POTENTIATION OF ANTICANCER DRUG EFFECT BY CANCER CELL GLYCOLYSIS: AN IN VITRO EXPERIMENT

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A series of in vitro experiments have been carried out with the commercially available anticancer drugs in order to find which drug, when used in the acidic condition induced by tumor cell glycolysis, displays the strongest tumoricidal activity not to be expected from the conventional mode of administration. For this purpose, Ehrlich ascites tumor cells were incubated, without shaking, together with the test drug dissolved in various vehicles such as 5% aqueous glucose solution, 5% glucose in phosphate buffer, and phosphate-buffered saline. The cytocidal effect of these drugs on the tumor cells was assayed by transplanting the treated cells intraperitoneally in mice.

Among the drugs tested, Carbazilquinone revealed the strongest cytocidal effect, especially when dissolved in aqueous glucose solution but not in other vehicles. This result shows that Carbazilquinone has responded to our anticipation as being cytocidally the most effective when employed in combination with cancer cell glycolysis, probably because of its chemical composition of containing aziridine groups.

One of the most important improvements yet to be effected in the present cancer chemotherapy is the discovery of an effective means for attacking cancer cells exclusively keeping normal cells unaffected by anticancer drugs administered. For this purpose, biochemically different properties between tumor and normal cells, if present, may be advantageously utilized. It has long been generally accepted that tumor cells have higher glycolytic activity than normal cells, and that the vigorous glycolytic activity of cancer cells leads to a production of a greater amount of lactic acid, resulting in the difference of extra- and intra-cellular pH between the two kinds of cells.

Subsequent to the report of Nakahara and Fukuoka concerning the carcinostatic liver factor, Hozumi et al. found that the subcutaneous transplantability of Ehrlich ascites tumor cells in mice was lost by preincubation of such cells in aqueous glucose solution at 37°C for 1 hr. They explained this phenomenon by the acceleration of autolysis of the tumor cells due to decrease in intra- and extra-cellular pH resulting from the production of lactic acid. Undoubtedly the accumulation of lactic acid around them killed the tumor cells themselves. This finding suggests that, if there were an anticancer drug whose action is potentiated in an acidic state, we might be able to make it exert selective cytocidal effect on tumor cells under the condition of tumor cell glycolysis.

In the present study, a series of in vitro experiments were carried out with the commercially available drugs in order to determine which drug, when used in the acidic state induced by tumor cell glycolysis, displays the strongest tumoricidal activity not to be expected from the conventional mode of administration.

MATERIALS AND METHODS

Tumor Cells Ehrlich ascites tumor cells used were those that had been maintained in DDD mice by weekly passage in our laboratory. Mostly, about 7-day-old ascites was used in the present experiments.

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Animals Two- to 5-month-old inbred DDD mice of both sexes propagated in and supplied from the Animal Center of our university were used.

Test Drugs The anticancer drugs used were as follows: Carbazilquinone (Sankyo Co., Tokyo), thio-TEPA (Sumitomo Chemical Co., Osaka), triethylenemelamine (supplied by Dr. Harry Gelboin of N.I.H., U.S.A.), nitrogen mustard N-oxide (Nitromin, Yoshitomi Pharmaceutical Ind., Ltd., Osaka), Mitomycin-C and Adriamycin (Kyowa Hakko Kogyo Co., Tokyo).

As a device for comparing the effectiveness of various drugs, we defined the "unit concentration" in the present study as follows: As a standard, LD_{50} of each drug for a mouse weighing 20 g was dissolved in 1 ml of various vehicles, and this drug concentration was designated as "1 unit concentration." The standard solutions in various vehicles prepared as above were then diluted with corresponding vehicles to the desired concentrations. The vehicles used in the present study were 5% glucose in redistilled water (aqueous glucose solution), 5% glucose in 0.05M phosphate buffer of pH 7.4 (glucose-containing buffer), and phosphate-buffered saline of pH 7.4 (PBS).

