Is There a Cell-to-Cell Contact Effect on the X-Ray Dose-Survival Response of Mammalian Cells?

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IS THERE A CELL-TO-CELL CONTACT EFFECT ON THE X-RAY DOSE-SURVIVAL RESPONSE OF MAMMALIAN CELLS?

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Abstract

While a cell-to-cell contact effect has been reported for a Chinese hamster subline V79-171B, this was not observed for another subline V79 171-S. Therefore, we tested whether the cell-to-cell contact effect on cell survival depended on the cell line or the experimental conditions used. We have cultured and compared both sublines under identical conditions. Both sublines, cultured in Eagle's minimal essential medium (MEM) with 15% serum, had nearly identical cell doubling times and radiosensitivities. For both sublines, the survival of spheroid and monolayer cells subcultured immediately after irradiation were nearly the same, i.e., **a radio-protective contact effect for spheroid cells was absent**. Under conditions favorable for the repair of radiation induced damage, cell survival was higher for cells in monolayers than for cells in spheroids. Potentially lethal damage (PLD) repair and sublethal damage (SLD) repair were present in both sublines. However, the magnitude of expression of PLD by hypertonic saline was higher for monolayer than for spheroid cells. We conclude that: 1) the reported differences between V79 sublines (contact effect on survival) appear to be dependent on differences between experimental conditions rather than on cell type; 2) delayed plating technique does not detect PLD repair in round spheroid cells; and 3) detection of repair by split dose is independent of cell shape and/or two- or three-dimensional culture conditions.

Key Words: Chinese hamster V79 cells, radiosensitivity, sublethal damage repair, potentially lethal damage repair, cell-to-cell contact, X-rays, spheroid cells, monolayer cells.

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Introduction

The *in vitro* radiosensitivity of mammalian cells can be determined by culturing cells either as monolayers or as spheroids in Eagle's minimal essential medium (MEM) supplemented with fetal calf serum (FCS) (Durand and Sutherland, 1972; Olive and Durand, 1985; Rockwell, 1986; Reddy and Lange, 1991). It has been reported that V79-171B cells grown as spheroids were more resistant to cell killing by X-rays (higher quasi-threshold dose, D_q) than cells cultured as monolayers. Such a difference in radiosensitivity between monolayers and spheroids has been interpreted to be due to a cell-to-cell contact effect enhancing damage repair in spheroid cells (Durand and Sutherland, 1972). For V79 171-S cells, such a difference between monolayer and spheroid cells was reproduced only when the serum concentration for spheroids (5%) was lower than that for monolayers (15%) but not when the same serum concentration was used for both types of cultures (Reddy and Lange, 1991). In fact, for any given serum concentration, the delayed plating (DP) survival of monolayer cells was always higher than the DP survival of spheroid cells. Since the results reported by Reddy and Lange (1991) and those published previously by others could have been due to cell line differences, we have compared the radiobiological aspects of both sublines cultured under identical conditions.

The supposed cell-to-cell contact effect on radiosensitivity does not appear to be a universal phenomenon; while a cell contact effect was reported for the CaSki cell line, no such effect was seen for A431 cells (Kwok and Sutherland, 1991) or for EMT6-Rw cells (Rockwell, 1986). Therefore, the purpose of this study was to: 1) see whether cells of the same origin exhibit the same biological and radiobiological responses when cultured under identical experimental conditions; and 2) to test whether the reported cell-to-cell contact effect on survival is dependent on cell line.

We have compared the following endpoints in Chinese hamster V79 171-S and V79-171B sublines cultured as monolayers and as spheroids in MEM with

15% fetal calf serum (FCS): cell doubling time, immediate plating (IP) and delayed plating (DP) survival (the difference being defined as PLD repair), response to post-irradiation treatment with hypertonic saline, and split dose recovery (SLD). Subline V79 171-S has been maintained in our laboratory for the past 8 years. The other subline, V79-171B, for which the cell-to-cell contact effect was first reported, was obtained from Dr. Danuta Wlodek, then in Vancouver.

Materials and Methods

Cell lines and subculturing

The subline designated as V79 171-S was obtained from Dr. G. Iliakis (Iliakis, 1985) in 1983, and since then the cells were maintained in our laboratory (Reddy et al., 1989, 1992). The other subline, V79-171B, was obtained from Dr. Danuta Wlodek in 1991 from Vancouver, Canada. V79-171B cells were the original cell line for which cell-to-cell contact effects on cell survival and DNA damage repair were first reported (Durand and Sutherland, 1972; Olive and Durand, 1985; Wlodek and Olive, 1992).

