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E19-82-589

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# MULTICELLULAR SPHEROIDS AS AN IN VITRO TUMOR MODEL

Submitted to "Acta Radiol. Oncology"

1982

Sutherland et al.[1] developed the method of cell cultivation as multicellular spheroids. The spheroids represent one of the best in vitro tumor models comparable to nodules of many solid carcinomas and are intermediate in complexity between standard cell culture systems and animal tumors[2]. Morphological studies showed development of an anoxic component in the center of spheroids (or even necrosis).

The response to ionizing radiation was investigated with single exposures to $\gamma$-radiation, neutrons, heavy ions or $\pi^-$-mesons or with fractionated irradiation ($\gamma$-radiation and neutrons) by Erzgräber et al.[3,4]. The survival of single cells of spheroids was studied and typical multicomponent curves were obtained. Fractionated irradiation showed that spheroids exhibit similar behaviour as in vivo carcinomas, particularly, similar slopes of Stransquist's graphs were determined. The possibilities of investigation of an NSD concept or similar relations were suggested by Erzgräber et al.[4]. Such relations are of great interest as they may be utilized in radiotherapy of malignant tumors. Some new formulae have been proposed for fractionated radiotherapy (Kozubek[5,6,7]). These new relations will be utilized for quantitative evaluation of spheroid data.

## MATERIAL AND METHODS

For growth as multicellular spheroids cells of Chinese hamster (V 79-4) were harvested during the exponential phase of growth by trypsinization, separated in individual cells. About $10^4$ single cells were seeded in 10 ml medium, supplemented with 25 $\mu$g/ml dextran sulfate. To obtain large spheroids, after 4 days the growing spheroids were seeded into a roller tube with new medium, one spheroid in each tube, and cultured at 1 rpm. On the day of irradiation the spheroids with diameter $500 \pm 50$ $\mu$m were rated out. After irradiation the diameters of the spheroids were measured at two-day intervals up to 25 days and the volume of each spheroid was calculated. For one dose 10 spheroids were investigated.

## THE GROWTH OF SPHEROIDS

The growth is exponential at first but an average doubling time gradually decreases with time. Owing to the existence of

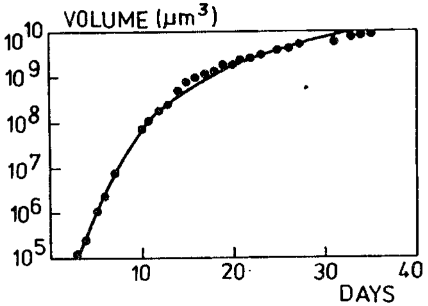



Fig.1. The growth of multicellular spheroid. Experimental data points (Kopp[8]) are compared with the solution of eqs.(1),(2) ($T = 0.58$ day$^{-1}$, $V_0 = 3.4\times10^5 \mu m^3$).

surface oxygen gradient, we can assume that only the surface layer consists of dividing cells. Spheroids grow exponentially up to some critical volume $V_0$ and then some non-growing fraction arises in the center. The growth curves are well described by the differential equations:

$$\frac{dV}{dt} = \frac{\ln 2}{T} \cdot V \qquad \text{for } V \leq V_0 , \qquad (1)$$

$$\frac{dV}{dt} = \frac{\ln 2}{T} \cdot [V - (V^{1/3} - V_0^{1/3})^3] \qquad \text{for } V > V_0 , \qquad (2)$$

where V is an immediate spheroid volume at time t, T is doubling time and $V_0$ is the critical volume. The experimental curve is compared with the theoretical one for $T = 0.58$, $V_0 = 3.4\times10^5 \mu m^3$ in Fig.1.

The parameters T and $V_0$ differ for various conditions of experiment and individual spheroids of the same experiment may have different parameters, too. The correlation between growth parameters and the sensitivity to irradiation was investigated but no dependence could be established on the basis of our data.

