Eur J Cancer Clin Oncol, Vol. 25, No. 8, pp. 1127–1133, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Commentary

Tumour Spheroid Technology in Cancer Therapy Research

JÖRGEN CARLSSON* and THORE NEDERMAN†

**Department of Radiation Sciences, Uppsala University, Box 535, S-75121 Uppsala, Sweden and †Department of Drugs Pharmacological Division, National Board of Health and Welfare, Box 607, S-75125 Uppsala, Sweden*

(A COMMENT ON: Cook JM, Florence AT, Russel J, Wheldon TE. Etoposide (VP-16) uptake by tumour spheroids and activity in the presence of Brij 30, formulation additives and sodium salicylate. *Eur J Cancer Clin Oncol* 1989, **25**, 311–318.)

THIS COMMENTARY is focussed on recent publications about tumour spheroid technology in cancer therapy research and mainly on those publications describing the effects of cytotoxic drugs and the possible penetration problems. Reports older than one or two years are essentially referred to through previously published reviews [1–4].

GENERAL GROWTH PATTERN

Spheroids have an outer layer of proliferating cells and an inside layer of mainly quiescent cells. In the central areas of spheroids with a diameter greater than 400–700 μm, there are often massive necrotic areas. This growth pattern is similar to that of solid tumour nodules containing proliferating cells close to the capillaries, quiescent cells next to these and necrotic areas at larger distances. Spheroid cells can be further divided into subpopulations as, for example, quiescent cells close to the necrotic centre which are irreversibly growth inhibited and quiescent cells closer to the surface that have the capacity to enter the cell cycle. The pattern is general although there are large cell-type dependent variations in growth rate, cell morphology and the thickness of the viable cell layers [1–7]. The spheroids contain an extensive extracellular matrix composed of, for example, fibronectin, laminin and collagen in a proteoglycan gel. There are differences between different types of spheroids also in this aspect and the differences correlate with differences in growth [8–11].

Accepted 30 March 1989.

CULTURE METHODS

Two different methods are mainly used for spheroid culture; liquid overlay culture and spinner flask culture. Both culture techniques are described in the book *Spheroids in Cancer Research*, edited by Acker *et al.* [2]. The techniques are often combined so that aggregates are formed in liquid overlay culture and, after some days, transferred to spinner flasks for long term culture. The spheroids might be grown in spinner flasks and later transferred to liquid overlay culture for individual handling. In the liquid overlay culture technique the spheroids are cultured in a static medium above an agarose gel which prevents cell attachment to the bottoms of the dishes. The method is especially well suited for tests of the cells' capacity to form and grow as spheroids. The spinner culture technique employs siliconated spinner flasks in which the tumour cell aggregates are formed and thereafter grown as spheroids. The spinner flask technique is especially suitable when large amounts of similar sized spheroids are needed. However, not all types of tumour cells can grow as spheroids in liquid overlay or spinner flask culture. Further methodological developments are needed, maybe using other culture techniques or new medium supplements.

A METHOD TO STUDY PENETRATION

A problem in studies with cytotoxic drugs is to measure penetration accurately. Most studies claiming that penetration problems exist have not really demonstrated penetration barriers. Instead they have most often applied conventional histology

in which most of the unbound drug has been washed away during fixation and dehydration. Thus, in these cases only binding was studied and it is not possible to know whether peripheral binding is dependent on a penetration barrier or if binding sites existed only in the peripheral cell layers. To really study penetration it is necessary to use a method which preserves the distribution of unbound drugs. The first experiments on spheroids with such a method was described by Nederman *et al.* [12] and showed that vinblastine had difficulties in penetrating into human glioma spheroids. The method is described in some detail below because it is still the only method by which the distribution of unbound substances can be demonstrated with high resolution (the resolution is given by the resolution in the autoradiographic process). The method is based on freeze drying and vapour fixation to preserve the distribution of soluble drugs in the spheroids. After incubation the spheroids are placed on cover slips and frozen in a bath of propane–propene cooled by liquid nitrogen. They are thereafter freeze-dried and vapour-fixed in a paraformic–aldehyde saturated atmosphere and finally immersed in xylene and embedded in wax. The embedded spheroids are sectioned and dry mounted on object glasses and then unwaxed in xylene. Autoradiographs are obtained avoiding contact between the specimen and hydrophilic solvents. An object glass with dried photoemulsion and the glass with the dry spheroid section are placed together in a dark room for the exposure period. The glass with the photoemulsion is then removed and processed for development and fixation. The autoradiographs are photographed and evaluated through densitometric evaluations in a scanning device.

