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DETECTION OF X-RAY DAMAGE REPAIR BY THE IMMEDIATE VERSUS DELAYED PLATING TECHNIQUE IS DEPENDENT ON CELL SHAPE AND CELL CONCENTRATION

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Abstract

A method commonly used to measure the ability of cells to repair potentially lethal damage (PLD) is to compare immediate plating (IP) and delayed plating (DP) survival. Lower cell survival under IP conditions relative to that after DP conditions has been interpreted to indicate a higher ability of cells to repair potentially lethal damage (PLD) under DP conditions. However, this IP radiosensitization has not been observed in several cell lines and tumor models. IP conditions involve treatment of cells with trypsin and plating them into fresh growth medium. We have investigated the possibility that radiosensitization under IP conditions may be related to both the cell-shape and the nutrient concentration in growth medium (GM, MEM+15% serum). This idea predicts that the IP and DP survival of spheroids will show a response similar to the IP survival of cells in monolayers and that the IP and DP survival of crowded monolayer cells in high densities will be the same. Chinese hamster V79 cells grown in monolayers (spread cells) and spheroids (clumps of round cells) were used. The IP survival was lower than the DP survival for spread log phase monolayer cells but not for round log phase cells in spheroids. Radiosensitization of cells by fresh (as opposed to spent) growth medium was absent for high density plateau phase cells in monolayers at or above $2x10^6$ cells/ml. However, PLD repair could be demonstrated in spheroid cells and in high density plateau phase cultures by exposing cells to hyperthermia or hypertonic saline.

Comparison of immediate plating versus delayed plating survival detects PLD repair only in well spread low density monolayer cells, but not in round spheroid cells nor in dense monolayer cells at $> 10^7$ cells/25 cm^2 flask/5 ml medium. The absence of a difference between IP and DP cell survival does not mean that PLD repair is absent. Incorrect prediction of tumor response to radiotherapy can occur when PLD repair capacity is assayed as a ratio of DP/IP survival. More than one method must be used to measure the capacity of cells to repair their PLD.

Key Words: Cell shape, potentially lethal damage repair, Chinese hamster V79 cells, trypsin effect on radiosensitivity, spheroid cultures, monolayer cultures, repair capacity, hyperthermia.

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Introduction

The dose-effect curve in cellular radiation biology is represented by either a simple exponential or a shouldered exponential curve of surviving fraction of cells with reproductive integrity versus dose. In an attempt to explain the shape of these curves, taking into account that cells are known to have repair processes, it has been hypothesized that ionizing radiation randomly (Poisson distribution) distributes potentially lethal lesions or damage (PLD) (26) amongst the cells of a population, and that all PLD in a cell must be repaired if it is to survive (PLD Models: 5, 27). As shown in Fig. 1, depending on the postirradiation milieu, different survival curves can be obtained for the same cell line. These curves are interpreted as showing amounts of PLD repair varying from almost none (repair deficient mutants) or very little (anisotonic treatment, hypertonic saline, HS), to more (immediate plating, IP), still more (delayed plating with incubation in growth medium, DPGM, or in conditioned medium, DPCM), or complete repair [initial slope: the alpha component of the linear-quadratic (LQ) model of cell survival; same as the limiting low dose rate survival due to irreparable, and therefore lethal, lesions]. This hypothesized division of cells into viable, those with PLD, and those with lethal lesions and the interconversions of cells between these groups is diagrammed on the right side of Fig. 1. In the literature, absence of a difference between IP and DP survival has been interpreted to mean the absence of PLD repair (16, 22, 42). Since we have found that the amount of PLD repair is related to cell morphology, we have examined the application of the Lethal Potentially Lethal (LPL) model concepts (5, 27) to data for different cell morphologies and environments.

A comparison of the immediate plating (IP) and delayed plating (DP) survival of mammalian cells exposed to X-rays is the technique most commonly used to measure PLD repair and to estimate the PLD repair capacity of a cell line (8, 20, 33, 40). Cell survival under IP conditions has been generally observed to be lower than that obtained under DP conditions. Under IP conditions cells in growth medium immediately postirradiation can progress through cell cycle; whereas under

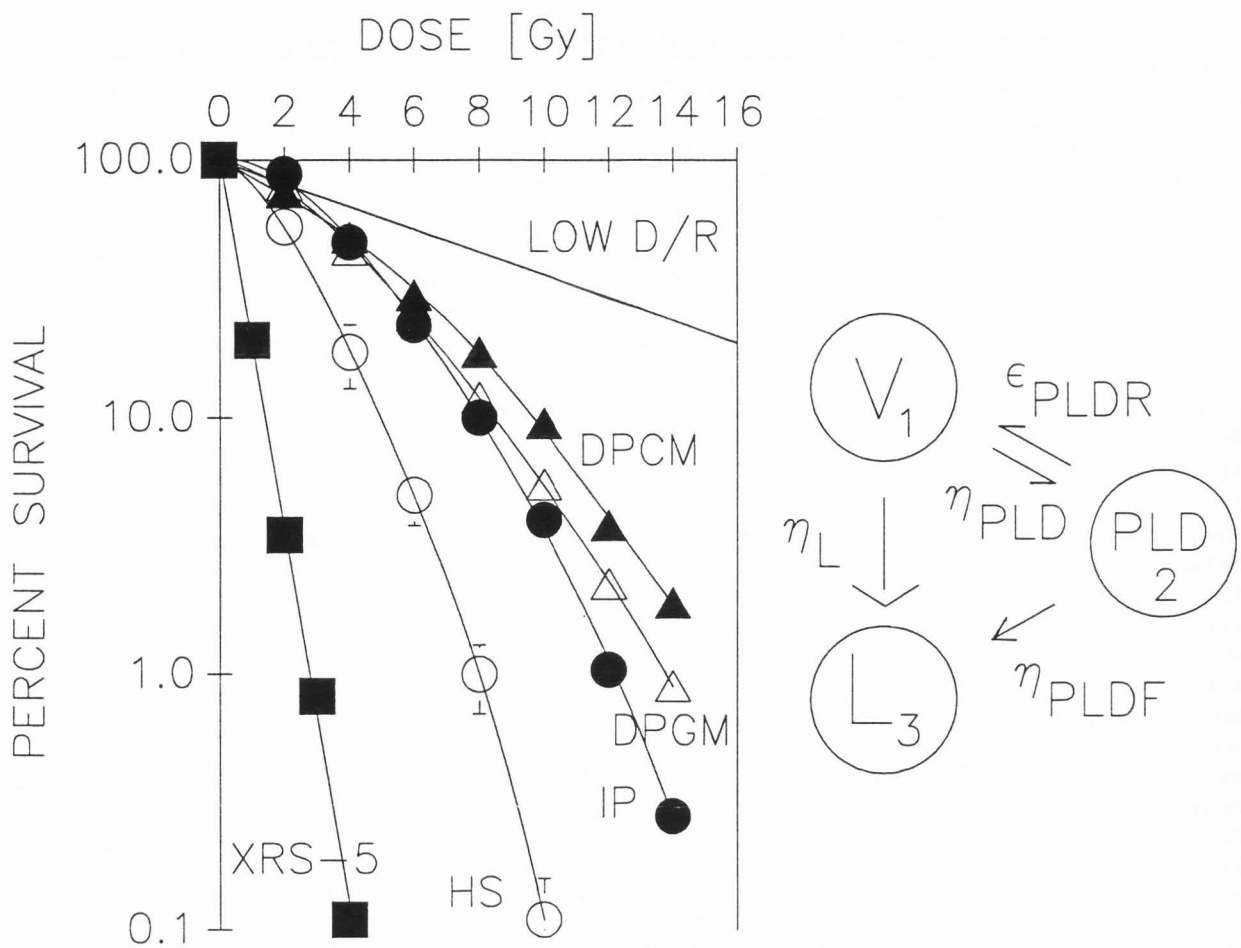

Fig. 1. Survival curves illustrating the basis and concepts underlying the Lethal Potentially Lethal Damage (LPL or PLD) model (5, 27). The figure represents the Cybernetic (PLD) model proposed by Pohlit and Heyder (27). All the survival curves are for V79 Chinese hamster lung fibroblasts obtained in our laboratory, except for the radiosensitive mutant, xrs-5 - (solid squares), (37), of Chinese hamster ovary K1 cells. According to the model, the differences in the survival are due to differences in the amounts of PLD induced (η_{PLD} = rate of induction) and repaired (ϵ_{PLDR} = rate of PLD repair); the more repair, the higher the fraction of cells with complete repair and hence returned to (reproductive) viability (V). L represents cells with lethal or irreparable lesions, and the rates of direct formation and of conversion from unrepaired PLD are shown. HS = cells treated with hypertonic saline (0.5 M NaCl for 20 minutes) immediately after irradiation (hollow circles); IP = immediate plating (trypsinization and plating) of cells after irradiation (solid circles); DPGM = delayed plating of cells after incubation in growth medium (hollow triangles); DPCM = delayed plating of cells after incubation in conditioned (nutritionally exhausted) medium (solid triangles); low D/R = limiting low dose rate survival, which is the same as the initial slope of the acute dose rate response curves (solid line).

