Environmental Mutagens & Carcinogens 17-1 : 7-11 (1997)

Reduced Adriamycin Cytotoxicity in RIF-1 Multicell Spheroid Due to an Acidic Microenvironment

Kyung Il Um[1], Sally B. Cheston, Mohan Suntharalingam and Juong G. Rhee

Department of Biology, [1]College of Natural Sciences, Dong-A University, Pusan, Korea, and Department of Radiation Oncology, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A.

(Received, March 15, 1997)

ABSTRACT : **Variations in adriamycin uptake and cytotoxicity were studied in tumor cells that were grown in different growth states and microenvironments. RIF-1 tumor cells were maintained in an RPMI 1640 medium, and grown in either a monolayer or multicell spheroids. For exponentially growing cells, adriamycin cytotoxicity increased with increased dosage up to 2.5 μg/ml, and this cytotoxicity was reduced when the cells were grown in a plateau phase or in an acidic microenvironment (pH 6.6). This reduced cytotoxicity was correlated with the uptake of the drug. For multicell spheroids, the cytotoxicity of the drug was reduced dramatically, and this reduction was also correlated with a reduced uptake of the drug and an acidic pH inside of the spheroids. When the drug cytotoxicity was evaluated at different locations within the spheroids, the cells in the inner regions were least affected by the drug, suggesting that both an acidic microenvironment and non-cycling plateau phase cells are contributing factors in decreasing the efficacy of the drug in an organized tissue, such as multicell spheroids.**

Keywords : **Adriamycin, Drug Resistance, Acidic pH, Multicell Spheroid, Cell Sorting**

INTRODUCTION

Since the first report by Sutherland *et al.* (1970), the multicell spheroid system has been adopted for a variety of human and rodent tumor cells (Kwok *et al.*, 1988; Schwachöfer, 1990; Kunz-Schughart *et al.*, 1996). This system has been considered as an *in vitro* tumor model, because the microenvironment and the three-dimensional organization of the cells are very similar to tumors *in vivo* (Sutherland *et al.*, 1979; Schwachöfer, 1990). This multicell spheroid system is particularly appropriate for studying the parameters determining the efficacy of treatment with ionizing radiation (Sutherland and Durand, 1976; Pourreau-Schneider and Malasie, 1981) and chemotherapeutic agents (Sutherland *et al.*, 1979; Twentyman, 1980; Wilson *et al.*, 1981; Kwok *et al.*, 1988; Erlichman and Wu, 1992). Since the microenvironment within a spheroid is heterogeneous, variations in viability, metabolism, acidity and oxygen content appear to be natural (Mueller-Klieser, 1987; Sutherland, 1988; Kunz-Schughart *et al.*, 1996).

It has been demonstrated that cells grown as multicell spheroids exhibit resistance to chemotherapeutic agents (Sutherland *et al.*, 1979; Wilson *et al.*, 1981; Erlichman and Wu, 1992) and ionizing radiation (Rodriguez *et al.*, 1988), when compared to identical cells grown in the monolayer. Radioresistance may be caused by hypoxia and cell-to-cell contact within the spheroids (Durand and Sutherland, 1972; Olive, 1989). Unlike most chemotherapeutic agents, the cytotoxic effect of adriamycin is dramatically reduced in cells grown as multicell spheroids (Sutherland *et al.*, 1979). This report postulates that altered metabolic and/or microenvironmental states in the inner spheroid cells could be the possible causes. Since this pioneering work, there has been no report delineating the cause for the resistance to adriamycin. In the present study, we chose growth state and an acidic microenvironment as possible factors, and the effects of these factors on the uptake and cytotoxicity of adriamycin have been investigated employing RIF-1 multicell spheroids.

MATERIALS AND METHODS

Cell and spheroid cultures

Radiation induced fibrosarcoma (RIF-1) cells of C_3H mice were maintained *in vivo* and *in vitro* as originally described

This study was supported, in part, by ABROAD RESEARCH FUND, Dong-A University, 1996.

