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Increased Radioresistance of Cells in Cultured Multicell Spheroids. I. Dependence on Cellular Interaction

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Summary. Monolayers of six different cell lines were investigated with respect to ionic coupling using micro-electrode techniques. In parallel, survival after Co-γ-irradiation of monolayer- and spheroid cultures of these lines was compared. It was found that spheroids of coupled cell lines were more radioresistant than monolayers ("contact effect"). However, cell coupling did not enhance the survival of monolayers over single cells. This suggests that the contact effect is a "tissue phenomenon" requiring cellular interaction but is expressed only under conditions of three-dimensional growth.

Introduction

The first demonstration in vitro that mammalian cells cultured as three dimensionally growing spherical clones (multicell spheroids) may become more radioresistant than monolayers has been given in the pioneering work by Sutherland and Durand [4, 5]. It has been hypothesized [4] that this increased resistance would be due to extensive intercellular communication within the three-dimensional cell "matrix" and the possible exchange of substances related to DNA-repair ("contact effect"). Although this hypothesis has not yet been critically tested it still described the phenomenon quite correctly in terms of a "tissue effect". Since the contact effect is most probably a major determinant of tissue radiosensitivity this series of investigations was initiated to study the underlying molecular mechanisms.

The first communication represents an interspecies approach of the problem to answer the questions whether (*1*) the contact effect is a universal phenomenon and (2) can be related to the cell's ability to communicate. Communication between cells is mediated by the so-called gap junctions [7, 15]. Both ions and small molecules can be exchanged via these "molecular pores", and usually ionic conductivity and metabolic exchange are found to occur in parallel [7, 13]. Thus,

measurements by micro-electrode techniques of electrical coupling through these "low-resistance" junctions provide a reasonable and accurate way of probing cell interaction.

Materials and Methods

1. Micro-Electrode Measurements

Six different cell lines were investigated with respect to ionic coupling using the micro-electrode setup described earlier [9]. Coupling was measured in 2 ways: (*1*) by monitoring the transfer of an ionic current from one cell to its neighbouring cell, (*2*) by comparison of the cellular input resistance R′ of a single isolated cell with the input resistance R″ of a cell within a monolayer. Cell coupling was expressed for each cell line by the ratio R′/R″. All measurements were performed with cells attached to the bottom of plastic Petri dishes overlayed with Hepes-buffered medium [9].

2. Cell Culture and Irradiation

The cell lines used in this investigation (see Table 1) were cultured as monolayers (on 6 cm plastic Petri dishes) and as spheroids applying spinner culture as described elsewhere [3]. For all cultures and cell lines the same growth- and irradiation medium was used: Eagle MEM with Hank's salts, supplemented with 15% fetal calf serum and antibiotics. γ-irradiation was carried out at 37 C under aerobic conditions (dose rate: 1.8 Gy/min). Two-days old monolayers (cell density $\sim 3 \times 10^4$ cm^{-2}) and spheroids of ~ 270 µm in diameter (3–8 days old depending on cell line) were irradiated, subsequently trypsinized and plated for colony assay. The plating efficiency varied between 25 and 80% depending on the cell line. Survival was determined as the fraction of colony formers relative to unirradiated controls.

In some experiments the outer and inner spheroid cells were analysed separately applying the technique of fractionated trypsinization [3]. Cell cycle

Table 1. Survival parameters and micro-electrode data of the cell monolayers (see "Materials and Methods" for definition of the quantities). R′ and R″ are mean values from 100 – 150 cells

Cell line	α/Gy^{-1}	β/Gy^{-2}	$\bar{D}_{ML}$/Gy	ϱ	R′ ± SD/MΩ	R″ ± SD/MΩ	R′/R″
L (Mouse)	0.31	0.032	2.31	1.03	18.3 ± 5.9	18.6 ± 6.7	0.98
HELA (Human)	0.23	0.034	2.68	1.17	16.0 ± 5.4	14.9 ± 5.3	1.08
V79 (Chin. Hamster)	0.17	0.018	3.66	1.28	13.4 ± 5.7	11.1 ± 6.8	1.21
3T3 (Mouse)	0.11	0.062	2.83	1.60	14.4 ± 4.5	8.4 ± 3.8	1.71
B14 FAF28 (Chin. Hamster)	0.13	0.017	4.20	1.82	11.9 ± 3.7	5.8 ± 2.9	2.04
BICR/M1R-K (Rat)	0.38	0.017	2.22	1.95	10.1 ± 3.0	2.2 ± 0.9	4.50

distributions were measured by flow-microfluorometry as described elsewhere [7].

