UNEXPECTED SENSITIVITY OF TUMOR CELLS TO CYCLOPHOSPHAMIDE IN ONE-DAY-OLD SOLID EHRlich ASCITES TUMORS

Muneyasu Urano, Norimoto Tanaka, and Ichiro Shirakura
(Department of Radiology, Kyoto Prefectural University of Medicine*)

Synopsis

The survival curve of tumor cells in one-day-old solid tumors was investigated as a function of the dose of cyclophosphamide. Serially diluted tumor cells were transplanted intracutaneously into six sites on the dorsal skin of mice for TD$_{50}$ assay. The drug was injected intraperitoneally 24 hr later when the inoculum was expected to have formed microcolonies or one-day-old solid tumors. Each TD$_{50}$ assay group received a different dose of cyclophosphamide. The TD$_{50}$ was computed from tumor-take frequencies by logit analysis. Survival fractions were calculated from the ratio of TD$_{50}$ of control to that of cyclophosphamide treated.

The dose-cell survival curve resembled the multi-component cell survival curve after X-ray irradiation, the cyclophosphamide-sensitive part being followed by a cyclophosphamide-resistant tail. The resistant cell fraction is discussed and a hypothesis advanced that a small fraction of slowly-proliferating cells may be present probably in hypoxic foci in one-day-old tumors. The cyclophosphamide-resistant tail in the survival curve is considered to represent the sensitivity of such tumor cells. This may mean that cyclophosphamide is incorporated into the hypoxic tumor cells.

INTRODUCTION

The use of chemotherapeutic or radiosensitizing agents together with ionizing radiation in the treatment of malignant tumors has been attempted with the hope that such agents would kill some radioresistant cells or sensitize their response to radiation. Hypoxic or oxygen-deficient cells would be the resistant cells. It is well established that radiation sensitivity of a tumor depends largely on the sensitivity of its hypoxic cells and their number. Therefore, our question is whether oxygen-deficient cells are affected by such agents. Irregularity of the vascular system in malignant tumors would limit the effect of drugs by interfering with their reaching these cells, along with limiting the oxygen supply.

An unexpected dose-survival curve was obtained in a series of experiments which was designed to test the effect of some drugs on hypoxic cells and this is described in this paper.

MATERIALS AND METHODS

Animal and Tumor System Eight- to twelve-week-old female ddYF mice supplied by Funabashi Nojo Co., Chiba, were used in all the experiments. They were kept in small animal facilities at a constant temperature. Animals were housed in groups of 8~12 in a cage with wood shavings and were provided with standard cube diets

* Kawaramachi-hiroköji, Kamigyo-ku, Kyoto 602 (高倉通保、才中紀元、白倉一路)
and water *ad libitum*. The tumor cells used were Ehrlich ascites carcinoma cells propagated in our laboratories for more than three years in the same strain of mice.

**Experimental Assay Method** The investigations were based on assays of TD$_{50}$ or determination of a number of cells expected to produce tumor in one-half of the inoculated sites.

**Transplantation Method** Animals with 7- to 10-day-old ascites tumor were sacrificed by cervical dislocation and tumor cells were removed from the peritoneal cavity for transplantation. After the viable cells were counted by Trypan Blue staining method in a hemocytometer, the tumor cells were diluted serially in Hanks media containing 5% fetal calf serum (Difco, Mich.) for TD$_{50}$ assays. Test tubes containing these cell suspensions were kept in ice water until termination of the transplantation. For each assay, six to eight different concentrations of cells in 1:1 or 1:2 dilution were employed. Three microliters of tumor cell suspension containing a fixed number of viable tumor cells was injected intracutaneously in six sites on the dorsal skin of a mouse. To avoid leakage of the inoculum, needles were first inserted subcutaneously and then the injection was made intracutaneously. The recipient animals received a whole body irradiation of 400 rads 24 hr before the transplantation (see Ref. 19 for details). After the irradiation, they were assigned by a random number scheme into one of the dose levels in one of the assays. For each assay 12~15 mice were used.