In the present study, following doses of each drug were set up as LD_{50} for mice: Carbazilquinone, 5 mg/kg; thio-TEPA, 10 mg/kg; triethylenemelamine, 2.8 mg/kg; nitrogen mustard N-oxide, 75 mg/kg; Mitomycin-C, 5 mg/kg; Adriamycin, 12 mg/kg.

Experimental Procedures Ehrlich ascites tumor cells were aspirated from the peritoneal cavity of a mouse and were washed once with cold physiological saline. After centrifugation at 2,000 rpm for 5 min, the supernatant was discarded and the cells were resuspended in physiological saline. Then, the cell suspension was divided into several test tubes so that each tube contained $2 \times 10^7$ viable Ehrlich tumor cells. After recentrifugation, the supernatant of each tube was carefully pipetted out, care being taken not to permit the cells to adhere to the glass wall. After 2 ml of each test drug solution was added to the tumor cell pellets in each tube, the tubes were rapidly shaken to bring each cell into direct contact with the drug solution. The tubes were then quickly set up in an incubator for treatment at 37°C without shaking for 30 min unless otherwise mentioned.

The treatment was terminated by a rapid addition of ca. 5 ml of cold PBS into each tube and by transferring it into an ice box. The tubes were then centrifuged at 2,000 rpm for 5 min and the supernatant in each was carefully pipetted out. Finally the cell pellets in each tube were resuspended in 2 ml of cold PBS. Each 0.4 ml of the cell suspension containing over $2 \times 10^6$ treated cells was inoculated intraperitoneally into groups of five mice for the bioassay. As a control, an additional group of mice was simultaneously inoculated with the same number of Ehrlich tumor cells treated with PBS by the same procedures as mentioned above. All the procedures were performed under aseptic conditions. All the mice thus inoculated were daily inspected for 60 days and the survival rate of each group was recorded.

Determination of Lactic Acid In order to realize whether the drug potentiation observed is really concerned with lowering of pH due to tumor cell glycolysis, amount of lactic acid was determined in our assay system. Process for preparation of the tumor cells in each test tube was identical as described above. The pellets of the tumor cells were resuspended in 2.0 ml of the following test solutions: (1) 5% aqueous glucose solution without Carbazilquinone, (2) 5% aqueous glucose solution containing 1/100 unit concentration of Carbazilquinone, (3) 5% aqueous glucose solution with 1/10 unit of Carbazilquinone, (4) 5% aqueous glucose solution with 1/1 unit of Carbazilquinone, and (5) phosphate-buffered saline without glucose. Incubation was carried out for 30 min at 37°C without shaking. Lactic acid in each tube was determined according to the method of Barker and Summerson.

Results

The cytoidal effect on Ehrlich tumor cells of each drug dissolved in various vehicles of graded concentrations was assessed by the survival rate of mice that had been inoculated intraperitoneally with the drug-treated Ehrlich tumor cells. It was clearly demonstrated that the cytoidal activity of each drug was markedly influenced by the kind of vehicle used. Each of the graphs attached here gives a representative result illustrative of those of our repeated experiments on the same drug.

Carbazilquinone Carbazilquinone showed the most interesting and powerful cytoidal effect on Ehrlich tumor cells in the present series of in vitro experiments. As it had been revealed by a preliminary test that the effect of the drug was more than 10 times stronger than other kinds of drugs tested, the data given in Fig. 1 are those of an experiment in which the concentration
level of doses was lowered to 1/10 of that of other drugs. As shown in Fig. 1, the treatment of Ehrlich tumor cells with Carbazilquinone in aqueous glucose solution for 30 min in vitro at 1/100 unit and units higher than that kept 100% of mice alive without tumor growth throughout the entire period of the experiment, and even at 1/200 unit 60% of mice could survive free from tumor. On the contrary, when the vehicle aqueous glucose solution was replaced by PBS, striking decrease in the survival rate was observed. Namely, when Ehrlich tumor cells were treated with 1/10 unit of Carbazilquinone in PBS, only 20% of the mice survived, while none of the mice survived when the cells were treated with 1/100 unit. The survival rates of the animals inoculated with the Ehrlich tumor cells treated with the drug in glucose-containing buffer solution were midway between those of the above two groups. In short, Carbazilquinone exhibited the strongest cytocidal effect in vitro among the drugs tested, especially when dissolved in aqueous glucose solution but not in other vehicles.