DP conditions, cells may not progress through cell cycle because they are generally incubated in conditioned (depleted) medium. Therefore, the cause of the lower survival under IP conditions has been suggested to be due to the fixation of PLD during DNA synthesis and/or cell cycle progression under growth conditions (5, 9, 13, 19). Hence, one could expect to observe differences between IP and DP survival when the incubation conditions and physiological state of cells allow different rates of progression of cells through the cell cycle. While a difference between IP and DP survival was observed for V79 (10, 29, 30, 38), LICH (19), HA-1 (9), normal human diploid fibroblast (4, 16, 22) and xenografted NCI-H226 (33) cells, such a difference was minimal or absent for fibrosarcoma (16), some melanoma cell lines (22), transformed C3H 10T1/2 cells (42), xenografted A549 cells (33), and Rhabdomyosarcoma tumor cells irradiated *in situ* (1). Furthermore, while an effect (increase in survival) of conditioned medium (CM) on V79 cell survival (increased) was observed for cells at

densities of 1 (29), 2, 4 (30) and $5x10^6$ cells/flask (10), it was absent in cells at a density of $9x10^6$ cells/flask (12). A hybrid cell line ESH5L (HeLa x human skin fibroblast) showed a decrease in the difference between IP and DP survival with an increase in cell density (35). However, in cases where IP and DP survival were the same, repairable damage was observed by using dose fractionation (12, 16) or by treating cells with hypertonic saline (12) or with repair inhibitors such as β-arabinofuranosyladenine (β-araA) (1, 12). Hence, radiosensitization under IP conditions may not be entirely due to postirradiation cell cycle progression (30, 35). Moreover, the expected correlation between DNA synthesis and cell survival has not been observed for several cell lines (3, 18, 24).

The above analysis led us to investigate other factors associated with IP and DP conditions, such as the effects of trypsin (11, 15, 29), cell shape (28-30, 36) and nutrient concentration in the growth medium (30). We have compared the IP and DP survival of cells in spheroids and in monolayers. The effect of cell concentration was studied by comparing the DP survival of cells at concentrations of 0.2, 0.8 and $2x10^6$ cells/ml in growth medium (GM, DPGM) versus in conditioned medium (CM, DPCM). In addition to using the IP-DP conditions, irradiated cells were exposed to hypertonic saline or to hyperthermia to detect the presence of repairable damage (by its reduced repair under these conditions).

Results presented here support the suggestion that cell lines with relatively higher radiosensitivity, and cells with an absence of a difference between IP and DP survival, may not be repair deficient (1, 25, 34).

Materials and Methods

Details of the cell line and techniques used for this study have been described extensively elsewhere (28-32). Briefly, Chinese hamster V79 (S-171) cells were used. Cells were routinely maintained as monolayer cultures in log phase. Growth medium (GM) was composed of Eagle's MEM with Earle's salts, supplemented with 2.2 g/l sodium bicarbonate, 15% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml)(all GIBCO products).

Monolayer cultures

Exponential (log) phase cells were obtained by growing $5x10^5$ cells/25 cm^2 flask in 5 ml GM for 18 ± 1 hours (28-32). Plateau phase cultures with different cell concentrations/flask were obtained by growing $1x10^5$ cells/flask containing 1 to 15 ml GM/25 cm^2 flask until they reach the plateau phase due to nutrient depletion (19, 30, 38). The relationship between final cell concentration at mid plateau phase versus volume of cell culture medium is shown in Results in Fig. 7.

Trypsin effects on cell shape

After removing the growth medium, cells were rinsed with physiological saline and then 1 ml of 0.05% trypsin + 0.02% EDTA solution was added and incubated at 37 °C for 10 minutes. The effect of trypsin on the shape of a group of cells was monitored through an inverted microscope for the first 10 minutes. Photomicrographs of cells were taken at 0.5 minutes intervals during the treatment of cells with trypsin so as to study the time course of trypsin effects on cell morphology.

Progression of cell spreading

Trypsin-treated round cells in 5 ml of fresh GM were plated into 25 cm^2 Corning tissue culture flasks and incubated in GM at 37 °C up to 6 hours to allow them to attach, spread and become monolayer cells. Photomicrographs of cells were taken at different time intervals after plating to study the progression of changes in cell morphology.

Spheroid cultures with round cells

The purpose of these cultures is to make the cells remain round as opposed to flat as in monolayer cultures. Exponential phase cells in monolayers, growing in MEM with 15% serum, were trypsinized and resuspended as single cell suspensions in MEM with 15% serum in (polycarbonate) Corning Erlenmeyer conical flasks at a concentration of $2\text{-}4x10^4$ cells/ml of medium (23, 32). Conical flasks were flushed with a mixture of 5% CO_2 in air, capped tightly to maintain pH, and incubated overnight (18 ± 1 hours) in a water-bath shaker at 37 °C to allow formation of spheroids containing 5 to 25 round cells.

Cell cycle distribution

Cells in monolayers and in spheroids were trypsinized, resuspended in growth medium, fixed in 50% ethanol, and stained with propidium iodide (500 μg/ml). The DNA content was determined using an Ortho flow cytometer (31, 32). Cell cycle distributions for monolayer and spheroid cultures were nearly the same (32). G_1, S and G_2+M: Spheroids - 42.7, 46.4 and 10.9%; monolayers- 45.5, 40.9 and 13.6%, respectively. It has been reported that the plateau phase cultures of this cell line have more than 90% of their cells containing a G_1 DNA content (12).

Irradiation

Cells were irradiated at room temperature in 5 ml medium with a Philips RT 250 X-ray machine (250 kVp X-rays, 15 mA, 0.39 mm Cu HVL, with 2 mm Al inherent filtration, dose rate with full back scatter 2.5 Gy/min) (28-32).

Immediate and delayed plating

To obtain immediate plating (IP) survival, cells are

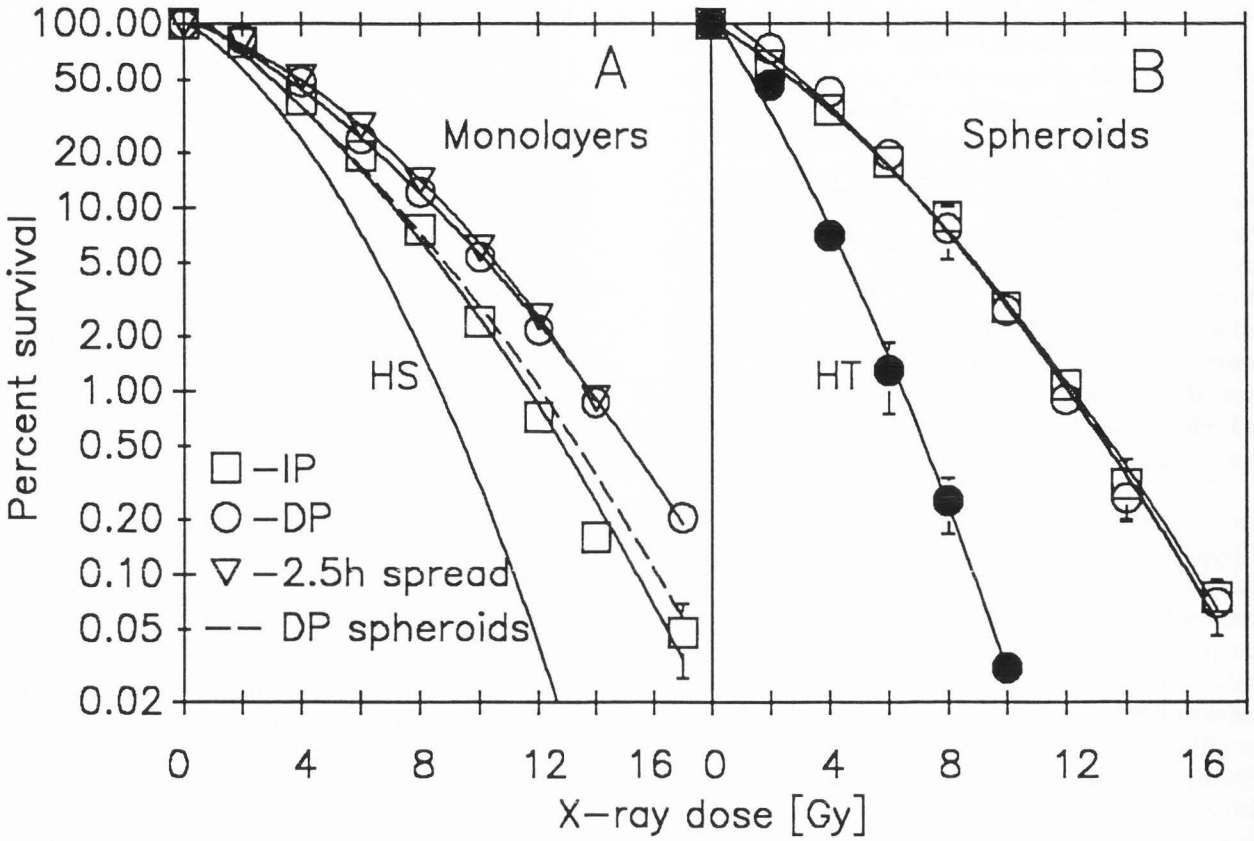

Fig. 2. Immediate and delayed plating survival of cells in exponential phase monolayers (panel **A**) and in spheroids (panel **B**). Each symbol represents a given condition in both panels: immediate plating (hollow squares); delayed plating (hollow circles); the survival of cells which were allowed to spread for 2.5 to 3 hours before irradiation and incubation for colony formation (hollow triangles). The solid line shown without data points in panel A represents the survival of cells treated with hypertonic saline for 20 minutes at 37 °C immediately after X-rays (28). The long dashed line in panel A represents the delayed plating survival of cells in spheroids, same as in panel B (hollow circles), for comparison. Survival of cells in spheroids exposed to X-rays and then to hyperthermia (43 °C for 20 minutes) is represented by solid circles in panel B. Delayed plating was 2-3 hours after irradiation and *in situ* incubation (see immediate and delayed plating under **Materials and Methods** section).

