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SINGLE-DOSE AND FRACTIONATED IRRADIATION OF FOUR HUMAN LUNG CANCER CELL LINES IN VITRO

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Abstract

Four established human lung cancer cell lines were exposed to single-dose irradiation. The survival curves of 2 small cell lung carcinomas (SCLC) were characterized by a limited capacity for repair with small and moderate shoulders with extrapolation numbers (n) of 1.05 and 1.60 respectively. Two non-small cell lung carcinoma (NSCLC) cell lines, one squamous cell (SQCLC) and one large cell (LCLC) had large shoulders with n-values of 73 and 15 respectively. The radiosensitivity when measured as D_0 did not, however, differ as much from cell line to cell line, with values from 1.22 to 1.65. The surviving fraction after 2 Gy (SF2) was 0.24 and 0.42 respectively in the SCLC cell lines and 0.90 and 0.88 respectively in the NSCLC cell lines. Fractionated irradiation delivered according to 3 different schedules was also investigated. All the schedules delivered a total dose of 10 Gy in 5 days and were applied in 1, 2 and 5 Gy dose fractions respectively. Survival followed the pattern found after single-dose irradiation; it was lowest in the SCLC cell line with the lowest SF and highest in the two NSCLC cell lines. In the SCLC cell lines all schedules were approximately equally efficient. In the LCLC and in the SQCLC cell lines, the 5 Gy schedule killed more cells than the 1 and 2 Gy schedules. The results indicate that the size of the shoulder of the survival curve is essential when choosing the most tumoricidal fractionation schedule.

Key words: Lung cancer, cell lines, in vitro, irradiation, single dose, fractionated dose, survival curves.

The optimal way of delivering radiotherapy to lung cancer with respect to dose per fraction, time between fractions or total dose has been settled neither in small cell (SCLC) nor in non-small cell lung carcinoma (NSCLC). This clinical reality formed the background to the present radiation study of human lung cancer, which was performed in vitro since radiobiological studies of tumour cell survival are difficult or impossible to perform in patients. The problem with the in vitro approach is that there is no simple way in which the results can be extrapolated to the clinical situation and applied to radiotherapy. However, a correlation between the SF2 of human tumour cell lines in vitro and the clinical radioresponsiveness respectively, has been demonstrated (1, 2). This indicates that conclusions drawn from in vitro studies may be relevant in in vivo conditions and, with caution, can be applied clinically in the search for improved radiotherapy.

The need for improved treatment results in human lung cancer is apparent both in SCLC and NSCLC. SCLC is clinically considered a radioresponsive tumour (3), clearly more so than NSCLC. Very few patients with SCLC have, however, been cured by radiotherapy alone (4) and the benefit of adding radiotherapy to chemotherapy, which is considered the main treatment modality in SCLC, has been questioned. However, the frequency of local cure with chemotherapy alone in cases with limited disease was 12–15% but approximately 40%, when radiotherapy, 40 Gy, was added (5, 6). A dose of 55–60 Gy resulted in a higher frequency of local cure than 40 Gy (7, 8) and some studies indicate a correlation between total dose and local cure (9, 10).

In squamous cell carcinomas, adenocarcinomas and large cell carcinomas of the lung, irradiation of the primary tumour has been applied with a curative intention in patients with limited disease and unresectable tumours but the frequency of local cure has been very low (11–13). There are results indicating that 50–60 Gy is an optimal total dose. Doses above 60 Gy do not seem to prolong survival (12); probably partly due to the fact that the limits of normal tissue tolerance are reached or transgressed.

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Data from in vitro radiation studies of human lung cancer cell lines have previously been presented (14–19). Many SCLC cell lines have survival curves with small shoulders (16–19), suggesting an insufficient capacity to repair sublethal radiation damage. In such tumours multiple small irradiation fractions may be as tumoricidal as the same total dose delivered with larger fractions and clinical radiotherapy may perhaps be optimized by reducing the dose per fraction and thereby reduce the risk of damage to normal late reacting tissues (20).

The aim of the present study was to investigate the effect of radiation on four human lung cancer cell lines, which have previously not been characterized radiobiologically. The effects of single dose as well as fractionated dose schedules were studied and compared.

