

Meeting Report

Spheroids in Cancer Research¹

Abstract

The purpose of the meeting was to exchange information on spheroid culture methods and to review the role of spheroids in cancer research. Techniques and results presented at this meeting suggested many areas of promising research applications using the multicellular spheroid tumor model system to better understand certain aspects of basic tumor biology and responses to different agents with therapeutic potential. Multicellular spheroids provide a system of intermediate complexity between standard two-dimensional culture systems and tumors *in vivo* and, when applied in experiments in conjunction with studies at other levels of complexity in the other systems, can produce important insight in a variety of areas of tumor biology. Accurate interpretation of results from experiments using multicellular spheroids requires detailed characterization of the particular spheroid culture system being used. Furthermore, care must be exercised to avoid generalizations from the study of only one or a few spheroid systems, since significant differences among different types of spheroid cells have been reported, as is often the case for tumors *in vivo*. As with any model system, it is important to be continually aware of its strengths and its limitations.

Introduction

Multicellular spheroids are composed of tumor cells growing in a 3-dimensional structure simulating the growth and microenvironmental conditions of real tumors. Spheroids can be used in a variety of research areas as an *in vitro* tumor model system of intermediate complexity between standard monolayer or suspension cultures and tumors *in vivo*.

Workshops, demonstrations, and discussions at the meeting centered on the various techniques and assays used for growing multicellular spheroids and for measuring responses to different types of therapeutic agents. Workshop themes included: (a) initiation and growth of spheroids; (b) characterization of spheroid cultures; and (c) uses of spheroids: principles and practice. Different techniques for growing spheroids were demonstrated, and the necessity to carefully characterize spheroid cultures because of differences among different types of tumor cells was emphasized. A wide variety of research applications of spheroids of tumor cells were demonstrated and discussed including studies of growth properties, tumor-like environments, and responses to different forms of cancer therapy.

¹ The First International Workshop/Conference on Spheroids in Cancer Research was held at the Cancer Center, University of Rochester, Rochester, New York, June 16 to 20, 1980. Because of the requirement to be concise, it has been necessary to eliminate experimental details and specific citations in this report. In addition, certain areas covered at the meeting have been mentioned only briefly or omitted entirely. Details and citations of the research presented at the conference can be obtained by writing to Dr. Robert Sutherland, to whom requests for reprints should be addressed.

Received January 23, 1981; accepted April 14, 1981.

The formal conference consisted of presentations of papers arranged in 2 major sections: (a) basic biology of spheroids; and (b) responses to therapy modalities. Research progress on the fundamental biology of spheroids related to growth control, cell interactions, microphysiology, and host-spheroid relationships was described. Also, this model system has been used by many researchers to investigate more applied questions involving responses of cells in a tumor-like structure and environment to different cancer therapy modalities such as radiation and drugs used both separately and in combination.

Initiation and Growth

Initiation and growth of multicellular spheroids have been accomplished with many different types of tumor cells using suspension culture in spinner flasks or growth of cells in liquid overlay cultures on nonadherent surfaces such as agar or special non-tissue culture plastic dishes. Initial formation of spheroids often involves aggregation of cells which then grow into larger 3-dimensional structures. Effects of different media and sera on growth of spheroids were discussed. The rate and extent of initial aggregation of cells and the thickness of the viable rim of cells surrounding the necrotic center which develops in large spheroids depended on the amount and the quality of the serum used. Spheroids of relatively homogeneous sizes approaching 1 mm diameter and containing 0.5 to 1.0×10^5 cells/spheroid can be obtained after growth for several weeks in culture. Growth curves from measurements of populations of spheroids in suspension culture or individual spheroids in microwells exhibited different kinetics for different cell types but, in general, Gompertzian kinetics similar to tumors *in vivo* were obtained.

It was concluded from a review of the literature that, of about 40 different types of malignant cells which have been tested thus far, approximately half could be grown as spheroids, while there were only limited reports of apparently nonmalignant cells which can be grown as spheroids; these, however, were usually established cell lines such as hamster V79 lung cells. The ability to grow human tumor cells as spheroids is a major interest. Papers were presented in which it was clearly demonstrated that it is possible to routinely grow many types of human cells as spheroids including tumors of the cervix, breast, thyroid, and other organs. Most established cell lines of human tumors which have been tested readily grow as spheroids. Some laboratories have obtained success rates as high as 40% for growth of spheroids directly from surgical specimens of a variety of human tumor types. Criteria for successful growth of spheroids include both initiation and continued growth of the spheroids to sizes of at least $500 \mu\text{m}$ in diameter. Other laboratories reported success rates for spheroid growth directly from surgical specimens of only about 5% for human lung and brain tumor cells. Preliminary studies of cells from surgical specimens of several different types of human ovarian tumors initially formed spheroids on nonadherent culture dishes, but continued growth has not yet been achieved.