plated either immediately before or immediately after irradiation (6, 23, 29, 37). The differences in survival between cells plated immediately before or immediately after irradiation are minimal (6, 23, 29). To obtain IP survival, cells in monolayers, or in spheroids, or in monolayers obtained after plating round cells, were trypsinized, counted, diluted and an appropriate number of cells plated into 4 flasks per dose, each with 5 ml fresh growth medium. Flasks were then immediately irradiated and incubated for colony formation (29).

To obtain DP survival, cells in monolayers were irradiated in the attached and spread state in tissue culture flasks and incubated at 37 °C in 5 ml of GM (DPGM) or of depleted medium [generally called conditioned medium, CM (DPCM)], irrespective of the cell number/flask. In the literature, cells have been incubated in 5 ml medium during the postirradiation repair incubation, independently of cell density and growth phase (exponential, unfed or fed plateau phase) at the time of irradiation (12, 16, 19, 28, 30, 38). Spheroids were irradiated in 5 ml GM in 35 mm bacteriological petri dishes (32). This was to maintain the same depth of medium and irradiation conditions as that of monolayer cultures and to prevent spheroid attachment during irradiation. Immediately after irradiation, spheroid cultures in GM were transferred to conical tubes, flushed with 5% CO_2 in air and incubated at 37 °C (DPGM).

Repair kinetics indicate that while exponential phase cells require 2-3 hours to reach a plateau survival, plateau phase cells require 4-6 hours (10, 19, 28, 29, 30, 31, 38). When exponential phase cells were incubated for 6 hours, their survival was the same as that of cells incubated for 2-3 hours (data not shown).

Table 1. D_0, n and D_q values for the survival curves shown in Figures 2 and 6.

Experimental condition	D_0 [Gy ± SEM]	n	D_q [Gy]
Log phase monolayer cells (Fig. 2A)			
Immediate plating	1.71 ± 0.05	7.88 (5.3-11.7)	3.53
Delayed plating	2.20 ± 0.03	4.94 (4.2-5.9)	3.52
2.5 hours after plating	2.18 ± 0.05	5.97 (4.8-7.5)	3.88
HS assay (28)	1.15 ± 0.14	3.20 (1.9-7.9)	1.33
Spheroid cells (Fig. 2B)			
Immediate plating	1.84 ± 0.08	6.79 (4.0-11.6)	3.54
Delayed plating	1.89 ± 0.05	4.87 (3.5-6.8)	3.00
X-rays + hyperthermia	1.18 ± 0.05	1.92 (1.3-2.9)	0.77
Plateau Phase (Fig. 6)			
$0.8x10^6$ cells/ml			
DPGM	2.04 ± 0.09	2.6 (1.6-4.2)	1.95
DPCM	2.46 ± 0.12	3.4 (2.2-5.4)	3.30
$2x10^6$ cells/ml			
DPGM	2.58 ± 0.09	3.14 (2.4-4.1)	2.95
DPCM	2.54 ± 0.08	3.10 (2.5-4.0)	2.88
HS assay	1.25 ± 0.11	9.44 (3.4-25.9)	2.81
IP	2.23 ± 0.16	4.39 (2.2-8.9)	3.25

SEM is standard error of the mean. Numbers in parentheses represent the 95% confidence limits. DPGM and DPCM are the delayed plating of cells after incubation in growth medium or conditioned medium, respectively. HS assay: cells were treated with 0.5 M NaCl, for 20 minutes, immediately after irradiation.

Hence, exponential phase cells irradiated *in situ* were incubated *in situ* for 2-3 hours (monolayers and spheroids) and plateau phase cells were incubated for 6 hours, before trypsinization and subculturing for colony formation.

Hypertonic saline assay

Exponential and plateau phase cells were exposed to graded doses of X-rays in GM and CM, respectively. Immediately after irradiation cells were treated with 5 ml of 0.5 M saline at 37 °C for 20 minutes (12, 28, 29, 31, 38). Hypertonic saline treatment was terminated by aspirating the saline and rinsing the cells with 0.9% saline. The cells were then trypsinized and plated.

Hyperthermic assay

Control and irradiated spheroids in 5 ml GM were incubated at 43 °C for 20 minutes. After this hyperthermic treatment, they were incubated at 37 °C for 2 hours before trypsinization and plating. The toxicity due to heat *per se* was 30% and this was taken into account when calculating the survival after combined irradiation and hyperthermia.

Computation of cell survival, D_0 and n

Cells were plated into four 25 cm^2 flasks (at concentrations sufficient to score 100-200 colonies per flask), and incubated for 7 to 8 days. Plating efficiency (PE) for exponential phase cells in monolayers and in spheroids was the same, 89±5%, and that of plateau phase monolayer cells was 61±8%. Colonies were stained with crystal violet and counted. PE was taken into account for the purpose of calculating percent survival (28-32). Survival curves were drawn by fitting a second order polynomial (by the Sigmaplot computer program, Jandel Scientific, Corte Madera, CA) (32). Data for a given condition were combined and multivariate least squares regression was applied using the Systat program (Systat, Inc., Evanston, IL) to determine the values of survival parameters D_0 and n. All the data points in the exponential region, (doses 8 Gy and above), for a given condition, were combined to determine the D_0 and n (28-32).

Rate of depletion of nutrients in GM by irradiated cells during the period of repair incubation

These experiments were performed to determine the cell-concentration dependence of the rate of depletion of nutrient concentration in GM during the period of repair incubation. This was done by comparing the strength (number of cells/ml) of fresh GM with that of GM incubated with irradiated cells for periods up to 6 hours. A series of flasks with $0.8x10^6$ or $2x10^6$ cells/ml were exposed to 10 Gy in CM. Immediately after irradiation, CM was replaced with 5 ml of GM and incubated at 37 °C for up to 6 hours, i.e., similar to DPGM conditions. At 1 hour intervals, the GM was removed and transferred to an empty flask and stored at 20 °C. After 6 hours, $1x10^5$ cells were added to each flask containing used GM and incubated at 37 °C until cell growth had reached a plateau. The number of cells in each flask was then determined by trypsinization, dilution and counting.

All experiments were repeated two to four times. The data points represent the mean plus or minus one standard deviation.

Results

The IP and DP survival of exponential phase monolayer cells, and the survival of cells after treatment with hypertonic saline, are shown in Fig. 2A. Mathematically, the curves are described by their negative reciprocal slope (D_0), extrapolation number (n, the zero dose intercept of the $-1/D_0$ slope). The quasithreshold dose (D_q) is $D_0 \ln(n)$. The D_0 of cells after IP is lower than that of the same cells after DP (Table 1). This indicates the failure of some cells, which repair all their PLD under DP conditions, to do so under IP. However, the IP and DP survival of cells in spheroids is nearly the same (Fig. 2B and Table 1). Since cells in spheroids are radiosensitized by hyperthermia as shown in Fig. 2B and Table 1, they obviously have repaired some PLD.

Trypsin effects on the cell morphology of exponential phase monolayer cells are shown in Fig. 3. It can be seen that while spread cells become round, round mitotic cells remain round after exposure to trypsin. Treatment of cells in spheroids resulted in cell dissociation without a noticeable change in their morphology (data not shown). Plating of round cells resulted in cell attachment to the substratum and spreading as shown in Fig. 4. By 2-3 hours most of the cells are in various stages of spreading. The radiosensitivity of these spread cells is nearly the same as that of the DP survival of overnight exponential phase monolayer cells (Fig. 2A and Table 1). However, when cells were incubated in suspension for 18 hours, they formed spheroids with round cells and their survival was the same as the IP survival of monolayer exponential phase cells (Fig. 2 and Table 1).