**thio-TEPA** As shown in Fig. 2, at 1 unit concentration of thio-TEPA, 100% of the mice survived free from tumor when they were inoculated with over $2 \times 10^6$ Ehrlich tumor cells that had been treated in vitro with the drug dissolved in glucose-containing buffer. However, the survival rates of the mice inoculated with the Ehrlich tumor cells treated with the drug dissolved either in aqueous glucose solution or in PBS decreased in that order. At 1/10 unit, the survival rate of mice challenged with the Ehrlich tumor cells treated with the drug in aqueous glucose solution was only 20%, while none survived in the other two groups for which different vehicles...
Fig. 2. Cytocidal activity of thio-TEPA in various vehicles on Ehrlich ascites tumor cells in vitro (37°C, 30 min)

Fig. 3. Cytocidal activity of triethylenemelamine in various vehicles on Ehrlich ascites tumor cells in vitro (37°C, 30 min)
POTENTIATION OF ANTICANCER DRUG EFFECT

were used. At 1/100 unit, all the mice in each group succumbed to the tumor, with no significant prolongation of life span being observed compared to that of the control.

Triethylenemelamine The survival rates of the mice inoculated with Ehrlich tumor cells treated with triethylenemelamine are shown in Fig. 3. At 1 unit concentration, all the mice inoculated with the Ehrlich tumor cells treated with triethylenemelamine dissolved in any of the 3 vehicles survived without tumor growth throughout the experimental period. At 1/10 unit, the same results as above were obtained in the two groups of mice for which aqueous glucose solution and glucose-containing buffer solution were used as vehicles. However, the mice inoculated with the cells treated with triethylenemelamine in PBS could not survive beyond the life span of the control. At 1/100 unit, no significant difference in survival rate and life span was observed among all the three groups and the control.

Nitrogen Mustard N-Oxide At 1 unit concentration, the treatment of Ehrlich tumor cells with this drug in aqueous glucose solution, in glucose-containing buffer, and in PBS resulted in 100% survival of the mice in each case. At 1/10 unit, the survivors were 80% in the case of aqueous glucose solution, 40% in the case of glucose-containing buffer, and 0% in the case of PBS. At 1/100 unit, the survival rates were 40%, 0%, and 20%, respectively. In short, the cytocidal effect of nitrogen mustard N-oxide was the strongest at every concentration tested, when dissolved in aqueous glucose solution (Fig. 4).

Mitomycin-C As shown in Fig. 5, at 1 unit concentration, 80~100% survival of mice was obtained in three different vehicle groups. At 1/10 unit, the survival rates of mice were 100% for aqueous glucose solution, 40% for glucose-containing buffer, and 0% for PBS. At 1/100 unit, no survivor was obtained in any of the groups.
Fig. 5. Cytocidal activity of Mitomycin-C in various vehicles on Ehrlich ascites tumor cells in vitro (37°C, 30 min)

Fig. 6. Cytocidal activity of Adriamycin in various vehicles on Ehrlich ascites tumor cells in vitro (37°C, 30 min)
**POTENTIATION OF ANTICANCER DRUG EFFECT**

Adriamycin  At both 1 and 1/10 unit, 100% survival was obtained in every group, irrespective of the kind of vehicle used. Contrarily, at 1/100 unit, neither survivors nor prolongation of life span compared to the control was observed in the 3 groups of mice (Fig. 6).

