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DOSIMETRY OF IRRADIATION MODELS

The 96-well clonogenic assay for testing radiosensitivity of cell lines

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Radiation experiments with cells in single cell suspension in test tubes and on 96-well plates were carried out and compared. The cells originated from cell lines established from carcinomas of the floor of the mouth and from endometrial carcinoma. Two irradiation models were constructed. Both models allowed the absorbed doses to the cells to be administered with a high accuracy in both experimental settings (better than 5.0%). These irradiation models were compared on cancer cell lines with dissimilar inherent radiation sensitivity and histologic type (UM-SCC-1 resistant, UM-SCC-14A sensitive, and UT-EC-2B highly sensitive); various radiation doses were used. The fractions of surviving cells as a function of radiation dose were compared: there was no significant difference between cells irradiated in test tubes and cells irradiated in 96-well plates. Thus, if the absorbed doses in cells suspended in a tube and in a plate were the same, the survival was similar regardless of the type of irradiation model.

One of the current focuses of interest in radiobiology concerns the effects of radiation on the surviving fraction of cells in vitro. Appropriate techniques may allow a quick assay of the radiation sensitivity of malignant cells. The accuracy and homogeneity of absorbed doses in cells have only seldom been studied (1, 2). The basic issue is the same as in clinical radiotherapy: how are cells to be irradiated if a homogeneous absorbed dose is to be delivered to the target cells or tissue?

We have performed radiation experiments on single cell suspensions in test tubes and in 96-well plates. We have constructed irradiation models for both experiment types which make it possible to irradiate the target cells repeatedly and with good accuracy with a clinical treatment unit. These models are used to describe the irradiation effects on cell lines as reported in the literature (3–5). We have also tested the model with same cell lines as has been done in the Cancer Research Laboratory, Department of Otolaryngology — Head and Neck Surgery of the University of Michigan; the results were comparable (6).

In the present paper we describe the radiation geometry and the determination of the absolute administered absorbed dose in two models using a clinical radiation unit. We have also compared how well these models describe the radiobiological effects of different absorbed doses on cancer cell lines with different inherent radiation sensitivity and histology.

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Material and Methods

Cell lines. The cell lines consisted of two squamous cell carcinoma (SCC) lines of the head and neck and one adenocarcinoma cell line of the uterine corpus. The SCC lines were selected on the basis of their dissimilar mean inactivation radiation dose tested earlier in our laboratory (6). The cell line UM-SCC-14A is relatively sensitive to radiation while the UM-SCC-1 is relatively resistant. Both cell lines have been established from carcinomas of the

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floor of the mouth (7). The endometrial adenocarcinoma cell line UT-EC-2B has recently been established from a lymph node metastasis of a patient with endometrial adenocarcinoma and characterized. UT-EC-2B is inherently highly radiosensitive and has a survival fraction of 0.044 at 2.0 Gy. Before the experiments, the cells were cultured in T 25 culture flasks in Dulbecco's minimal essential medium supplemented with 1% non-essential aminoacids, 2 mM L-glutamine, streptomycin and penicillin and 10% fetal bovine serum (FBS) and kept in logarithmic growth by weekly passages. Cells in midlogarithmic phase (40–60% confluency) were used for the experiments, and the cells were fed with fresh medium on the day before plating. The cells were harvested with trypsin-EDTA, counted manually using hemocytometer chambers and diluted to make a standard stock single cell solution of 4 167 cells/ml. If necessary, this cell concentration was increased according to the plating efficiency (PE) of the individual cell lines.

Further dilutions of this single cell stock suspension were made in 6 ml of Ham's F-12 medium supplemented with 15% FBS. The final cell number per well in 96-well plates was adjusted according to the expected cell kill as follows: control, 2 c/w (c/w = cells/well); 0.75 Gy, 3 c/w; 1.25 Gy, 4 c/w; 2.50 Gy, 10 c/w; 5.00 Gy, 20 c/w; 7.50 Gy, 100 c/w. The higly radiosensitive adenocarcinoma cell line was irradiated with 0.40 Gy, 0.75 Gy, 1.25 Gy, 1.75 Gy and 2.50 Gy. The suspensions were irradiated in tubes (see below) and diluted immediately after irradiation with 44 ml of the same culture medium; 200 ml of the suspension was plated into each of the 96 wells on culture plates in duplicate. The tubes were inverted and mixed with a whirl mixer several times to avoid sedimentation. The plates were placed in an incubator saturated with water vapor containing 5% CO_2 at 37°C. After 4 weeks, the number of positive wells containing living, coherent colonies of at least 32 cells was counted through an inverted phase contrast microscope (8). The number of cells plated per well could thus be adjusted by multiplying the concentration of the stock solution and also by adjusting the cell concentration at an individual radiation dose. The aim of this was to get approximately an equal amount of positive wells in controls and irradiated plates. Later, the assay was modified so that single cell suspensions of the same concentrations as above were made in 50 ml of culture medium and plated immediatelly into the 96-well culture plates. The plates were incubated at 37°C in a humid atmosphere containing 5% CO_2. After 24 h, when the cells had attached to the wells and resumed logarithmic growth, the cells were irradiated while in situ in the plates. Incubation after irradiation and counting of the colonies was performed as mentioned above. When we compared radiosensitivity between different cell lines the effect of radiation was expressed as fraction survival and the overall radiosensitivity was given as area under the survival curve (9).