Fig. 5 shows the DPGM and DPCM survival of plateau phase cells exposed to 14 Gy, plotted as a function of cell concentration. The data show that the difference between DPGM and DPCM survival is dependent on cell concentration and that this difference decreases with increasing cell concentration and disappears at concentrations $\geq 2x10^6$ cells/ml. This is because these cells greatly deplete the nutrients in the growth medium and make it equivalent to (convert it into dilute growth medium) the so-called conditioned medium.

The dose-survival relationships of plateau phase cells at two concentrations, for DPGM and DPCM conditions, are shown in Fig. 6. Panel A shows the results at a low cell concentration ($0.8x10^6$ cells/ml) and panel B at a high cell concentration ($2x10^6$ cells/ml). These results show that for cells at low concentration (panel A) the DPCM survival is higher than that for DPGM, while for cells at high concentration the DPGM survival is the same as that for DPCM. Furthermore, the IP radiosensitization of cells at high concentration is minimal, as shown in Fig. 6. Absence of an IP-DP survival difference for V79 cells (2) and fibrosarcoma cells (16) has been reported by others. DPGM and DPCM survival of V79 cells at a density of $9x10^6$ cells/25 cm^2 flask has been reported to be the same (12). However, cells in high density are radiosensitized by hypertonic saline, as shown in Fig. 6B, indicating the presence of cells with repairable damage. The D_0, n and D_q values for the survival curves are presented in Table 1. These results, and those presented in Fig. 2, indicate that absence of a difference between IP and DP, or between DPGM and DPCM, survival does not mean that cells have no repairable damage nor that cells are unable to repair/recover under certain conditions.

The cell number/25 cm^2 flask, in the plateau phase, is plotted against the quantity of fresh GM/flask in Fig. 7A. The relationship is linear and indicates that cell division and final concentration are determined by the nutrient content of the medium and are not limited by the surface area of the flask. Cell number/ml (the slope) was independent of whether flasks received 1 to 15 ml of GM at the beginning (unfed plateau phase) or were refed with 5 ml each, on days 4 and 5 of subculture (fed plateau phase).

The data in Fig. 7A can also be used to study the cell-concentration dependence of the rate of nutrient depletion by irradiated cells. The rate of nutrient depletion is estimated in terms of the number of cells in plateau phase in each 5 ml of GM which had previously been incubated with irradiated cells for 0-6 hours (Fig. 7B). This graph shows that the rate of dilution of nutrient

strength (or of a critical nutrient) by metabolic utilization is cell-concentration dependent and that at higher cell concentrations, irradiated cells are gradually shifted from a high to a low nutrient environment during the repair incubation. For example, in 5 ml of GM, which had been incubated with $1x10^7$ cells for 6 hours, the cell number attained is $< 4x10^6$ cells/flask, instead of $1x10^7$ cells/flask in a flask with 5 ml fresh GM. This indicates that the strength of this 5 ml GM is equivalent to 2 ml of fresh GM (see Fig. 7A) and that cells at a concentration of $2x10^6$ cells/ml utilized nutrients equivalent to those present in 3 ml of fresh GM during 6 hours of repair incubation. In other words, the nutrient concentration in this 5 ml of GM was gradually reduced to less than 35% of its original concentration. Whereas, cells at a concentration of $0.8x10^6$ cells/ml utilized close to 1 ml equivalent of the GM, the nutrient concentration remaining high, at about 80% of fresh GM, by 6 hours.

Comparison of the survival data (Figs. 5 and 6) and the rate of depletion of nutrient strength for cells at 0.8 and $2x10^6$ cells/ml (Fig. 7B) indicates a correlation between nutrient concentration in the repair medium and cell survival as reported earlier (30).

Discussion

In the literature, absence ($S_{IP} = S_{DP}$) or presence ($S_{DP} > S_{IP}$) of a difference between IP and DP survival has been taken as an indication of the respective ability or failure of cells to repair PLD (9, 16, 19, 22). $A > 1$ S_{DP}/S_{IP} ratio was taken to indicate a cell line with higher repair capacity than that of cells with an S_{DP}/S_{IP} ratio of 1 (8, 16, 20, 21, 40). Such an interpretation could be misleading if the IP-DP technique can not detect repair under all experimental conditions (33). For cells which have the capacity to repair X-ray induced damage, the cell shape and cell-concentration dependence of the differences between IP and DP survival can explain the presence or absence of a difference between IP and DP survival of the same cell line, or for different cell lines, or for tumor cells.

Trypsin treatment and growth conditions being the same, the IP survival of monolayer exponential phase cells was lower than the DP survival; whereas the IP and DP survivals were the same for round cells in spheroids (Fig. 2). Such an absence of difference between IP and DP survival of cells in spheroids could be misinterpreted to mean that cells in spheroids do not repair PLD. The fact that PLD repair could be detected in spheroid cells by hyperthermia (Fig. 2A) indicates that such an interpretation is not valid. Moreover, the same cells in spheroids, when converted to monolayers, show radiosensitization under IP conditions (32). In addition to differences in cell shape, cells in monolayer and spheroid cultures may also differ in their metabolic state. Radiosensitization by trypsin has been shown to be independent of trypsin-concentration and treatment duration, i.e., the effect of 0.05% trypsin was the same as that of 0.25% trypsin and the effect of 1.5 minutes of trypsin treatment duration was the same as that of 10 minutes (29). Hence, we suggest that: **1)** IP radiosensitization is not related to trypsin effects *per se*, **2)** trypsin alters the shape of spread, but not round, cells (Fig. 3), **3)** radiosensitization under IP conditions may be related to trypsin-induced cell-shape changes, **4)** the IP-DP technique may fail to detect PLD repair when cells are round, and **5)** the absence of a difference between IP and DP survival does not mean the absence of an ability to repair damage (1, 25, 34).

Plating efficiencies being the same, the survival of round cells from monolayers was similar to that of round cells in spheroids (Fig. 2). This observation suggests an absence of a contact effect for this cell line as reported earlier (29, 32). The higher radiosensitivity of cells in monolayers in relation to that in spheroids, as reported by others (6, 23), may be due to cell line dependent differences in cell-to-cell contact, cell junctions, and serum concentration in the culture medium for spheroids and monolayers (32), and to their differential response to radiosensitization by trypsin (29, 32 and Fig. 2). It has been recently shown that of two human squamous cell lines, A431 and CaSki, the former showed a cell-cell contact effect, the latter did not (17).

The idea of a cell-shape dependence of radiosensitivity is a recent one (14, 15, 29, 30, 36). Differences in cytoskeletal and chromatin organization could influence the accessibility of DNA damage to repair enzymes (11, 15, 28-32, 36, 41). Recent data show that the higher radiosensitivity of trypsin-treated round cells from monolayers, and of round cells from spheroids under immediate plating conditions, as opposed to delayed plating conditions, are associated with correlated changes in cell shape and chromatin structure (14).

The DPGM survival is higher for cells at $2x10^6$ than at $0.8x10^6$ cells/ml (Figs. 5 and 6, and Table 1). The survival of plateau phase V79 cells, with $2x10^6$ cells/ml, incubated in growth medium or in conditioned medium have been reported to be the same (12). Cell-concentration-dependent dilution of nutrient concentration (Fig. 7) correlates with the increase in DPGM survival (Figs. 5 and 6). Furthermore, addition of fresh GM after 6 hours of repair incubation in GM eliminates the increase in survival observed beyond 6 hours for plateau phase LICH cells (19). Two reasons can be suggested to explain this phenomenon. **1)** In high cell concentration cultures ($2x10^6$ cells/ml), nutrients are depleted faster and accumulation of metabolic products that inhibit