Basic Biology

When processed and sectioned for histology, differences were seen among different types of spheroids, although at large sizes, all developed the general characteristic of a viable rim of cells surrounding a necrotic core. The cell organization as well as the thickness of the viable cell layers surrounding the necrotic center in larger spheroids differed significantly for different cell types. The cell packing density and, consequently, the amount of extracellular space also differed. In general, cells near the periphery were more loosely attached, the intermediate zone was tightly packed, and there was significant extracellular space near the necrotic center. Differences in extracellular space and packing density can theoretically greatly affect concentration gradients of oxygen and nutrients and, consequently, growth and other properties of cells in different regions.

Detailed light and electron microscopy studies of some tumor cell culture lines were reported. Where comparisons have been made, such as the EMT6 mammary tumor, the cells in spheroids appear morphologically very similar to the same cells when grown as tumors *in vivo*. Often the cells on the outer spheroid surface appear to align in a more ordered fashion with tighter cell-to-cell contacts. Different types of microprojections are prominent on these cells as revealed by scanning and transmission electron microscopy. Cells in RIF fibrosarcoma spheroids often exhibited 2 distinct morphologies: rounded cells in the outer few layers; and very elongated spindle-shaped cells in the more central regions. Other than these findings, no detailed reports on morphogenesis in spheroids or on comparisons of specific cellular characteristics of spheroids *in vitro* and tumors *in vivo* were presented. It is to be expected that, as the spheroid technique becomes applied more for research on primary cultures of tumors, especially of human origin, more detailed investigations of morphogenesis will be carried out.

Different types of intercellular junctions have been reported in spheroids. The frequency of such cell-to-cell contacts varied widely among different types of spheroids. The types of junctions observed in the spheroids frequently were similar to those observed in solid tumors of the same histological type.

In the presentations of cell growth kinetics in spheroids, it was demonstrated that the growth fraction decreases as the spheroids grow. The decrease of cycling cells in central areas of spheroids appears to be a general phenomenon. In the few types of spheroids which have been studied in detail, the cells stop cycling mainly in G₁ phase of the cell cycle, although cells in a quiescent phase in S and G₂ sometimes also occur. These alterations in cell growth kinetics appear similar to many tumors *in vivo*.

Data were presented showing that cells could detach from surfaces of some types of spheroids (EMT6 mammary, V79 lung, and human thyroid). Detailed studies of the cells shed from EMT6 spheroids showed that they were mainly mitotic cells. Measurements of cell shedding and factors which influence it might be helpful for understanding early events in metastases.

By introducing radioactively labeled tumor cells on to EMT6 spheroid surfaces and using autoradiography, it was found that cells could migrate within the spheroids. The migration rate of the cells which were introduced into the spheroids was greatly influenced by pretreatment with various surface active agents

such as trypsin or EDTA. These studies were considered to be important for interpretation of kinetic studies involving locations of different subpopulations of cells and because high migration capacity might be correlated with cell spreading and metastasis *in vivo*. In other related experiments, small spheroids of mammary tumor cells were used to explore the interaction with endothelial cells cultured on collagen gels. Cells from the spheroids extended cellular cords on top of the endothelium, moved underneath the edges of the endothelial cells, and invaded into the collagen matrix. These interactions could be modified by pretreating the spheroid cells with heparin, dextran sulphate, and similar surface active agents. This system should be highly useful in exploring factors influencing invasion of malignant cells into normal tissues.

New Techniques

There was considerable interest in the use of techniques to sequentially dissociate spheroids and thereby obtain cells from different regions. The methods used are not all the same but basically rely on using proteolytic enzymes either at or below room temperature and, at different time intervals, removing cells from the spheroid surface. Cells obtained from different regions within the spheroids can then be analyzed for different properties such as clonogenicity, cell cycle state, and response to different drugs. Caution is, however, required with the use of certain proteolytic enzymes to dissociate the spheroids since they may in some cases produce loss of cells during the procedure.