**Comparative Cytocidal Effect of Carbazilquinone and Nitrogen Mustard N-oxide in Aqueous Glucose Solution in vitro after 10-min Incubation**  Since these two drugs had exerted excellent cytocidal effect on Ehrlich tumor cells when incubated in vitro at 37° for 30 min (Figs. 1 and 4), further experiments were carried out to find if similar results could be obtained by shortening the incubation period. As illustrated in Fig. 7, the incubation of only 10 min at 1/10, 1/20, and 1/40 unit concentration of Carbazilquinone in aqueous glucose solution killed all Ehrlich tumor cells in vitro, with all the mice inoculated surviving without tumor growth throughout the entire period of observation. Among the animals challenged with Ehrlich tumor cells treated with nitrogen mustard N-oxide, however, only 20% of them survived and no significant prolongation of life span was observed at the above three drug concentration levels. At 1/80 and 1/160 unit, the cytocidal effect of Carbazilquinone still remained, while nitrogen mustard N-oxide employed at the same concentration levels showed no cytocidal effects at all.

Table I summarizes the comparative cytocidal effects of the anticancer drugs dissolved in aqueous glucose solution on Ehrlich tumor cells. When the drugs were employed at 1 or 1/10 unit concentration, all the mice inoculated with the Ehrlich tumor cells treated with any of the drugs, Carbazilquinone, triethylenemelamine, and Adriamycin, survived without tumor growth.

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Fig. 7. Comparison of cytocidal activity between Carbazilquinone and nitrogen mustard N-oxide in aqueous glucose solution on Ehrlich ascites tumor cells in vitro (37°, 10 min)
growth. When challenged with cells treated with either nitrogen mustard N-oxide or Mitomycin-C, 100% of the mice survived at 1 unit concentration and 80% at 1/10 unit. On the other hand, in the case of cells treated with thio-TEPA, there were 60% survivors at 1 unit and only 20% at 1/100 unit. It should be emphasized that, when the concentration was reduced to as low as 1/100 unit, 100% survivors were obtained only in the case of treatment with Carbazilquinone. Even at much lower concentrations, some survivors were still observed exclusively in the case of Carbazilquinone.

In Table II, effect of the presence of Carbazilquinone in various concentrations on tumor cell glycolysis is shown, based on the comparative efficiency of lactic acid formation. At the concentration of 1/100 unit, the drug exerted a practically insignificant influence on tumor cell glycolysis. At 1/10 unit, the glycolytic activity of the tumor cells was still much reserved; the amount of lactic acid produced was only reduced to 70% of the control in which tumor cells were incubated in 5% aqueous glucose solution without Carbazilquinone. At 1/1 unit, though too high a concentration of the drug in the present assay system, the glycolytic processes seemed to be almost totally interfered.

**DISCUSSION**

It can easily be presumed that the extra- and intra-cellular pH of cancer cells would be decreased by lactic acid produced by their vigorous glycolytic action. For that matter, a number of workers has so far conducted and reported a work along this line.

In an *in vitro* experiment, Sähler calculated the decrease in the local extracellular pH, using Ehrlich tumor cells suspended in saline containing 5.5mM of D-glucose at a concentration of 5 x 10^7 cells/ml, and incubating the cells at 37° without shaking for 1 hr. The fall in pH value was found to be 2.16 to 2.42.
Poole\textsuperscript{7)} also reported the fall of extra- (pHe) and intra-cellular pH (pHi) of Ehrlich tumor cells incubated in a buffer containing 11mM glucose in a metabolic shaker at 37° for 30 min. His experiment showed that, in a Krebs-Ringer-0.025M phosphate buffer, the pHe fell from 7.2 to 6.06 and the pHi from 7.02 to 6.45.