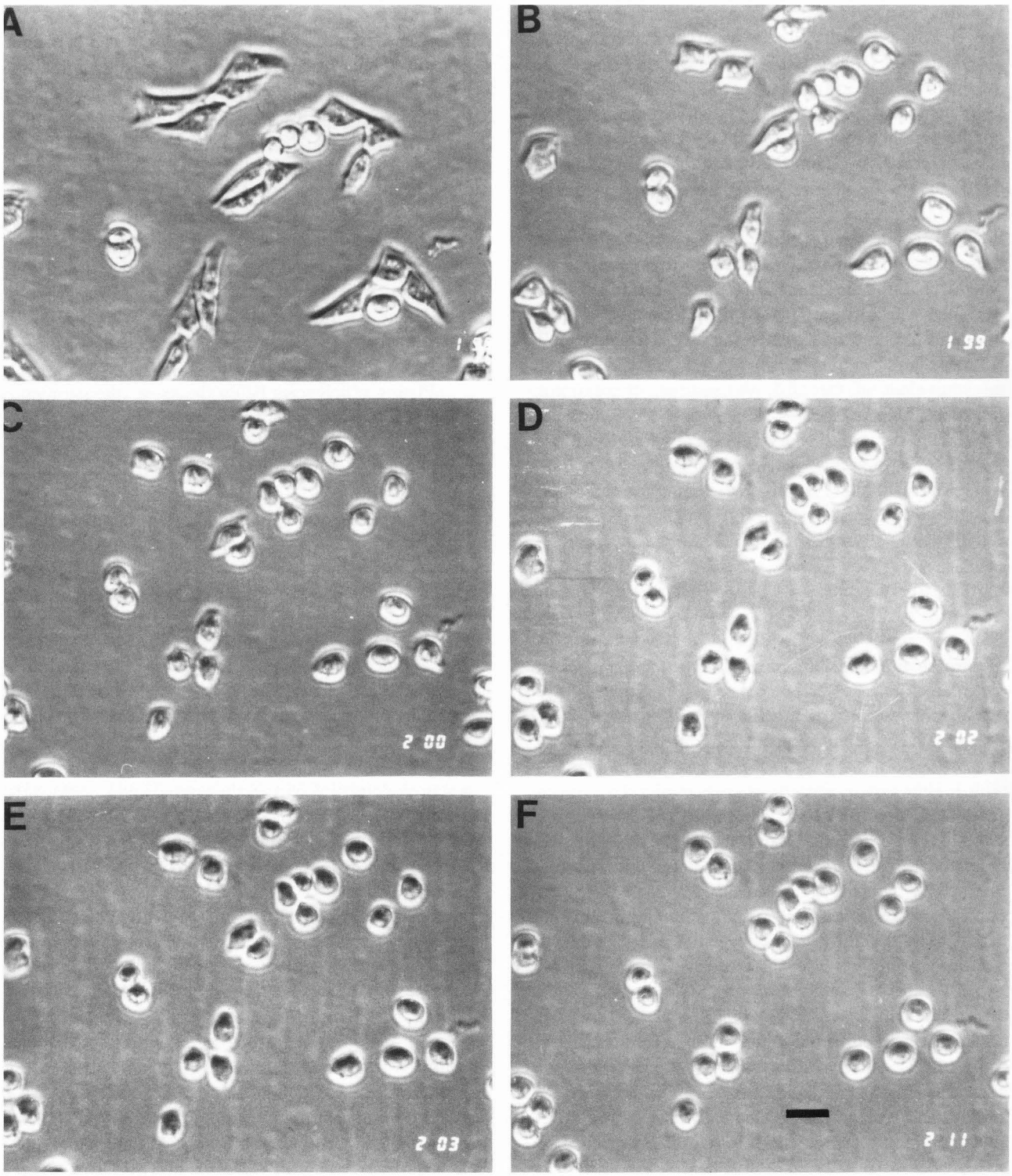

Fig. 3. The kinetics of the trypsin effect on cell morphology. Photomicrographs: **A** - A group of spread log phase cells with round mitotic cells; **B** - 0.5 minute, **C** - 1.0 minute, **D** - 1.5 minutes, **E** - 2 minutes, and **F** - 10 minutes after addition of trypsin to cultures. While spread cells become round by about 2 minutes, the shape of mitotic cells is not affected by trypsin. Bar = 20 μm.

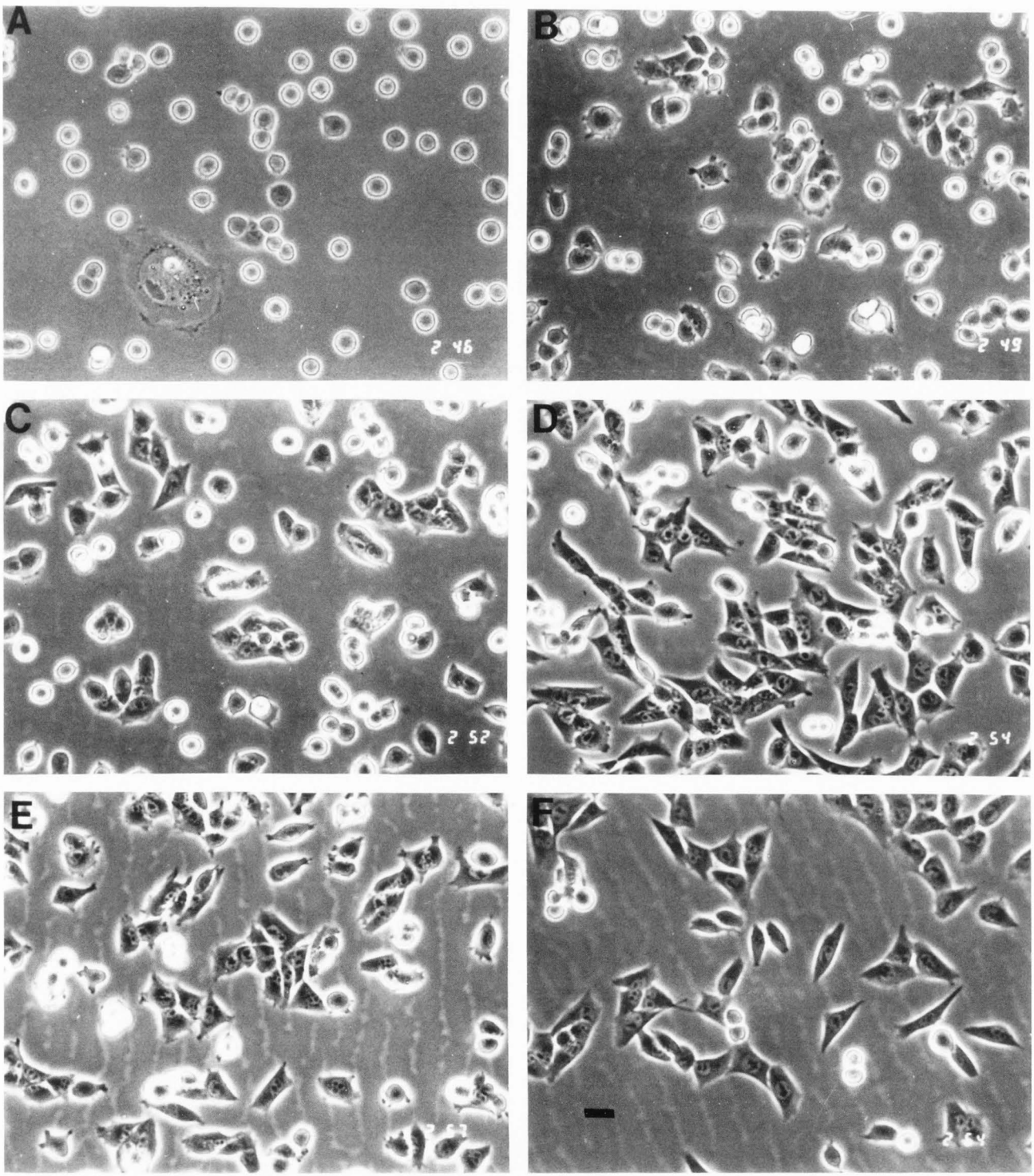

Fig. 4. The kinetics of cell spreading during incubation of cells in tissue culture flasks in growth medium. Photomicrographs: **A** - 1 hour, **B** - 2 hours, **C** - 2.5 hours, **D** - 3 hours, **E** - 4 hours, and **F** - 6 hours after plating and incubation. Round cells attach and the degree of cell spreading increases with the time of incubation. Most cells are nearly spread by 2-3 hours and appear similar to overnight exponential phase cultures. Bar = 20 μm.

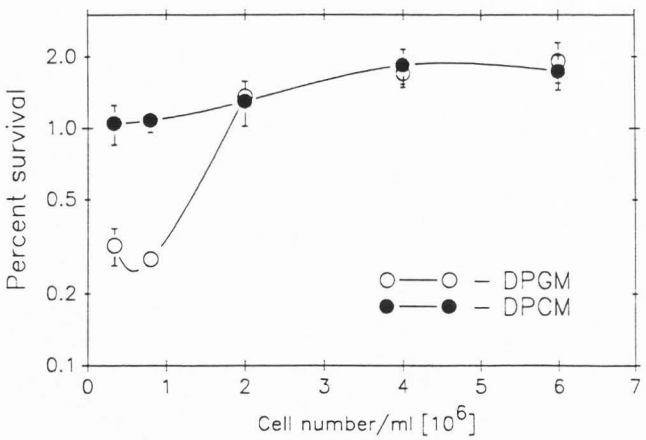

Fig. 5. Delayed plating survival of monolayer cells, at various densities, exposed to 14 Gy and then incubated in growth medium (DPGM) or in conditioned medium (DPCM) for 6 hours.

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proliferation may also be higher. Differences in metabolic states may affect intracellular ultrastructure. Consequently, the rate of progression through cell cycle is slower compared to cells at low concentration. Such a difference in cell cycle progression, between low and high cell density cultures, might be invoked to account for the differences in cell survival (5, 9, 13, 19, 40). However, doubling times for cells cultured in full-strength growth medium (100%) or in diluted growth medium (40%) were nearly the same, indicating that the doubling time, and hence progression, were not affected by nutrient concentration (30). Therefore, it appears that the survival differences between DPGM and DPCM are not due to progression of cells in growth medium. 2) An alternative suggestion is that the microenvironment under DPGM and DPCM conditions - in terms of cell volume, concentration of serum proteins, glucose, and other nutrients - is different. The observation that cell survival was higher in medium with 5% versus 15% serum (32) and in dilute (40%) versus fresh (100%) growth medium (30) indicates a correlation between nutrient concentration in the growth medium and radiosensitivity. Experiments are in progress to analyze the differences in cell cycle progression in low and high cell density cultures under different microenvironments.

