for approximately 12 hours to permit the cells to adhere to the glass surface. Then the culture medium in the tubes was discarded and the experiment was started by the addition of 1.5 ml of fresh medium with and without oxamate. After a given period of incubation, the medium was poured off and analyzed for lactic acid and glucose content. The cells which remained adhering to the glass surface were washed three times with 5 ml of 0.1 M phosphate buffer, pH 7.4, and further treated for protein analysis. Each protein, glucose, and lactic acid determination reported is the average of three individual tube cultures identically prepared.

Chemicals and Analyses—Lactic acid determinations were done according to the method of Barker and Summerson (10). Proteins were determined by the Lowry method as modified by Oyama and Eagle for tissue cultures (11). The glucose determinations were made by the method of Nelson (12). Sodium oxamate was prepared by partial ammonolysis of diethyl oxalate.
Fig. 1. Protein data showing the growth of HeLa 22a cells in the presence of $8 \times 10^{-2}$ M sodium oxamate. The production of lactic acid and utilization of glucose corresponding to protein data are shown. The amount of lactic acid produced and glucose utilized is expressed as micromoles per milliliter of medium.

(13), followed by hydrolysis of ethyl oxamate with sodium hydroxide and crystallization from alcohol. Sodium pyruvate was obtained from the Mann Research Laboratories.

RESULTS

The effect of $8 \times 10^{-2}$ M oxamate on the time course of growth and glycolysis of HeLa 22a cells, over a period of 220 hours is shown in Fig. 1. The concentration of oxamate used has been found in previous experiments to result in 90 to 95% inhibition of glycolysis by Ehrlich ascites tumor cells, in vitro (1). These data show that in the presence of $8 \times 10^{-2}$ M oxamate the growth of HeLa 22a cells is completely inhibited. In fact, there is a significant drop in protein from the initial value of 78 μg of total protein to a constant value of approximately 40 μg of total protein. Microscopic examination of these cultures revealed that there were some cells which remained attached to the glass surface at the end of the experiment. These cells appeared unchanged morphologically.

The lactic acid production and the glucose utilization data are also shown in Fig. 1. These data essentially reflect the protein data. Inasmuch as in the presence of oxamate, both growth and lactic acid production were very strongly inhibited, it is impossible to determine from these data whether or not the inhibition of glycolysis was a secondary effect resulting from the inhibition of growth, or vice versa.

The effect of varying concentrations of oxamate on the growth and metabolism of HeLa S-3 cells is shown in Fig. 2. In this experiment, 10 cultures were set up for each concentration of oxamate used. The cultures were permitted to grow for 136 hours and at the end of this incubation period, the protein concentration, lactic acid production, and glucose utilization were determined. The protein data indicate that there is a slight inhibition of growth by $2 \times 10^{-5}$ M oxamate, and complete inhibition by $4 \times 10^{-4}$ M oxamate. Again it can be seen that the glucose and lactic acid data correspond to the protein data, i.e. that a given concentration of oxamate causes corresponding decreases in the growth and glycolysis of HeLa S-3 cells. The concentrations of oxamate correspond roughly to those needed for inhibition of glycolysis in resting ascites tumor cells (1), suggesting that the growth inhibition observed here may be due to inhibition of glycolysis.

In our earlier experiments (1), it was shown that Ehrlich ascites tumor cells in vitro will accumulate pyruvate in the presence of oxamate under anaerobic conditions, and that this pyruvate accumulation results in the reversal of the oxamate inhibition of glycolysis. This ability of pyruvate to reverse the effect of oxamate can be explained by the fact that oxamate is a competitive inhibitor of lactic dehydrogenase. Because this reversal of inhibition of glycolysis by pyruvate was observed with Ehrlich ascites tumor cells in vitro, it was thought possible that the same effect might be observed with growing cells, and that oxamate might be competitive with pyruvate with respect to cellular growth as well as with lactic dehydrogenase.

By the addition of $8 \times 10^{-2}$ M sodium pyruvate to the culture medium containing $8 \times 10^{-2}$ M oxamate, it was found that pyruvate could indeed reverse the inhibitory effect of oxamate on growth and glycolysis of HeLa S-3 cells. The protein, lactic acid, and glucose uptake data from this time course experiment are shown in Fig. 3. The protein data show that the rate of growth of HeLa S-3 cells in the cultures containing oxamate plus pyruvate is the same as that of the control cultures. There is a complete reversal by pyruvate of the oxamate effect on growth, and the data in Fig. 3 also show that pyruvate causes a complete reversal of the oxamate effect on glycolysis. The glucose uptake data indicate that less glucose is used in the
FIG. 3. Protein data showing the effect of $8 \times 10^{-2}$ M sodium oxamate on the growth of HeLa S-3 cells and the ability of $8 \times 10^{-2}$ M sodium pyruvate to reverse the inhibitory effect of oxamate. The production of lactic acid and utilization of glucose corresponding to the protein data are also shown. The amount of lactic acid produced and glucose utilized is expressed as micromoles per milliliter of medium. The cells were grown in 1.5 ml of complete medium composed of nutrients, balanced salts, and 20% human serum. The initial glucose concentration was 6.31 mM. The cultures were incubated at 37° in a humidified incubator gassed with 5% CO$_2$-95% air.

It should also be noted that the protein, lactic acid, and glucose data in Fig. 3 for the samples with oxamate alone show a definite reversal of the oxamate effect with time after 80 hours of incubation. This might be due to a gradual accumulation of pyruvate which after 80 hours is high enough in concentration to reverse the oxamate effect. It is also possible that the growth of these cells after 80 hours is a result of the selection of a small group of oxamate-resistant cells.