Material and Methods

Cell lines

Four human lung cancer cell lines which have been established at our laboratory were investigated (21, 22). Two cell lines, U-1285 and U-1906, had an in vitro morphology of SCLC and other phenotypic SCLC characteristics, such as the presence of neurofilaments, dense core granules and production of certain polypeptide hormones. U-1285 grew in flat clusters mainly confined to the suspension whereas U-1906 mainly grew attached to the vessel but also with some floating cell clusters. Both cell lines were established more than three years before the start of these experiments.

The squamous cell lung cancer (SQCLC) cell line, U-1752, had an in vitro morphology of an epithelial tumour with tonofilaments and desmosomes, produced keratin and grew firmly attached to the bottom of the vessels in monolayers (22).

The large cell lung cancer (LCLC) cell line, U-1810, consisted of large epithelial cells with microvilli, cellular junctions and tonofilaments. It produced both neurofilaments and keratin and grew firmly attached in monolayers. Both NSCLC cell lines have been characterized previously (22).

The culture medium used was RPMI-1640 (Flow Laboratories, Stockholm, Sweden) enriched with 10% newborn calf serum (Flow) and supplemented with penicillin 100 IU/ml and streptomycin 50 μg/ml. The cells were grown in humidified air containing 5% CO_2 at 37°C.

Irradiation

The cell cultures were irradiated with photons from an 8 MeV linear accelerator at a dose rate of 4 Gy/min in air at room temperature. Controls and irradiated cells were exposed to room conditions for the same period of time and then returned to the incubator. Single doses ranging from 0 to 15 Gy were delivered. The cell cultures were covered with bolus during irradiation in order to achieve an adequate dose build-up.

For the fractionated irradiation, three schedules were applied, all delivering a total dose of 10 Gy in 5 days. It started on a Monday and continued until Friday. Schedule I: 1 Gy twice a day, 6 h apart for 5 consecutive days; schedule II: 2 Gy once a day for 5 consecutive days and schedule III: 5 Gy on Monday and 5 Gy on Friday.

The extrapolation method

This method was applied to all the cell lines. Half a million cells in 5 ml medium were placed in plastic culture dishes (diameter 50 mm, Falcon). After irradiation, the cell cultures were regularly evaluated with an inverted light phase contrast microscope (Leitz-Diavert) over a period of 5–6 weeks. At a cell confluence of 50–100%, the cultures were subcultivated 1 : 2 or 1 : 4 depending on the density of the culture. At subcultivation the NSCLC cultures (U-1752) and U-1810) were first treated with 0.2% EDTA (Titriplex III, Merck) in PBS (pH 7.4) for 0.5–1 min and with trypsin (0.25% w/v in PBS) for 2–4 min and thereafter gently sucked up and down a pipette to loosen the cells from the dishes and dissociate them from each other in order to obtain a single cell suspension. The SCLC cultures did not need chemical treatment. The number of cells was counted using a Buerger chamber. Afterwards the cell concentration was adjusted and an appropriate volume saved for further cultivation. Staining with trypan-blue was always performed for counting, and regularly demonstrated less than 5% dead cells. Within an hour after subcultivation, the cells from the bottom growing cell lines had attached. Proliferation was highest when the cultures were neither too dense nor too sparse, i.e. the subcultivations were performed with the purpose of obtaining an exponential growth throughout the entire experiments. Subcultivation and counting, or in the case of sparse growth, only a change of medium, were performed twice a week.

Growth of irradiated cultures was delayed and the length of that period depended on the dose and the sensitivity of the cell line. After a time lag, the cultures grew exponentially. The extrapolated 'total' number of cells in the culture was obtained by multiplying the cell number of the dilution factor. Thus, growth curves were constructed as if all the cells were saved and cultured continuously. The growth after each dose was plotted on a log-linear diagram with time on the x-axis and number of cells on the y-axis. The number of surviving cells immediately after an irradiation was estimated by extrapolating the exponential part of the curve back to the interception with the y-axis. The surviving fraction after each dose was calculated as the quotient between this extrapolated value of the treated cultures and of the untreated controls.

Radiation survival curves were constructed by log-linear plotting of the ratios as a funcion of the dose. This procedure has been used in previous studies on the radiosensitivity of cultured human cells (23, 24). The radiobiological parameters D_0, D_q and n were graphically read from the survival curves.

The result after each dose schedules and in each cell line were derived from 2–4 investigations with duplicates and each dish was studied twice a week for at least 5 weeks.

The clonogenic assay

This assay was performed on the SCLC cell lines but not on U-1752 or U-1810, since the clonogenic efficiency of these cell lines was too low (< 1%).