## SURVIVAL CURVE OF SPHEROID CELLS

The survival curves after X (or neutron) irradiation of spheroids of various diameters were found to be different from survival curves of monolayer cells[8]. Small spheroid cells are more resistant than monolayer cells. The survival curves of larger spheroids are multicomponent. The fraction of hypoxic cells was estimated to 30% for the spheroids 300-500 μm in diameter, 40% for 600-700 μm diameters and 70% for 1100-1200 μm diameters[8]. The survival curve can be described as two-component. The slopes $D_0$ and extrapolation numbers m estimated from[8] are shown in the Table. The value of extrapolation number of resistant component was estimated to $m_r = 1.75$, which gives anoxic frac-



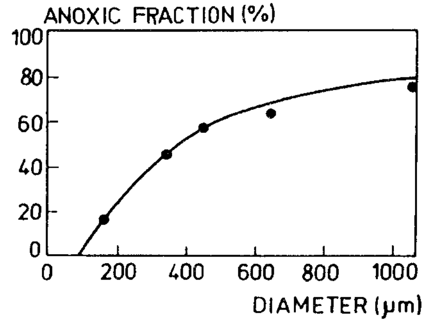
Table

The parameters of survival curves of spheroid cells and anoxic fractions for spheroids of various diameters

| Diameter (μm) | $D_0$ (Gy) | m | $f_a$(%) |
|---|---|---|---|
| 150-200 | 8.89 | 0.3 | 17 |
| 300-400 | 7.86 | 0.8 | 46 |
| 400-500 | 8.58 | 1.0 | 57 |
| 600-700 | 8.73 | 1.1 | 63 |
| 1100-1200 | 7.08 | 1.3 | 74 |

m - extrapolation number; $f_a$ - the fraction of anoxic cells.

Fig.2. The dependence of the anoxic component on the spheroid diameter shown. The points have been determined from survival curves of spheroid cells (Kopp[8]). Theoretical curve calculated assuming constant oxic fraction on the surface (with the thickness of about 40 μm)




tions shown in the Table. These values of anoxic fractions are in agreement with the values given by Kopp[8] and they are also in agreement with the assumption that oxic cells form some surface layer (Fig.2). The anoxic cells may form some layer between the surface oxic layer and necrotic center in great spheroids.

The survival curve of oxic cells has been well determined from small spheroids and will be parametrized in further paragraphs.

## CONTROL OF SPHEROID GROWTH AFTER SINGLE EXPOSURE

The survival of spheroids follows typical tumor - control curve with 50% control after 45 Gy for X irradiation and 500 μm diameters. The probability curve can be easily described by analytical function assuming that an irradiated spheroid may grow from one cell but the probability is less than one - any surviving cell in an irradiated spheroid has only some probability that it will form new spheroid. The probability of control P is then connected with an average number of cells M persisting in irradiated spheroid:

$$P = \exp(-pM). \tag{3}$$

The number of surviving cells can be calculated from the initial cell number $M_0$ and survival S. As the doses are fairly great, we can write

$$M = M_0 \cdot S = M_0 \cdot m \cdot e^{-D/D_0}, \tag{4}$$

where m is the extrapolation number for the corresponding diameter. Inserting eq.(4) into eq.(3) we obtain

$$P = \exp(-pM_0 \cdot m \cdot e^{-D/D_0}). \tag{5}$$

The parameters are mostly known: m=1 for 500 $\mu$m diameters used in further experiments, $D_0$ = 8.23 Gy and $M_0$ = $3.5 \times 10^4$ cells /2/ The only unknown parameter p can be determined from 50% control probability point (45 Gy), which gives p = 0.0047. Function (5) gives immediately the correct value of the slope of the dose-response curve for P = 50%:

$$\frac{dP}{dD} = P \cdot (-\ln P) \frac{1}{D_0}; \tag{6}$$

for P = 0.5 and $D_0$ = 8.23 we have dP/dD = 0.041 in good agreement with the experimental value of about 0.045 $Gy^{-1}$.

Function (5) turns quickly to zero for doses less than 30 Gy. On the contrary there exists significant probability of spheroid death even for very small doses (e.g., for 10 Gy of X rays or 1 Gy of neutrons). This fact suggests that some spheroids are extremely sensitive to irradiation and for these ones even low doses are lethal. They represent approximately 10% of all spheroids cultivated.

This correction changes somewhat the value of parameter p as 50% control of normally responding spheroids is shifted to 55%. The optimal values of the parameters of the final formula

$$P = (1-f).\exp(-a.e^{-D/D_0}) + f, \tag{7}$$

where f is the sensitive fraction: f = 0.08, $a = p \cdot m \cdot M_0 = 194$. The theoretical curve is compared with experimental data points in Fig.3. ($\chi^2$= 0.78 per degree of freedom).

