Radiation Sensitivity in Vitro of Cells Isolated from Human Tumor Surgical Specimens

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ABSTRACT

The radiation sensitivity of cells isolated directly from human tumor surgical specimens was studied using the Courtenay soft agar colony assay. Aerobic cell survival curves covering 2–3 decades were achieved for eight melanomas, seven ovarian, six cervix, five breast, four bladder, and four squamous cell carcinomas of the head and neck and two seminomas. Cell survival following exposure to 2.0 Gy was measured also for several other tumors of these histological types. Experiments repeated with cells stored in liquid nitrogen showed that the survival assay gave highly reproducible results. The D0 (0.61–1.65 Gy) as well as the surviving fraction at 2.0 Gy (0.12–0.66) differed considerably among individual tumors of the same histological type. Neither of these parameters was therefore significantly different for the seven tumor categories. However, about one-third of the melanomas showed a higher surviving fraction at 2.0 Gy than the highest value measured for the other tumors. Two of three seminomas showed surviving fractions at 2.0 Gy in the absolute lower range, i.e., below 0.20. Altogether the data were consistent with the suggestion recently put forward that the clinical radiocurability of tumors may be correlated to the cell surviving fraction in vitro at 2.0 Gy. However, it was not possible on the basis of individual tumors to investigate whether the surviving fraction at 2.0 Gy was correlated to the clinical radiocurability, since adequate clinical data were not available for the parent tumors. It is suggested that melanomas may be especially suitable for prospective studies aimed at establishing whether such a correlation really does exist. If a significant correlation can be verified, then a very important conclusion may be drawn from our data: the radiocurability of human tumors may differ almost just as much among individual tumors of the same histological type as among individual tumors of different histology.

INTRODUCTION

Clinical investigations show clear evidence that the radiation dose required to eradicate human tumors differs significantly among individual patients (1, 2). Human tumors have therefore been divided into groups and ranked in clinical radiocurability by using histological type as parameter (3, 4). However, although clinical radiocurability may be correlated to histological type, histology is possibly not a major determinant of radiocurability. There is significant evidence that different tumors of the same histological type may require totally different doses to be locally controlled (5, 6).

Radiation survival curves in vitro have been reported for cell lines established from a large number of human tumors including most histological types (4, 7). Several authors have claimed that the variability in clinical radiocurability among human tumors cannot be explained from such in vitro survival curves. However, some recent observations have led to a renewed interest in cellular radiation sensitivity as a parameter for clinical radiocurability. Fertil and Malaise (7, 8) analyzed published survival curves for human tumor cell lines and found evidence that the cell survival at 2.0 Gy as well as the mean inactivation dose (D) for a given cell type were correlated to the 95% control dose for tumors of corresponding histology. Deacon et al. (4) reanalyzed the same survival curves using an altogether different approach, and in agreement with the conclusion drawn by Fertil and Malaise (7, 8), they found a clear positive correlation between the initial slope of the cell survival curves and the clinical radiosensitivity. These analyses were mainly based on survival curves for cell lines established in monolayer culture, and it is well known that the radiation sensitivity of some lines may change significantly during serial subculture in vitro (9). Survival curves for cells isolated directly from human tumors may therefore shed new light on possible correlations between cellular radiation sensitivity and clinical radiocurability of tumors.

Much effort has been concentrated on development of colony assays for cells isolated from human tumors (10). Comparative studies have indicated that the Courtenay assay (11) is superior to other assays reported so far (12, 13). The Courtenay assay has been used widely to study the sensitivity of human tumor xenografts to treatment with radiation (14) and chemotherapeutic agents (15). Moreover, extensive chemosensitivity testing of cells from human tumor biopsy specimens has been carried out using the Courtenay assay (16, 17).

In our laboratory, the Courtenay assay has been used to establish radiation survival curves for cells isolated from human melanoma surgical specimens (18). Eleven of the 14 melanomas studied showed a sufficiently high plating efficiency that cell survival over at least 2 decades could be measured (18). Radiation survival curves for cells isolated from surgical specimens of human ovarian, cervix, breast, bladder, and squamous cell carcinomas of the head and neck, seminomas, and 8 additional melanomas are reported in the present communication. The main purpose of the work was to search for possible relationships between in vitro radiation survival curve parameters and clinical radiocurability in an attempt to identify major causes of radiation resistance in human tumors.