For both sublines, within two weeks of entering our laboratory, a large stock was grown under standard monolayer culture conditions, trypsinized and resuspended in Eagle's MEM supplemented with 15% FCS (both from Gibco, Grand Island, NY) plus 10% dimethyl sulfoxide (DMSO) and frozen at -1°C/min, and stored in liquid nitrogen. Thereafter, every 2 to 3 months, fresh aliquots of cells from the liquid nitrogen storage system were thawed and used both to replenish the frozen stock and to set up the stock cultures from which cells were obtained for experiments. This procedure minimizes genetic drift.

Log phase cells in monolayers and in spheroids

Monolayer cultures were obtained by incubating 5×10^5 cells in 25 cm^2 flasks for 18-20 hours in growth medium consisting of Eagle's MEM supplemented with 15% FCS (Reddy et al., 1989). Spheroid cultures were obtained by incubating 50 ml of cell suspension containing 2×10^4 cells/ml in Corning Ehrlenmayer conical flasks. Flasks were flushed with 5% CO_2 in air, sealed and then incubated in a shaker water bath at 37°C for 18-20 hours. By 18-20 hours, small spheroids (agglomerates) with 5 to 25 cells had formed (Olive and Durand, 1985; Reddy and Lange, 1991).

Cell doubling times

Cells were seeded at 2×10^5 cells per flask with 5 ml growth medium and incubated at 37°C. Cell number per flask was determined as a function of incubation time (Reddy and Lange, 1989a).

Irradiation, immediate plating (IP), and delayed plating (DP)

Cells were irradiated at room temperature using a Philips RT 250 X-ray machine [250 kVp (i.e., X-rays with a maximum energy of 250 kV), 15 mA, 2 mm Al inherent filtration with full back scatter, dose rate 2.5 Gy/min) (Reddy et al., 1989).

Cells in monolayers or spheroids were treated with trypsin and plated either immediately before irradiation (IP) or 2-3 hours after irradiation and in situ incubation at 37°C in growth medium (DP) (Reddy and Lange, 1991; Reddy et al., 1989, 1992).

Split dose recovery (SLD repair) studies

Cells were exposed to 7 Gy, incubated at 37°C for different recovery intervals, and then exposed to a second dose of 7 Gy (Iliakis, 1985; Reddy and Lange, 1989b; Reddy et al., 1989; Kwok and Sutherland, 1991). Cells were incubated for 2 hours at 37°C after the second dose to minimize the effects of trypsinization (cell rounding and detachment) on radiosensitivity (Reddy and Lange, 1989b; Reddy et al., 1989). Cells were then trypsinized, diluted and plated.

Hypertonic saline (PLD) assay

Monolayer cells, with traces of medium, were irradiated, and, immediately post-irradiation, 5 ml of 0.5 M NaCl (hypertonic saline in PBS without calcium and magnesium) was added to the flasks which were then incubated for 20 minutes at 37°C. After 20 minutes, the hypertonic saline was aspirated, the cells were rinsed with normal saline (PBS without calcium and magnesium), trypsinized and plated (van Ankeren and Wheeler, 1985; Iliakis, 1985; Utsumi and Elkind, 1985; Reddy and Lange, 1989b; Reddy et al., 1989, 1990). Spheroid cultures in plastic centrifuge tubes also were irradiated with traces of medium, 5 ml of 0.5 M NaCl was added immediately after irradiation, and the tubes were incubated for 20 minutes at 37°C. The last 5 minutes of incubation included centrifugation (800 g) at 37°C. Following centrifugation, hypertonic saline was aspirated, and the cells were rinsed with normal saline, trypsinized, and plated.

Cell survival assay

After a given treatment, cells were trypsinized and plated into 25 cm^2 flasks, at cell concentrations sufficient to score 100-200 colonies/flask. Flasks were incubated for 7-8 days for colony formation. Colonies were stained with crystal violet and counted. Plating efficiencies (PE) of cells from monolayer or spheroid cultures

were the same, $89 \pm 5\%$. PE was taken into account when calculating post-irradiation survival (Reddy et al., 1989, 1992).

Four 25 cm² flasks were used for each data point in each experiment. Experiments were repeated 3 to 5 times. Error bars on data points represent ± 1 standard deviation of the mean.

Results

Cell doubling times for both V79 cell sublines were essentially the same (p = 0.53): 8.4 ± 0.3 and 8.9 ± 0.6 hours, for V79 171-S and V79-171B cells, respectively (data not shown).

