DIFFERENTIAL THERMO-RESISTANCE OF MULTICELLULAR TUMOR SPHEROIDS[*]

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Abstract – Many cell lines, when cultured under proper conditions, can form three dimensional structures called multicellular spheroids. These spheroids resemble in vivo tumor models in several aspects. Therefore, studying growth characteristics and behavior of spheroids is beneficial in understanding the behavior of tumors under various experimental conditions. In this work, we have studied the growth properties, along with the thermal characteristics of spheroids of DU 145 human prostate carcinoma cell lines and compared the results to monolayer cultures of these cells. For this purpose, the DU 145 cells were cultured either as monolayer or spheroids. At various times after initiation of cultures, the growth properties of spheroids as a function of seeding cell number was determined. To evaluate the thermal characteristics of spheroids, they were heated at various stages of growth at 43 °C for various periods. The thermal response was judged by the survival fraction of colony forming cells in spheroids or monolayer culture following heat treatment. The results showed spheroids were more resistant to heat than monolayer cultures at all stages of development. However, the extent of this thermal resistant was dependent on the age, and consequently, the size of the spheroid. The result suggests that the differential thermal resistance of the spheroid cultures develop gradually during the growth of spheroid cultures of DU 145 cell line.

Keywords – Hyperthermia, multicellular spheroids, thermal resistance, DU 145 prostate cell line

1. INTRODUCTION

The multicellular spheroid represents an *in vitro - in vivo* transition model which exhibits important *in vivo* solid tumor correlates [1]. These include intimate cell-cell contact [2], individual hypoxic cell populations [3], and cycle times that range from comparable to exponential monolayer rates through an essentially nondividing state [4]. In brief, they combine the relevance of organized tissues with the accuracy of *in vitro* methodology [5-10].

It has been suggested that some physiological differences may exist between cells grown in two-dimensional contact (monolayer) and cells grown in three-dimensional contact (multicellular tumor spheroids) [11, 12]. Research showed that growth of human glioma cells in these two systems led to different degrees of sensitivity to radioionated iododeoxy uridine (IUdR) [13]. Several authors have reported higher radioresistance of cells in spheroids compared with monolayer cultures [14-17].

Prostate carcinoma is the second most frequent cause of cancer related death in the world. The rate at which prostate cancer grows and metastasizes is highly variable from patient to patient. Progression of this disease is associated with genetic changes within the tumor [18] and interactions between the tumor and host environment [19]. In vitro models provide a well defined environment for cancer studies, in contrast to the complex host environment of an *in vivo* model. Several experimental models, including multicellular spheroids of prostate cancer have been developed and studied [7, 20-23].

In this work we have studied the differential thermal sensitivity of prostate carcinoma cell line DU 145 [24] multicellular spheroids. DU 145 is an established cell line that can self-assemble into large, stable spheroids though a combination of intracellular communication and diffusion [25]. We studied the correlation between the size and number of spheroids with the concentration of cultured cells. Then, the influence of three-dimensional contact between DU 145 cells and the size of spheroids on their sensitivity to hyperthermia at $43\,^{\circ}C$ was compared with monolayer cultures.

2- MATERIALS AND METHODS

a) Cell Line

Human prostate carcinoma cell line, DU 145, was established from a metastatic lesion of poor to moderately differentiated prostatic adenocarinoma in the central nervous system of a patient who also had leukemia [24]. This cell line was maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS) (obtained locally), 500 U/ml of penicillin (SIGMA) and 200 mg/lit of streptomycin (JABEREBN-HAYAN).

b) Growth curve

Cells were cultured at a density of $2x10^4$ per well in multiwell plates (24 wells/plate) (NUNC). At 24 hr intervals, the cells from triplicate wells were removed by 1mM EDTA/ .25% Trypsin (w/v) treatment and counted in a hemocytometer. An average of nine counts was used to define each point. Mean $\pm$ SEM (Standard Error of mean)

c) Monolayer culture

Cells were cultured as monolayer at a density of 10^4 cells/cm^2 in T-25 tissue culture flasks (NUNC). Cultures were maintained at $37\,^{\circ}C$ in a humidified atmosphere of 7.5% CO_2. Cultures were propagated or cells were harvested by trypsinizing cultures with 1mM EDTA/ 0.25% Trypsin (w/v) in Phosphate Buffer Saline (PBS).

d) Spheroid Culture

Spheroids were initiated using the Liquid Overlay technique [26]. Different concentrations of DU 145 cells from 10^5 to 10^6 cells were seeded into 100 mm dishes coated with a thin layer of 1% agar (Bacto Agar, Difco, Detroit, MI) with 10ml of RPMI 1640 supplemented with 10% FCS. The plates were incubated at $37\,^{\circ}C$ in a humidified atmosphere of 7.5% CO_2. Half of the culture medium was replaced with fresh medium twice per week.