MATERIALS AND METHODS

Tumor Tissue. Tumor tissue was obtained from patients admitted to The Norwegian Radium Hospital, Oslo, and The National Hospital, Oslo, Norway. Immediately after surgery, the tumor tissue was put into culture medium (4°C) and brought to the laboratory. Normal tissue and necrotic areas were removed with scalpels. Tumor fragments were suspended in 20 ml culture medium in a plastic bag and treated for 30 s with a stomacher (Lab-Blender 80; Seward Laboratory, London, England). After the mechanical disaggregation, some of the tumor specimens were disaggregated further by treatment with an enzyme suspension. The suspensions were then filtered through 45-7-μm nylon mesh before centrifugation and resuspension in culture medium. The quality of the suspensions was examined using a phase contrast microscope. A hemocytometer was used to determine the fraction of single cells, doublets, and cell aggregates. Only morphologically intact, viable cells, i.e., cells having an intact and smooth outline with a bright halo, were counted. Radiation sensitivity experiments were performed only if the fraction of doublets was <5% and the
fraction of larger aggregates was <0.1%. Other cell suspensions were discarded. The probability of obtaining an acceptable cell suspension increased with increasing size of the surgical specimen. It was generally difficult to isolate cell suspensions of sufficient quality from specimens ≤1–2 cm³. When the cell yield was high, some of the cells were frozen in liquid nitrogen and stored. The remaining cell suspension was diluted in culture medium and used in survival experiments within a few hours.

Irradiation. A Siemens Stabilipan X-ray unit, operated at 220 kV, 19–20 mA, and with 0.5 mm Cu filtration, was used for irradiation. The cell suspensions, which were irradiated under aerobic conditions at a dose rate of 3.0 Gy/min, were kept in glass Carrel flasks during exposure. The irradiation was performed at room temperature.

Colony Assay. Cell survival was measured using the Courtenay soft agar colony assay (11). The soft agar was prepared from powdered agar (Bacto agar; Difco, Detroit, MI) and culture medium [Ham’s F12 medium with 20% fetal calf serum, penicillin (250 mg/liter), and streptomycin (50 mg/liter; Gibco-Biocult, Glasgow, Scotland)]. Erythrocytes from August rats and tumor cells were added as previously described (19). Aliquots of 1 ml of soft agar with the appropriate number of tumor cells were seeded in plastic tubes (Falcon 2057 tubes; Falcon Labware, Becton Dickinson and Co., Oxnard, CA). The cells were incubated at 37°C for 4–5 weeks in an atmosphere of 5% O₂, 5% CO₂, and 90% N₂. Culture medium (2 ml) was added to the top of the agar 5 days after seeding and then changed weekly. Normally, cells giving rise to colonies containing more than 50 cells were scored as clonogenic. Colonies were counted using a stereomicroscope. Plating efficiency was calculated from the number of colonies counted, and the number of morphologically intact, viable single cells seeded, which was determined as described above.