3. *Evaluation of the Contact Effect*

The magnitude of the contact effect was determined for each cell line from the survival curves of monolayers and spheroids and expressed by the quantity;

$$\varrho = \left.\frac{S_{SPH}}{S_{ML}}\right|_{\bar{D}_{ML}} \tag{1}$$

where S_{SPH} and S_{ML} are the surviving fractions of spheroid- and monolayer cells measured at the mean inactivation dose $\bar{D}_{ML}$ of the monolayer survival curve. This statistical parameter was proposed by Kellerer [10] and is defined as:

$$\bar{D}_{ML} = -\int_0^\infty D \frac{dS_{ML}}{dD} dD \tag{2}$$

with $\frac{dS_{ML}}{dD}$ being the density function of the monolayer survival curve.

The advantages of the definition of ϱ by (1) are: (*1*) $\bar{D}_{ML}$ is a cell-specific quantity which depends on the parameters of the individual survival curves. (*2*) As can be seen from Table 1 the $\bar{D}_{ML}$ values are always in the shoulder region of the survival curves. For this reason our presentation of the contact effect is compatible with the finding of other authors that it is a "shoulder effect" [6]. In this way also a possible confusion of the contact effect with hypoxia is eliminated which in some of the spheroid survival curves gives rise to a second shoulder at higher doses (Fig. 2). (*3*) Finally, the commonly adopted expression of contact resistance in terms of an extrapolation number or other shoulder parameters of the survival curve turned out to be inadequate in our case, since no unequivocal extrapolation numbers could be attributed to the monolayer survival curves. In fact the best fit to these curves was obtained with the linear-quadratic model [1, 11]:

$$S_{ML} = e^{-(\alpha D + \beta D^2)}. \tag{3}$$

The quality of fit to (3) can be visualized from the dotted lines of Figs. 2 and 3, representing the regression lines to this model. Calculation of $\bar{D}_{ML}$ by (2) and (3) is based on the parameters α and β (Table 1) obtained by regression analysis.

Results

Table 1 gives a compilation of the cell lines investigated; the monolayer survival curve parameters α, β, and $\bar{D}_{ML}$, as well as ϱ, the factor by which survival in spheroids is enhanced relative to the monolayer at the dose $\bar{D}_{ML}$. In addition,

the resistances R′ and R″ and the ratio R′/R″ (coupling ratio) obtained from the microelectrode measurements are included, showing the degree of coupling to increase from L cells (uncoupled) to the rat-tumor line BICR/M1R-K [6].

These coupling data are basically confirmed by those obtained by the signal-transfer method. Figure 1 shows that only 3T3, B14 and BICR/M1R-K cells allow signal transfer whereas L, HELA and V79 which according to the resistance method are only weakly coupled do not. Figures 2 and 3 show survival curves of the uncoupled L and the most strongly coupled BICR/M1R-K cells. The monolayer survival points are connected by dotted curves representing the regression lines according to (3). For L cells the initial portions of the

