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Relationship between Intercellular Communication and Radiosensitivity of Human Tumor Xenografts*†

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Abstract—*Micro-electrode techniques permit the detection of electrical coupling between adjacent cells if they are connected by intercellular junctions (gap junctions). This technique was applied to four human tumors xenografted onto nude mice. In three of the tumors, which showed a 'contact resistance' after irradiation* in vivo, *electrical coupling could be established. No coupling was found in the other tumor, which did not exhibit contact resistance. These results are similar to those obtained recently with cultured spheroids of mammalian cell lines in which only the electrically coupled cell types developed contact resistance to ionizing radiation.*

INTRODUCTION

SEVERAL years ago Durand and Sutherland observed that irradiation of Chinese hamster cells *in vitro* as multicell spheroids resulted in a higher survival than irradiation of monolayers of the same cell line [1]. This reduced radiosensitivity was attributed to the three-dimensional contact of cells within the spheroids and thus denoted as 'contact resistance' or 'contact effect' (CE). The CE has since been reported or suggested in different rodent tumors [2–4]. In xenografts of two human colorectal carcinomas and one melanoma it has been recently shown that CE is probably the primary cause of radioresistance *in vivo* [5]. These results suggested that the CE, originally discovered in rodent cell spheroids *in vitro* [1], could also play a role in the radiosensitivity of some human tumors irradiated *in vivo*.

In vitro, a systematic study of CE revealed that only spheroids of cells capable of forming specialized intercellular junctions (gap junctions) exhibited CE [6, 7]. Since the gap junctions provide electrical low-resistance connections between adjacent cells they can be detected by means of micro-electrode techniques [8].

The aim of this investigation was to search for a possible correlation between CE and intercellular electrical coupling in four human tumor xenografts growing in nude mice, in analogy to the findings in mammalian cell spheroids *in vitro* [6]. In addition, two of these human tumor cell lines were irradiated as multicell spheroids, and their survival curves were compared to those of the xenografts.

MATERIALS AND METHODS

Two colorectal adenocarcinoma (HT29 and HRT18) and two melanoma (Nall and Bell) cell lines were used. The transplantation and cell-handling techniques have been described in detail elsewhere [5, 9, 10]. The main features are mentioned briefly here.

Cell culture

Cells were maintained in monolayer culture in Eagle's MEM (Earle's salts) supplemented with 20% foetal calf serum (FCS) and antibiotics. For subculture and colony assay, cells were dispersed with a calcium-free salt solution containing either 0.2% trypsin (Nall and Bell) or 0.1% trypsin plus 0.4% EDTA (HT29 and HRT18). HT29 and Nall cell lines were also grown as spheroids in

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spinner flasks. At the time of irradiation, the average diameter of the spheroids was 300 μm and the cell density of the Nall monolayers was approximately $10^5/cm^2$. As soon as possible after irradiation, cells (spheroids or monolayers) were trypsinized and plated for colony assay.

Irradiation

Cells were irradiated at 37°C under aerobic conditions with ^{137}Cs γ-rays at a dose rate of 2.4 Gy/min.

Tumors

For tumor formation in nude mice, cells were cultured as monolayers and trypsinized towards the end of the exponential growth phase. Cells of the four tumor cell lines were then injected subcutaneously (2×10^6 in 0.1 ml per mouse) into both flanks of the nude mice as previously described [10]. The resulting solid tumors were excised for analysis when they had reached a volume of approximately 200 mm^3 [5, 9, 10].

Micro-electrode techniques

This method uses micro-capillaries saturated with KCl to pass an electrical current between pairs of cells and to measure a possible voltage deflection due to coupling by gap junctions [11]. If no current passes between neighboring cells, the cells are not coupled. As is common practice, we used three electrodes (see Fig. 1): an injector electrode, which was connected to a rectangular current signal source (80 Hz; 10 nA), and two monitor electrodes, recording the voltage deflections in the neighboring and in the injected cell respectively. The voltages detected by the electrodes were amplified and displayed on a dual-trace oscilloscope. Details of the electrical set-up are given elsewhere [12].