In the present study, Carbazilquinone displayed the strongest cytocidal effect on Ehrlich tumor cells when it was employed as a solution in 5% aqueous glucose solution, but the effect weakened when it was dissolved in a buffer solution (Fig. 1). This finding suggests that the enhanced action of Carbazilquinone may have resulted from the decrease in the extra- and intra-cellular pH due to cell glycolysis.

Here a question arises; does the tumor cell glycolysis actually proceed even in the presence of Carbazilquinone? The evidence shown in Table II solves this question, i.e., even in the presence of Carbazilquinone at the concentration of 1/10 or 1/100 unit in 5% aqueous glucose solution, the lactate formation by tumor cell glycolysis was almost as high as that in the control of simple 5% aqueous glucose solution. Since these drug concentrations exerted satisfactory cytocidal effect on the tumor cells under identical conditions (Fig. 1), it may be safely concluded that the main mechanism involved in the potentiation of tumoricidal action of Carbazilquinone in glucose solution is coupled with tumor cell glycolysis.

Now another question is, does the intratumoral pH actually decrease in vivo, too, following a systemic administration of glucose as it does in vitro? If so, does a difference in pH value due to the higher glycolytic activity of cancer cells exist between cancer and normal tissues? By means of the direct insertion of microelectrodes into the tumor mass of the living animal, Voegtlin \textit{et al.}\textsuperscript{10)} in fact, demonstrated years ago that the pH of tumor tissue dropped very quickly from 6.9 to 6.3 following glucose administration without any occurrence of systemic acidosis.

As an \textit{in vivo} experiment, Kahler and Robertson\textsuperscript{5)} also compared the pH of normal liver and hepatoma tissue in rats. The pH of normal tissue was 7.4 and that of hepatoma 7.0 and, after glucose administration, the former remained unaffected whereas that of the latter fell to 6.4. According to Coris\textsuperscript{3)}, the content of lactic acid of the mouse mammary carcinoma increased to as much as over 4 times of the initial one following glucose administration.

The differences of 1~2 in the pH value in tumor between \textit{in vitro} and \textit{in vivo} experiments mentioned above may be due to several reasons. The pH value \textit{in vitro} indicates that of the local extracellular fluid around the cancer cells, while the value \textit{in vivo} indicates that of the interstitium of the tumor. Furthermore, lactic acid accumulated around the cancer cells \textit{in vivo} may partly be carried away through the blood stream, and partly be neutralized by buffers that are continuously supplied. On the other hand, lactic acid produced by cells \textit{in vitro} remains around the cells, because the cancer cells were stationarily incubated without shaking. Especially in the Sahler's experiment, in which no buffer was used, all the lactic acid produced seemed to be directly responsible for the decrease in the pH value. This may be the reason why the subcutaneous transplatability of Ehrlich tumor cells incubated with glucose in physiological saline without shaking was markedly diminished whereas that of the cells incubated with shaking or in buffer solution was not.\textsuperscript{4)}

As Ross\textsuperscript{8)} once remarked, it is possible to induce an artificial, selective difference in pH between tumor and normal tissues by glucose administration. Based on this principle, we carried out the present study to find the kind of anticancer drugs whose action would be potentiated in an acidic state. It was our anticipation that, among a number of alkylating agents, the chemicals having an aziridine ring in their composition would probably satisfy our requirements. In fact, our present effort of screening has revealed that, among the chemicals containing...
aziridine group in their chemical structure, Carbazilquinone was the drug which proved
to be cytocidally the most effective when employed in combination with tumor cell glycolysis.

We have conducted an experimental cancer chemotherapy in vivo by using Carbazilquinone
based on the above principle and, as a striking result was also obtained from this experiment,
a preliminary communication has been published.\(^1\)

It is earnestly hoped that the above principle be further exploited for the development of
ideal anticancer drugs along the line of the present study.

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References

4) Hozumi, M., Tateno, Y., Matsuoka, K., Mori, T., Sugimura, T., Gann, 56, 267 (1965).
    164, 15 (1935).