(Twentyman *et al.*, 1980). The cells were cultured in RPMI 1640 medium with 10% calf serum in a humidified incubator equilibrated with 5% (v/v) CO_2 at 37°C. In order to produce multicell spheroids, approximately 10^6 cells were cultured in a petri-dish, of which some cells adhere to one another rather than adhering to the dish. After 1 to 2 days, the small cell clumps (50-150 μm) were collected and cultured in a spinner flask. Spheroids of 0.8-1.2 mm in diameter were obtained in about 3 weeks of culture and used in the present study.

Drug treatment and survival assay

Adriamycin (Sigma, MO) was dissolved in distilled water and mixed with an equivalent volume of double strength RPMI 1640 medium. This mixture (100 μg/ml) was sterilized with a Millipore filter (0.22 μm), and diluted to necessary concentrations just prior to use. The mixture containing adriamycin was handled under a yellow light illumination, and the cells or spheroids were exposed to the solution at 37°C, in the dark.

For our drug uptake studies, fluorometric analysis was adopted in accordance with Schwartz (1973). One milliliter of adriamycin treated cells (10^5 cells) was added to tubes containing 0.2 ml 33% (w/v) $AgNO_3$ and extracted at 4°C with 2.0 ml iso-amyl alcohol. The organic phase was analyzed using a Sequoia-Turner fluorometer (Abott Diagnostics, IL) by exiting at 490 nm and by collecting the emitting data maximally at 585 nm. Blank and standard adriamycin solutions were prepared for each set of experiments.

Cell survival was determined by employing an *in vitro* clonogenic assay. Adriamycin treated cells and spheroids were dispersed with a 0.25% trypsin solution, washed twice, and an appropriate number of cells (10^2-10^4 cells) was plated in culture flasks. These flasks were incubated for 6-7 days and stained with 1% crystal violet. Clones containing more than 50 cells were included for survival determination.

Cell sorting

In order to distinguish cell locations within a multicell spheroid, a fluorescence-activated cell sorting technique (Durand and Olive, 1981) was employed. Spheroids were exposed to 5 μM of Hoechst 33342 (Molecular Probes, OR) for 30 min and dispersed into single cells with a 0.25% trypsin solution. A Becton-Dickinson FACSTAR cell sorter was used in sorting. Ten fractions with equal numbers of cells were sorted by using excitation at 350-360 nm (40 mW) with emissions monitored with a 449 nm band pass filter. Cells sorted for odd number fractions were collected and subjected to survival assays.

pH determination within a multicell spheroid

Glass capillaries were pulled to 5 μm tips, and the tips were treated for hydrophobicity. These tips were filled with a H^+ selective ion-exchanger (World Precision Instruments, FL). The response of each electrode was tested for pH 6, 7 and 8 prior to use. Under a dissecting microscope, a spheroid was immobilized by using a microforcep, and the spheroid were placed on a meshed platform in a small fountain. Medium of the fountain was constantly overflowed to keep the spheroid in a constant growing condition while measuring pH. Using a hydraulic micromanipulator, the electrode was advanced through a spheroid at the speed of 15 μm/sec, and the signals were digitized every second and stored on a disk.

RESULTS

The influence of growth state on the cytotoxicity of adriamycin is shown in Fig. 1. For exponentially growing cells (Expo), adriamycin cytotoxicity increased with increased dosage up to 2.5 μg/ml. Cytotoxicity was reduced when the cells were grown in a plateau phase (Plat) and further reduced when grown as multicell spheroids (Whole Spheroids). When the spheroids were dispersed and the dispersed cells were exposed to the drug, the cytotoxicity increased (Dispersed Cells) and became an intermediate state between exponential and plateau phase cells. This marked difference in drug efficacy between whole spheroids and dispersed cells was correlated with the uptake of the drug (Fig. 2).