Cell impalement with the electrodes was performed by means of Leitz-micromanipulators under a microscope (Leitz 'Laborlux') equipped with long working-distance objectives. Measurements were conducted with cells on 6-cm Petri dishes with cell aggregates that were of 20–50 cells in diameter. The cell aggregates were obtained by fine dissection with scissors of tumors excised from the flanks of the mice. Prior to electrical coupling measurements, the tumor cell aggregates were washed twice in HEPES-buffered medium [12] supplemented with 20% FCS, which was also used as medium during the micro-electrode measurements. In addition, coupling was measured in spheroids (200–300 μm in diameter) of HT29 and Nall cells, for which radiation survival curves were also determined.

RESULTS

Micro-electrode measurements

Figure 1a shows the configuration of the micro-electrodes used for the measurement of electrical coupling. In Fig. 1b the results of the measurements performed on specimens of the human tumors are summarized. Oscilloscope readings of the electrodes 1 and 2 show that the cells of HT29, HRT18 and Bell tumors are coupled whereas those of Nall are not.

Electrical coupling measurements performed on HT29 and Nall spheroids provided results consistent with those obtained with the solid tumors: coupling in HT29, no coupling in Nall.

In addition, coupling of Nall cells was determined in the absence of serum as well as in the presence of prostaglandin E_1 (10 μg/ml), which in coupled cell lines was found to enhance coupling above the basal level [13]. Although this treatment induced a considerable modification of the morphological appearance (elongation of cells and formation of spindles), cell coupling was never detected with Nall cells.

Contact effect and electrical coupling in HT29, HRT18 and Bell tumors

As previously stated, recently-obtained survival curves of these three human tumors exhibited a

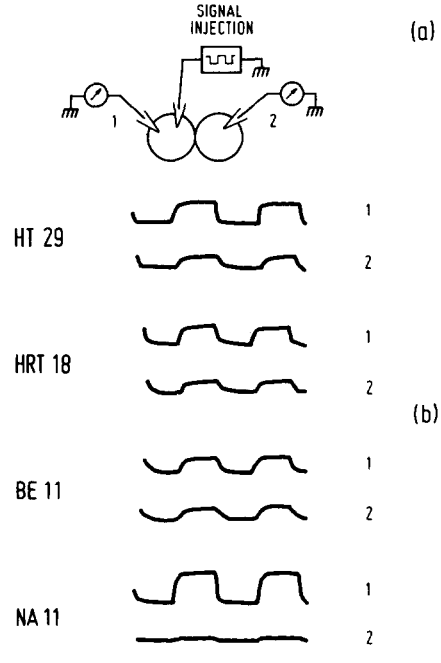

Fig. 1. (a) Schematic drawing of two adjacent cells showing the configuration of micro-electrodes used for the coupling measurements. A rectangular current signal of 80 Hz and 10 nA is passed into the left cell and the voltage deflections in this and the neighbouring cell are monitored by electrodes 1 and 2 respectively. (b) Oscilloscope traces of the signals detected by electrodes 1 and 2 in small tumor specimens. HT29, HRT18 and Bell are coupled; Nall is not coupled.

decreased radiosensitivity at low doses that was assigned to a CE [5]. In order to quantify the magnitude of CE a curve-fitting analysis based on the linear-quadratic model was carried out [5]. The 'contact enhancement ratio' (CER) was defined as the ratio of doses at the 10% survival level of oxic cells in air-breathing mice to tumor cells taken from the animal and irradiated *in vitro:*

$$\mathrm{CER} = \frac{D_{10}\ (\text{oxic tumor cells irradiated in air-breathing mice})}{D_{10}\ (\text{cells from tumor irradiated } \textit{in vitro})}. \quad (1)$$

In Table 1 the CER values are reproduced [5] along with the results of the corresponding microelectrode measurements given in Fig. 1b. HT29, HRT18 and Bell tumors exhibit both cell coupling and CE, with a CER of about 2.