Radiation. Cell survival was determined after irradiation with 4 MV x-rays produced by a linear accelerator (Clinac 4/100, Varian, Palo Alto, USA). The radiation quality was specified according to the NACP-protocol (10). The ratio of ionization at depths of 100 mm and 200 mm (J_{100}/J_{200}) with an SSD 100 cm and a field size of 10×10 cm^2, was 1.84. The ionization ratio could be correlated to the radiation quality specification TRP^{20}_{10}, as recommended by IAEA (11). The corresponding value of TRP^{20}_{10} was 0.63. All irradiations were made with a dose rate of 2.0 Gy/min (SSD 100 cm), which conforms to clinical practice.

Irradiation techniques. A special Plexiglas ($\rho = 1.19$ kg/dm^3) phantom (Fig. 1a) was constructed to be used for the irradiation experiments. The tubes (15 ml, COSTAR 3216) were irradiated with one horizontal beam, SSD 100 cm and a field size of 20×40 cm^2. The center lines of the tubes were located in the dose maximum area. Ten tubes could be irradiated simultaneously, since there was a line of holes along the Plexiglas phantom.

In the experiments with the 96-well plates (microwell plate 96 F with a lid, NUNC 1-67008) one vertical beam was used from below (Fig. 1b) with an SSD 100 cm and a field size of 40×40 cm^2. A special tray of Plexiglas ($\rho = 1.19$ kg/dm^3) was designed. The plates were placed on a water layer on the tray. The dose maximum was located in the cell suspension area. No less than 8 plates could be irradiated simultaneously with this arrangement. The radiation doses for the SCC lines were 0.75 Gy, 1.25 Gy, 2.50 Gy, 5.00 Gy and 7.50 Gy per fraction. Because the adenocarcinoma cell lines were highly radiosensitive, the doses in subsequent experiments were reduced to 0.40 Gy, 0.75 Gy, 1.25 Gy, 1.75 Gy and 2.50 Gy.

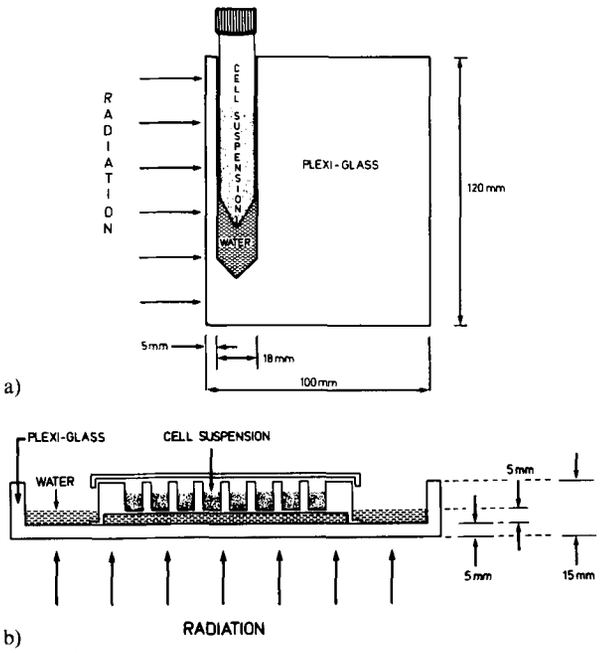

Fig. 1. Geometry of experimental setting. Two stages for exposure of cells to irradiation: a) tube and b) plate.

