Effects of Hypoxia, pH, and Growth Stage on Cell Killing in Chinese Hamster V79 Cells in Vitro by Activated Cyclophosphamide

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ABSTRACT

Several factors which influence the sensitivity of Chinese hamster V79 cells to cyclophosphamide (CY) have been studied in vitro in both suspension and monolayer cultures. Activated CY was obtained from the blood of mice 15 to 30 min after i.p. injection of CY (400 mg/kg).

At pH 7.4, hypoxia rendered the cells more sensitive to activated CY. At lower values of pH (6.6 and 7.0), there was no difference between the sensitivities of oxic and hypoxic cells, although cells in both conditions were more sensitive to CY than at pH 7.4.

Drug sensitivity was markedly affected by the stage of cell growth. Monolayer cultures were most sensitive to CY within a few hours of plating. Cultures then rapidly became less sensitive, with maximum resistance occurring between 24 and 48 h after plating, while the cells were still exhibiting rapid exponential growth. This development of resistance paralleled the formation of small colonies (2 to 4 cells), implying that intercellular contact may confer resistance to killing by activated CY.

INTRODUCTION

Hypoxic cells are known to exist in both experimental animal and human tumors. They have been found for many years to be considerably more resistant than oxic cells to X-rays. In recent years, evidence has been presented of drug resistance of hypoxic cells in some experimental solid tumors, which could compromise effective chemotherapy (6, 10, 17). Some possible mechanisms are: (a) hypoxic cells may be noncyling and therefore resistant to cycle active drugs; (b) hypoxia may affect drug metabolism; (c) a low pH, often associated with hypoxic cells in vivo, may also affect drug metabolism; and (d) hypoxic cells may receive a lower drug exposure due to poor penetration of the compound.

It is therefore important to establish the nature and extent of factors determining the sensitivity of hypoxic cells to chemotherapeutic agents. This study was designed to determine the magnitude of some of the modifying effects described above on cellular sensitivity to the alkylating agent CY. Investigations have been performed in vitro to take advantage of the greater independent control of variables such as oxygen tension and pH in comparison with that possible in vivo studies.

Both we and others (6, 17) have found that hypoxic tumor cells in vivo appear to be more resistant to CY, although this finding is not observed in all tumors (10). There have been several in vitro investigations on the relative sensitivities of oxic and hypoxic cells to various cytotoxic drugs (9, 19, 21, 22, 25, 28). With the exception of Tannock (25), all have omitted CY, because this drug is noncytotoxic without metabolic activation by liver enzymes (3, 20). We have taken advantage of a bioassay for activated CY in blood, developed independently by Tannock (25) and ourselves (2). By using activated CY taken from the blood of treated mice, we have investigated the effects of hypoxia and pH, both separately and together, and the effect of stage of growth of the sensitivity of V79 cells to CY.

MATERIALS AND METHODS

Cells. Chinese hamster V79-379A cells were used throughout and were maintained in suspension culture in Eagle's MEM plus 7.5% FCS. Cyclophosphamide (Cytoxan) used in these experiments was a gift from Ward Blenkinsop Pharmaceuticals (Bracknell, Berks, England). The method of obtaining activated CY was as described previously (2). Briefly, WHT/GyBSVS mice were given injections of CY (400 mg/kg) intraperitoneally; 15 to 30 min later, blood was extracted and heparinized. The blood was centrifuged (3000 rpm, 15 min), and the plasma was extracted and diluted 1 to 6 in complete medium (MEM + 10% FCS). This provided the stock solution of activated CY, which was frozen in aliquots until required. A dilution of the stock by a factor of 2, the highest concentration used in these experiments, was assigned a value of 1.0. The concentration of cytotoxic CY metabolites was thus expressed in arbitrary units relative to this standard dilution of plasma. The stability of the cytotoxic CY metabolites in the diluted plasma was tested by performing cell survival assays after storage of the plasma for various times and temperatures. The results of three separate experiments are shown in Table 1. Cytotoxic activity decreased rapidly at room temperature (cell survival increased by approximately 1.5 decades after 4 h of storage), relatively slowly at refrigerator temperature (survival increase of approximately 2 decades after days of storage), and very slowly at freezer temperature (survival increase of less than 1 decade after 11 days of storage). The maximum storage time in the present series of experiments was 6 days at freezer temperature, which would therefore have little influence on the reported results.