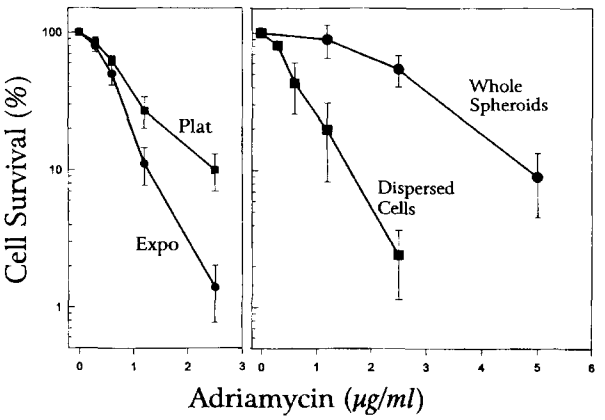

Fig. 1. The influence of growth state on the cytotoxicity of adriamycin. Left panel: RIF-1 cells were grown in either an exponential (Expo) or plateau (Plat) phase. Right panel: Whole spheroids were much more resistant to adriamycin treatments for 30 min than the dispersed cells from the spheroids. Each symbol represents 4-7 experiments and the bars are S.E.M.

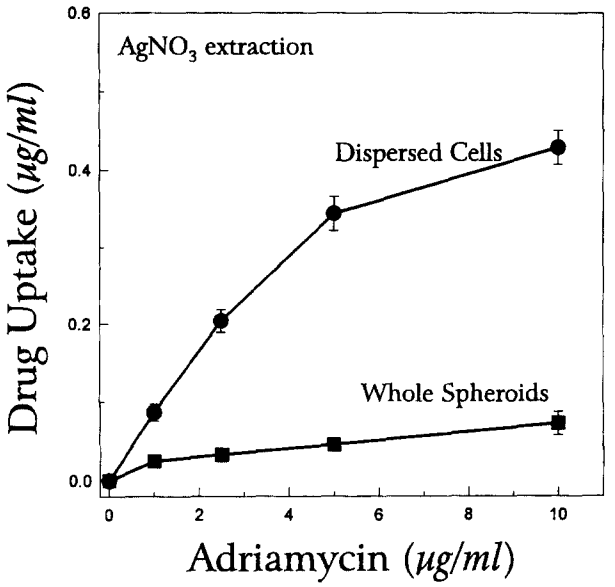

Fig. 2. Differences in uptake of adriamycin. Spheroids or cells were exposed to adriamycin for 30 min, and the drug bound to DNA was extracted with the use of $AgNO_3$ and isoamyl alcohol and detected with a fluorometer. Each symbol represents 8-15 experiments, and the bars are S.E.M.

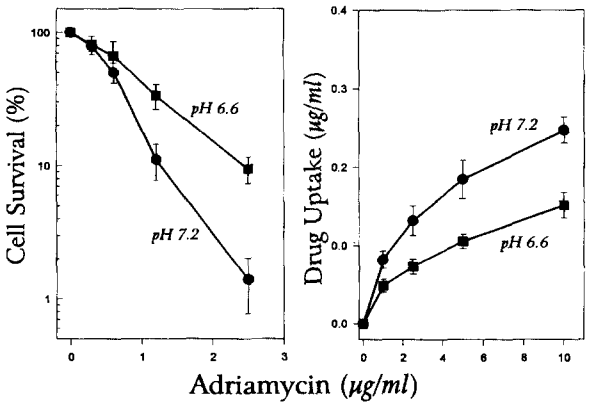

Fig. 3. The influence of pH on the effect of adriamycin. Exponentially growing cells were acutely exposed to pH 7.2 or 6.6 and exposed to adriamycin for 30 min. The survival of drug treated cells (left panel) was compared with the uptake of the drug (right panel). Each symbol represents 4-9 experiments, and the bars are S.E.M.

The influence of an acidic pH on the cytotoxicity of adriamycin is shown in Fig. 3. The cytotoxicity of the drug was reduced when the pH of the medium used for exponentially growing cells was changed from 7.2 to 6.6 (left panel). This difference was also correlated with the uptake of the drug (right panel), suggesting an important role of an acidic microenvironment in reducing drug efficacy.