Dosimetry. The absorbed dose was calculated, first, by using a conventional dose planning method. The dose distribution in the water-filled tube and plate was calculated with a CT-based radiotherapy treatment planning system (Cadplan, Dosetek, Espoo, Finland). A point dose in the center of the target cells was defined using a 0.25×0.25 cm^2 pixel size (ICRU-point) (12). An ionization chamber was used for controlling the calculated absorbed dose in case of the plastic tubes. The irradiation set-up was the same as with the cells. This small cylindrical ionization chamber (0.14 cm^3, RK chamber, Scanditronix, Uppsala, Sweden) was positioned inside the water filled tube in the tube phantom. An integrating dosimeter (Farmer 2570, Nuclear Enterprises LTD, Reading, England) was used. The dose was determined by comparing the measured values in the tubes with the values measured in reference geometry (SSD 100 cm, field size 10×10 cm^2, depth of 5 cm in water), where the absolute absorbed dose was determined (10). The TLD-measurements were used on the 96-well plastic plates to control the calculated absorbed doses. The wells were filled with water and the TLD-pellets were dropped in the wells. Lithium borate ($Li_2B_4O_7$) pellets, diameter 4.6 mm and thickness 0.8 mm (Alnor, Turku, Finland) were used with sensitivity corrections. The pellets were read by a Dosacus TLD reader (Alnor, Turku, Finland) (13). The absorbed dose was determined by comparing the TLD-values measured in the wells of the plate and in reference geometry (SSD 100 cm, field size 10×10 cm^2, depth 5 cm in water).

Plating efficiency. The plating efficiency (PE) was calculated according to the formula PE = $-\ln$ (number of negative wells/total number of wells)/number of cells plated per well (14).

Statistical methods. The effects of the culture model on survival values were analyzed by a three-way mixed model analysis of variance (ANOVA). The factors used in the ANOVA were the irradiation model, irradiation dose and batch. The batch was considered as a random factor, the others were fixed. The analyses were carried out on the BMDP2SV program (BMDP Statistical Software, Inc., Los Angeles, USA).

Results

Radiation dose. The Table shows the calculated point doses and the measured doses in the tubes and in the wells of the plates. Linearity was studied with three dose values within the area of cell irradiation. The values of the S.D. in the tubes were calculated from measurements in each tube along the whole Plexiglas phantom. The values of the S.D. were determined from measurements in eight wells of the plate on different parts of the phantom tray. The values of the S.D. include also the precision of the accelerator-produced absorbed dose. The differences between the calculated and measured data were insignificant and therefore correction factors were not required. The calculated absorbed dose distributions on the target cell area in the tube and plate were 102–98% and 102–99% respectively. The calculated mean absorbed doses in both models were 101%. The results correlated strongly with the measured data.

Table

Simulation of cell irradiation. Calculated and measured absorbed doses with 4 MV x-ray-beam in water

Model	Calculated (Gy) absorbed point dose	Measured ± SD (Gy)	
		ionization chamber	TLD
Tube	0.75	0.76 ± 0.02	
	2.50	2.51 ± 0.07	
	5.00	5.00 ± 0.14	
Well	0.75		0.74 ± 0.03
	2.50		2.48 ± 0.08
	5.00		4.95 ± 0.17

Survival data. The pooled results of survival of the UM-SCC-1 cells as a function of radiation dose are shown in Fig. 2a. The values are collected from 6 parallel experiments. By ANOVA, the p-values of the main effects of the irradiation model and the interaction effects between absorbed dose and model were 0.88 and 0.92 respectively. These p-values show that the survival curves do not differ statistically significantly from each other, i.e. the data are from the same biological population. The survival of the UM-SCC-14C cells is shown in Fig. 2b. The values are collected from 4 parallel experiments. The p-values of the main effects of the irradiation model and the interaction effects between absorbed dose and model were 0.13 and 0.18 correspondingly. Thus, the survival curves do not differ statistically significantly. The pooled results of the survival data of the UT-EC-2B cells irradiated by both models are presented in Fig. 2c. The values stem from 4 parallel experiments. The data from the two models were similar: the interaction effects between absorbed dose and model had a p-value of 0.42 and the main effects of $p = 0.06$.

Discussion

The accuracy of the determination of absorbed dose in a water phantom that can be achieved with ionization chambers and the TLD is better than 2.0% (13, 15–16).

The present results show that a similar degree of accuracy (3%) can be achieved with irradiation of cells in tubes and on plates, provided that the density of the stage on which the culture lies is taken into consideration. The overall accuracy can be estimated at better than 5%; the accuracy is influenced, in addition to the physical characteristics of the stage, by the repeatability of the physical