Hypoxia and pH Experiments. 10^6 cells were plated in 60-mm glass Petri dishes and allowed to grow for 16 to 18 h under standard conditions (37°C, pH 7.4), air. The medium was then replaced with 1 ml of medium containing the bicarbonate concentration required to give a particular pH (see below), and 1 ml activated CY was added. The volume of medium was kept low to facilitate rapid deoxygenation. The dishes were transferred immediately to aluminium chambers connected to a 95% N₂ + 5% CO₂ cylinder (<10 ppm O₂) via copper pipes. They were gassed for 30 min at a flow rate of 4 liters/min at 37°C. The chambers were then sealed and incubated for a further 3½ h, giving a total exposure of CY and hypoxia of 4 h. The cells were then reseeded and washed twice with phosphate-buffered saline. After trypsinization and counting, known

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4 The abbreviations used are: CY, cyclophosphamide; FCS, fetal calf serum; MEM, minimal essential medium.

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numbers of cells were plated into plastic Petri dishes. The fraction of surviving cells was assessed by colony formation after 7 days.

The pH was regulated by using different NaHCO₃ concentrations in the medium while maintaining the CO₂ concentration at 5%. The final bicarbonate concentrations were 0.15, 0.55, and 2.2 g/liter to maintain pHs of 6.6, 7.0, and 7.4, respectively. The activated CY was made up without bicarbonate (1 ml/dish), and therefore the additional 1 ml of "normal" medium required to bring the final volume to 2 ml was made up with twice the required bicarbonate concentration.

Controls were exposed to plasma from untreated mice and subjected to the same pH and oxygen concentrations as the CY-treated cells.

Growth Stage Experiments. For the suspension culture experiments, cells were inoculated into 500-ml "serum" bottles containing 120 ml of suspension medium (MEM + 7.5% FCS) to give a concentration of 10⁶ cells/ml. Plateau phase (1 to 2 x 10⁶ cells/ml) was reached 4 days later. At various times up to 6 days later, 30 ml of stock activated CY solution were added. At intervals ranging from 1 to 8 h after adding the CY, 5- to 10-ml aliquots of the suspension were taken; the cells were washed and counted, and known numbers were plated to assess the surviving fraction by colony formation.

For the monolayer experiments, cells from suspension-maintained cultures were taken when in log phase and either 10⁴ or 10⁵ cells plated into 50-mm plastic Petri dishes. At times ranging from 2 h to 6 days later, the cells were exposed to graded concentrations of activated CY, obtained by diluting the stock CY solution in complete medium. The exposure time was either 4 or 6 h at 37°C, at which time the cells were washed, trypsinized, counted, and plated for colony formation.

Cells in control dishes were also trypsinized and counted at various times after plating in order to establish growth curves. Cell multiplicity was also assessed at each time interval by scoring the incidence of single cells, doubles, quadruples, etc. using an inverted microscope.

RESULTS

Hypoxia. The effect of acute hypoxia on the sensitivity of log phase V79 cells to activated CY is shown in Chart 1. Hypoxia markedly sensitized these cells to the killing by cytotoxic metabolites of CY at physiological pH (7.4). For example, to produce a surviving fraction of 10⁻², the hypoxic cells required approximately 1.6 times less dose.

pH. Further experiments were performed in which the effects of acute hypoxia were tested on cells held at 3 different levels of pH. The sensitizing effect of hypoxia on cells at pH 7.4 was again seen (Chart 2). At pH 7.0 and 6.6, however, oxic and hypoxic cells were equally sensitive to activated CY.

Chart 3 shows isoeffective CY doses required to produce a surviving fraction of 10⁻³. The doses were read off the curves in Chart 2 and plotted as a function of pH for both gassing conditions. Oxic cells became progressively more sensitive to killing by CY as the pH was lowered. The sensitivities of oxic and hypoxic cells were indistinguishable at pH values of 6.6 and 7.0. Only with pH 7.4 was there a difference in sensitivity to CY.