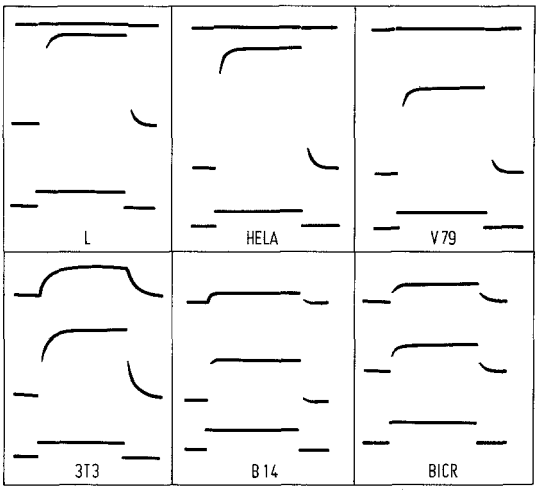
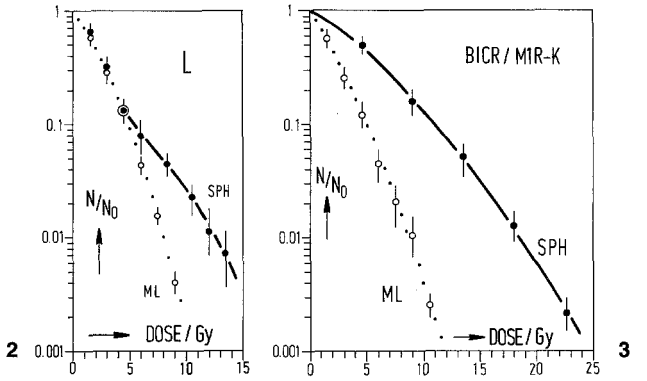


Fig. 1. Oscilloscope recordings of signal transfer in monolayers. Lower trace: Injected constant current pulse (20 nA/50 ms). Middle trace: Voltage deflection in the cell with the current electrode (monitored by a second electrode). Upper trace: Voltage deflection in a neighbouring cell



2 3

Fig. 2. Survival curves for monolayers (ML) and spheroids (SPH) of L cells. Standard errors refer to independent experiments; . . . . = regression line obtained by fitting (3) to the monolayer survival points

Fig. 3. Survival curves for monolayers (ML) and spheroids (SPH) of BICR/M1R-K cells. Standard errors refer to independent experiments; . . . . = regression line obtained by fitting (3) to the monolayer survival points

monolayer- and spheroid survival curves coincide thus indicating the absence of a contact effect. However, a "hypoxic shoulder" below 10% survival is observed. In contrast to the L cells a strong contact effect is found with the BICR/M1R-K line (Fig. 3).

In Fig. 4 monolayer- and spheroid survival curves of B14 FAF28 cells are shown as solid or dashed lines, respectively. In addition, survival points obtained when only the outer spheroid cells were plated or when single cells were irradiated instead of monolayers, are included. Apparently, these points closely match the corresponding monolayer- and spheroid survival curves within the shoulder region. The small divergence (mainly between single cell- and

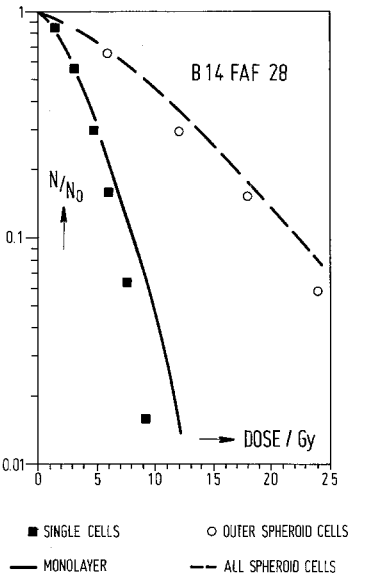


Fig. 4. Line B 14 FAF28: Survival of monolayers or single cells on plates, outer and total spheroid cells

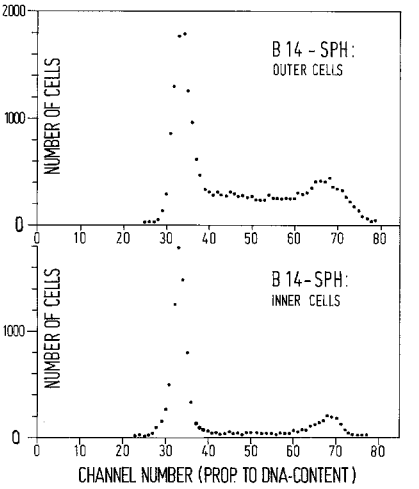


Fig. 5. Cycle distribution of outer and inner spheroid (SPH) cells of line B 14 FAF28 obtained by flow-microfluorometry. Outer cells: $G_1 = 34\%$; $S = 45\%$; $G_2 + M = 21\%$. Inner cells: $G_1 = 77\%$; $S = 8\%$; $G_2 + M = 15\%$