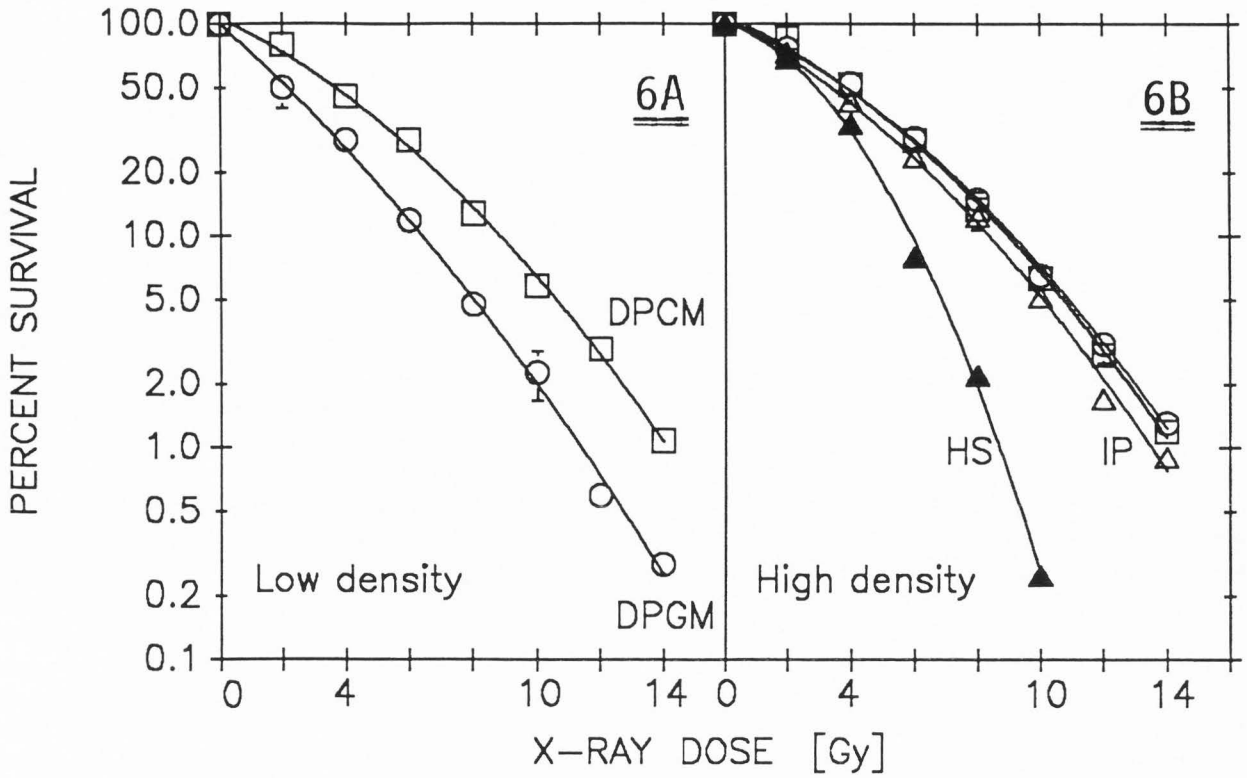
Hence, we have shown that the magnitude of difference between IP and DP survival correlates with cell morphology and cell density for V79 (S-171) cells. Cell shape and density can be different for "normal" (contact inhibited) and transformed (no contact inhibition) cell lines. For normal cell lines, such as diploid fibroblasts and C3H 10T1/2 cells, the limiting cell density depends on the growth surface area because of contact inhibition of cell division (42). The shape and density of these

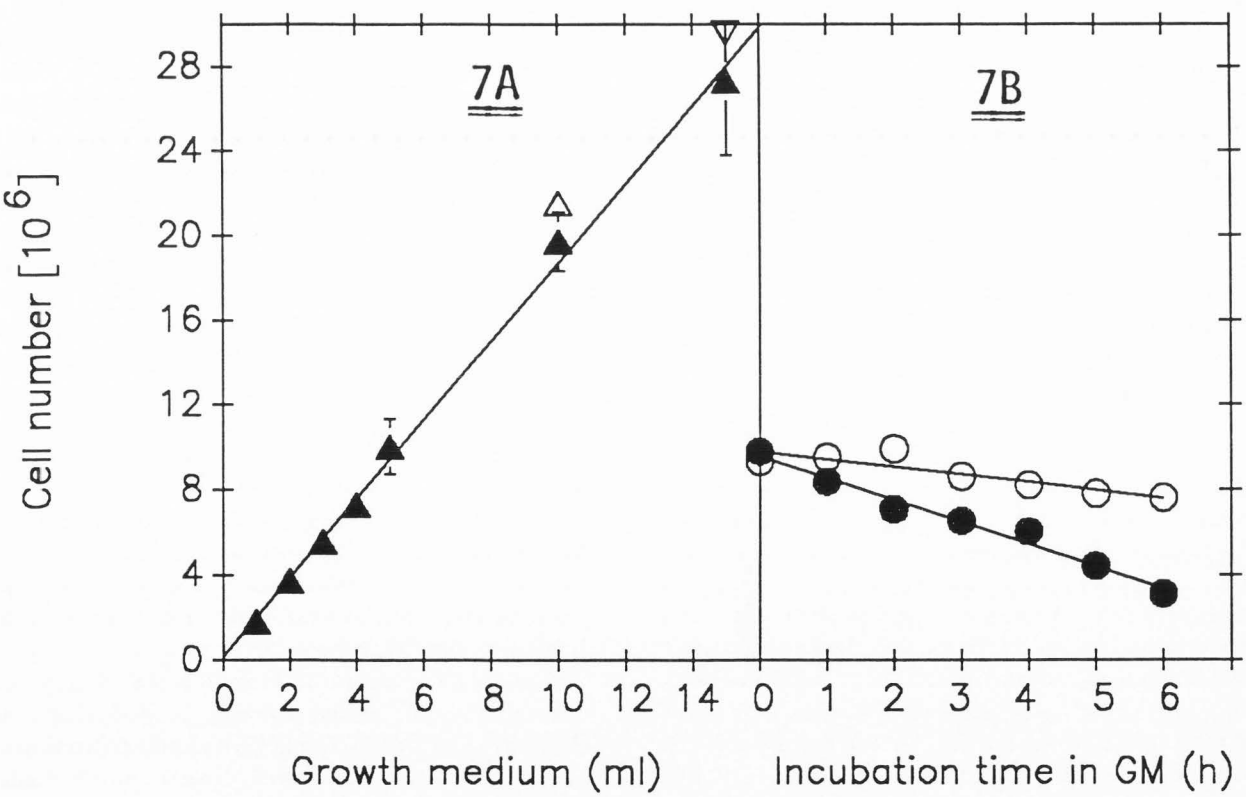
Fig. 6 (facing page, top). Delayed plating survival of plateau phase cells at concentrations of $0.8x10^6$ (panel **A**) and $2x10^6$ cells/ ml (panel **B**). Each symbol represents a given condition in both panels. Incubated in growth medium (DPGM) - hollow circles, or incubated in conditioned medium (DPCM) - hollow squares. Immediate plating survival of cells at a concentration of $2x10^6$ cells/ml - hollow triangles. Survival of cells at a concentration of $2x10^6$ cells/ml, exposed to 0.5 M hypertonic saline for 20 minutes immediately after exposure to X-rays (panel B) - solid triangles. Delayed plating was 6 hours after irradiation and *in situ* incubation (see immediate and delayed plating under **Materials and Methods** section).

Fig. 7 (facing page, bottom). The relationship between the quantity of growth medium (GM) and the cell number per flask in the plateau phase. **Panel A:** Unfed, where 1 to 15 ml of GM was added to flasks at the start of culture - solid triangles, refed with 5 ml of GM on day 4 (total of 10 ml) - hollow triangles, and on day 5 (total of 15 ml) - hollow inverted triangle. **Panel B:** The decrease in the strength of 5 ml of GM incubated with $0.8x10^6$ (hollow circles) or $2x10^6$ (solid circles) irradiated (10 Gy) cells as a function of duration of incubation with cells. The capacity of 5 ml GM was estimated in terms of the number of cells each 5 ml could yield from an initial inoculum of $1x10^5$ cells.

Cells are not influenced by the nutritional state of the medium. Therefore, the IP survival levels of normal fibroblasts and of C3H 10T1/2 cells are invariably lower than those for DP (16, 22, 42), because they are subject to cell-shape-related radiosensitization by trypsin (11, 15, 28, 29, 32, 36) and to nutrient-concentration-related radiosensitization by the culture medium (30 and Figs. 5-7).