**Drug Administration** The drug tested was an alkylating agent, cyclophosphamide of 96% purity (Shionogi & Co.). Cyclophosphamide was dissolved in distilled water. The amount of the solution to be injected was fixed at 0.01 ml/g body weight by dissolving different dosages of the drug in a fixed volume of distilled water. A fixed amount of the drug (one of the drug dose levels) was injected intraperitoneally 24 hr after the transplantation when the inoculum would be expected to have formed microcolonies or one-day-old solid tumor, except in the second series of experiments. For example, 13 mice which had received varying doses of viable tumor cells for one TD$_{50}$ assay were injected a fixed amount of cyclophosphamide (say 0.05 mg/g) 24 hr after transplantation, and 13 mice for another TD$_{50}$ assays were injected with another fixed amount of the drug (say 0.1 mg/g) 24 hr after the challenge, and so on.

**Scoring Tumor Take and Analysis of TD$_{50}$** The inoculated sites were palpated for possible tumor growth every 5 or 7 days after the transplantation. This examination was started on the 7th day after transplantation and was continued for 30 days. If a tumor grew to more than 5 mm in diameter, it was scored as a "tumor take". If an animal died before the termination of scoring, it was excluded from the TD$_{50}$ assay unless it had tumors in all the sites. TD$_{50}$ was computed from tumor take frequency by logit analysis.

The survival fraction of tumor cells after drug administration was calculated from the ratio of TD$_{50}$ of the control to that of cyclophosphamide group.

**RESULTS**

The results of several separate experiments are summarized in Fig. 1. An unexpected finding was that the dose-survival curve is composed of two different cellular drug sensitivities, resembling the multicomponent cell survival curve after X-ray irradiation. Tumor cells in one-day-old solid tumors were sensitive to dose levels up to 0.05
SENSITIVITY OF TUMOR CELLS TO CYCLOPHOSPHAMIDE

Fig. 1. Survival of Ehrlich ascites carcinoma cells in one-day-old solid tumors after single administration of cyclophosphamide

(different symbols indicate separate experiments)

Fig. 2. Survival of Ehrlich ascites carcinoma cells in 6- to 54-hour-old solid tumors after a single administration of cyclophosphamide

(different symbols indicate separate experiments)

mg/g and were resistant to higher doses. The $D_{0}$, or a dose to reduce the survival fraction from 1 to $1/e$ in the straight portion of the dose-survival curve, was calculated according to the multi-target theory of cellular radiation response. It was 0.011 mg/g in the cyclophosphamide-sensitive portion and 0.033 mg/g in the resistant tail of the survival curve. The ratio of $D_{0}$ (resistant)/$D_{0}$ (sensitive) was 3.

The extrapolation number ($m$) or number extrapolated from the straight portion of the survival curve to the ordinate was calculated in the same manner. This does not
mean the actual number, because cellular multiplicity was not analysed in the present series. However, the ratio of $m_{\text{resistant}}/m_{\text{sensitive}}$, i.e., 0.07, may represent the fraction of resistant cells in the tumor. Thus, one-day-old solid tumor might contain 7\% cyclophosphamide-resistant cells.

Fig. 2 demonstrates surviving fractions after one injection of 0.1 mg/g cyclophosphamide given at various intervals after transplantation. Increase in the surviving fraction is due to the increased number of tumor cells or of cells in a certain cell population in the tumor. The doubling time of tumor cells according to this curve was 8.3 hr. This is shorter than the generation time of Ehrlich ascites tumor cells in the ascitic form 1~2 days after transplantation; 10~12 hr.\textsuperscript{5,17,20} Note that 0.1 mg/g of cyclophosphamide reduces cell survival to the resistant portion, if it is administered 24 hr after transplantation (see Fig. 1). Therefore, the curve in Fig. 2 seems to represent an increase of resistant cells in the tumor rather than the increase of the total number of tumor cells.

**DISCUSSION**

Our original expectations were that (1) one-day-old solid tumors or microcolonies might be well-oxygenated and might not contain any hypoxic cell fraction, so that tumor cells would respond to cyclophosphamide in a single component cell survival curve, i.e., an exponential survival curve expressed by a single $D_0$, and that (2) older tumors, say 10- or 20-day-old tumors, would show a limitation in the effectiveness of cyclophosphamide because of the poorly vascularized cell population within such large tumors. In other words, the administration of an alkylating agent such as cyclophosphamide might decrease cell survival as a function of the drug dose only to a certain value which would represent the limit of efficacy of the agent.

The present results, demonstrated in Fig. 1, failed to confirm either of these. The dose-cell survival curve shows two components of cyclophosphamide sensitivity. One-day-old solid tumors contained both cyclophosphamide-sensitive and -resistant cell populations. The question arises as to what kind of cells are in the cyclophosphamide-resistant fraction.