Care was taken to avoid potential pitfalls inherent in the Courtenay assay when used to study survival of cells isolated directly from human tumor surgical specimens. To begin with, preliminary experiments had shown that for some tumors the plating efficiency depended on the number of cells seeded. An equal number of cells per tube was therefore seeded for all dose groups, usually in the range 50,000–100,000, depending on the cell yield. In addition, one or two lower levels, usually in the range of 2,000–15,000 cells/tube, were seeded for unirradiated cells and cells given low radiation doses. Generally, the plating efficiency and all survival levels were calculated from the number of colonies in the tubes with the highest cell number. However, in a few cases when the plating efficiency was high, the plating efficiency and the survival levels for the lower radiation doses were calculated from the tubes with one of the lower cell numbers, whereas the survival levels for the higher doses were calculated from the tubes with the highest cell number. A linear relationship between number of colonies and number of cells seeded was always verified when this procedure was used. Moreover, irradiated cells from some tumors could give rise to some compact, relatively large abortive colonies that might be difficult to distinguish from vital, real colonies. In these few cases, counting criteria based on careful examination of the size, color, and morphology of the colonies from unirradiated cells were established. It was not possible to establish reliable counting criteria for 2 such tumors, and they were therefore discarded from the study. Furthermore, preliminary investigations had revealed that cell clumps present in irradiated suspensions could erroneously lead to survival curves with a shallow tail. Tumors giving cell suspensions with a significant fraction of doublets and aggregates were therefore not subjected to radiation sensitivity studies (see above). Moreover, artificial colonies due to cell clumps used. Moreover, irradiated cells from some tumors could give rise to some compact, relatively large abortive colonies that might be difficult to distinguish from vital, real colonies. In these few cases, counting criteria based on careful examination of the size, color, and morphology of the colonies from unirradiated cells were established. It was not possible to establish reliable counting criteria for 2 such tumors, and they were therefore discarded from the study. Furthermore, preliminary investigations had revealed that cell clumps present in irradiated suspensions could erroneously lead to survival curves with a shallow tail. Tumors giving cell suspensions with a significant fraction of doublets and aggregates were therefore not subjected to radiation sensitivity studies (see above). Moreover, artificial colonies due to cell clumps were scored for by examining soft agar cultures of cells given a lethal radiation dose (20 Gy), but were never seen. It was not found necessary to correct measured survival levels mathematically for multiplicity. These pitfalls in the Courtenay assay have been discussed in detail previously (18).

RESULTS

Radiation survival curves for 8 melanomas, 7 ovarian carcinomas, 6 cervix carcinomas, 5 breast carcinomas, 4 bladder carcinomas, 4 squamous cell carcinomas of the head and neck, and 2 seminomas are presented in Figs. 1–7. The plating efficiency was calculated from the number of colonies counted, and the number of cells seeded. An equal number of cells per tube was therefore seeded for all dose groups, usually in the range 50,000–100,000, depending on the cell yield. In addition, one or two lower levels, usually in the range of 2,000–15,000 cells/tube, were seeded for unirradiated cells and cells given low radiation doses. Generally, the plating efficiency and all survival levels were calculated from the number of colonies in the tubes with the highest cell number. However, in a few cases when the plating efficiency was high, the plating efficiency and the survival levels for the lower radiation doses were calculated from the tubes with one of the lower cell numbers, whereas the survival levels for the higher doses were calculated from the tubes with the highest cell number. A linear relationship between number of colonies and number of cells seeded was always verified when this procedure was used. Moreover, irradiated cells from some tumors could give rise to some compact, relatively large abortive colonies that might be difficult to distinguish from vital, real colonies. In these few cases, counting criteria based on careful examination of the size, color, and morphology of the colonies from unirradiated cells were established. It was not possible to establish reliable counting criteria for 2 such tumors, and they were therefore discarded from the study. Furthermore, preliminary investigations had revealed that cell clumps present in irradiated suspensions could erroneously lead to survival curves with a shallow tail. Tumors giving cell suspensions with a significant fraction of doublets and aggregates were therefore not subjected to radiation sensitivity studies (see above). Moreover, artificial colonies due to cell clumps were scored for by examining soft agar cultures of cells given a lethal radiation dose (20 Gy), but were never seen. It was not found necessary to correct measured survival levels mathematically for multiplicity. These pitfalls in the Courtenay assay have been discussed in detail previously (18).

Fig. 1. X-ray survival curves for cells from melanomas. Circles and triangles in the same panel refer to different tumors. ◦, △, cells from newly prepared suspensions; ○, cells stored in liquid nitrogen. Each survival level was calculated from the mean number of colonies in 4 tubes with irradiated and 4 tubes with unirradiated cells.

Fig. 2. X-ray survival curves for cells from ovarian carcinomas. Circles and triangles in the same panel refer to different tumors. ◦, △, cells from newly prepared suspensions; ○, cells stored in liquid nitrogen. Each survival level was calculated from the mean number of colonies in 4 tubes with irradiated and 4 tubes with unirradiated cells.

Fig. 3. X-ray survival curves for cells from cervix carcinomas. Circles and triangles in the same panel refer to different tumors. ◦, △, cells from newly prepared suspensions; ○, cells stored in liquid nitrogen. Each survival level was calculated from the mean number of colonies in 4 tubes with irradiated and 4 tubes with unirradiated cells.