Figure 1 shows that the IP survival of cells in monolayers and in spheroids was very similar. However, the DP survival was higher than IP survival for monolayer cells but not for spheroid cells for both V79 171-S (Fig. 1A) and V79-171B (Fig. 1B) sublines. The D_0, n and D_q values obtained for the different experimental conditions are presented in Table 1 and compared in Table 2. These results indicate that: 1) under the experimental conditions used here, the cell-to-cell contact has no effect on the radiosensitivity of cells in spheroids; and 2) delayed plating appears to promote recovery in cells in monolayers but not in spheroids. Kwok and Sutherland (1991) and Olive and MacPhail (1992) have also reported that the differences in the IP and DP survival of cells in spheroids were minimal.

The survival of monolayer cells treated with hypertonic saline was lower than the IP survival of cells, for both sublines (Fig. 2). The expression of damage by hypertonic saline in monolayer cells was similar to that reported for several other cell lines (Dettor et al., 1972; Raaphorst and Dewey, 1979; van Ankeren and Wheeler, 1985; Iliakis, 1985, 1988; Utsumi and Elkind, 1985; Reddy et al., 1989, 1990, 1992). However, the effect of hypertonic saline on cells in spheroids was small for both sublines compared to that on monolayer cells (Fig. 2). Hypertonic saline treatment of cells in spheroids appears to reduce the shoulder of the survival curves of both V79 sublines, hence sensitizing slightly at low doses. However, the same treatment appears to enhance the repair of damage and protect the cells at high doses, more so in V79-171B cells. A possible effect of hypertonic saline on the radiosensitivity of cells in small spheroids has not been reported in the literature.

Split-dose recovery (repair of sublethal damage) was seen in both monolayer and spheroid cells and was nearly identical for both sublines (Fig. 3). Comparison of the data in Figures 1, 2 and 3 shows that although the split-dose assay can detect the repair of damage in both monolayer and spheroid cells, both trypsin and hypertonic saline distinguish between cells in monolayer and those in spheroids by demonstrating more recovery from potentially lethal damage in the former than the latter.

Discussion

The data presented here indicate that there is no cell-to-cell contact effect on the dose-survival relationship for both V79 171-S and V79-171B cells (Fig. 1, Tables 1 and 2). However, V79-171B cells were reported to exhibit a cell-to-cell contact effect on radiosensitivity (Durand and Sutherland, 1972; Olive and Durand, 1985), while the V79 171-S cells did not (Reddy and Lange, 1991). In the later case, both monolayer and spheroid cells were cultured in growth medium with 15% FCS. However, in the case of V79-171B cells, while the monolayers were cultured in MEM with 15% FCS (Sutherland et al., 1970, 1971; Durand and Sutherland, 1973; Durand, 1976) or 10% FCS (Olive and Durand, 1985), spheroids were cultured in 5% FCS (Sutherland et al., 1970, 1971; Durand and Sutherland, 1973; Durand, 1976; Olive and Durand, 1985). It has been observed that the serum and nutrient concentration in the culture medium influences the radiosensitivity of cells (Luk and Sutherland, 1987; Reddy and Lange, 1991; Schwachofer et al., 1990) and that the spheroid or monolayer cells cultured in 15% FCS were more radiosensitive than those cultured in MEM with 5% FCS (Reddy and Lange, 1991). Other culture conditions such as spheroid size, the batch of FCS and Eagle's MEM have been the same (see **Materials and Methods**; Olive and Durand, 1985; Reddy and Lange, 1991). Therefore, it appears that the reported contact effect on survival for V79-171B cells may have been related to differences in serum and nutrient concentrations in the culture medium.