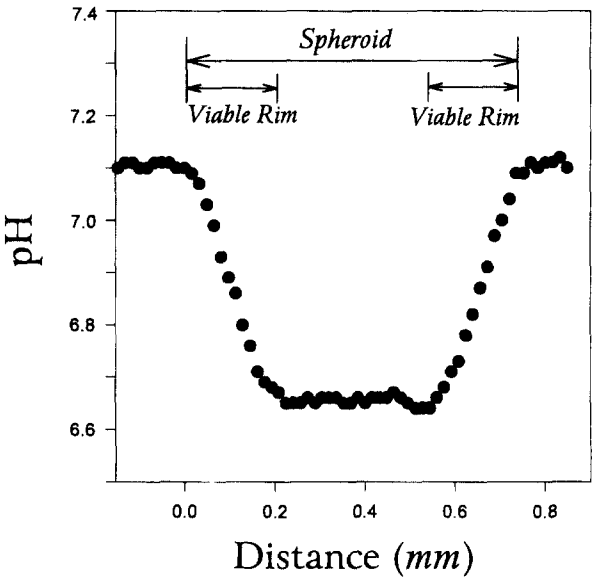

Fig. 4. pH distribution within a RIF-1 multicell spheroid. Using a hydraulic micromanipulator, a glass microelectrode (5 μm tip diameter) was advanced through a spheroid. pH signals are plotted as a function of the distance from the surface of a spheroid.

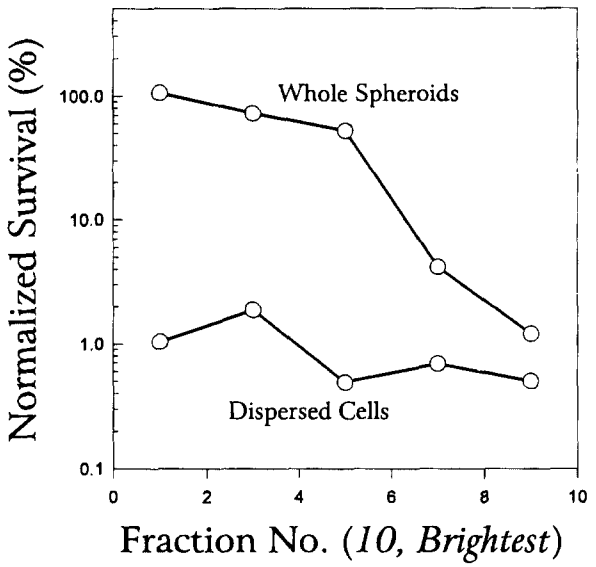

Fig. 5. Varying effects of adriamycin within multicell spheroids. The location of the cells within a spheroid was distinguished by using a Hoechst 33342 and by sorting them. Among 10 fractions of equal numbers of cells, the cells from odd number fractions were collected and assayed for survival. The survival of the drug treated cells was normalized to that of untreated cells.

When a pH microelectrode was inserted through a multicell spheroid, the pH signal gradually decreased from 7.1 to 6.6

while passing through the viable rim (200-250 μm), remained low through the necrotic center (300-400 μm), and gradually increased from 6.6 to 7.1 through the opposite viable rim (Fig. 4). The microenvironment inside of a spheroid was apparently acidic. In order to evaluate the cytotoxicity of adriamycin for different locations within a spheroid, a fluorescence-activated cell sorting technique was employed (Fig. 5). In cell sorting, RIF-1 spheroids were exposed to adriamycin, stained with Hoechst dye, and then dispersed with a trypsin solution (Whole spheroids). Cytotoxicity was minimal for dimly stained inner cells (fractions 1, 3, and 5) and significant for brightly stained outer cells (fractions 7 and 9). When the dispersed and sorted cells were exposed to adriamycin at pH 7.2, the dim and bright cells were equally sensitive to the drug (Dispersed cells). These results suggest that adriamycin only works for cells in the outer regions of spheroids, although cells in the deeper regions, once dispersed, have similar sensitivities to the drug.

DISCUSSION

In the present study, we observed a reduced cytotoxicity of adriamycin for RIF-1 cells grown as multicell spheroids (Fig. 1), and the reduced effect was correlated with the uptake of the drug (Fig. 2). Our observations for RIF-1 spheroids are consistent with the observations for EMT-6 spheroids as previously reported (Sutherland *et al.*, 1979). For instance, when exponentially grown EMT-6 cells were treated with 0.5 μg/ml adriamycin for 1 h, the surviving fraction was approximately 0.001, but it increased to 0.3 when the cells were grown as spheroids. This difference also correlated with the uptake of the drug. Therefore, it is likely that the effect of adriamycin dramatically decreases when tumor cells are grown in an organized tissue, such as multicell spheroids.