Before irradiation, 5×10^3 cells were suspended in a mixture of RPMI-1640 medium (enriched with serum and antibiotics as described above) and agarose (Sigma type VII LTG agarose) to a final concentration of 0.33% agarose and seeded on plastic dishes (35 mm diameter, Falcon). Before this seeding, the dishes were covered with 2 ml of 1% agarose mixed with RPMI-1640 medium 1 : 1, enriched with serum and antibiotics as described above to a final concentration of 0.5% agarose. After irradiation, the cells were incubated for 3 weeks under standard growing conditions. The number of cell clones was estimated regularly. After 2–3 weeks, the cultures were in good condition and the final counting was performed. After 3 weeks the number of large clones did not increase and the viability of certain clones started to decrease. Since the outline of the cell-membranes of the U-1285 and U-1906 tumour cells were very difficult to observe, we used 0.125 mm as the minimum diameter of a large clone. By picking out such a clone and counting the stained nuclei it was estimated that such a clone contained at least 50 cells. The number of clones in relation to the non-irradiated control cells was plotted on a log-linear diagram and D_0, D_q and n were graphically extrapolated from the survival curves.

Results from single-dose irradiation according to the clonogenic assay were derived from 4 investigations in duplicate or triplicate, amounting to a total of 9–11 dishes for each dose. In the fractionated studies, the investigations were performed twice with triplicates. In a few experiments, heavily irradiated 'feeder' cells were added to the bottom-layer of agarose; however, no difference in clonogenic activity was seen when compared with cultures without feeder cells.

Results

Survival values after single-dose irradiation of U-1285 according to the extrapolation method and the clonogenic assay concorded excellently (Fig. 1). This cell line had a very small shoulder with a D_q of 0.2, indicating a low capacity to repair irradiation-induced damage. In addition,

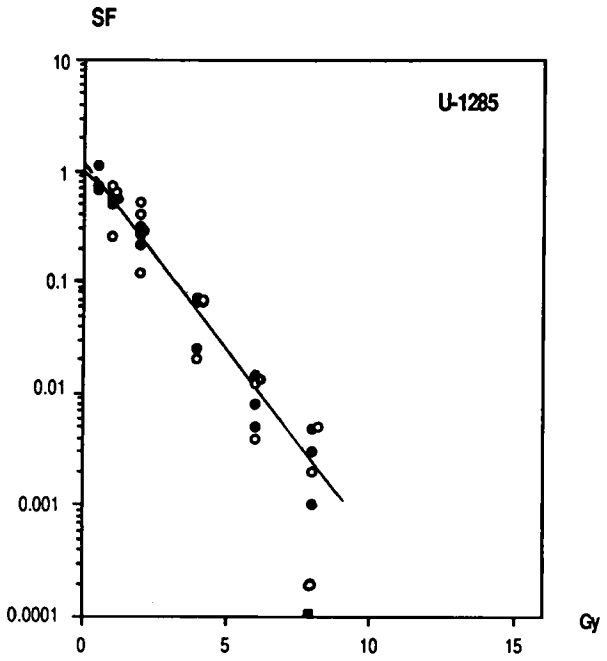

Fig. 1. Single dose irradiation of the SCLC cell line U-1285. Data obtained using the clonogenic assay, each point the medium of one set of experiments with 2 or 3 dishes: ●; with no clones at all: ■. Data obtained using the extrapolation method: ○.

Table 1

Radiobiological data of 2 human SCLC cell lines, U-1285 and U-1906, an SQCLC cell line, U-1752, and an LCLC cell line, U-1810

Cell line	D_0	D_q	n	SF2	Doubl. time	Clonog. eff.
U-1285						
cl.ass.	1.30	0.20	1.20	0.25	—	0.060
e.pol.	1.30	0.25	1.15	0.24	61 h	—
	1.30	0.20	1.15	0.25		
U-1906						
cl.ass.	1.70	0.60	1.45	0.45	—	0.044
e.pol.	1.60	0.50	1.45	0.40	61 h	—
	1.65	0.55	1.45	0.43		
U-1752	1.22	5.5	73	0.90	60 h	<0.01
U-1810	1.65	4.5	15	0.88	52 h	<0.01

Cl.ass. means the clonogenic assay and e.pol. means the extrapolation method.

its radiosensitivity was fairly high, with a D_0 of 1.30. These characteristics led to a SF2 of only 0.24 (Table 1). U-1906 had a larger shoulder with a D_q of 0.6 and was less radiosensitive than U-1285 with a D_0 of 1.65 (Table 1, Fig. 2). This resulted in a SF2 of 0.43. Survival data obtained by the clonogenic assay were in accordance with those from the extrapolation method (Table 1).