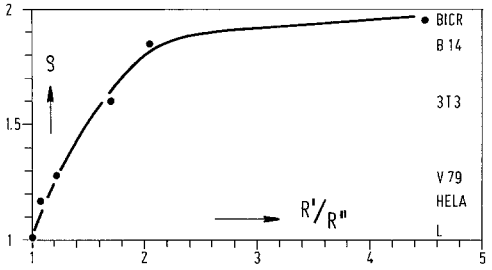


Fig. 6. "Contact enhancement" factor ϱ defined by (1) as function of coupling ratio R′/R″ (data taken from Table 1)

monolayer survival) at low survival rates deserves no consideration in this context since the contact effect is estimated from the shoulder region, i.e., for $N/N_0 < 0.1$. Thus the contact effect is not observed in the monolayer system, but is already fully developed in the outer spheroid cells.

Figure 5 shows DNA-distributions for outer and inner spheroid cells. In contrast to the outer cells representing a cycling population, the inner cells are plateau-like cells arested predominantly in the G_1 (G_0) phase. However, according to Fig. 4 the contact effect is largely independent of the proliferative status of the spheroid cells plated.

In Fig. 6 ϱ is plotted against R′/R″ revealing a correlation between the magnitude of the contact effect and the amount of ionic coupling.

Discussion

These results indicate that only coupled cells exhibit the contact effect. Other parameters of the cell lines investigated such as doubling time, DNA-content or chromosome number could not be correlated with this phenomenon. However, coupling apparently is only a necessary but not a sufficient criterion for the effect to occur. Three-dimensional growth under this condition appear to be required for the expression of the contact phenomenon.

Two hypotheses of the contact effect are compatible with our data: (*1*) it depends on the exchange of certain substances (preferentially molecules related to DNA repair); (*2*) the contact effect is a property of the individual cell acquired during three-dimensional growth under the influence of intercellular communication. The following arguments are in favour of hypothesis (*2*): Exchange processes are known to proceed very efficiently in monolayer culture [2, 14], yet a contact effect is not observed in monolayers (Fig. 4). Even an enhanced cell – cell communication in the three-dimensional spheroid matrix seems to be unlikely: Micro-electrode measurements performed with B 14 spheroids at the time of irradiation yielded an input resistance of 6.5 MΩ (monolayer: 5.8 MΩ; see Table 1). Thus the contact effect is a single-cell property in the sense of hypothesis (*2*) rather than a "helper-function".

Another factor to be discussed in this context is the cell density in the individual cultures. Investigations of contact-inhibited cell lines revealed an increased radioresistance of confluent plateau phase monolayers relative to exponentially growing cultures [12]. We have investigated the influence of cell

density and confirmed the findings of Kim et al. [12]: For B14 monolayer cells, survival after 10 Gy progressively increased to 0.12% with increasing cell density. However, this survival level which could not be enhanced further by serum deprivation is much lower than the corresponding figure for spheroids (0.36%; see Fig. 4). Furthermore, even the outer (proliferating!) spheroid cells show contact resistance. Thus, the contact effect of spheroid cells is not only larger than the "plateau effect", but, in addition, is not restricted to the plateau-like inner spheroid cells.

Unlike the "classical" radiobiological phenomena influencing cellular radiosensitivity such as oxygen effect, recovery, cycle distribution etc., the contact effect is a characteristic of both cell interaction and three-dimensional growth. It is not restricted to tissue culture but has recently been demonstrated in a transplantable mouse tumour system [8]. Thus, whenever the radiation response of cells within a tissue has to be estimated as for example in radiotherapy the contact effect may be an important criterion.

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