The difference between DP and IP cell survival was found to be minimal or absent for several V79 sublines, a fibrosarcoma cell line, and a few melanoma cell lines (Iliakis, 1985; Iliakis et al., 1985; Marchese et al., 1985; Konefal and Taylor, 1989; Antoku and Kura, 1990; Reddy et al., 1992). These observations show that a two-dimensional cell contact under DP conditions does not appear to affect survival at all. Moreover, a difference between IP and DP survival has been reported for log phase cells at low cell densities (Little, 1973; Utsumi and Elkind, 1985; Sun et al., 1986; Reddy et al., 1989). Therefore, it appears that other factors, such as: 1) trypsin-induced changes in cell shape and chromatin structure; and 2) nutrient, serum, and amino acid concentration, may be associated with the radiosensitization observed under IP conditions (Kaufmann and Briley, 1987; Luk and Sutherland, 1987; Reddy et al., 1989, 1992; Kapiszewska et al., 1991; Hill and Hill, 1991).
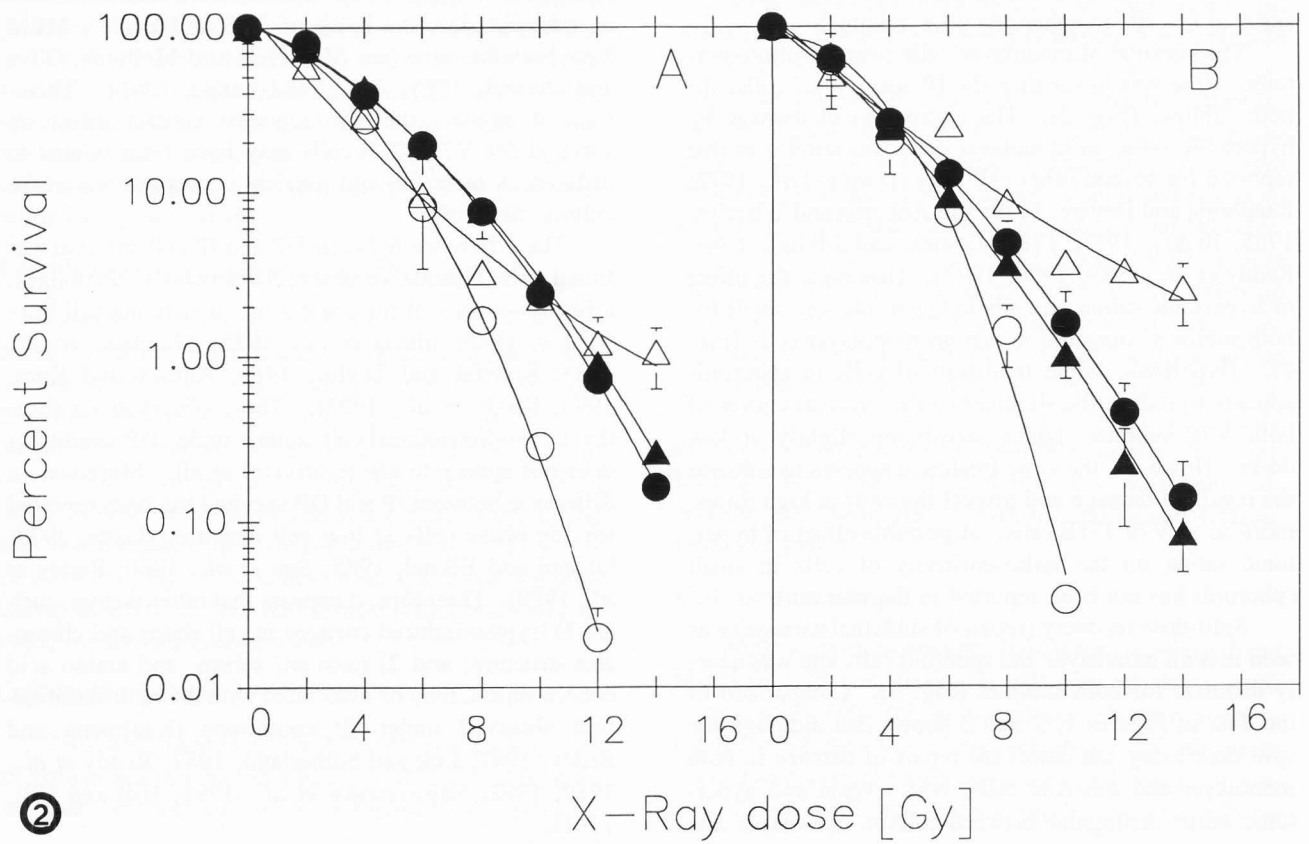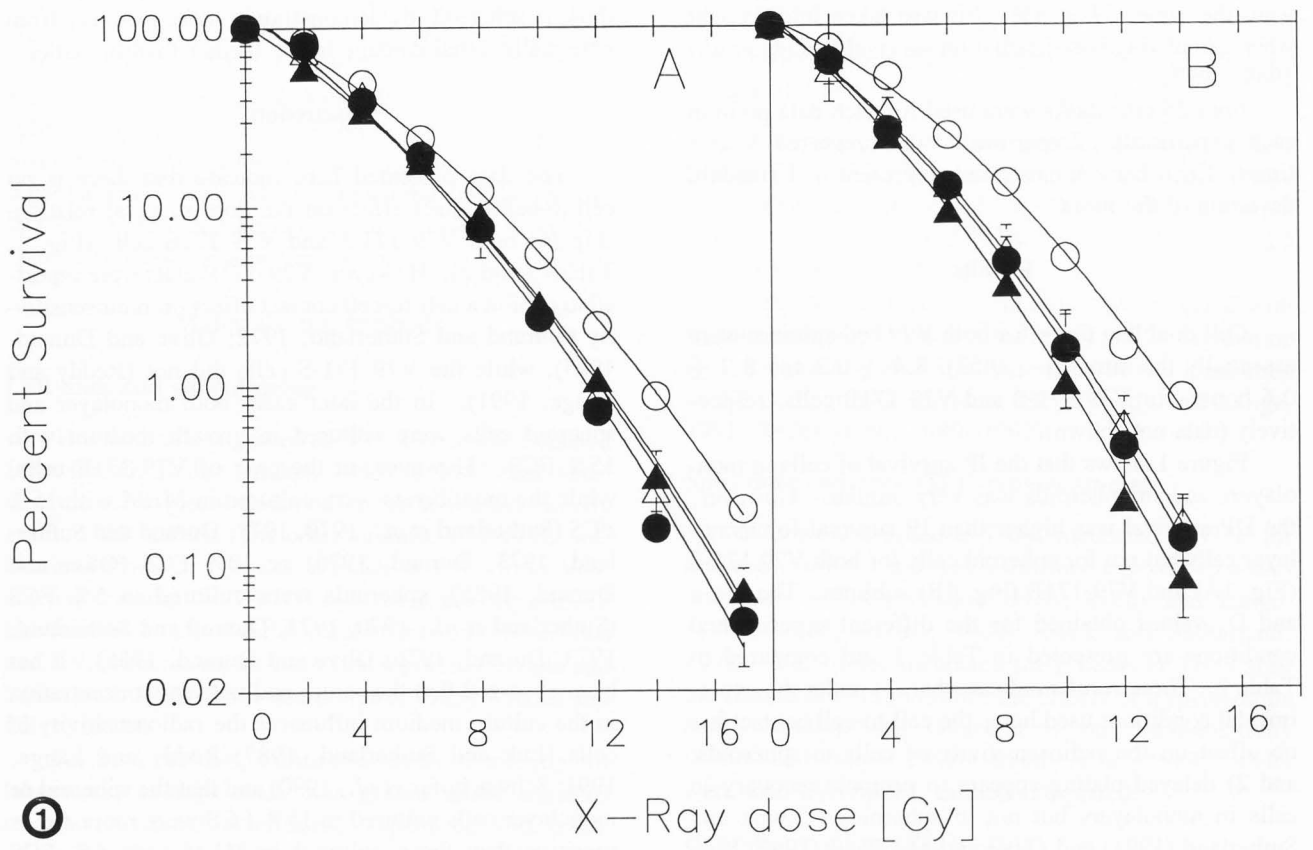
Table 1. Survival curve parameters, D_0, n and D_q, of V79 171-S and V79-171B cells cultured as monolayers or as spheroids (Fig. 1). SEM = standard error of mean.