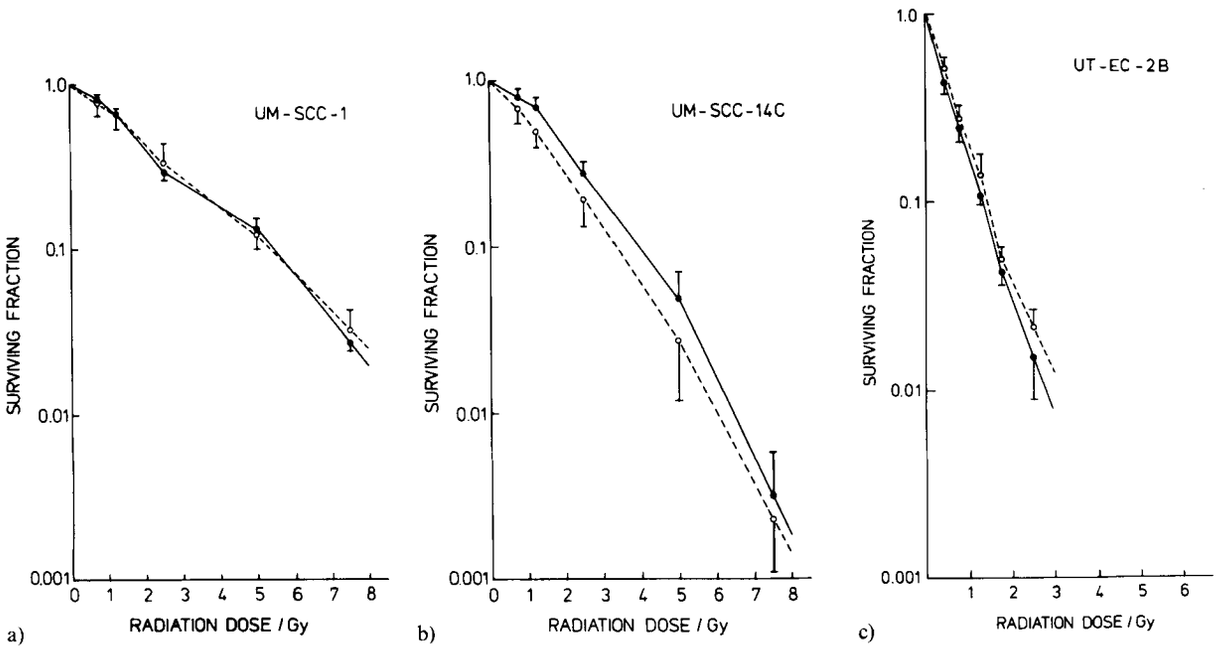

Fig. 2. Cell survival as a function of radiation dose. a) UM-SCC-1, b) UM-SCC-14C and c) UT-EC-2B cells in tube (●) and plate (○) irradiation. Data points ± error bars represent the mean value ± 1 S.D. from different experiments.

set-ups in the irradiation field and by the repeatability of the administered absorbed dose.

Besides accurate dosimetry, also the cell preparation is important if one wants to obtain reproducible results in tests for radiation sensitivity in vitro. Cancer cells must be kept in logarithmic growth by weekly passages to prevent overconfluency. Cultures in mid-logarithmic growth dissociate easily into single cells and have uniform growth characteristics after passage. On the day before plating the cells were fed with fresh medium, and cells from one single culture flask were used. The cells were cautiously trypsinized to get a single cell suspension. Further suspending with a needle was done to avoid clusters. The number of cells was counted manually in hemocytometer chambers, and the mean of several counted chambers was used. All irradiated tubes and plates as well as controls contained cells from the same stock suspension of single cells. The cells were irradiated at room temperature. Both control tubes and plates and those aimed for irradiation were taken from the laboratory to the radio-therapy unit, and all cells were kept at room temperature an equal time. All the tubes were inverted several times before plating to prevent hypoxia due to sedimentation. Each experiment was made in duplicate, and at least two or three experiments were made from the same cell line. The number of cells plated per well was adjusted to get enough and approximately equal amount of positive wells both in controls and irradiated plates. This minimizes the effects of reading errors.

The plating efficiency (PE) values of ten SCC lines tested with the 96-well plate assay were 0.01–0.30 corresponding to 1–30% (17). The PE values of 6 endometrial adenocarcinoma cell lines tested with the same method were 0.005–0.45 (5). We have earlier tested SCC lines in the semi-solid agar and the 96-well assay, and the PE values obtained in the 96-well assay were clearly higher (8). The present values are thus highly comparable to those achieved in other assays for testing of the radiosensitivity of cell lines derived from different tumor types (18–19). The 96-well plate clonogenic assay is thus suitable for in vitro radiosensitivity testing of both SCC lines and adenocarcinoma lines. This method has given reproducible radiation sensitivity testing results with tens of cell lines (3–8, 20).