e) Efficiency of spheroid formation

To determine the correlation between spheroid number and the efficiency of spheroid formation with the number of cells seeded, different concentrations of single cells were cultured as described above. The number of spheroids growing in dishes was determined using an inverted phase microscope in days 10 and 20 after culturing. To count spheroids, the number of spheroids were counted within seven random squares, each with 1 cm^2 area, and then extrapolated to the area of 100 mm Petri dishes. The efficiency of spheroid formation was determined as the ratio of the number of spheroids formed to the number of cells cultured.

f) Clonogenic assay

Single cell suspensions, either from spheroid or monolayer cultures, were diluted so that different concentrations of cells were seeded in 60 mm Petri dishes (NUNC), grown in RPMI 1640 and supplemented with 10% FCS. The cells were incubated at $37\,°C$ in a humidified atmosphere of 7.5% CO$_2$ for ten days. The colonies were counted using an inverted phase microscope (ZEISS, Axiovert 405M) on day 10 and clonal plating efficiency (PE) was determined.

g) Heat treatment of monolayer cultures

Cells were cultured at 5 x 10^5 cells per flask in T-25 culture flasks in RPMI-1640 culture medium supplemented with 10% FCS for monolayer culture. After 48 hours, the culture medium was replaced with the RPMI-1640 culture medium which was used during the period of heat treatment. Hyperthermia was applied at $43\,°C$ for periods of 0-120 min in a precision water bath (HAKKE F3) with $\pm\,0.1\,°C$ accuracy. Controls were exposed to $37\,°C$. The cells were then harvested, counted and tested for viability as described above. The cells were also assayed for colony forming ability by plating 500 cells per dish in 60 mm Petri dishes. The colonies were counted on day 10.

h) Heat treatment of spheroid cultures

To study the effect of hyperthermia on spheroids, cells were cultured at 5 x 10^5 cells per Petri dish in 100 mm dishes coated with a thin layer of 1% agar for multicellular spheroid formation. On day 3, and day 26, the spheroids were gently precipitated. The culture medium was replaced with RPMI 1640 medium and spheroids were resuspended and heated as described for monolayer cultures. They were then treated with 300 μl of PBS containing 1mM EDTA/ 0.25% trypsin (w/v) for 10 minutes at $37\,°C$. Trypsin was neutralized by the addition of 700 μl of the culture medium containing FCS. Spheroids were mechanically disaggregated. Single cells were counted and tested for viability. Cells were then seeded at a density of 3000 cells per Petri dish for colony formation assay.

i) Survival curves

Survival curves were generated by plotting the log of the ratio of the number of colonies produced at a given heating condition to the number of colonies produced by related control cells versus the heating time at the given temperature.

j) Statistical analysis

Data are given as mean values $\pm$ SEM (Standard Error of Mean), with n denoting the number of experiments. Student's t test was considered appropriate and applied. A value of $p \leq 0.05$ was considered significant.

3- RESULTS

The growth curve of DU 145 cells in monolayer culture is shown in Fig. 1. The population doubling time calculated from this curve was approximately 23 hours.
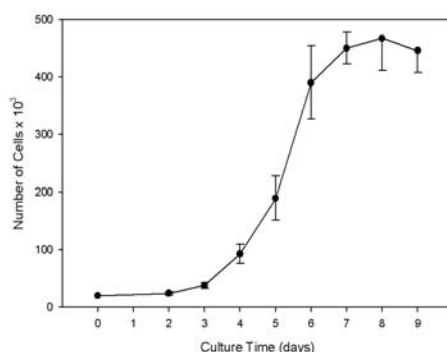

Fig. 1. The growth curve of DU 145 cell line in the monolayer cultures. $2x10^4$ cells were seeded per well in 24 wells microplates. At 24 hr intervals, the cells from triplicate wells were removed by Trypsin/ EDTA treatment and counted. An average of nine counts was used to define each point.
Mean $\pm$ SEM (Standard Error of mean) of 3 experiments