The plating efficiency of the SQCLC cell line U-1752 was below 1% and the extrapolation method was therefore

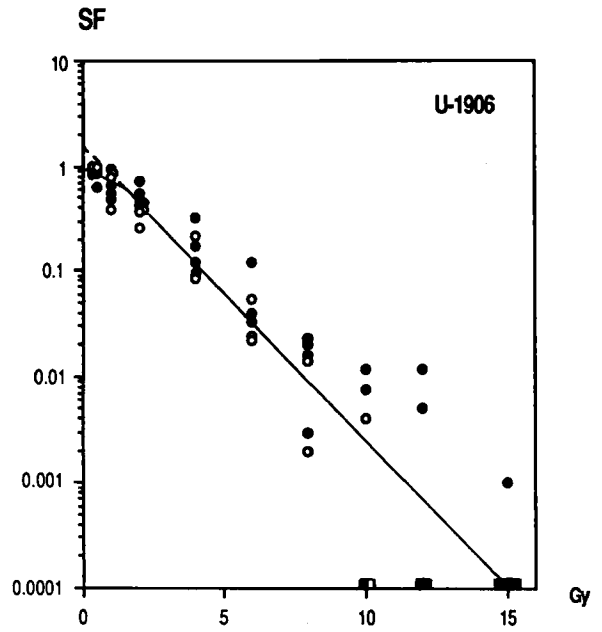

Fig. 2. Single dose irradiation of the SCLC cell line U-1906. Data obtained using clonogenic assay, each point the medium of one set of experiments with 2 or 3 dishes: ●; with no clones at all: ■. Data obtained using the extrapolation method: ○.

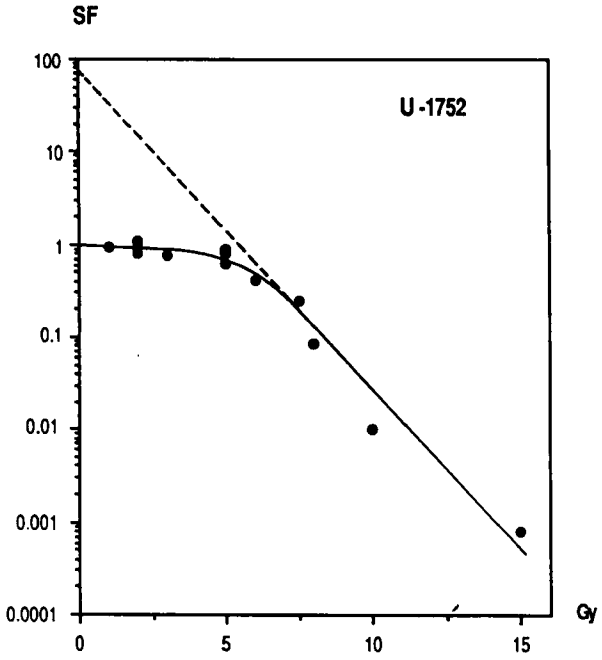

Fig. 3. Single dose irradiation of the SQCLC cell line U-1752. Data obtained using the extrapolation method.

applied. This cell line had a considerable shoulder with a D_q of 5.5 Gy indicating a pronounced capacity to repair. Its radiosensitivity was, however, fairly high with a D_0 of 1.22 (Fig. 3). The large shoulder of U-1752 had a major impact on the SF2 which was very high, 0.90. The radiosensitivity measured as D_0 was 1.65.