Table 1. Electrical coupling and contact enhancement ratio (CER) for human tumor xenografts

Cells	Coupling	CER
HT29 tumors	+	1.9
HT29 spheroids	+	2.0
HRT18 tumors	+	1.8
Bell tumors	+	2.2
Nall tumors	-	1
Nall spheroids	-	1

For HT29, HRT18 and Bell, the value of the CER defined by (1) are reproduced from [5]. No CE (CER = 1) could be detected in Nall. For details see text.

To determine whether or not cultured spheroids of coupled tumor cells have the same response to irradiation as tumor xenografts of the same cells, spheroids of HT29 cells were subjected to irradiation. Figure 2 shows the survival curves of the HT29 tumor cells irradiated *in vitro* and in air-breathing nude mice respectively. Superimposed are the surviving fractions of HT29 spheroid cells; they closely fit the *in vivo* tumor curve, particularly along the initial part, which mainly reflects the *in vivo* radiosensitivity of the oxic cells.

Nall tumors and spheroids

Previously published survival curves [10] of Nall tumors irradiated under different conditions were not rigorously analyzed with respect to a potential CE. Since Nall tumors in air-breathing mice have an average of 85% hypoxic cells, it is not possible to reliably infer the radiosensitivity of the oxic cell fraction from the *in vivo* survival curve by simple means. Therefore we used two independent approaches to search for a possible

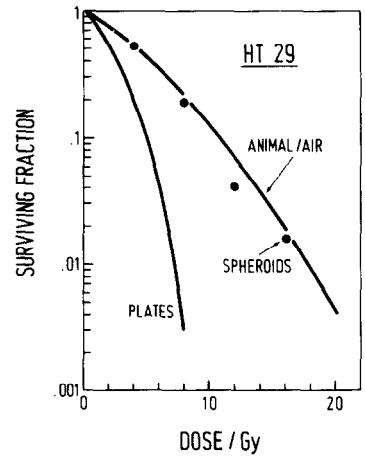

Fig. 2. Solid lines: survival curves of HT29 tumors irradiated in air-breathing nude mice ('animal/air') and of tumor cells taken from the animals and irradiated in vitro *('plates', both reproduced from* [5]*). Solid circles: survival of HT29 spheroids obtained in this work.*

CE *in vivo* and a third approach using Nall cells cultivated as spheroids.

In the first approach the survival of tumor cells irradiated *in vitro* and in asphyxiated animals [10] were fitted to the linear-quadratic model [14] by linear regression analysis:

$$S = \exp(-\alpha D - \beta D^2), \quad (2)$$

where S = surviving fraction and D = radiation dose. The resulting best-fit values α and β are given for oxic cells on lines 1 and 2 and for hypoxic cells on line 4 in Table 2. Using these values a two-population survival curve was computed. It was thus assumed that the radiosensitivity of the oxic cells can be expressed by the *in vitro* survival parameters, so that finally the composite survival curve is:

$$S = 0.85\exp(-0.191D - 0.0035D^2) + 0.15\exp(-0.110D - 0.044D^2), \quad (3)$$

with 0.85 and 0.15 referring to 85% hypoxic and 15% oxic cells, as derived previously [2]. This computed curve is represented as the solid line in Fig. 3, and it fits the experimental survival points. Attempts to modify the oxic component of this curve to allow for a possible CE yield significantly poorer fits to the data. This finding is consistent with the absence of CE in the Nall tumor.