The DU 145 cells were able to form spheroids in liquid overlay cultures. Fig. 2 shows the phase contrast micrographs of these spheroids during the course of development. During the first 24 hours of the culture, only loose aggregates of cells were formed (Fig. 2a). At longer time periods, 48 – 72 hours, spheroids began to take shape, but the cells were still loosely organized (Fig. 2b). With still more time, 7 and 25 days, spheroids formed completely and became well rounded structures composed of numerous highly compacted cells in which it was difficult to distinguish individual cells from each other (Figs. 2c and 2d, respectively). In general, the time of formation of spheroids depends on the initial number of cells which are plated. For instance, when $5x10^5$ cells were plated in the 100 mm Petri dishes on a thin layer of agar, the spheroids were formed within 2 to 3 days.
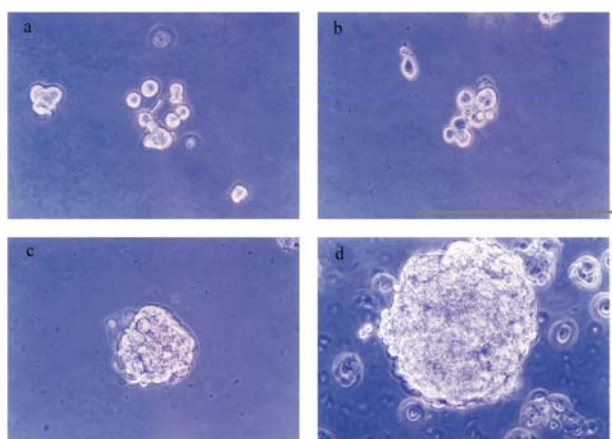

Fig. 2. Phase contrast micrographs of DU 145 cells spheroid formation. (a) Cell aggregates 24 hrs after initiation of the spheroid culture. (b) Primary nucleus of spheroids at 72 hrs. (c) Spheroids at day 7

of the culture. (d) Spheroids at day 25 of the culture. Magnifications: x32 for all micrographs

Figure 3 shows the number of spheroids formed as a function of the number of cells cultured per Petri dish. The efficiency of spheroid formation was calculated as described in the method section and the result is shown in Fig. 3. The efficiency of spheroid formation initially increased rapidly as the seeding concentration of cells increased, but then reached a plateau at higher concentrations of seeding cells.
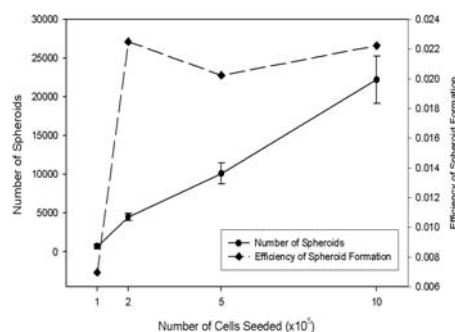

Fig. 3. The efficiency of spheroid formation by DU 145 cell line. Cells were seeded in different numbers in 100 mm Petri dishes coated with a thin layer of 1% agar. Spheroids were counted in seven randomly chosen squares of 1 cm^2 area and then extrapolated to the area of the 100 mm Petri dish. The efficiency of spheroid formation was calculated by dividing the number of spheroids by the number of cells seeded. Mean $\pm$ SEM of 3 experiments

The effect of spheroid formation and intimate cell-cell contact on the colony forming ability of DU 145 cells was studied by comparing the colony forming ability of cells harvested from the monolayer culture, with the colony forming ability of cells harvested from spheroids of various ages. For this purpose, a similar number of cells from the two cultures were harvested and used to assay the colony forming ability as described in the method section. The results are shown in Fig. 4. It is evident that cells lose their colony forming ability when they come in contact with one another and form multicellular spheroids. The PE for monolayer culture was 0.129, that of the 3 day spheroid culture was 0.0139, and that of the 26 day spheroid culture was 0.006. In all cases, the PE of spheroid cultures was significantly smaller than the monolayer cultures.
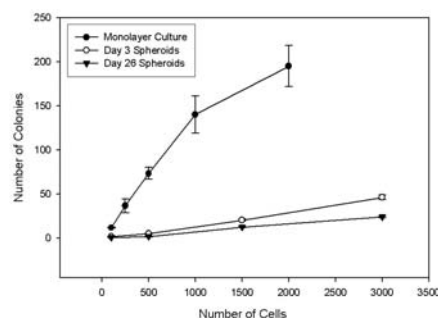

Fig. 4. The colony forming ability of DU 145 cell line from monolayer and spheroid cultures. Cells were harvested from monolayer cultures, day 3 and day 26 of spheroids and plated in 60 mm Petri dishes at various concentrations. The colonies formed 10 days after initiation of cultures were counted. Mean $\pm$ SEM of 3 experiments

The response of spheroids to hyperthermia was studied by applying hyperthermia at 43 °C for various periods of time to spheroids of 3 days and 26 days old and also to monolayer cultures. The

number of colonies formed by cells from each group: monolayer, 3 day spheroids, and 26 day spheroids as functions of heating time is shown in Fig. 5. As can be seen, hyperthermia reduced the colony forming ability of both monolayer cells and spheroid cells. However, the extent of reduction in the number of colony forming cells from spheroids was much less than those of monolayer cultures. The plot of survival fractions versus heating time at 43 °C for monolayer and spheroid cultures is shown in Fig. 6. This Figure clearly shows that cells from spheroid cultures are more heat resistant than cells from monolayer cultures and the extent of thermal resistance is dependent on the age of spheroids.
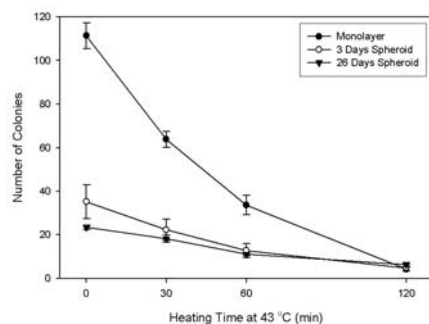