Flow cytometric analyses of cell populations derived by dissociation of spheroids are being used to measure cell cycle distributions and other parameters. The advantages and pitfalls of using these flow cytometric techniques for determining spheroid growth kinetics were discussed. These include the problems of discriminating between living and dead cells and the presence of multinucleated cells. Minimally toxic fluorescent stains of the Hoescht type were used to sort subpopulations from V79 spheroids to allow cloning experiments. Preliminary evidence was presented suggesting that the nonproliferating quiescent fraction could be identified in EMT6 spheroids using an acridine orange stain for both RNA and DNA.

Oxygen

Concentration gradients of oxygen, nutrients, and other factors develop within spheroids as they increase in size. A series of papers from different institutions considered the following topics: (a) the dependence of oxygen consumption rate of cells in spheroids on spheroid size; (b) the oxygen gradients in spheroids measured by microelectrodes; and (c) the effects of stationary *versus* flowing medium on growth and responses to radiation and drugs. The data and theoretical analyses suggested that concentration gradients in spheroids could be very important in determining growth kinetics and drug and radiation sensitivity and demonstrated clearly the necessity for more research on the role of diffusion and consumption-related factors.

Oxygen gradients have been measured using microelectrodes in spheroids of human osteosarcoma, human thyroid cancer, mouse EMT6 mammary tumor, and hamster V79 lung cells. The microelectrode data indicate that spheroids contain

hypoxic cells, but the oxygen gradients differ significantly among spheroids of different types and different sizes. It was apparent that oxygenation varies under different culture and experimental conditions. Three culture situations were considered: (a) the spinner flask culture; (b) liquid overlay culture; and (c) culture of attached spheroids in a slowly flowing medium. The diffusion boundary layer outside the spheroids differs when applying these different culture methods. Cellular metabolism causes concentration gradients of oxygen, nutrients, catabolites, and other products to develop in unstirred media, giving rise to lowered oxygen partial pressure and lowered concentrations of nutrients near the cells. Thus, absolute values of gradients inside spheroids would be affected. It was concluded that the influence of medium flow rates on the gradients inside spheroids should be investigated in detail since theoretical estimates of the influences of this parameter indicated that significant differences in oxygen concentration were possible.

The results obtained to date using microelectrodes and spheroids grown in different gas mixtures allow some conclusions to be drawn about the role of oxygen for induction of necrosis in spheroids. Based on earlier work and theoretical considerations from studies of tumors *in vivo*, it has generally been assumed that there is a complete lack of oxygen near the necrotic regions in malignant tumors and that hypoxia induces necrosis. On the basis of limited experiments, it appears that hypoxia plays a major role in the development of necrosis in spheroids, but nutrient depletion and other unfavorable environmental conditions (e.g., low pH) may play a significant role depending on the oxygen concentration in the culture medium. It also appears that significant levels of oxygen may be present in the necrotic region depending on the cell type, culture, and experimental conditions.

The oxygen distribution inside the spheroids could be modified by altering the respiration rate of the cells with hyperthermia or drugs. For example, moderate increases in temperature for short periods increased cell respiration rates and shifted oxygen distributions to lower levels within the spheroids as measured with microelectrodes. Several drugs, for example, BCNU,² nitrogen mustard, and chlorambucil, also inhibited respiration at concentrations and exposure times which were otherwise nontoxic. These changes in oxygen concentration profiles in spheroids were found to significantly affect the radiation response of spheroids of V79 hamster lung cells.

Radiation

Several types of spheroids contain cells which are hypoxic and, consequently, are radiation resistant. However, the survival curves obtained by clonogenic assay of cells obtained by dissociating irradiated spheroids do not always indicate the level of resistance expected of severely hypoxic cells. Many types of spheroids, even at large sizes with central necrosis, contain cells which exhibit either intermediate levels of resistance or normal aerobic radiosensitivity. This may be related to cell packing densities, culture conditions, and other inherent properties of the cells which determine the oxygen concentration gradients as discussed previously. Survival curves of cells

obtained from irradiated animal tumors generally exhibit the radiation response expected from a subpopulation of severely hypoxic cells. Thus, these experiments with spheroids suggest that limitations of vascular perfusion and not chronic oxygen diffusion limitations may be the main cause of radiobiologically significant hypoxia in many tumors *in vivo*.