However, the growth of transformed cell lines, e.g., V79 cells, fibrosarcoma and the transformed counterpart of C3H 10T1/2 cells, is not inhibited by cell contact (42). This can result in a difference between the cell density of normal and transformed cell lines, independent of surface area (Fig. 7). For example, the maximal cell density for normal and transformed C3H 10T1/2 cells was ca. $1x10^6$ and $7\text{-}10x10^6$ cells/25 cm^2 flask, respectively (42). High cell densities in plateau phase cultures result in cell crowding and loss of fibroblastic morphology, i.e., cells become tightly packed and rounded (7). The difference between IP and DP survival of plateau phase "transformed" cell lines at high cell densities [such as melanoma (22), fibrosarcoma (16), C3H 10T1/2 (42) and V79 (2)] has been reported in the literature as minimal or absent. This absence of an IP-DP survival difference for high density plateau phase


PERCENT SURVIVAL
100.0
50.0
20.0
10.0
5.0
2.0
1.0
0.5
0.2
0.1
6A
6B
DPCM
DPGM
HS
IP
Low density
High density
0
4
10
14
X–RAY DOSE [Gy]


Cell number [10^6]
28
24
20
16
12
8
4
0
7A
7B
0 2 4 6 8 10 12 14
0 1 2 3 4 5 6
Growth medium (ml)
Incubation time in GM (h)

transformed cells can be explained as due to the lack of a cell-shape and/or nutrient-concentration related reduction of PLD repair under IP conditions by trypsin and growth medium, respectively (Figures 2, 3 and 6). Treatment of these cells with other PLD repair inhibitors such as hypertonic saline (13, 28, 38), hyperthermia (39) or β-araA (1, 12) should shed more light on these phenomena.

Thus, the reported failure to detect PLD repair by the IP-DP technique in many tumor models irradiated *in situ* (33) may either be due to the cells of these tissues having a round shape (as in spheroids), or be a result of the limitation of cell dissociation enzymes to reduce PLD repair. For example, it takes only minutes for trypsin to alter cell-shape and dissociate monolayer cells from the substratum (Fig. 3), whereas alteration of shape and dissociation of cells in tumor tissue often take more than an hour (1). However, the rate of repair in both cases may be the same, on the order of minutes/Gy (29, 31). As a consequence, repair of PLD in tumor tissue may well be completed before trypsin, collagenase, or other dissociative enzymes, affect the cells. Such a difference in rates of PLD repair versus tissue dissociation may explain why trypsin-related PLD repair reduction under IP conditions is reported to be absent in cells in tissues but present when the same tumor cells are grown in monolayers (1).

Conclusions

Results presented here suggest that: **1**) cell architecture, cell concentration and medium nutrient condition can affect the level of potentially lethal damage repair and PLD repair detection by the IP-DP technique and, **2**) studies of IP versus DP and DPGM versus DPCM will not always reveal the capability of cells to repair PLD. Therefore, cell survival differences between immediate plating and delayed plating should not be used as the only method for measuring whether cells have the capacity to repair PLD.

Acknowledgements

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Discussion with Reviewers

K.T. Wheeler: To prepare single cells from either monolayer or spheroid cultures, trypsinization has been carried out at 37 °C and not 4 °C. Therefore some repair/recovery would have taken place during this time. Authors use this as IP survival. I wonder if they compared the IP survival when cells were trypsinized at both these temperatures? Could the lack of a difference between IP and DP survival of cells in spheroids be due to some repair taking place during trypsinization?

Reviewer V: Have the authors attempted-to measure the recovery from PLD using an ice-cold trypsinization procedure where both molecular repair and cellular repair processes are stopped within a few second of reaching 4 °C?

Authors: It is true that a very small fraction of cells can repair their damage during the 10 minutes of trypsinization at 37 °C (29). However, the fraction of cells involved is insufficient to result in the lack of a difference between IP and DP survival in spheroids. Even if the absence of a difference could be thus explained, such data would not mean that such cells had no PLDR, because exposure of these cells to hyperthermia resulted in failure to repair some of the PLD which would otherwise have been repaired.

An attempt was made to trypsinize V79 cells at 4 °C. However, cold trypsinization for up to 30 minutes resulted only in detachment of cells from the substratum with most of the cells remaining in clumps. This appeared to be due to a strong attachment of V79 cells to the substratum and to each other.

Reviewer V: In Figure 3, the cells are rounded to their fullest extent by 1.0 to 1.5 minutes after trypsinization. If the cells are irradiated at this time, does one get the same survival as after 10 minutes of trypsinization?

Authors: Yes. The survival of monolayer log phase cells treated with 0.05% trypsin for 1.5 minutes or for 10 minutes was uniformly the same and the radiosensitizing effect of 0.05% and 0.25% trypsin was identical (see text and ref. 29). We have recently shown that mouse cells (L5178Y-S) cultured as round cells in suspension are not radiosensitized by trypsin and compared this to monolayer V79 cells when the trypsin treatment for both cell lines was 10 minutes (14). These results indicate that the IP radiosensitization is not related to trypsin effects *per se* (for short to moderate exposure times and concentrations) and that it is related to trypsin-induced cell shape changes (14,29).

R.M. Sutherland: In the case of monolayers obtained after plating round cells, the cells underwent trypsinization twice within two to three hours. What are the effects due to double trypsinization on cell survival (PE) and radiosensitivity of cells? What is the cell survival (PE) if these round cells were kept round during the time for PLDR?

Authors: Double trypsinization did not affect the plating efficiency (PE) or radiation response of cells. Their response was similar to that of cells undergoing single trypsinization. PE generally refers to unirradiated cells. If round cells were kept round during PLDR, an increase in survival was not observed (data not shown). LICH cells also did not show an increase in cell survival when they were kept round during PLDR (19).

K.T. Wheeler: Does the density of cells at the time of irradiation affect radiosensitivity?

Authors: The difference between IP and DP survival of cells at low density (250-30,000 per 25 cm^2 flask) versus at a relatively high density (1-2x10^6 per 25 cm^2 flask) at the time of irradiation was minimal (29). The IP survival was lower than the DP survival for cell densities less than 4x10^6 cells per 25 cm^2 flask, as long as the cells were spread and amenable to cell shape changes by trypsin. However, the differences between IP and DP survival at densities greater than 1x10^7 cells per flask was minimal.

K.T. Wheeler: Why is there a difference in the time of incubation between log and plateau phase cells irradiated *in situ*? Was there any difference in the results obtained when identical incubation times were used?

H.Z. Hill: How long was the delay? Could you clarify how (trypsin) and when the cells were replated for colonies?

Authors: While log phase cells require about 2-3 hours, plateau phase cells require about 4-6 hours to complete repair. Incubation of log phase cells for 6 hours, as is done for plateau phase cells, did not result in an increase in survival above that obtained for 2 hours of incubation.

K.T. Wheeler: Is it possible that the rates of cell cycle progression and DNA synthesis are dependent on cell density?

Authors: We are currently studying the rates of DNA synthesis and cell cycle progression in cells at low and high density, incubated in conditioned medium or fresh growth medium. Preliminary results indicate that the growth kinetics, cell doubling time and the rate of DNA synthesis in growth medium are independent of cell density (provided that they do not exhaust the medium during the incubation period). Not surprisingly, cell cycle progression and DNA synthesis in low and high density cultures incubated in conditioned medium was minimal.

H.Z. Hill: Does the expression "conditioned medium" imply that the medium is in some way enriched?
R.M. Sutherland: Do the authors have evidence that the observed effect with conditioned medium (CM) is due to nutrient depletion rather than to substances released from the cells?
Authors: No. We have introduced the term "depleted medium" to denote the generally used term "conditioned medium"; such medium does not support cell growth. Yes. The same effect, seen with depleted/conditioned medium, is seen with fresh growth medium (or MEM) diluted to 40% with normal saline and is seen with Hank's balanced salt solution (HBSS). All three treatments yield the same result.

Reviewer V: Why not measure the glucose concentration to define the strength of the growth medium?
Authors: The concentration of glucose in the medium would be one way to characterize the capacity of a given medium. However, other components, such as serum and sodium bicarbonate, also modify cell survival after radiation (unpublished data). The concentrations of the latter components of the medium also keep changing with duration of incubation.

H.Z. Hill: Why did you use hyperthermia for spheroids and hypertonic saline for monolayers?
Authors: Our intent was to show that more than one technique is available to test PLD repair and to demonstrate that if one technique fails to express PLD it does not mean that there was no PLD repair.

Reviewer III: What is the role of cell contact and of cell junctions in cell survival following radiation?
Z. Somosy: How do your results for spheroids compare with the results reported recently by Kwok and Sutherland for human squamous cell lines A431 and CaSki?
Authors: Some reports have claimed that cell contact affects radiosensitivity. We have shown that cell contact does not affect the radiosensitivity of our V79 cells, hence any cell contact effect may depend on cell line and experimental conditions (29, 32). Similarly, Kwok and Sutherland (17) have shown that the cell contact effect can be cell line dependent. They have shown that of the two human cell lines - A431 and CaSki - the former, but not the latter, showed a cell contact effect on radiosensitivity.