Fig. 5. The effect of hyperthermia on the colony forming ability of DU 145 cells from monolayer and day 3 and 26 of spheroid cultures. Hyperthermia at 43 °C was applied to monolayer and spheroids as described in the method section. Cells were then harvested and assayed for colony formation as described in the legend to Figure 4. Mean $\pm$ SEM of 3 experiments
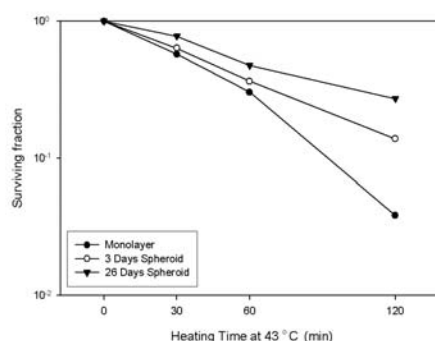

Fig. 6. The hyperthermic survival curve for DU 145 cell line in monolayer and spheroid cultures. Data extracted from Fig. 5

4- DISCUSSION

In this work, we have studied the growth and thermal properties of human prostate carcinoma cell line DU 145 in spheroid and monolayer cultures. Spheroid cultures are interesting and valuable in vitro model systems, which allow many properties of in vivo tumor systems to be studied quantitatively [27, 28]. They have been used extensively to study the effect of various agents such as biological factors [29], drugs [5, 30], radiation [27, 31-33], and hyperthermia [12, 34-36] on tumor systems.

Many cell lines can form multicellular spheroids [8, 37]. This property is highly dependent on the adhesion molecules such as the integrin and cadherin families [38]. Prostate cell spheroids have been used extensively as a model system to study various biochemical mechanisms involved in prostate malignancies [7, 20].

The kinetics and growth properties of spheroid formation is highly dependent on the culture condition and technique [26, 39].The liquid Overlay technique is a reliable and easy technique for the generation of spheroids, and has been extensively used and described for the production of spheroids from the DU 145 cell line [25]. The results presented here show that although the number of spheroids formed is apparently a function of the number of cells which are seeded for spheroid production, there is, however, a limit in the efficiency of spheroid formation (Fig. 3). In our results, the efficiency of spheroid formation reached a plateau value of 0.02 when the seed number of cells was 2×10^5 or higher. This value might well be dependent on the culture vessel geometry, the ingredients of the culture medium, and even on the nature of the cell line used for the study [40].

The extent of the ability of a cell line to produce colonies in vitro has been considered as a measure of its malignancy in vivo. The results presented here show that cells cultured in a monolayer condition are more clonogenic than cells cultured in a multicellular spheroid condition. Obviously important regulatory systems are lost when cells lose their extensive intercellular contacts in monolayer cultures, and therefore produce more colonies. The plating efficiency of cells cultured in a monolayer condition was at least an order of magnitude larger than the plating efficiency of cells cultured in spheroids. As the spheroids grew in age and size, the PE decreased accordingly. This extra loss of clonogenecity is probably due to limitations in nutrient and oxygen supply to larger sized spheroids [31].

Hyperthermia is now a well established modality in cancer treatment, either alone or in combination with other modalities such as chemotherapy and radiotherapy [41, 42]. By a similar reasoning mentioned above, cells in monolayer cultures, by losing intercellular communication, may have lost protective systems against environmental stresses such as hyperthermia [12, 43]. Our results presented here (Figs. 5 & 6) support this hypothesis. Hyperthermia reduced the clonogenecity of cells from monolayer and spheroid cultures (Fig. 5). However, the extent of reduction in clonogenic cells from monolayer cultures was significantly larger than cells from spheroid cultures (Fig. 6).

Several agents used to treat cancer are subject to a contact effect similar to that seen for hyperthermia [29]. Spheroids with ~100μm mean diameter are about twice as resistant to photodynamic therapy, even when reduced to single cells prior to light exposure [31, 44]. This phenomenon could be explained on the grounds of a hypothesis, which assumes an existence of a pool of a substance exchangeable between cells which would be required for the repair of radiation induced damage [45]. Although such a substance has not been identified so far, it is interesting to note that the relationship between ionic coupling and radiosensitivity has been demonstrated [15], and the mechanism of increased heat resistance of DU 145 cells in spheroid cultures as compared with monolayer is not yet known.

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