A method that uses plastic dishes after irradiation rather than the semi-solid agar assay was chosen due to of better PE values. The problems with cell migration on big dishes, e.g., Petri dishes, could be overcome by 96-well plates. In these small microtiter dishes the colonies grow coherently. The 96-well plates are easy to read by microscope and the result is based on the number of positive wells. Wells are considered to be positive if they have a coherent colony consisting of 32 cells or more. A negative well may consist of a few single cells or of an abortive or incoherent colony. A positive well may have several colonies, but the accuracy of the assay is not affected by the number of colonies per well or the exact size of colonies.

These irradiation models provide excellent control of the absorbed dose of irradiation. Likewise, the management of

the cells before and after irradiation is also reliable. When the absorbed doses are equal in different kinds of cell lines, the survival data is comparable regardless of the model used.

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REFERENCES

1. Weeks KJ, Buchsbaum DJ, Terry VH, Ten Haken RK. Effect of backscatter on cell survival for a clinical electron beam. Radiother Oncol 1991; 21: 269–72.
2. Zackrisson B. Biological effects of high energy radiation and ultra high dose rates (Dissertation). Umeå, Sweden: University of Umeå, 1991.
3. Pekkola-Heino K, Kulmala J, Grénman S, Carey TE, Grénman R. Radiation response of vulvar squamous cell carcinoma (UM-SCV-1A, UM-SCV-1B, UM-SCV-2, and A-431) cells in vitro. Cancer Res 1989; 49: 4876–8.
4. Pekkola-Heino K, Kulmala J, Klemi P et al. Effects of radiation fractionation on four squamous cell carcinoma lines with dissimilar inherent radiation sensitivity. J Cancer Res Clin Oncol 1991; 117: 597–602.
5. Rantanen V, Grénman S, Kulmala J, Salmi T, Grénman R. The radiation sensitivity of endometrial carcinoma in vitro. Gynecol Oncol 1992; 44: 217–22.
6. Grenman R, Burk D, Virolainen E, Wagner JG, Lichter AS, Carey TE. Radiosensitivity of head and neck cancer cells in vitro. Arch Otolaryngol Head Neck Surg 1988; 114: 427–31.
7. Krause CJ, Carey TE, Russell WO, Herbis C, McClatchey KD, Regezi JA. Human squamous cell carcinoma. Establishment and characterization of new permanent lines. Arch Otolaryngol 1981; 107: 703–10.
8. Grenman R, Burk D, Virolainen E, et al. Clonogenic cell assay for anchorage-dependent squamous cascinoma cell lines using limiting dilution. Int J Cancer 1989; 44: 131–6.
9. Fertil B, Dertinger H, Courdi A, Malaise EP. Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. Radiat Res 1984; 99: 73–84.
10. The Nordic Association of Clinical Physics (NACP). Procedures in external radiation therapy dosimetry with electron and photon beams with maximum energies between 1 and 50 MeV. Acta Radiol Oncol 1980; 19: 55–79.
11. International Atomic Energy Agency (IAEA). Absorbed dose determination in photon and electron beams. An international code of practice, Technical reports series No. 277, 1987, IAEA, Vienna.
12. ICRU report 29. Dose specification for reporting external beam therapy with photons and electrons, Issued April 1, 1978, ICRU, Washington.
13. Haaslahti J. The design philosophy for automatic TLD system to meet current international specifications. Radiat Prot Dosim 1986; 17: 275–8.
14. Thilly WG, DeLuca JG, Furth EE, et al. Genelocus mutation assay in diploid human lymphoblast lines. In: de Serpes FJ, Hollaender A, eds. Chemical Mutagens. New York: Plenum Publishing Corp, 1980: Vol. 6, 331–64.
15. Johns H, Gunningham J. Measurement of radiation. In: Johns H, Gunningham J, eds. The physics of radiology, 4th ed. Springfield: Charles et Thomas, 1983: 290–335.
16. Khan F. Measurement and calculation of absorbed dose. In: Stamathis G, ed. The physics of radiation therapy. Baltimore: Williams & Wilkins, 1984: 122–56.
17. Pekkola-Heino K, Kulmala J, Grénman R. Sublethal damage repair in squamous cell carcinoma cell lines. Head Neck 1992; 14: 196–9.
18. Rotmensch J, Schwartz JL, Atcher RW, Grdina DJ, Toohill M, Weichselbaum RR. The inherent cellular radiosensitivity of epithelial ovarian carcinoma. Gynecol Oncol 1989; 35: 282–5.
19. Weichselbaum RR, Schmit A, Little JB. Cellular repair factors influencing radiocurability of human malignant tumors. Br J Cancer 1982; 45: 10–6.
20. Grenman R, Carey TE, McClatchey KD, et al. In vitro radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck. Cancer 1991; 67: 2741–7.