CONVENTIONAL HISTOLOGY

For comparison with the special technique applied in the penetration studies, conventional histological processing can be performed with, for example, glycol–methacrylate or paraffin embedding after formalin fixation and alcohol dehydration. This is done to study the distribution of substances that are bound to the cellular structures in the spheroids.

AVERAGE DRUG CONCENTRATIONS

The average drug concentration in single spheroids can be measured using the oil centrifugation method described by Freyer and Sutherland [13]. Single spheroids are positioned in microwells, their diameters measured and their volumes calculated. They are then incubated for different times with a radioactive drug. After incubation each spheroid, together with the radioactive medium, is placed on top of oil in a small centrifugation tube. The tube is immediately spun forcing the spheroids to pass through the oil and down to the bottom of the tube. The radioactive medium remains above the oil surface. The bottom of the tube is cut off and placed in a plastic vial for liquid scintillation counting.

METHODS OF STUDYING EFFECTS OF THERAPEUTIC TREATMENTS

The method of first choice in the analysis of therapeutic effects is the test of the capacity for clonogenic survival of single cells dispersed from the spheroids. Two alternative methods have been applied. *The first* is to totally trypsinize apart the spheroids and one such example is the original study by Sutherland *et al.* [14], where the survival of cells from V79 spheroids was studied after irradiation. A subpopulation of radioresistant cells was, in this case, seen at high doses. Fluorescence activated cell sorting based on gradients of intracellular probes for viable cells has been used to distinguish peripheral from central cells [15]. Olive and Durand [16] have recently described a carbocyanine derivative (DiOC7-3) which seems even better than the previously used Hoescht 33342 for characterizing the original positions of cells in spheroids after enzymatic treatment. *The second* method is to make sequential trypsinization to directly obtain cell populations from different radial positions in the spheroids. This is possible for some spheroid systems while others are difficult to separate with trypsination. For example, some types of human glioma spheroids are extremely difficult to disintegrate although different combinations of degradation enzymes were tested [17]. The explanation is probably that the differences in extracellular matrix composition influence the possibilities for enzymatic cell separations [10].

An alternative or complementary method to clonogenic survival is to analyse the possible outgrowth of cells from spheroids. This method, which has been applied in only a few cases, is based on the seeding of intact spheroids in conventional culture dishes. The cells have to migrate out of the spheroid and form a growing monolayer. If the spheroid cells are successful, then the spheroid is said to have escaped the treatment but if they do not form a growing monolayer the spheroid is said to be sterilized. The requirement for migration is not necessarily a strong disadvantage because *in vivo* tumour cells subjected to therapeutic agents do, in fact, have to experience similar conditions. They can stay in their original position or migrate to a position with better nutritional conditions for growth. Thus, clonogenic survival of individual cells and cloning of intact spheroids might give complementary information. A detailed comparison between these methods, when applied to V79 cells, has been published by Durand [18].

Growth curves have been measured to quantify the effects of different therapeutic modalities but this method is not very powerful because all spheroids

reach a plateau whether they are treated or not, and this makes the differences between the control and treated spheroids less clear. Several studies on disturbances in growth curves, mostly quantified as growth delays, have however been published. Promising attempts have recently been made to connect data from growth curves with data from single cell survival experiments by using calculation methods, thereby allowing comparisons to be made and raising hope for future work with growth curves [19]. Other methods, such as analysis of thymidine labelling, are of course only for studies of acute radiation effects.