Growth Stage. The effect of stage of growth on cellular sensitivity to CY was first investigated in suspension culture. After inoculation of approximately 10⁴ cells per ml, the cells grew exponentially for 3½ days and remained at approximately the
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A similar set of experiments was carried out in monolayer culture (Chart 5). The growth rate of the cells was very similar to that in suspension culture, i.e., a doubling time of approximately 10 h (Chart 5). Cells were treated 2 h after plating 10⁴ cells or 2, 4, or 6 days later. The cells treated at 2 h were highly sensitive to the drug, while cells at all other times were considerably more resistant (Chart 5). Cells on Day 2, which were in the middle of the log phase of growth, were as resistant as the plateau cells. In contrast to the suspension culture experiments, it appears that a simple determination of whether the cells are in log or plateau phase does not predict their sensitivity to CY.

The time course of development of resistance was further investigated by giving a constant drug dose at various intervals after plating 10⁴ or 10⁵ cells (Chart 6). The amount of cell killing decreased rapidly with time after plating so that, by 1 day, almost the maximum level of resistance had been attained. Again, this resistance occurred when the cells were in the middle of log phase growth.

To test whether intercellular contact influenced sensitivity to CY, the development of small colonies was followed with time after plating (Chart 7). One day after plating single cells, over 90% appeared as small colonies of 2 cells or more. The time scale of development of these microcolonies was therefore similar to that for the development of resistance to killing by CY.

DISCUSSION

We have studied some of the factors governing cellular sensitivity to CY in an in vitro model. We have chosen to study the state of oxygenation, pH, and the stage of growth, since tumors are known to be heterogeneous in these parameters and all have been shown capable of affecting cellular sensitivity to some cytotoxic agents (e.g., 1, 8, 21, 28, 29).

The plasma from Cy-treated mice used for the in vitro treatments will contain cytotoxic and non cytotoxic metabolites of CY. The advantages of this bioassay method are that, firstly, the

same cell numbers for 1 day, after which numbers began to decline (Chart 4). The cells were treated 1, 2, or 4 days after inoculation, i.e., in early log, mid-log, and plateau phase, respectively. Long-phase cells were found to be more sensitive than those in plateau phase (Chart 4). Mid-log phase cells (Day 2) were also more sensitive than early log phase cells (Day 1), for reasons which are not clear.

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Chart 6. Time course for development of resistance to CY. At different times after plating $10^5$ or $10^6$ cells per dish, cells were treated with a fixed concentration of CY plasma (1/4 dilution of "stock") for 4 h. Resistance developed rapidly within the first 24 h after plating.

metabolites are easy to obtain, and secondly, the cells being treated will be exposed to the same mixture of metabolites in the same proportions as occurs in vivo. The disadvantage from a mechanistic viewpoint is that the exact concentration of each metabolite is not known and was not determined in this study. This was not regarded as an important disadvantage, since of primary interest was the relative sensitivities of cells under various conditions when exposed to all of the metabolites of CY likely to occur in vivo.

Hypoxia. The data available on the sensitivity of cells in tumors to killing by CY suggest that hypoxic cells in situ are either less sensitive (6) or equally sensitive (10) compared with oxic cells. It was therefore a surprise to find that hypoxia sensitized V79 cells in vitro to killing by activated CY. There are a few drug types that have been found to be more cytotoxic to hypoxic cells. The electron affinic hypoxic cell radiosensitizers, e.g., misonidazole, are known to be toxic specifically to hypoxic cells (16, 23). This is due partly to the formation of toxic product(s) after nitroreduction (27, 30), a pathway inhibited by oxygen (14, 15). Mitomycin C and other quinones have also been shown to be more effective against hypoxic cells. It has been postulated that bioreductive processes in the cell form the toxic, in this case alkylating, species, and that the redox state in hypoxic cells favors this process (11, 13). There is a similarity between mitomycin C and CY in that they are both alkylating agents requiring enzymatic activation to become cytotoxic. It is therefore possible that CY is more toxic to hypoxic cells for reasons similar to those for mitomycin C.