Different end points for assessing spheroid responses to radiation and other cytotoxic agents were compared. End points that are being used for radiobiological responses include (a) spheroid survival, (b) single cell survival, (c) spheroid regression and regrowth rates, (d) spheroid cellularity, and (e) cell cycle redistributions. Dissociation of the spheroids after treatment followed by clonogenic assay appeared to be the most informative end point. On the other hand, methods such as cloning of whole spheroids or measurements of spheroid volumes after treatment have the advantage that the spheroid structure is kept intact after the treatment in a way which might be more similar to the situation in a solid tumor. Cellularity and outgrowth of cells from intact spheroids to determine sterilizing or curative doses were considered to be rather good end points, while regression rates in many cases did not correlate well with cell kill measured by clonogenic assay of cells from dissociated spheroids. Whenever growth curves are utilized, the conclusions about spheroid cell survival should be supported by other measurements. For one type of spheroid studied, the rat 9L gliosarcoma, cell survival after radiation was affected significantly by the presence of feeder (heavily irradiated) cells in the clonogenic assay. Feeder cells also promoted repair of potential lethal damage produced by irradiation in cells from both monolayer and spheroid cultures.

The effect of intercellular contact on responses to ionizing radiation, hyperthermia, and ultrasound was investigated using small EMT6 or V79 spheroids (multiplicity, ≈ 5 to 20 cells/spheroid). The increased radiation resistance due to growth of cells in close intercellular contact in EMT6 spheroids was reported to be qualitatively similar to, but quantitatively less than, that found previously in V79 spheroids. Cells in contact are also more resistant to hyperthermia, but, unlike the response to radiation, this resistance is exhibited mainly as a change in the slopes of the survival curves. Exposure of small EMT6 spheroids to ultrasound intensities of 1 to 5 w/sq cm produced lysis of external cells and decreased clonogenic capacity, but the spheroid cells were again more resistant than cells grown in monolayer or suspension cultures. There was little or no effect on growth, survival, and detachment of cells from large spheroids (200 to 500 μm) exposed to up to 50 w/sq cm ultrasound. Investigations of the mechanism of increased cell resistance associated with intercellular contact have shown that, in monolayer cultures, there seems to be a correlation between occurrence of low-resistance contacts between cells as measured by microelectrodes and resistance to radiation when grown as small spheroids. Increased resistance could be detected also when using other end points such as chromosome damage, mutagenesis, and cell cycle progression.

Drugs

Spheroids are useful for tests of the cytotoxicity of different drugs, not only because of cell cycle growth phase redistributions and heterogeneous microenvironments but also because the penetration barriers which might exist in poorly vascular-

² The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MAMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidine.

ized regions of tumors can in part be simulated. The drugs must penetrate through several layers of cells without the aid of capillaries. Studies were reported on both the primary sensitivity to different drugs and development of resistance associated with repeated exposures.

Drug resistance based mainly on limited capacity to penetrate spheroids was found for BCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037), Adriamycin, melphalan, carminomycin, vinblastine, vincristine, methotrexate, and MAMSA. This was shown by direct studies such as fluorescence or autoradiography. The only exception reported was 5-fluorouracil which seemed to penetrate efficiently at least through glioma spheroids.

Several nitroimidazoles have been evaluated and determined to preferentially kill hypoxic cells in spheroids. Because these may be resistant to other forms of therapy including radiation and other drugs, experiments were carried out to determine the potential usefulness of this preferential cytotoxicity. Cells located in the central areas of EMT6 spheroids were resistant to several anthracyclines tested including Adriamycin. Experiments were done to determine the mechanism of this resistance. Poor drug penetration was a major factor, although other unknown mechanisms possibly related to the metabolic state of the internal cells appear to be involved. When the nitroimidazole misonidazole and Adriamycin were combined, it was possible to produce an apparent supraadditive response. Favorable interactions of misonidazole with several drugs have now been reported for different animal tumor systems.

Spheroids of V79 cells exposed to MAMSA after dissociation of intact spheroids in trypsin were much less sensitive than log-phase monolayer cells. However, the shape of the MAMSA survival curve suggested that the majority of noncycling spheroid cells were more sensitive than cells from noncycling plateau-phase monolayers. When intact spheroids were exposed to MAMSA, cell killing was less extensive than for cells from disaggregated spheroids, possibly as a result of drug diffusion barriers. When a sequential trypsinization procedure was used to recover populations of cells from various depths within the viable rim, cell survival was shown to increase rapidly with distance from the surface of MAMSA-treated spheroids. These results can be interpreted as indicating that noncycling cells in V79 spheroids are less sensitive to MAMSA than are cycling cells. However, these cells become more sensitive as they approach the necrotic core while, in intact spheroids, these innermost cells are partially protected by a drug penetration barrier.