Fig. 4. X-ray survival curves for cells from breast carcinomas. Circles and triangles in the same panel refer to different tumors. ◦, △, cells from newly prepared suspensions; ○, cells stored in liquid nitrogen. Each survival level was calculated from the mean number of colonies in 4 tubes with irradiated and 4 tubes with unirradiated cells.
efficiency for these tumors was high enough that cell survival could be measured over about 2–3 decades (Table 1). Some of the tumors gave a sufficiently high cell yield that the experiments could be repeated with cell samples stored in liquid nitrogen. Experiments with stored cells gave similar results as experiments with newly prepared cell suspensions. Survival curves were fitted to the data using the multitarget-single-hit model. The parameters of the curves are presented in Table 1. The parameters of the curves are presented in Table 1. The survival curves differed considerably among individual tumors of the same histological type, and thus there were no statistically significant differences between the survival curve parameters for the 7 tumor types. Mean $D_0$ for example, was in the ranges 0.74–1.48 Gy (melanomas), 0.61–1.60 Gy (ovarian carcinomas), 0.64–1.65 Gy (cervix carcinomas), 0.66–1.36 Gy (breast carcinomas), 0.80–1.35 Gy (bladder carcinomas), 0.73–1.44 Gy (head and neck carcinomas), and 0.75–1.06 Gy (seminomas). The majority of the survival curves possessed a small but significant shoulder. Except for the B. K. and X. A. melanomas, which had broad-shouldered survival curves, all tumors showed survival curves with mean extrapolation numbers in the range 1.0–5.0. None of the survival curve parameters $D_0$, $D_s$, and $n$ was positively correlated to the plating efficiency. The tumors in Table 1 constituted 73% (melanomas), 27% (ovarian carcinomas), 38% (cervix carcinomas), 28% (breast carcinomas), 36% (bladder carcinomas), 33% (head and neck carcinomas), and 40% (seminomas) of the total number of irradiated tumors. Complete survival curves could not be established for the remaining tumors because the plating efficiency was too low.

Fig. 8 shows the surviving fraction at 2.0 Gy for the tumors in Table 1 grouped according to histological type. The surviving fraction at 2.0 Gy could also be measured for some of the other tumors studied, although it was not possible to establish complete survival curves. These values as well as the surviving fraction at 2.0 Gy for 11 melanomas reported previously (18) are included in Fig. 8. The data for the surviving fraction at 2.0 Gy in Fig. 8 represent measured values and not values calculated from the multitarget-single-hit model. Fig. 8 shows that the surviving fraction at 2.0 Gy differed considerably among individual tumors of the same histological category, as did the
survival curve parameters $D_0$, $D_n$, and $n$. The surviving fractions at 2.0 Gy were not significantly different for any of the 7 tumor categories.

**DISCUSSION**

The radiation sensitivity of cells derived directly from surgical specimens of human tumors was studied using the Courtenay soft agar colony assay. The plating efficiency varied considerably among different tumors, but was in some cases sufficiently high that survival curves over 2–3 decades could be established. In other cases, colonies were not formed at all or the plating efficiency was so low that cell survival could be measured after exposure to 2.0 Gy only. Previous work has revealed some pitfalls in the Courtenay assay when used to study survival of cells isolated directly from human tumor surgical specimens (18). When the necessary precautions are taken to avoid the pitfalls, the assay gives reliable survival curves. Thus, the shape of the survival curves established in the present work was similar to that of survival curves reported for tumor cell lines established in monolayer culture. The reproducibility of the assay was adequate as indicated by the coinciding results in independent experiments performed with cells from the same tumor. Moreover, cells from different tumors showed individual and characteristic survival curves varying significantly in $D_0$, $D_n$, and $n$. These observations suggest that differences in radiation sensitivity among cell populations isolated directly from human tumor surgical specimens can be identified by using the Courtenay soft agar colony assay.