Reviewer V: Treatment with either hypertonic salt solution or hyperthermia usually causes tissue culture cells to round up, similar to the effect of trypsin. If so, should not the hypertonic saline assay give you essentially the same survival curve as your immediate plating assay?
Authors: Treatment of monolayer V79 cells with 0.5M hypertonic saline (for 20 minutes) causes cell shrinkage but not cell rounding; the cells remain spread. There was no detachment of cells after treatment with hypertonic saline. Cell survival after high salt assay is lower than that after treatment of cells with trypsin [immediate plating assay (Fig. 2A and ref. 28)]. This indicates that high salt treatment affects the repair of PLD to a higher degree than does trypsin. This may be related to a higher magnitude of alterations in chromatin structure (possibly resulting in increased DNA fragment separation and loss from the chromosome) due to osmotic shock after high salt treatment versus cell rounding after trypsin treatment.

Reviewer V: Are not V79 cells normal lung fibroblasts and not tumor cells?
Authors: V79 cells are normal only in the sense of being non-tumorigenic. However, they are transformed as far as contact inhibition is concerned. These are immortalized cells and can multiply indefinitely, unlike normal fibroblasts. The latter have only a limited life span and are characterized by contact inhibition of growth. With reference to cell transformation, three stages can be defined: **1)** normal (contact inhibited, non-transformed and non-tumorigenic), **2)** transformed but non-tumorigenic (e.g., V79), and **3)** transformed and tumorigenic (e.g., fibrosarcoma cells). More information can be obtained on the three types from the book **Radiobiology for the Radiologist**, by Eric. J. Hall, 3rd edition, J.B. Lippincott Company/Philadelphia, (1988).

H.Z. Hill: Are there several step to PLDR, as exemplified by the transitions between high radiosensitivity when exposed to high salt and IP (step 1), and from IP to DP (step 2)?
Authors: This line of reasoning could be extended to additional steps between DP in GM and DP in CM (step 3), with additional steps for each additional postirradiation treatment as Iliakis (12, 13) argued for α and β PLDs after β-araA and HS treatments. We believe that it is more profitable to interpret these survival differences as due to different proportions of the cell population which can repair **all** of their damage under each of these different postirradiation conditions. The different proportions could be due to repair of the same lesion (e.g., DNA DSBs) but with different times available for repair (for all cells) under each of these conditions, due to different metabolic events or demands made upon the cell by each of these conditions. HS treatment could also cause chromatin conformation changes which could result in DNA fragment loss and hence misrepaired DSBs (a lethal event). Ostashevsky uses the same inter-

pretation and successfully treats these types of data quantitatively in the DSB model (Radiat. Res. **118**, 437-466, 1989).

Alternatively, the different proportions could be due to different subpopulations, each with its own ability to repair all damage (e.g., DSBs) under some - but not other - environmental conditions. This would be consistent with our finding that the fraction of cells which do not repair all their damage when delay plated in growth medium (DPGM), but which do so in conditioned (depleted) medium (DPCM), can repair this damage and survive when switched from DPGM to DPCM - even after no further survival increase in DPGM can be observed.

H.Z. Hill: As a predictor of radiosensitivity, would not the most meaningful determination be the difference in response to high salt (HS) versus response to delayed plating (DP)?

Authors: Not necessarily. It depends on what the underlying lesions and repair processes determining survival are. If they are DSBs which can be rendered irreparable (i.e., misrepaired) by fragment loss as after HS treatment, or be blocked in their repair for a fraction of their time available for repair (a time determined within the cell) as with β-araA treatment, then (as in the DSB model, Ostashevsky, Int. J. Radiat. Biol. **57**, 523-536, 1990 and Radiat. Res. **118**, 437-466, 1989) survival probability can be predicted from a knowledge of the efficiency of DSB induction (G-value), the time constant for DSB repair (τ_{DSBR}), the time available for repair (t_{rep}), and the probability of DNA fragment loss before repair. If G_{DSB} and τ_{DSB} differ only minimally with cell type (there is evidence for a wide range of eukaryotic systems that this is so), then, it is probably t_{rep} which is most sensitive to modulation by postirradiation conditions. In this case the HS treatment is only relevant as a very crude estimator of initial damage (not all, or even most, DNA fragments between two DSBs are lost after HS treatment, and differences in chromatin conformation could yield different susceptibilities to HS treatment). Thus, it is the final survival under conditions which mimic those applicable *in vivo* which is most relevant for radioresistance determination. One cell population would be more radioresistant than another if it had a higher final survival probability (DP) even if it had a smaller difference between responses in HS versus DP. Comparison to less optimal repair conditions is unnecessary for radiosensitivity determination, but may be useful for the understanding of repair processes.

R.M. Sutherland: How can one exclude all the influences from factors affecting ability to detect PLDR in order to define the basal level of cell kill by radiation and then to subsequently define the PLDR level? PLDR can be modified by external agents but the effects are cell system dependent. In other words, what is the definition of PLDR?

Authors: Let us note that potentially lethal damage (PLD) is just a historical definition to distinguish it (postirradiation milieu change altering survival) from sublethal damage (SLD) (an operational definition for split dose alteration of cell survival as opposed to its earlier target theory meaning of accumulation of sublethal lesions which become lethal and irreparable only when their number becomes equal to the target number) and that since the repair kinetics of both are identical (28, 31) they probably represent the same molecular lesion, e.g., DNA DSBs. If the potentially lethal lesion is the unrepaired DSB then, when this can be measured reliably for mammalian cells, PLD is the number of DSBs remaining. This is the approach used in the DSB model (Ostashevsky, Radiat. Res. **118**, 437-466, 1989) with considerable success, and perhaps is the only valid approach for the long run.

Alternatively, one could define unrepaired damage as: $-\ln(S) = \alpha D + \beta D^2$; if one assumes that the linear component is irreparable (lethal damage, LD), then unrepaired PLD = βD^2. If a condition existed in which no PLD could be repaired, then the LD and PLD would both be lethal and the slope of this exponential survival curve times dose (α'D) minus αD would be the initial amount of PLD, this minus βD^2 would be the amount of PLD repaired, and βD^2 would be the amount of unrepaired PLD. This is essentially the approach taken in the Cybernetic (27) and LPL models (5). However, several problems exist for this approach. First, it is not clear what condition, if any, completely blocks **all** PLDR, and hence the total amount of PLD+LD is not readily ascertainable [i.e., the amount measured depends on assumptions about the effectiveness of the most nontoxic radiosensitizing treatment known (HS)]. Second, if a single unrepaired DSB were a lethal event (as successfully modeled in the DSB model), then repair-absent mutants should have a D_0 (reciprocal slope) of only 4 cGy (Ostashevsky, Radiat. Res. **118**, 437-466, 1989)! Since this is not seen, repair-deficient mutants with D_0 = 50-65 cGy (e.g., xrs-5 of CHO-K1 cells) must still repair a considerable amount of PLD. Third, since the molecular lesion responsible for PLD is not specified in a way which allows a potentially destructive test of the Cybernetic or LPL models, they are not really testable.

R.M. Sutherland: Why did you use the survival response of a mutant of a different cell line in Fig. 1? Where did the other data in this figure come from?

Authors: The xrs-5 cell line is a mutant of CHO-K1. Ideally one should compare the response of a mutant

with that of its parent wild type. In Figure 1, the response of xrs-5 and V79 cells has been compared to show qualitatively the various factors (such as mutation, cell culture, and environmental conditions) which could modify the response of cells. We have not derived any quantitative information from this comparison. All the other data in this figure have been obtained for V79 cells in our laboratory, as noted in the figure legend.

R.M. Sutherland: How old were the cells in monolayer plateau phase cultures?
Authors: Cells were in mid plateau phase. Depending on the quantity of growth medium, cells inoculated at 10^5 cells/flask reach plateau phase at different times. Cells were used between 6-10 hours after they had reached plateau phase.

R.M. Sutherland: What are the repair kinetics for cells in spheroids? Also state how you controlled pH and what the pH was?
Authors: We have not yet studied the repair kinetics of cells in spheroids. It is true that PLDR in some cell lines is affected by pH. We have not controlled pH. This is so because the comparisons were made for identical pH conditions: log phase spheroids versus monolayers in fresh medium and low versus high density plateau phase cells in fresh medium or conditioned medium. Therefore, we believe that the pH effect on cell response in our study is minimal, if any. The pH of fresh growth medium was 7.3 to 7.5. The pH of conditioned medium was 6.8 to 7.0. It is worth remembering that these spheroids (5-25 cells) are too small to have hypoxic or acidic centers.

R.M. Sutherland: Should you be comparing current data with historical data (1989) since the cells and/or serum could have changed?
Authors: Current measurements are in agreement with our more extensive historical data. The cells were grown into a large stock population, upon receipt of our original cultures from Dr. Iliakis in 1985, and frozen in aliquots in liquid nitrogen. Every three months a fresh aliquot is taken, grown to provide it's replacement, and used to provide stock populations for experiments. Thus genetic drift is negligible. The growth-tested serum batches appear to be highly uniform and results of experiments are highly repeatable (ca. $\pm$ 5%) from one batch to another.