Misonidazole data were used in the second approach [15]. After injection of misonidazole into mice bearing Nall tumors, no significant difference between *in vivo* and *in vitro* survival can be detected at 3 and 4.5 Gy (lines 6 and 7, Table 2), again suggesting the absence of a CE in this tumor at these low doses. Increased resistance in the tumor, had it occurred, would have been

Table 2. Nall survival curve parameters and surviving fraction after 3 and 4.5 Gy of Nall cells

	α (Gy^{-1})	β (Gy^{-2})	Percentage surviving fraction 3 Gy	4.5 Gy
Monolayers*	0.110	0.0440	—	—
Spheroids/oxic cells*	0.110	0.0440	—	—
Spheroids/hypoxic cells†	0.041	0.0097	—	—
Tumor/asphyxiated mice‡	0.191	0.0035	—	—
Tumor/oxic cells‡	0.111	0.0660	—	—
Tumor/cell irradiated *in vitro*‡	0.111	0.0660	47%	16%
Tumor/misonidazole§	—	—	40%	18%

α and β are the best-fit values of (2) to the experimental data (see text for details). For the oxic fraction of the spheroid cells, the radiosensitivity was expressed using the monolayer values. For the oxic fraction of tumor cells the radiosensitivity was calculated using the values obtained with tumor cells first plated *in vitro* and then irradiated.

*See Fig. 4.

†Data of Fig. 4, calculated from hypoxic fraction.

‡Data from [10].

§Data from [15].

due to incomplete sensitization by either misonidazole or CE.

In the third approach Nall cells were irradiated as spheroids and monolayers. Figure 4 shows that a second component of resistant cells was present on the spheroid survival curve, but at doses smaller than 6 Gy the survival curves of monolayers and spheroids coincide, as previously observed [16]. Thus no CE is superimposed on the initial (oxic) part of the spheroid survival curve, and we may assign to the oxic spheroid cell fraction the same radiosensitivity as that of the Nall monolayer. The survival points for spheroids were fitted to a two-population curve similar to (3), assuming that the second population of the curve was radioresistant due to hypoxia, in order to estimate the fraction of hypoxic cells. The survival curve of the oxic component of this population was represented by the best-fit α and β values of the curve for monolayers (line 1, Table 2). Minimizing the least-squares sum yielded 9.5% hypoxic cells, with the corresponding α and β values given in Table 2, line 3. The curves connecting the points in Fig. 4 are computed using the best-fit parameters in Table 2.

The results of these three different approaches thus consistently suggest the absence of a CE in tumors and spheroids of the Nall melanoma, in which, according to Fig. 1, cells are not electrically coupled.

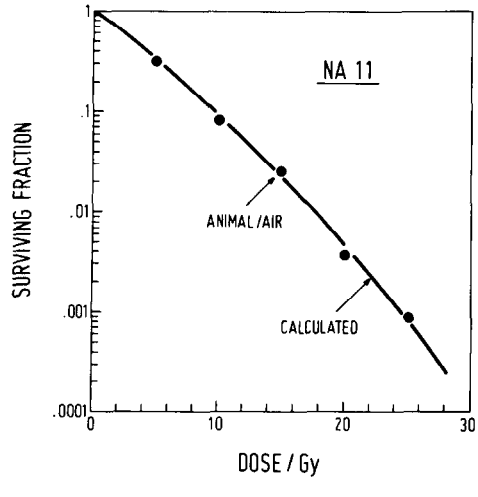

Fig. 3. Solid line: computed two-population survival curve (3) for Nall tumors irradiated in air-breathing nude mice, assuming 15% oxic cells (α = 0.111/Gy, β = 0.066/Gy^2) and 85% hypoxic cells (α = 0.191/Gy, β = 0.0035/Gy^2; see Table 2). Solid circles are experimental survival points from [10].