Assuming the same values of f and a for neutrons, we can calculate the value of $D_0$ for corresponding survival curve. We have obtained $D_0$ = 0.81 Gy for 50% control probability at 4.5 Gy. The value of RBE should be the same for both spheroids and cells



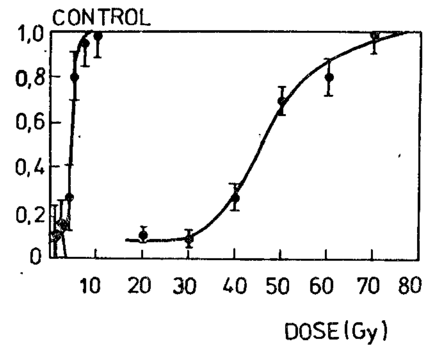



Fig.3. The dependence of spheroid growth control on the dose of X-rays (on the right) or neutrons (on the left). Theoretical curves calculated according to eq.(7) (f = 0.08, a = 194, $D_0$ = 8.23 for X-rays or $D_0$ = = 0.81 for neutrons). Our experimental data points shown with 95% confidence intervals (average values from several experiments taken).

from spheroids. The value of RBE is near to the experimentally determined one for cell survival of monolayer cells: RBE = 8.

## FRACTIONATED IRRADIATION OF SPHEROIDS

The low dose region of survival curve is important in the case of fractionated irradiation and so the multitarget formula cannot be used. Several other formulae have been used in low dose region and the most appropriate one seems to be Huggett's formula /6,7/

$$S = e^{-\alpha d^{\gamma}}, \tag{8}$$

where d is the dose, $\alpha$, $\gamma$ are free parameters. In the case of fractionated irradiation the survival at the end of schedule will be

$$S = e^{-N.\alpha.d^{\gamma}} \tag{9}$$

(N is the number of fractions), providing that there are no changes of the survival curve. The parameter $\gamma$ determines the slope of corresponding Strandquist's graph $\kappa$:

$$\kappa = \frac{\gamma}{\gamma - 1}. \tag{10}$$

The formulae given above are concerned with homogeneous population of the same sensitivity during the regime. Some generalization should be done for spheroids.

A substantial fraction of spheroid oxic cells is killed even after the first fraction (or several first fractions). Then the process of reoxygenation begins and some sensitized cells may be effectively killed by radiation again. It may be, however, generally assumed that dominant cells are in some layer between oxic surface layer and necrotic center. This layer may be reoxygenated to some degree during fractionated irradiation. The parameters of eq.(9) may vary.

The parameter $\gamma$ of eq.(9) has been shown not to depend significantly on the conditions of irradiation. So we shall assume changes of $\alpha$ only. The survival at the end of a schedule is then

$$S = e^{-\sum_{i=1}^{N} \alpha_i \cdot d^{\gamma}}, \quad (11)$$

where $\alpha_i$ is the value of $\alpha$ at the time of i-th fraction. An average value of $\bar{\alpha}$ can be introduced for the given schedule

$$\bar{\alpha} = \frac{1}{N} \cdot \sum_{i=1}^{N} \alpha_i \quad (12)$$

and so

$$S = e^{-N \cdot \bar{\alpha} \cdot d^{\gamma}}. \quad (13)$$

The values of $\gamma$ and $\bar{\alpha}$ for 5 fractions ($\bar{\alpha}_5$) and for 10 fractions ($\bar{\alpha}_{10}$) can be determined from available experimental material. Formula (13) can be inserted into eq.(7) for probability dependence:

$$P = (1-f) \cdot \exp\left(-p \cdot f_d \cdot M_0 \cdot e^{-\bar{\alpha} N \left(\frac{D}{N}\right)^{\gamma}}\right) + f, \quad (14)$$

where $f_d$ is the fraction of dominant cells; and the slope of dose-response curve is

$$\frac{dP}{dD} = -(P-f) \cdot \ln \frac{P-f}{1-f} \cdot \bar{\alpha} \cdot \gamma \cdot \left(\frac{D}{N}\right)^{\gamma-1}, \quad (15)$$

50% control probability was determined at 45 Gy (5 fractions) or at 59 Gy (10 fractions). The slopes were determined to 0.055 $Gy^{-1}$. Optimum parameters determined from these experimental data are:

$$\gamma = 1.58 \qquad \bar{\alpha}_5 = 0.0312 \qquad \bar{\alpha}_{10} = 0.0304,$$

$f_d$ was assumed to be equal to $f_a$ - anoxic fraction. The two values of $\bar{\alpha}$ are practically the same, which suggests that reoxygenation takes place at the very beginning of a schedule. Function (14) with parameters f = 0.08, p = 0.0055, $M_0 = 3.5 \times 10^4$, $\bar{\alpha} = 0.031$ and $\gamma = 1.58$ is compared with experimental points in Fig.4.