The only other in vitro study on the effects of CY and hypoxia is that of Tannock (25), who showed no difference between the sensitivities of oxic and hypoxic cells in suspension culture. Sensitization by hypoxia would therefore appear to be either cell line dependent [Chinese hamster ovary (Tannock) versus V79 (present report)] or determined by the growth conditions of the cells (α-MEM versus MEM; suspension culture versus monolayer). Hypoxia-induced sensitization may therefore not occur with all cell types.

pH. Gullino et al. (7) have reported that interstitial fluid in tumors is in a permanent state of acidosis, with average pH levels of 6.9 to 7.2. Intercellular pH in experimental animal tumors has been shown by microelectrode studies to vary from values as low as 6.0 up to 7.4, with a substantial fraction of the cells existing in pH regions of less than 7.0 (31). Based on in vivo evidence of hypoxic cell resistance, it was expected that a lowered pH may protect against killing by CY. This was based on the assumption that the hypoxic cells would be at the lowest pH, due to a greater dependence on anaerobic glycolysis with concomitant production of lactic acid. In fact, the opposite was observed in this in vitro study, with a sensitization of cells as the pH was lowered to 6.6.

A possible explanation for this finding may lie in the stability of the cytotoxic metabolites. Low et al. (14) have found that both 4-hydroxy-cyclophosphamide and phosphoramidate mustard, the major cytotoxic metabolites of CY (18), were stable at lower
Thus be higher and result in greater cell killing. Whether this is the full explanation and whether this would also occur in vivo are not known.

Studies on the effects of pH on cytotoxicity by drugs are few, and none have been reported for CY. Hahn and Shiu (8) found little difference in killing by bleomycin or methotrexate at 37°C with changes in pH between 6.5 and 8.5. They did, however, find a large pH dependence for drug cytotoxicity at elevated temperatures. The present study and that by Hahn and Shiu (8) show that, in some circumstances, pH can markedly influence cell killing by drugs.

**Growth Stage.** CY has been shown to kill proliferating cells up to 4 times more efficiently than nonproliferating cells (4, 12). We also found that plateau phase cells in suspension culture were less sensitive to CY than exponentially growing cells. The present study, however, also showed that the greatest change in sensitivity in attached cells occurred during exponential growth and before the plateau phase was reached. The rate of proliferation was not therefore the important factor in determining sensitivity to the drug.

In a concurrent study, hypoxia was shown to halt the proliferation of V79 cells. The cessation of proliferation did not reduce the sensitivity of hypoxic cells to CY (Charts 1 and 2). In fact, sensitivity was increased, confirming the lack of importance of proliferation rate in determining V79 cell killing by CY.

The resistance to CY developed at a rate closely similar to that for the appearance of small colonies. A comparison of Charts 6 and 7 indicates that, by the time the cells are in colonies of at least 2 to 4 cells, they have fully developed their resistance. It is therefore possible that CY resistance results from a cell-to-cell contact phenomenon. This would also explain the lack of such a rapid rise in resistance in suspension culture where the cells remain separated.

Other factors could be important, however. For example, Cullen et al. (5) also showed significant changes in intracellular sulphydryl levels after subculture which were large enough to affect radiosensitivity (5). Such changes, in particular a sulphydryl depletion after plating, may also affect drug sensitivity (24, 26).

The sensitization of cells to CY killing by both hypoxia and reduced pH would lead to the expectation that hypoxic cells in vivo would not limit the tumor’s response to chemotherapy with CY. The fact that hypoxic cells in some experimental tumors have been found to preferentially survive CY therapy would therefore imply that the reduced killing resulted from the drug concentrations in hypoxic cells being lower than in oxic cells. Since many of the hypoxic cells will be those furthest from the blood vessels, a lower drug concentration in hypoxic cells could understandably result from a diffusion limitation and/or preferential uptake and binding by the intervening oxic cells.

In conclusion, these results show that pH, hypoxia, and growth stage all influence the sensitivity of V79 cells to killing by activated CY. The sensitization by pH and hypoxia implies that the resistance of hypoxic cells seen in vivo may result from a drug supply limitation. Other studies are necessary to test whether the increased sensitivity seen in vitro could be exploited therapeutically, for example, by modifying tumor acidity as described by Giulino et al. (7).

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