BASIC BIOLOGY

Basic aspects of spheroid growth must be studied to obtain a good understanding of the effects of therapeutical agents. Several factors seem to modify the growth conditions. Gradients in oxygen tension [2–4], pH [20, 21] and nutrients like glucose and amino acids are at hand. Catabolic products from the necrotic centre and the internal cells must form outward gradients and the excreted products might be more or less toxic. It has recently been shown by Freyer *et al.* [7, 22] that spheroids excrete cell degenerating factors. The lack of oxygen does not by itself induce necrosis, but the combination of glucose deficiency and hypoxia might do so [2–4, 23–25]. Several questions about the basic biology remain to be answered, for example, concerning the detailed relationships betwen hypoxia, hypoglycaemia and pH. Different types of spheroids have different pH and oxygen gradients and there is a tendency for those with low central pH values to have high central oxygen values [21]. The role of extracellular pH on the growth conditions is an interesting topic because it has been shown that changes in the buffer capacity, and thereby changes in the extracellular pH gradients, influence the thymidine labelling index preferentially in the inner regions of spheroids [20]. New methods for studying the metabolism in cellular spheroids have recently been described. Bioluminescence and photon counting were used for metabolic imaging of the spatial distribution of glucose, lactate and ATP in cryosections of spheroids [26]. A recent methodological development has demonstrated that ultrasound backscatter microscopy allows the central necrotic core and the rim with viable cells to be seen with good contrast [27].

The local conditions in the spheroids influence the metabolic state of the cells in several ways. For example, the amplification of the activated c-H-ras oncogene increased when human bladder cancer cells were grown as spheroids [28]. Melanoma spheroids has been shown to differentiate, seen as an increased melanogenesis, in the central regions [9]. Differentiation has also been demonstrated in colon carcinoma spheroids by formation of acini structures [29].

CHEMOTHERAPY AND PENETRATION

Reports have indicated that drugs such as methotrexate, vinblastine and the anthracyclines Adriamycin®, daunomycin and deoxydoxorubicin might penentrate poorly into the studied types of spheroids and that, in the tested cases, the spheroids were more resistant to the drugs than the corresponding monolayers [12, 30–36]. An interesting question is whether the increased resistance is mainly dependent upon penetration difficulties or if other factors such as varying sensitivity during the cell cycle and varying cell cycle distribution between spheroids and monolayers also are of importance. Conventional histological and autoradiographical procedures have been used in many of the previously published reports on penetration and most of these studies have indicated insufficient penetration. Since only the covalent or otherwise strongly bound drug is retained when using conventional histological methods, these results might be somewhat misleading. A soluble drug might not have been detected and it is possible that the penetration had been good but that only the peripheral proliferative cells bound the drug. Previously published studies from our laboratories have described that, when 5-FU and vinblastine penetration were compared in spheroids, drug resistance of vinblastine could, to a large extent, be explained by a lack of penetration [32, 37]. Penetration was in this case studied with the method which preserves the distribution of the soluble fraction of drugs [12]. Scientists are encouraged to make use of this method, otherwise it is difficult to distinguish between penetration barriers and peripheral binding.

The optimal physical and chemical conditions, e.g. pH and ion strength, in the peripheral regions of tumour spheroids might provide good conditions for the binding of therapeutically interesting substances while the conditions for binding might be less favourable in the deeper regions where the pH is lower [21] and where, for example, toxic catabolic products are produced [7]. A decreased sensitivity to CCNU has, in EMT6 spheroids, been demonstrated by lowering the pH or the oxygen tension in the culture medium [38].

The penetration pattern can probably be modified by changing the lipophilicity of the drugs. For example, it was recently shown that lipophilic Adriamycin® analogues partitioned more rapidly than Adriamycin® in lung tumour spheroids and that the lipophilic derivatives were more toxic [36, 39]. The possibilities of increasing drug uptake and penetration in spheroids by coadministration with surfactants and hydrotrophic agents have been investigated in some cases [40–42]. These studies

have, for example, shown that Tween 80 and sodium salicylate increase the toxic effects of etoposide in neuroblastoma spheroids. In studies by Cook and Florence [41] and Cook *et al.* [42] it seemed as if increased uptake of etoposide was obtained due to interactions of the additives with the tumour cell membranes. Several different types of additives have to be tested in combination with different types of drugs to reveal if there are any general mechanisms by which penetration can be changed.

Variations in both penetration and sensitivity to treatment for single drugs have been demonstrated to depend also on the type of spheroids. For example, two different types of lung cancer spheroids showed differences in the penetration patterns and their response to doxorubicin [43]. Furthermore, it has, with the V79 spheroid system, been demonstrated that mitoxantrone resistance is not solely due to restricted drug penetration, but is also due to an induced altered intrinsic resistance of the cells when grown as spheroids [44]. In spite of all indications about penetration problems there are drugs that seem to easily penetrate into spheroids such as 5-fluorouracil [37, 45] and cisplatin [43, 45]. It is likely that interactions in the extracellular matrix are of importance for the penetration patterns. Substances which can only penetrate into the extracellular space and are not expected to interact with the matrix or the cell membranes, like insulin and sucrose, can effectively penetrate into different types of spheroids [46]. This means that extracellular transport is possible in spheroids and that low molecular substances like cytotoxic drugs should be able to diffuse if they do not interact with the extracellular matrix or the cells.