In the experiment shown in Fig. 4, the pyruvate concentration was varied from $8 \times 10^{-3}$ M to $8 \times 10^{-2}$ M, whereas the oxamate concentration was kept constant at $4 \times 10^{-2}$ M. The protein data show that in the presence of $8 \times 10^{-3}$ M pyruvate and $4 \times 10^{-2}$ M oxamate, a slight but significant reversal was obtained. The reversal is almost complete in the presence of $8 \times 10^{-4}$ M pyruvate, and complete reversal of oxamate inhibition of growth was obtained by $8 \times 10^{-3}$ M pyruvate. These protein data indicate that oxamate is competitive with pyruvate with respect to growth and also indicate that these cells may be dependent on glycolysis for growth.

FIG. 4. Protein data showing the effect of varying concentrations of pyruvate on the growth of HeLa S-3 cells in the presence and absence of sodium oxamate. The production of lactic acid and utilization of glucose corresponding to the protein data are also shown. The amount of lactic acid produced and glucose utilized is expressed as micromoles per milliliter of medium. The sodium oxamate concentration is $4 \times 10^{-2}$ M. The cells were grown in 1.5 ml of complete medium composed of nutrients, balanced salts, and 20% human serum. The initial glucose concentration was 6.85 mM. The cultures were incubated at 37° in a humidified incubator gassed with 5% CO$_2$-95% air.

DISCUSSION

The results show clearly that the oxamate ion, an inhibitor of glycolysis, can prevent the growth of HeLa cells in tissue culture. It is then natural to inquire as to what the same inhibitor would do to the growth of normal, nonglycolyzing cells. This is a difficult question to answer, since by ordinary techniques, embryonic or adult normal cells in growing cultures give rise to cell lines which exhibit a high rate of aerobic glycolysis (14-19). According to Eagle et al. (19), such "normal" cells are indistinguishable metabolically from tumor cells grown in culture. (However, see summary of (2), prepared by Burk.) As one might expect, such cells are also indistinguishable from HeLa cells with respect to oxamate inhibition of growth. Dr. Harold Nitowsky$^1$ has found that oxamate readily inhibits the growth of a culture derived by him from newborn human liver cells, which showed the same glycolytic and morphological characteristics as HeLa cells. Similarly, Mr. Edward Goldberg$^2$ has found that oxamate inhibits completely the growth of a culture of rapidly glycolyzing chick fibroblasts derived from the chick embryo, as well as the growth of Rous sarcoma cells. The question of whether or not oxamate will inhibit the growth of normal nonglycolyzing cells in culture must await the use of techniques for

$^1$ Personal communication.

$^2$ Unpublished experiments.
measuring growth in media which permit growth without dedifferentiation.

It has been tentatively concluded that oxamate inhibition of growth of animal cells is due to inhibition of lactic dehydrogenase. This conclusion is based on the following considerations. First, oxamate has been shown to act at the lactic dehydrogenase level when inhibiting the glycolysis of resting ascites tumor cells (1). Second, the concentration required for this inhibition of glycolysis by the ascites tumor cells corresponds closely to that required for growth inhibition with HeLa cells. Third, pyruvate in low concentration prevents the inhibition of growth, just as expected if oxamate were acting specifically at the lactic dehydrogenase level. The latter observation certainly suggests that, whatever the site of oxamate inhibition of growth, it must be a site at which pyruvate is also bound. Although such sites, other than lactic dehydrogenase, may of course exist, none has as yet been demonstrated.

Assuming that lactic dehydrogenase activity is somehow essential for growth, the next question is whether this requirement represents a need for glycolysis as a major energy source for growth. This seems not to be the case, since Eagle et al. (19) have shown that maximal growth of HeLa cells can occur at very low concentrations of sugar, where the rate of lactate formation appears to be very low. When oxamate effects on growth were compared at low and high glucose concentrations, e.g. 0.2 versus 5 mM, no significant difference in effectiveness was noted. Thus, although glycolysis may be quantitatively of less significance at low sugar concentrations, it appears to remain essential for growth.

One must keep in mind the possibility that lactic dehydrogenase may be necessary for growth because of a function distinct from the usual DPNH-linked glycolytic activity. Ernster et al. (20) have pointed out that the TPNH-linked activity of lactic dehydrogenase may be of quantitative significance as the major pathway for reoxidation of TPNH in liver. Wenner (21) has shown, in fact, that pyruvate addition to ascites tumor cells increases the oxidation of glucose via the phosphogluconate pathway.

Finally, Herzenberg (22) has noted that pyruvate is an essential growth factor for the clonal cultivation of lymphoma cells. That the latter effect is via lactic dehydrogenase is strongly suggested by Herzenberg's finding that \( \alpha \)-ketobutyrate, a good substrate for this enzyme, can replace pyruvate as growth factor. These findings indicate that other pathways for the oxidation of TPNH and DPNH (e.g. respiration, fat synthesis) are inadequate to initiate growth in these cells, and suggest an important role for lactic dehydrogenase in this process.

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1. Oxamate, a structural analogue of pyruvate, inhibits completely the growth of HeLa cells at the same concentration (0.04 to 0.08 mM) found previously to inhibit the glycolysis of resting ascites tumor cells.

2. The inhibition of growth at varying oxamate concentrations is paralleled by corresponding decreases in glucose utilization and lactate production.

3. The growth inhibition by 0.04 mM oxamate is prevented almost completely by the presence of \( 8 \times 10^{-4} \) mM pyruvate, and lactic acid production is also restored to the uninhibited level.

4. Inasmuch as oxamate is competitive with pyruvate with respect to both growth and lactic dehydrogenase activity, it is concluded that lactic dehydrogenase activity is somehow essential for the growth of the HeLa strains tested under the conditions of these experiments.

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