Experimental condition	D_0 [Gy ± SEM]	n (95% CL)	D_q [Gy]
V79 171-S monolayers			
1. Immediate plating	1.71 ± 0.05	7.88 (5.3-11.7)	3.53
2. Delayed plating	2.20 ± 0.03	4.94 (4.2- 5.9)	3.52
V79 171-S spheroids			
3. Immediate plating	1.81 ± 0.08	6.79 (4.0-11.6)	3.47
4. Delayed plating	1.90 ± 0.05	4.87 (3.4- 6.9)	3.00
V79-171B monolayers			
5. Immediate plating	1.62 ± 0.12	6.58 (2.35-18.4)	3.05
6. Delayed plating	2.13 ± 0.11	5.30 (3.09-9.10)	3.55
V79-171B Spheroids			
7. Immediate plating	1.54 ± 0.09	5.59 (2.42-12.8)	2.65
8. Delayed plating	1.73 ± 0.13	5.24 (2.01-13.6)	2.87

Table 2. Statistical comparison of D_0 values obtained for different experimental conditions, as presented in Table 1. Student's t-test was used to calculate p values for the Null hypothesis of NO difference between compared experimental conditions.

Experimental condition comparison of D_0 values and conclusion	Group numbers in Table 1	Contact Effect	PLD* Repair	p
V79 171-S				
Monolayer IP = spheroid IP	1 vs. 3	No	-	> 0.05
Monolayer DP > spheroid DP	2 vs. 4	No	-	< 0.001
Monolayer IP < monolayer DP	1 vs. 2	-	Yes	< 0.001
Spheroid IP = spheroid DP	3 vs. 4	-	No	> 0.05
V79-171B				
Monolayer IP = spheroid IP	5 vs. 7	No	-	> 0.05
Monolayer DP > spheroid DP	6 vs. 8	No	-	< 0.05
Monolayer IP < monolayer DP	5 vs. 6	-	Yes	< 0.005
Spheroid IP = spheroid DP	7 vs. 8	-	No	> 0.05

*PLD repair = potentially lethal damage repair measured as $S_{DP} > S_{IP}$. S = cell survival, IP = immediate plating, DP = delayed plating.