SHEDDING

Anticancer agents like vincristine, Adriamycin® and cisplatin have been shown to inhibit cell shedding from melanoma spheroids and, in the case of vincristine, also from squamous lung cancer spheroids. Inhibition of shedding took place at concentrations in the same range as in which they are active in producing cell lethality. Inhibition of shedding might be an important factor to inhibit metastasis [47].

COMBINATION TREATMENTS

Recently published results have indicated that complex interactions occur in combination treatments and that the time sequences of the treatments are of importance as well as the actual doses given. For example, by using the V79 spheroid system, Durand and Vanderbyl [48] investigated the response of cell subpopulations to radiation and different drug combinations. Marked sequence dependent differences in overall toxicity were observed and drug toxicity, reoxygenation and sensitization occurred to a different extent with each drug and sequence. Maximal interactions between all drugs and radiation were observed when drug exposure immediately preceded irradiation. Synergistic effects when combinations of cytostatic drugs have been used, for example cisplatin and cytarabine, have also been demonstrated [45] . One study showed that ACNU and 5-fluorouracil had complementary actions in that the former was more active on cells in the deeper layers while the latter was more active on the cells in the outer layers. If the drugs were used together synergistic effects were obtained [49]. A parallel example was that of hyperthermia, which had a greater effect on the inner cells while radiotherapy was more effective on the outer layers [50]. Misonidazole has previously been shown not only to be a radiosensitizer but also a chemosensitizer and synergistic effects have recently been demonstrated when V79 spheroids were treated with CCNU and misonidazole [51]. The spheroid system has also been applied in studies of the effects of ultrasound and the results in one recent study indicated that ultrasound influenced the effects of heat treatment [52].

ANTIBODY MEDIATED RADIOTHERAPY

Another interesting aspect of the spheroid model is that the effects of monoclonal antibodies labelled with therapeutically interesting radioactive nuclides, like for example 131-I, can be studied [53]. Other radioactive nuclides such as 90-Y or 211-At or stable nuclides like 10-B or 157-Gd, which can be activated by low energy neutrons, should be of interest in future studies. Analysis of tumour seeking 'missiles', using spheroids, will probably be of importance because penetration, binding and the therapeutic effects can be studied simultaneously. Recent reports have indicated that penetration barriers for monoclonal antibodies can exist in spheroids and that antibody fragments might penetrate better [54–56]. Proteins other than immunoglobulins might be valuable for administering nuclides. The nuclides 59-Fe and 239-Pu have, when bound to transferrin, been shown to accumulate strongly in hepatocyte spheroids [57].

RADIATION AND HYPERTHERMIA

Spheroids have been extensively used in studies of radiation effects and the modifying influence of hypoxia. Some studies on the effects of hyperthermia have also been published. These fields are well covered by previous reviews [1–4]. Generally, it has been shown that spheroid cells are more radiation resistant than the corresponding monolayers. This is also valid for small spheroids, where there are no problems with oxygenation. The latter phenomenon, which often is referred to as a cell-contact dependent effect, has recently been proposed to be

due to a slow reentry of quiescent spheroid cells into the cycle allowing for more time to repair radiation damage [58]. A new aspect concerning radiation and spheroids is the question about radiation induced fragmentation of spheroids which indicates that a risk for radiation induced metastasis might exist [59].

CONCLUDING REMARKS

There is, in the studied tumour spheroids, a strong heterogeneity regarding the effects of cytotoxic drugs. Peripheral cells are often more damaged than central cells. This heterogeneity is probably representative of the heterogeneity in real tumours which often have irregular vascularization. There are also cell-type dependent differences in the response to the cytotoxic agents which parallel the differences seen between different types of tumours. This means that the spheroid system will be useful in further studies of how tumour cell heterogeneity influences the effects of cytotoxic drugs. In addition, it will be of interest to analyse the possibility of using targeting substances, like antibodies, to specifically deliver toxic agents. The spheroid system can also be used in studies determining the factors that are of importance in the creation of heterogeneity.

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