The plating efficiency of the LCLC cell line, U-1810, was

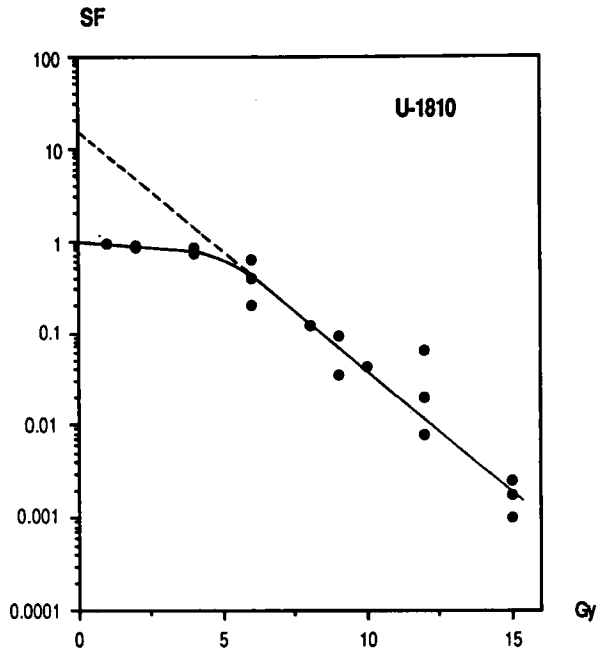

Fig. 4. Single dose irradiation of the LCLC cell line U-1810. Data obtained using the extrapolation method.

even lower than that of U-1752 and therefore also in this case only the extrapolation method was applied. This cell line was also characterized by a very large shoulder with a D_q of 4.5. SF2 was very high also in this cell line (Fig. 4).

The difference between NSCLC cell lines and the SCLC cell lines was striking with respect to repair capacity and SF2, however, their radiosensitivity estimated as D_0 was fairly similar (Table 1). The doubling time in vitro was approximately 60 h for U-1906 and U-1285 and U-1752 (Table 1). The doubling time of U-1810 was a little shorter, viz. 52 h.

Data from the fractionation experiments are presented in Table 2 as the surviving fraction (the quotient between irradiated and non-irradiated control cells). In U-1285 and U-1906, no statistical difference was demonstrated between the three schedules. In U-1810 and U-1752, the 5 Gy fractionation schedule was more efficient when compared with the 1 and 2 Gy schedules ($p > 0.05$). All schedules accomplished considerably more cell death in the SCLC cell lines than in the NSCLC cell lines (Fig. 5).

Table 2

Survival after fractionated irradiation of 4 human lung cancer cell lines

Cell line	Fractionation schedules		
	10 × 1 Gy	5 × 2 Gy	2 × 5 Gy
U-1285	0.005	0.015	0.014
U-1906	0.033	0.022	0.021
U-1752	0.49	0.50	0.21
U-1810	0.49	0.52	0.19

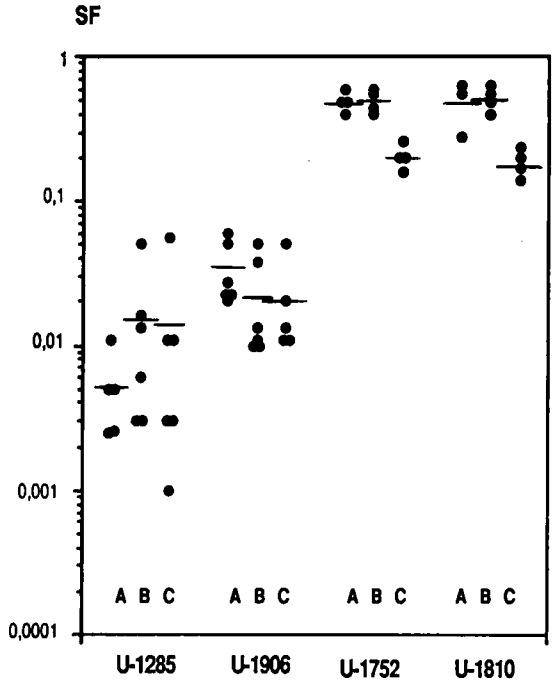

Fig. 5. Fractionated irradiation of the 4 cell lines. In each cell line the surviving fraction for the 3 fractionation schedules was applied: 1 Gy twice a day for 5 consecutive days; 2 Gy once a day for 5 consecutive days; 5 Gy on day 1 and day 5.

Discussion

The clonogenic assay is an established method for defining radiobiological properties of cell lines in vitro (25). It is fairly simple both with respect to the experimental set up and to the processing of raw data; however, the assay is difficult or impossible to apply to tumour cell lines which will not readily clone out on soft agarose (24). We considered the method appropriate for the SCLC cell lines with sufficient plating efficiency. Since this efficiency was very low in the two NSCLC cell lines, the extrapolation method only was applied for these cell lines. However, for comparison both methods were applied in the SCLC cell lines.