EMT6 spheroids were exposed for a constant time to graded doses of 8 different cytotoxic drugs, and the response was assayed using both growth delay and clonogenic cell survival as end points. For nitrogen mustard, BCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037), melphalan, and cisplatin, a considerable increase in measured cell survival was observed if spheroid dissociation was delayed for 24 hr after drug exposure. A reasonably good correlation was seen between growth delay and cell survival measured at 24 hr for the 5 agents. For Adriamycin, actinomycin D, and 5-fluorouracil, no recovery in measured survival occurred over a period of 24 hr. These 3 drugs produced longer growth delays than did the other 5 for an equal amount of cell killing.

Whether the response of mammary tumor spheroids to chemotherapy correlated with the response observed in the

respective animal tumor lines *in vivo* was also investigated. Growth delay induced by 4 drugs (Adriamycin, vincristine, L-phenylalanine mustard, and Cytosan) was compared in the 2 systems. In all cases, the sensitivity of the 2 systems was directly correlated, including the development of resistance after repeated drug exposures. It appears that spheroids might be useful as a predictor of *in vivo* sensitivity.

A new method for investigation of the penetration of radioactively labeled chemicals into spheroids was described. The method was based on rapid freezing, freeze-drying, vapor fixation, wax embedding, dry-sectioning, and contact autoradiography. The method did not significantly disturb the distribution of water-soluble test molecules. Investigations on human glioma spheroids showed that the low-molecular-weight substances thymidine and D-leucine equilibrated rapidly throughout the spheroids. Investigations on cytotoxic drugs showed that 5-fluorouracil penetrated the spheroids much more efficiently than vinblastine.

The preferential cytotoxicity to internal *versus* external cells was investigated by using V79 spheroids consisting of thioguanine-resistant and thioguanine-sensitive cells. Composite spheroids containing, for example, an outer layer of thioguanine-sensitive cells and internal thioguanine-resistant cells could be formed. Differential effects of drugs or radiation were assayed by plating cells from the spheroids in medium containing thioguanine (in which only the resistant cells could survive). By using this technique, it was shown that Adriamycin acts mainly on external cells, while drugs such as nifuroxime act mainly on internal hypoxic cells.

Immunology

The response of the immune system in mice to transplanted EMT6 spheroids was studied. The experiments showed that the spheroids recovered at different times after peritoneal implantation were penetrated by granulocytes, macrophages, and lymphocytes resulting in killing of cells within the spheroids. The kinetics and extent of this killing could be modified by sensitization procedures and the degree of immunological competence of the host animals. Allogenic implants showed very rapid killing kinetics with nearly all tumor cells dead in 4 to 5 days, while spheroids implanted into sensitized syngeneic mice showed much slower kinetics, taking 12 to 14 days to achieve the same level of tumor cell kill. The kinetics of penetration of the different host cell types was reported on in some detail. It was demonstrated that killing of tumor cells in the syngeneic system was due mainly to T-lymphocytes, although there also appeared to be a degree of T-cell-independent cytotoxicity.

Research into determining the interactions of the host and tumor cells in the spheroids and the role of different therapy agents on the host-tumor relationships is ongoing. In this regard, experiments using heated spheroids were presented. One interesting observation was that spheroids heated with low levels of hyperthermia (e.g., 42.5° for 1 hr) actually killed the animal earlier after *i.p.* implantation than did nonheated spheroids. The unexpected enhancement of toxicity of moderately heated spheroids cannot be explained by the kinetics and types of infiltrating host cells. The explanation may lie in the functional properties of the different host cells which are infiltrating or in the effects of heat on structure and properties

R. Sutherland et al.

of spheroids such as shedding of cells into the various peritoneal areas.

These experiments are in an early stage but show promise in that the immunologically active cells can be trapped in the spheroids and therefore analyzed further regarding functional properties in control of tumor-host interactions. Modifications of these interactions by different therapy modalities can also be readily evaluated.

Robert Sutherland
Department of Radiation Biology and Biophysics and Cancer
Center
University of Rochester
Rochester, N. Y. 14642

Jorgen Carlsson
National Defense Research Institute
Department FOA 4
S-90182 Umea, Sweden

Ralph Durand
Radiation Biology Department
The Johns Hopkins Oncology Center
Baltimore, Md. 21205

John Yuhas
University of Pennsylvania
Department of Radiation Therapy
Philadelphia, Pa. 19104