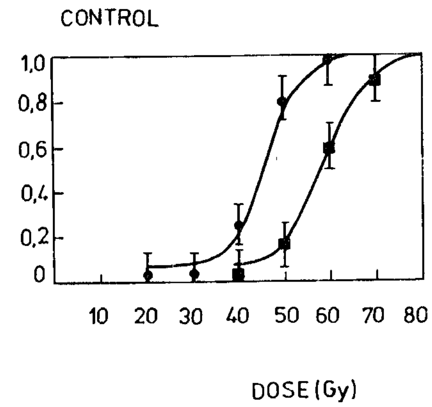



Fig.4. The dependence of spheroid growth control on the total dose of fractionated X-ray irradiation: 5 fractions (on the left) or 10 fractions (on the right). Theoretical curves calculated according to eq.(14) ($f = 0.08$, $p = 0.0055$, $f_d = f_a = 0.6$, $M_0 = 3.5 \times 10^4$, $\bar{\alpha} = 0.031$, $\gamma = 1.58$). Our experimental data points shown with 95% confidence intervals: ● 5 fractions and ■ 10 fractions.

Oxic and anoxic survival curves of spheroids can be described by Huggett's formula with the parameter $\gamma \doteq 1.6$; the values of $\alpha$ for oxic resp. anoxic conditions are $\alpha = 0.074$ resp. 0.017. The value obtained from fractionated irradiation $\bar{\alpha} = 0.031$ corresponds to some degree of hypoxia. If the dominant layer were only some part of anoxic cells then the conclusions remain the same, only the value of $\bar{\alpha}$ would be somewhat smaller (near to the value of anoxic component).

The value of $\gamma$ corresponds to the slope of Strandquist's graph of about $\kappa = 0.367$.

## DISCUSSION

Multicellular spheroids seem to be very similar to animal (or human) tumors in many respects: the growth of similar pattern, anoxic component or even necrosis, the response to single exposure (typical dose - response curves), the response to fractionated irradiation (typical Strandquist's graphs). On the other hand it is obvious that the results of the irradiation of spheroids can't be transferred to human radiotherapy immediately. Only general correlations can be carefully utilized. Radiobiologic models with an adequate degree of mathematical apparatus are the appropriate tool which should enable us to plan further experiments intelligently and to utilize the results of such experiments in radiotherapy in future.

We have tried to analyse the experimental data by means of simple formulae and we suggested several assumptions which are in agreement with the present data: 1) the spheroids may regrow from one surviving cell but the probability of such growth is much less than one, 2) oxic cells form surface layer which corresponds to growing fraction, 3) some spheroids are markedly more sensitive to radiation than the other (10%), 4) the reoxygenation of the dominant layer is finished very quickly at the very beginning of fractionated schedule but is not complete.

The formulae suggested and based on these assumptions give us quantitative predictions for a broad range of independent variables. Further experiments should precise the formulae as well as the assumptions.

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Received by Publishing Department
on August 2. 1982.




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Многоклеточные сфероиды как модель опухоли


Обсуждаются возможности математического моделирования действия излучения на многоклеточные сфероиды. Показано, что их ответ на однократное и фракционированное облучение разными типами излучений соответствует реакции опухолей. Определяется наклон графиков Страндквиста, обсуждается вопрос реоксигенации. Предлагается несколько гипотез для экспериментальной проверки.



Работа выполнена в Лаборатории ядерных проблем ОИЯИ.

Препринт Объединенного института ядерных исследований. Дубна 1982


Kozubek S., Erzgräber G. E18-82-589

Multicellular Spheroids as an in Vitro Tumor Model


Experiments with fractionated irradiation of multicellular spheroids were performed. Our data as well as the data of other works have been evaluated by means of simple mathematical formulae on the basis of several hypothesis. The spheroids are shown to exhibit similar behaviour as in vivo carcinomas. They offer the possibility of investigation of quantitative correlations for practical purposes.



The investigation has been performed at the Laboratory of Nuclear Problems, JINR.

Preprint of the Joint Institute for Nuclear Research. Dubna 1982