One important objective of the present work was to search for possible relationships between cell survival curve parameters in vitro and clinical radiocurability. The radiation doses required to achieve local control of human tumors cover a wide range and depend on the tumor volume as well as on the radiation fractionation pattern. Assessment of the relative radiocurability of various histological types of tumors must therefore be based on data for tumors of a specified size treated by a particular fractionation regimen. A detailed ranking of different histological types of tumors in clinical radiocurability is unfortunately not feasible on the basis of available clinical data (4, 7, 20). However, there seems to be general agreement that ovarian, cervix, breast, bladder, and squamous cell carcinomas of the head and neck are all of intermediate clinical radiocurability (3, 4, 7). Melanoma has often been classified as a typically radiation resistant tumor type (3, 4, 7), but the radiation resistance of melanomas has been questioned by several authors (21, 22). Recent reviews conclude that melanomas constitute a heterogeneous tumor type with highly variable radiocurability (23–25). Seminoma on the other hand is one of the most radiation sensitive tumor types (20) with a very high fraction of local control after treatment with radiation alone (26, 27).

The $D_0$ of the radiation survival curves in Figs. 1–7 differed considerably among individual tumors but was not significantly different for any of the 7 histological groups, suggesting that differences in clinical radiocurability, for example between melanomas and seminomas, probably cannot be attributed to differences in the $D_0$. Since radiation therapy usually is given in many small fractions, the shoulder region of cell survival curves is important for clinical radiocurability. The surviving fraction at 2.0 Gy differed considerably among individual tumors of similar histology but was not significantly different for any of the 7 tumor categories (Fig. 8). The ovarian, cervix, breast, bladder, and squamous cell carcinomas of the head and neck showed all surviving fractions at 2.0 Gy that were equally distributed within the range 0.14–0.50, in agreement with the general opinion that these tumor categories are of intermediate clinical radiocurability. The majority of the melanomas also showed surviving fractions at 2.0 Gy within the same range, but 6 of the 19 melanomas showed higher values, i.e., values within the range 0.50–0.70. These survival levels are consistent with the view that melanoma is a heterogeneous tumor category with very variable clinical radiocurability and may also explain why some melanomas are extremely resistant. The lowest surviving fraction at 2.0 Gy (0.12) was observed for one of the seminomas. Two of the 3 seminomas showed surviving fractions at 2.0 Gy below 0.20, a higher proportion than for any of the other tumor categories, in accordance with the high clinical radiocurability of seminomas. The third seminoma on the other hand showed a relatively high surviving fraction at 2.0 Gy (0.40) and a large $D_0$. The data in Fig. 8 are altogether consistent with the suggestion of Fértil and Malaise (7, 8) and Deacon et al. (4) that the clinical radiocurability of human tumors may be correlated to the cell survival in vitro at 2.0 Gy. If such a correlation can be proved to be true, then another very important conclusion may be drawn from Fig. 8: the radiocurability of human tumors may differ almost just as much among individual tumors of the same histological type as among individual tumors of different histology, i.e., histological category is possibly a poor parameter for assessment of the clinical radiocurability of human tumors.

The present work has revealed that melanomas may be especially suitable for prospective studies aimed at establishing whether a positive correlation really exists between clinical radiocurability and cell survival in vitro at 2.0 Gy. Clinical investigations (23, 24) as well as the cell survival studies in vitro (Figs. 1 and 8) indicate that melanomas are extremely heterogeneous in response to radiation treatment. The plating effi-
ciency in soft agar is generally higher for melanomas than for other tumor types (Table 1) and hence cell survival measurements in vitro can be performed for a high proportion of the melanomas. Moreover, cutaneous, s.c., and lymph node melanoma metastases are often easily accessible, and it should therefore be possible to measure the clinical radiotherapeutic response with sufficient accuracy. The relationship between the radiosensitivity in vivo of human melanoma xenografts and the initial slope of the cell survival curves in vitro is at present being studied at our institute. The results are encouraging and indeed suggest that corresponding clinical studies also should be undertaken.

ACKNOWLEDGMENTS

The skillful technical assistance of Gunnvor Anne Birkeland Olsen is gratefully acknowledged. The Departments of Surgery at The Norwegian Radium Hospital, Oslo and at The National Hospital, Oslo, Norway are thanked for supplying the human tumor surgical specimens.

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