It is known that there are cycling and noncycling cells within a spheroid (Kunz-Schughart *et al.*, 1996). The difference in the cell cycle does not account for the reduced uptake and reduced efficacy of adriamycin in multicell spheroids, since both exponential and plateau phase EMT-6 cells are about equally sensitive (Sutherland *et al.*, 1979). At different locations within a multicell spheroid, the cellular uptake of adriamycin is known to vary, decreasing from the outer regions to the inner regions, as demonstrated by histological sections (Sutherland *et al.*, 1979). Employing a fluorescence-activated cell sorting technique (Durand and Olive, 1981), a gradient of adriamycin uptake within V79 multicell spheroids has also clearly been shown (Durand, 1981 and 1989). This gradient is correlated with cell survival (Durand, 1989). We have also observed the gradient of cell survival within a spheroid (Fig. 5), suggesting that the growth of cells as spheroids seems to impart an additional degree of drug resistance relative to the cells grown as monolayers (Durand, 1981). Recently, the same author concluded that the delivery of the drug to organized tissues is a problem, particularly for adriamycin, probably because of a diffusion barrier within organized tissues (Durand, 1989).

In the present study, we showed that there was a pH gradient within a multicell spheroid (Fig. 4). The neutral pH (7.1) measured at the outer regions sharply shifted to an acidic pH (6.6) inside of the spheroid. This pH gradient appeared to parallel a reduced cytotoxicity to adriamycin for cells of the inner regions (Fig. 5), indicating a good correlation between a low pH and a reduced efficacy of the drug. As we demonstrated in the present study, uptake of adriamycin was reduced by a modification of the microenvironment to an acidic pH (Fig. 3). To our knowledge, there has been no report which clarifies the role of an acidic pH in modifying the efficacy of adriamycin. Our present observations strongly support not only the idea that the microenvironment within multicell spheroids is an important factor determining cellular metabolism, activity and vitality (Sutherland, 1988), but also the possibility that an acidic pH microenvironment is one of the leading factors that determines the uptake and efficacy of adriamycin in organized tissues, such as multicell spheroids.

REFERENCE

Durand, R.E., (1981): Flow cytometry studies of intracellular adriamycin in multicell spheroids *in vitro, Cancer Res.*, **41**: 3495-3498.

Durand, R.E., (1989): Distribution and activity of antineoplastic drugs in a tumor model, *J. Natl. Cancer Inst.*, **81**: 146-152.

Durand, R.E., and R.M. Sutherland, (1972): Effects of intercellular contact on repair of radiation damage, *Exp. Cell Res.*, **71**: 75-80.

Durand, R.E., and P.L. Olive, (1981): Flow cytometry studies of intracellular adriamycin in single cells *in vitro, Cancer Res.*, **41**: 3489-3494.

Erlichman, C., and A. Wu, (1992): Resistance of MGH-U1 bladder cancer spheroids to vincristine, *Anticancer Res.*, **12**: 1233-1236.

Kunz-Schughart, L.A., K. Groebe, and W. Mueller-Klieser, (1996): Three-dimensional cell culture induced novel proliferative and metabolic alterations associated with oncogenic transformation, *Int. J. Cancer*, **66**: 578-586.

Kwok, C.S., S.E. Cole, and S.-K. Liao, (1988): Uptake kinetics of monoclonal antibodies by human malignant melanoma multieell spheroids, *Cancer Res.*, **48**: 1856-1863.

Mueller-Klieser, W., (1987): Multicellular spheroids. A review on cellular aggregates in cancer research, *J. Cancer Res. clin. Oncol.,* **113**: 101-122.

Olive, P.L. (1989): Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids. *Radiation Research,* **117**: 79-92.

Pourreau-Schneider, N., and E.P. Malasie, (1981): Relationship between surviving fractions using the colony method, the LD50 and the growth delay after irradiation of human melanoma cells grown as multicellular spheroids, *Radiat. Res.,* **85**: 321-332.