Figure 1 (facing page 624, top). Comparison of immediate plating (IP) and delayed plating (DP) cell survival for V79 171-S (A) and V79-171B (B) sublines in monolayers and in spheroids. Each symbol represents the same condition in both parts of the Figure. Spheroids: IP - ▲, and DP - △. Monolayers: IP - ●, and DP - ○.

Figure 2 (facing page 624, bottom). Comparison of expression of potentially lethal damage by hypertonic saline in V79 171-S cells (A) and V79-171B cells (B). Each symbol represents the same condition in both parts of the Figure. Monolayer cell survival after treatment with hypertonic saline - ○, or after immediate plating - ●. Spheroid cell survival after treatment of cells with hypertonic saline - △, or after immediate plating - ▲.
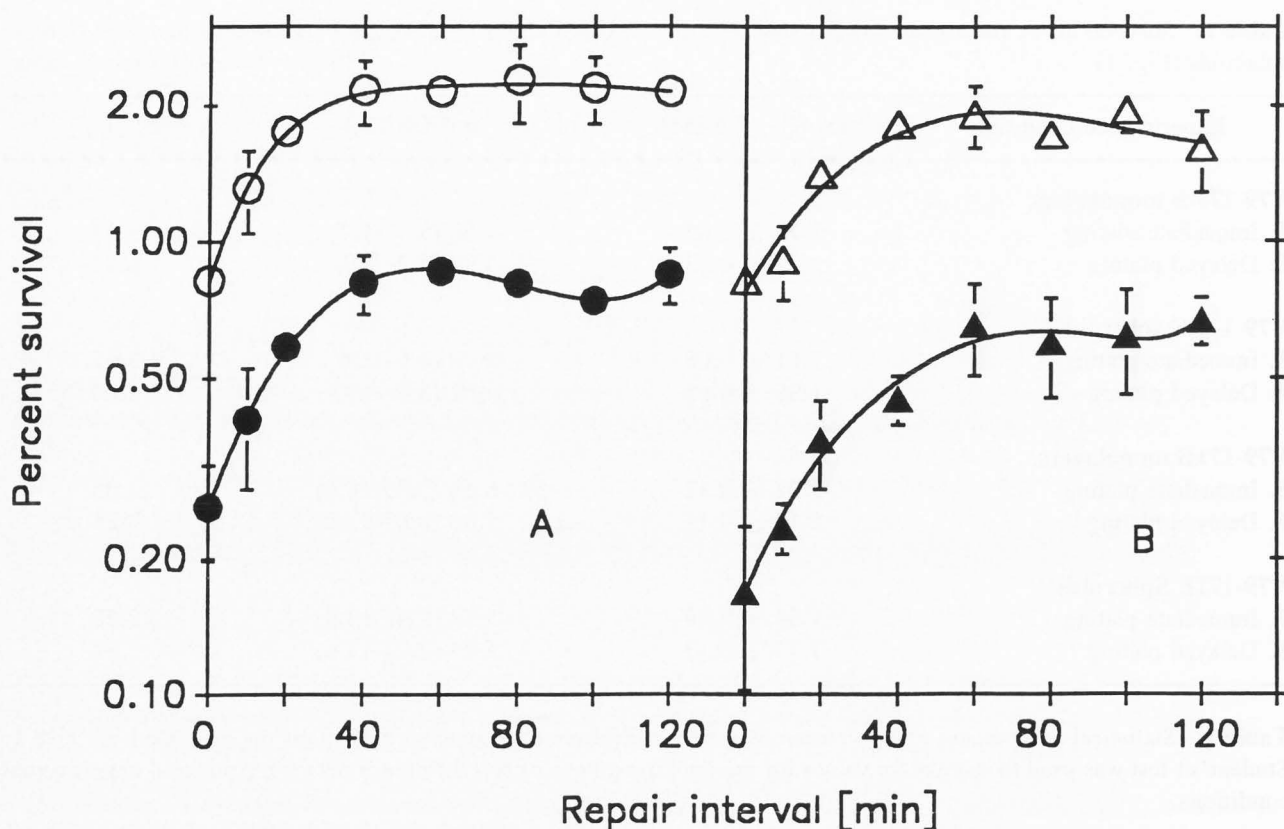
Figure 3. Comparison of split-dose recovery (sublethal damage repair) in monolayer and in spheroid cells of V79 171-S (**A**) and V79-171B (**B**) sublines. V79 171-S: monolayer cells - ○; spheroid cells - ●. V79-171B: monolayer cells - △; spheroid cells - ▲. As noted in **Materials and Methods**, cells were plated 2 hours after D1 and/or D2 doses (DP survival).