One of the answers to this question might be that some cells were originally resistant to cyclophosphamide. However, the fact that the cyclophosphamide-resistant cell fraction increased more quickly than the total cell population (Fig. 2) fails to support this assumption. If the resistant cell fraction increased spontaneously, only resistant cells would be left in the tumor cell line which is propagated by weekly transplantations. Therefore, it must be that a difference in the cell state in the tumor is the main factor inducing the development of a cyclophosphamide-resistant fraction.

A second explanation might be that cells of a certain stage in the cell generation cycle become resistant to cyclophosphamide. However, no cell lines have been reported in which age-responses to alkylating agents or to ionizing radiations differ by a factor of 3.\textsuperscript{9,10,14,21}

A third explanation is similar to that of the X-ray multicomponent cell survival curve; the initial portion represents sensitive aerobic or well-oxygenated cells, and this is followed by a tail representing the resistance of hypoxic or oxygen-deficient cells.
Bruce et al. demonstrated three different types of action of chemotherapeutic agents on colony-forming cells. Cyclophosphamide was in the third group, which showed an exponential survival curve, but had different effects on lymphoma and normal hematopoietic colony-forming cells, i.e., the sensitivity of cells to cyclophosphamide depended on the proliferative state of the cells. Fast-growing cells (lymphoma colony-forming cells) are more sensitive to cyclophosphamide than slowly proliferating cells (normal hematopoietic colony-forming cells). It was also demonstrated in vivo that the sensitivity of lymphoma cells to 5-fluorouracil, which also belongs to their third group and has an effect similar to that of cyclophosphamide, is lower in the transitional growth phase than in the exponential growth phase of the cell cycle.4)

Their data contributed to the present studies in that a small fraction of cells was cyclophosphamide-resistant might indicate the presence of slowly proliferating cells in one-day-old solid tumors. Tannock reported that the doubling time of two-day-old tumors was the same as the cell generation time and growth fraction was 100%. Belli and Andrews showed that P388 tumor cells in one-day-old ascites tumors were all aerobic. On the other hand, Lala and Patt showed that the growth fraction of one-day-old ascites Ehrlich tumors was already 82%. Hypoxic foci were demonstrated by Suit and Maeda in three-day-old C3H mouse mammary carcinoma. On the basis of these data, a small fraction of slowly-proliferating cells would be expected in one-day-old solid tumors.

Another fact that should be noted is that TD50 of Ehrlich tumor cells transplanted intracutaneously was usually 3~5 × 10² viable cells without any treatment and 5 × 10⁴ cells with treatment with 0.05 mg/g of cyclophosphamide (this is the dose which reduces the survival fraction to the point of intersection of sensitive and resistant cell survival curves). If the diameter of an Ehrlich ascites tumor cell is assumed to be ≈10 μ, then 5 × 10⁴ cells would form a solid tumor of ≈0.1 mm³ with a radius of 300 μ. Note that this radius is longer than the maximum distance that oxygen can diffuse from a capillary to cells, i.e., 160 μ. Therefore, it might be reasonable to consider that a one-day-old solid tumor contains a small population of oxygen-deficient cells and that slowly proliferating cells would be present in such hypoxic foci. The resistant tail in the dose-cell survival curve may represent the sensitivity of these cells which would be hypoxic and proliferate with a slower generation time than the aerobic cells.

In other words, it might be that cyclophosphamide is incorporated into the hypoxic cell population in the tumor, although the sensitivity of these tumor cells to cyclophosphamide depends largely on their proliferative state. 5-Iododeoxyuridine tested in C3H mouse mammary carcinoma was also able to increase the sensitivity of hypoxic cells in tumors 8 mm in diameter to radiation. This result gives some support to our considerations in the present studies.

We are grateful to the Section of Experimental Radiotherapy and Department of Biomathematics, M. D. Anderson Hospital and Tumor Institute, Houston, U.S.A., for the computation of TD50 assays. We would like to thank Professor Hiromu Kaneda for his constant support of this research and Miss Hiroe Morimoto for her technical assistance. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education.

(Received March 4, 1970)
M. URANO, ET AL.

REFERENCES

4) Bruce, W. R., Meeker, B. E., ibid., 38, 401 (1967).
10) Mauro, F., Madoc-Jones, H., personal communication.
19) Urano, M., Shirakura, I., Tanaka, N., in press.
20) Urano, M., Tanaka, N., unpublished data.