Earlier investigations have demonstrated comparable results with these two techniques (23, 24). However, in a summary of radiobiological data from 193 human tumours and tumour cell lines it was concluded that mass culture methods (such as the extrapolation method used in the present investigation) generally produced a higher average value for SF2s than the clonogenic assay for a given histological cell type (26). In our study, survival data from both the methods were in good accordance in the two SCLC cell lines.

An advantage with the extrapolation method is that culture conditions can be better controlled throughout the whole experiment, since the regular subcultivations deliver optimal conditions with respect to nourishment and space and thus permit exponential growth of tumour cell lines for an unlimited period of time. Repeated subcultivations may entail a risk of cell loss and disturbed growing conditions but these risks seem to be small since we have not observed increased numbers of dead cells during the procedure and the cells commenced proliferation within 1–2 h after subcultivation. The risk of disturbance of exponential growth seems to be much greater if the cultures are permitted to grow too densely.

Regarding the clonogenic assay, the clones were largest and most vital during the second and the third week when counting was performed but declined after the fourth week.

The present study demonstrated a greater cell kill in the SCLC compared with the NSCLC cell lines during the initial part of the survival curve, measured as SF2. This is consistent with the clinical experience of these two categories of lung cancer. Previous in vitro studies have demonstrated similar results. The median SF2 of 17 classic SCLC cell lines was 0.26 and of 4 large cell SCLC variant cell lines 0.57 whereas it was 0.56 for 12 NSCLC cell lines (Tables 2 and 3). The range of SFs was, however, considerable, from 0.02–0.53 for the classic SCLC cell lines, 0.47–0.66 for the SCLC variant cell lines and 0.20–0.90 for the NSCLC cell lines. Wide ranges of SF have been found also for other categories of tumours (1, 2, 27). However, even if the ranges overlap, the SF2 of the 17 classic SCLC cell lines was significantly lower than that of the 12 NSCLC cell lines (Table 2) ($p < 0.00001$).

An accordance between the radiocurability in vivo and SF2 in vitro of the corresponding tumours has been

Table 3

Radiobiological characteristics of 17 SCLC cell lines, classic subtype and 4 SCLC large cell variant cell type

Cell line classic	D_0	n	SF2	Ref.
OH-1	0.45	22	0.53	16
OAT-1975	0.85	10	0.42	14
HX-33	1.31	1.4	0.28	15
NCI-H209	1.37	1.43	0.32	17
NCI-69	1.40	1.08	0.25	17
NCI-H146	0.51	3.3	0.07	17
NCI-H249	0.80	1.49	0.12	17
NCI-H187	1.10	1.0	0.17	17
NCI-H345	0.91	2.0	0.23	18
NCI-H449	0.52	1.0	0.016	18
HC-12	1.23	2.75	0.45	19
HC-38	1.17	1.82	0.31	19
HC-39	0.89	2.19	0.22	19
HC-41	1.59	1.46	0.37	19
HX 149	1.19	1.51	0.26	19
U-1285	1.45	1.0	0.25	present
U-1906	1.75	1.1	0.42	present
Large cell variant				
NCI-H82	0.80	11.1	0.61	17
NCI-N417	0.91	5.6	0.539	17
NCI-H446	0.87	6.8	0.47	18
HX 149M	1.78	2.79	0.66	19

described (1, 2, 27). This accordance between in vitro SF2 and clinical radioresponsiveness may be due to the fact that dose fractions of 2 Gy are generally applied in clinical radiotherapy and the supposition may be that there is an accordance between the effect of a single 2 Gy fraction in vitro with multiples of 2 Gy fractions in vivo. This concept formed the background to the studies with fractionated irradiation. These experiments were designed to deliver the same total dose, 10 Gy, during the same total time, 96 h, but with different fraction sizes. The purpose of applying the same total treatment time was to offer all the cultures the same time for proliferation during the irradiation course. A period of mitotic delay has been demonstrated in cells exposed to ionizing irradiation. Its length depends on the dose and has been claimed to be 1.5 h/Gy (28). After the mitotic delay the cells start to proliferate again. Thus, all the fractionation schedules should result in a net mitotic delay of 15 h, and might thus be comparable with respect to time for proliferation during the treatment. However, in single-dose irradiation, the mitotic delay for high doses should be longer than for lower doses. Survival after higher doses might thus be falsely registered a little too low, since the mitotic delay leaves less time for proliferation. This phenomenon is probably of minor importance (28). Maybe it is still less important in the clonogenic assay, in which all clones above a certain size are counted equally, compared with the extrapolation method and correction for mitotic delay is generally not performed with the clonogenic assay (17–19).