Rodriguez, A., E.L. Alpen, M. Mendonca, and R.J. DeGuzman, (1988): Recovery from potentially lethal damage and recruitment time of noncycling clonogenic cells in 9L confluent monolayers and spheroids, *Radiat. Res.,* **114**: 515-527.

Schwachöfer, J.H.M., (1990): Multicell tumor spheroids in radiotherapy research (Review), *Anticancer Res.,* **10**: 963-970.

Schwartz, H.S., (1973): A fluorometric assay for daunomycin and adriamycin in animal tissues, *Biochem. Medicine,* **7**: 396-404.

Sutherland, R.M., (1988): Cell and environment interactions in tumor microregions; the multicell spheroid model, *Science (Wash.),* **240**: 177-184.

Sutherland, R.M., and R.E. Durand, (1976): Radiation response of multicell spheroids. An *in vitro* tumour model, *Curr. Top. Radiat. Res. Q.,* **11**: 87-139.

Sutherland, R.M., H.A. Eddy, B. Bareham, K. Reich, and D. Vanantwerp, (1979): Resistance to adriamycin in multicellular spheroids, *Int. J. Radiat. Oncol. Biol. Phys.,* **5**: 1225-1230.

Sutherland, R.M., W.R. Inch, J.A. McCredie, and J. Kruur, (1970): A multicomponent radiation survival curve using an *in vitro* tumour model, *Int. J. Radiat. Biol.,* **18**: 491-495.

Twentyman, P.R., (1980): The response to chemotherapy of EMT6 spheroids as measured by growth delay and by cell survival, *Br. J. Cancer,* **42**: 297-304.

Twentyman, P.R., Brown, J.M., Gray, J.W., Franko, A.J., Scoles, M.A., and F.R Kallman,. (1980): A new mouse tumor model system (RIF-1) for comparison of end-point studies. *JNCI,* **64**: 595-604.

Wilson, W. R., G. F. Whitmore, and R. P. Hill, (1981) : Activity of 4'-(9-Acridinylamino)methanesulfon-m-anisidic against Chinese hamster cells in multicellular spheroids, *Cancer Res.,* **41**: 2817-2822.

RIF-1 다세포 Spheroid에서 미세환경이 산성일때 감소되는 Adriamycin의 세포독성

엄경일[1], Sally B. Cheston, Mohan Suntharalingam, 이종길

[1]동아대학교 자연과학대학 생물학과, *Department of Radiation Oncology, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A.*

적 요

종양세포를 각기 다른 성장상태와 미세환경에서 배양한 후, **Adriamycin**섭취와 그 세포독성에 대한 변화를 연구하였다. **RIF-1** 종양세포를 **RPMI 1640** 배지에서 단층 또는 다세포 **spheroid**로 배양하였다. 지수적으로 성장하는 단층세포는 **Adriamycin**의 농도가 **2.5 μg/ml** 까지 증가됨에 따라 그 세포 독성이 증가했으나, **Adriamycin**의 효과는 세포가 **plateau** 상태이거나, 미세환경이 산성 **(pH 6.6)**이 되면 감소되었다. 감소되는 **Adriamycin**의 효과는 세포가 섭취한 **Adriamycin**의 양과 비례하였다. 다세포 **spheroid**에서는 **Adriamycin**의 효과가 현저히 감소하는데, 이는 **Adriamycin**의 섭취양이 감소되었거나 **spheroid**의 내부가 산성 **pH**가 되는 것과 깊은 관련이 있었다. **Adriamycin**의 효과를 **spheroid** 안의 여러 부위별로 측정하면, 내부에 위치한 세포들은 **Adriamycin**의 영향을 거의 받지 않았다. 이상의 결과를 종합하여 볼 때, **Adriamycin**의 효과는 다세포 **spheroid**와 같은 체제를 가진 악성종양의 경우 현저히 감소되는데, 그 주된 원인은 종양의 미세환경이 산성 **pH**가 되거나, 비대사적인 세포로 구성되어 있기 때문으로 추측된다.