DISCUSSION

Micro-electrode investigations of tumor samples of HT29, HRT18 and Bell xenografts show that they all have coupled cells and their *in*

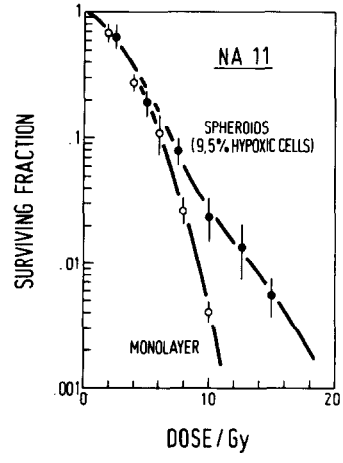

Fig. 4. Survival curves of Nall monolayers and spheroids. The solid lines are fitted curves, as described in the text.

vivo survival curves display a CE. Nall does not show coupling, even under various experimental conditions such as PGE_1-treatment, and no CE could be inferred from the *in vivo* survival characteristics. At least in the two established tumor cell lines that could be studied *in vitro* and *in vivo*, the spheroid system yielded results comparable to those in the tumor: in HT29 and Nall, spheroids and tumor specimens consistently indicate the presence and absence of coupling respectively. Also with respect to CE, spheroids and solid tumors give comparable results: CE was found in HT29 and not in Nall. Even though this conclusion is based on the study of only two human tumor cell lines, the prospect of deriving radiobiologically important properties of solid tumors from the study of spheroids *in vitro* is encouraging, as using spheroids provides the well-known advantages of experiments performed fully *in vitro*.

With respect to the proportion of hypoxic cells, we cannot expect a fully quantitative estimation in the spheroids. For example, the survival curve of the Nall spheroids reveals the presence of roughly 10% hypoxic cells (Fig. 4), whereas 85% are found in the xenografts [10]. One reason for this divergence is probably the 'geometry' of oxygen supply, which is different in spheroids and xenografts. Whereas the spheroid is a sphere of cells that receive oxygen and nutrients from the periphery, solid tumors can be considered as annuli or cylinders, with the tumor cells being fed and oxygenated from central capillary vessels. With increasing depth in spheroids, less cells are found per spherical layer, and the range of oxygen diffusion is considerably enhanced compared to that in the solid tumor geometry. In the solid tumor we can expect a considerable radial oxygen dilution since the number of cells per cylindrical cell layer around a capillary increases in proportion to the distance from the vessel. Thus one may expect hypoxia to be more pronounced in the tumor than in the spheroid. In spite of the different degree of oxygenation in the spheroids and the tumors, CE seems to be rather insensitive to geometrical factors and seems to be predictable in tumors on the basis of the spheroid model. Furthermore, the fact that CE is already expressed in the outer well-oxygenated spheroid cells speaks against a possible role of partial hypoxia in the radiosensitivity of these cells [6]. Further arguments that CE in these tumors is not likely to be the consequence of some particular form of hypoxia have been given elsewhere [5].

The results obtained here with solid tumors and spheroids of four human tumor cell lines, together with those obtained with spheroids of six different mammalian cell lines [6], consistently demonstrate a correlation between CE and electrical coupling. This correlation based on the analysis of ten different cell lines (six of them showing electrical coupling) leads us to believe that CE could be a basic property of cell aggregates of neoplastic or transformed cell lines composed of communicating (coupled) cells, i.e. cells with gap junctions.

A final comment should be devoted to the possible role of the gap junctions in the induction of CE. Clearly, the most elementary physiological function of the gap junctions resides in their buffering capacity of individual variations in channel-permeant molecules in tissue cells. The resulting equilibration among the cells of small molecules and ions creates the basis for tissue homeostasis [17]. The fact that CE is of rather uniform magnitude regardless of the position of the cells within the spheroids [6] could reflect this function of the gap junctions.

The gap junctions are also supposed to play a role in cellular differentiation [17]. The finding that induction of CE requires a critical age (maturation) of spheroids [18] together with the observation of drastic changes in cyclic AMP synthesis in coupled spheroids [7] are in favor of the view that some type of functional differentiation takes place in the spheroids. For this reason one can think of CE as being the result of a cellular differentiation process.

As, on the other hand, the degree of differentiation of a solid human tumor is generally accepted to correlate with its radioresistance, CE could be considered as a scientific basis for this empirical principle. In addition, the correlation between CE and gap junctions could possibly be used to improve the radiotherapeutic indicators of human solid tumors.

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