While the cells in monolayers were radiosensitized by both immediate plating conditions and hypertonic saline, the survival of cells in spheroids was not affected to the same extent (Figs. 1 and 2). There is a considerable literature reporting radiosensitization of monolayer cells by hypertonic saline (Dettor *et al.*, 1972; Raaphorst and Dewey, 1979; van Ankeren and Wheeler, 1985; Iliakis, 1985, 1988; Utsumi and Elkind, 1985; Reddy *et al.*, 1989, 1990, 1992). However, a possible influence of hypertonic saline on cells in small spheroids has not been reported. Therefore, at this stage, it can only be speculated as to why hypertonic treatment radiosensitizes cells in spheroids at low doses and enhances the repair of damage at high doses. The mechanisms of radiosensitization by treatment of cells with trypsin (detachment) and by hypertonic saline immediately post-irradiation appear to be qualitatively similar (Reddy and Lange, 1989b). Both trypsin and hypertonic saline cause anchorage-dependent monolayer cells to shrink and to retract their attachment points, which may lead to alterations in nuclear morphology and chromatin structure

(Raaphorst and Dewey, 1979; Utsumi and Elkind, 1985; Reddy *et al.*, 1989, 1992; Reddy and Lange, 1991; Kaufmann and Briley, 1987; Kapiszewska *et al.*, 1991; Hill and Hill, 1991). Such alterations in the nuclear morphology and chromatin structure due to trypsin and hypertonic saline may be minimal in round spheroid cells with a concomitant lack of radiosensitization.

Conclusions

1) The radiosensitivities of two sublines of V79 cells, maintained in two different laboratories, are nearly identical.

2) Apparent contact effects on radiosensitivity appear to be dependent on differences between experimental conditions rather than on cell type.

3) Comparison of the delayed and immediate plating survival levels does not detect PLD repair in round cells because trypsinization does not radiosensitize round cells.

4) Expression of PLD by hypertonic saline was

greater in monolayer cells than in small-spheroid cells.

5) Detection of repair by split-dose is independent of culture conditions.

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Discussion with Reviewers

G. Iliakis: What are the assumptions underlying the choice of experimental conditions for immediate plating and delayed plating and the interpretations of the results? **Authors:** Since cell survival can be interpreted (understood?) in terms of radiation-induced damage and its repair or lack thereof, changes in cell survival attendant upon altered post-irradiation conditions have been interpreted as representing increased or decreased levels of (potentially lethal) damage repair. Usually, survival, and hence this repair, is higher when subculturing is delayed for a few hours after irradiation (delayed plating) than when subculturing follows immediately after irradiation (immediate plating). This has been interpreted as due to the repair of PLD (Little, 1973; Utsumi and Elkind, 1985; Iliakis, 1988; Antoku and Kura, 1990; Reddy et al., 1992). Since this survival difference represents a difference in the amounts of damage repaired under each of the compared conditions, the absence of a difference in cell survival between immediate and de-

layed plating conditions does not necessarily mean the absence of repair of PLD; i.e., the same amount of damage could be repaired under both conditions, resulting in equal survival (Iliakis, 1985; Antoku and Kura, 1990; Reddy et al., 1992).

G. Iliakis: The observation that hypertonic solutions are ineffective in irradiated spheroids is interesting. However, the authors should provide evidence that there is no shielding effect due to cell aggregation. Are the authors certain that cells in the inner parts of the spheroids really "see" the hypertonic environment? Have the authors tried longer exposures to hypertonic solutions? **Authors:** The size of the spheroids used for these experiments was between ca. 30 to 60 μm, with 5 to 25 cells per spheroid. Because of the small size of the spheroids, we do not think that there was any shielding of cells from hypertonic effect. Our microscopic observations indicated that all cells in the small spheroids were affected by hypertonic saline. Cells became smaller and were shrunk within a few seconds of exposure to hypertonic saline and remained like that until the end of treatment. Once the hypertonic saline was removed and cells were resuspended in growth medium, cells quickly became normal in appearance (data not shown). These observations indicated that cells in inner parts of these small spheroids were not shielded from the effects of the hypertonic environment. In addition, cells in hybrid spheroids of 100 μm diameter, containing 107 $\pm$ 9 cells, showed no signs of being shielded from much larger molecules such as adriamycin or 5-Fluorouracil [Djordjevic B, Lange CS. (1991). Measurement of sensitivity to adriamycin in hybrid spheroids. Cancer Invest. **9**, 505-512; Djordjevic B, Lange CS, Allison, RR, Rotman, M. (1993). Response of primary colon cancer cells in hybrid spheroids to 5-fluorouracil. Cancer Invest. **11**, 291-298].

In order to keep the experimental conditions similar, the duration of hypertonic saline treatment of 20 minutes was the same for both monolayer and spheroid cells. Therefore, we did not expose the cells in spheroids for longer than 20 minutes.

H.G. Hill: I thought that hypertonic saline was supposed to inhibit repair enzymes; yet, there is no mention of that here. There should be some discussion of this, especially since hypertonic saline has such a strange effect on the spheroids. What effects does hypertonic saline have on the permeability of cells? Is that the key to the differences? **Authors:** It is not clear that hypertonic saline inhibits DNA repair enzymes per se. Dewey and coworkers [see Ostashevsky JY. (1992). Cellular recovery kinetics for post-irradiation treatments. Int. J. Radiat. Biol. **62**,

337-351 for more extensive discussion and references] showed that hypertonic saline treatment causes a condensation of DNA molecules which is correlated with increased radiosensitivity. This condensation may block DSB repair by two mechanisms: (1) it may block access of repair molecules to the DNA lesions (DSBs?) and (2) it can cause the loss of DNA fragments, seen as increased PCC (prematurely condensed chromosome) fragments and micronuclei. However, for unirradiated cells, the plating efficiency of hypertonic-saline treated cells is similar to that of control cells. Therefore, it can be inferred that hypertonic saline treatment does not damage intact DNA, nor permanently alter membrane permeability and other physiological functions.

If the hypertonic saline effect was due **only** to the inhibition of enzyme activity (by chromatin condensation), the effect of hypertonic saline on cells in both monolayers and in spheroids should have been the same. However, hypertonic saline is known to cause cells to lose water and shrink, which leads to alterations in nuclear morphology and chromatin structure (Raaphorst and Dewey, 1979; Utsumi and Elkind, 1985; Reddy *et al.*, 1989, 1991, 1992; Kapiszewska *et al.*, 1991). Such mechanical alterations in the nuclear morphology and chromatin structure can lead to the loss of DNA fragments (seen as increased micronuclei) and displacement of broken ends of the DNA. Thus the mechanism of radiosensitization by hypertonic saline is thought to involve both fragment loss and reduced time for repair due to the loss of the time during which DNA lesions remain inaccessible.

S. Kura: The data in Figure 2 demonstrate that the survival of cells in spheroids treated with hypertonic saline was higher than the immediate plating (IP) survival, especially in the case of V79-171B cells. I feel that the results should be interpreted in terms of the enhancement of the repair of damage rather than in terms of failure of radiosensitization.

Authors: The two interpretations represent opposite sides of the same coin. The survival of cells in spheroids treated with hypertonic saline is higher than the immediate plating (IP) from spheroids survival for V79 171-S and V79-171B cells beyond 10 Gy and 4 Gy, respectively, which are each higher than their respective survivals for hypertonic treated cells in monolayer. One can interpret the higher survival of hypertonic treated cells in spheroids than in monolayers in terms of the former having less of a change in shape of the cell and chromatin than the latter, and therefore, less disruption of DSB ends and less loss of chromatin/chromosome fragments. The somewhat similar initial slopes for these curves would be consistent with this interpretation in terms of the DSB model (Ostashevsky *op cit.*). How-

ever, the spheroid immediately plated cells should have the same time available for repair as do the hypertonic treated cells, since the time in hypertonic saline is subtracted from the time available for repair due to delayed plating, and the former do not have the disruption of DSB ends that would be expected for the latter. These data therefore suggest that spheroid cells exposed to hypertonic treatment have a longer time available for repair than do those which are immediately plated. The reasons for why this should occur are not clear. From this point of view, it can be argued that hypertonic saline enhances the repair of radiation-induced damage.

H.G. Hill: The effect of hypertonic saline on spheroids does not look small to me! The entire shape of the survival curves has changed, and the spheroids are considerably more resistant, especially in Fig. 2B.

Authors: It is true that the response to hypertonic saline treatment of cells in spheroids is not similar to that of cells in monolayers. Cells in spheroids are radiosensitized at low doses but are protected at high doses by the exposure of cells to hypertonic saline, more so for V79-171B cells (Fig. 2B). The reasons for this phenomenon are not yet understood, but our above reply to Dr. Kura may suggest some possibilities to test.