No significant difference in tumor cell survival was found between the 1, 2 and 5 Gy schedules respectively in the two SCLC cell lines. It indicates that 1 Gy fractions might kill SCLC tumour cells just as efficiently as 2 Gy and 5 Gy fractions, delivered to the same total dose during the same total treatment time. This may be valid for cell lines with no or only small shoulder. The application of dose fractions smaller than 2 Gy in clinical radiotherapy might perhaps improve the therapeutic ratio and reduce late normal tissue toxicity (20). This could be important since lethal toxicity is not uncommon in cases with SCLC treated with radiotherapy (6). However, this lack of difference between different fractionation schedules should be confirmed in more SCLC cell lines before attempts with altered clinical fractionation schedules might be suggested. Besides, certain SCLC cell lines have a shoulder, especially those of the variant cell type (Table 3) and in these tumours smaller doses per fraction than 2 Gy may be a disadvantage. Our results for the 2 SCLC cell lines are, however, in accordance with results on fractionated irradiation in vitro of a neuroblastoma cell line with similar characteristics (29).

In U-1752 and U-1810, both radioresistant tumours with very large shoulders, 5 Gy per fraction, beyond the shoulder of the survival curve, were more efficient than 2 Gy and 1 Gy fractions. Large shoulders characterized by high n-values and SF2s, have been demonstrated in many other NSCLC cell lines (Table 4).

Table 4

SF2 for 12 NSCLC cell lines

Cell line	Hist.	D_0	n	SF2	Ref.
HX 147	l	1.48	3.77	0.64	19
HX 148M	a-l	1.39	6.44	0.81	19
(HX 148	a	1.82	1.99	0.55	19)
HX 144	a	1.81	1.34	0.41	19
NCI-H157	l	0.90	14	0.80	18
NCI-H23	a	0.97	2.0	0.20	18
Calu6	a	1.30	1.5	0.42	18
NCI H324	a	1.04	1.3	0.30	18
NCI H125	a	1.13	2.0	0.37	18
A549	a	1.90	3.0	0.62	18
(LX1	e	1.14	1.2	0.18	37)
LX1-1		0.96	8.54	0.72	37
(LX1-2		0.96	2.48	0.32	37)
(LX1-3		0.68	20.3	0.53	37)
(LX1-9		1.12	1.0	0.18	37)
U-1752	s	1.22	73	0.90	pres.
U-1810	l	1.65	15	0.88	pres.

Abbreviations: Hist. means histopathological subgroup: a = adenocarcinoma, l = large cell carcinoma, s = squamous cell carcinoma, a-l mixed adenocarcinoma–large cell carcinoma and e = epidermoid carcinoma. From the cell lines HX 148 and LX1 radioresistant subclones have developed. The most radioresistant clones should be the most important ones from a clinical point of view and the other clones are placed in brackets.

In malignant melanomas, which in vitro are characterized by large shoulders (30, 31), an improved clinical effect has been described using large doses per fraction (32, 33). Such schedules may have a higher potential for local cure also in many patients with NSCLC.

The large shoulders of U-1752 and U-1810 with SF2 close to 0.9 indicate that very high numbers of 2 Gy fractions will be needed to cure these tumours, considering the high number of clonogenic cells that must be killed in a clinical tumour. If a 1 cm^3 tumour consists of 10^9 cells (34), of which 0.1% or more are clonogenic (35), probably more than 99.9999% of the tumour cells must be killed in order to achieve cure. Ten Gy delivered as 2 Gy fractions during 5 days killed only 48% of the cells of U-1810, 50% of U-1752, 97.8% of U-1906 and 98.5% of U-1285 according to the fractionation experiment (Table 2). This may explain why certain lung carcinomas, especially NSCLC, are not cured with radiotherapy, assuming similar characteristics in the clinical tumours. If tumours proliferate during fractionated radiotherapy this should make cure even more difficult.

Proliferation in vitro of the four cell lines was characterized by doubling times between 52 and 60 h. Cell loss was low during exponential growth in vitro, viz. below 5%. This indicates that the estimated doubling time in vitro should be comparable with the potential doubling time

(36). This latter parameter investigated in biopsies of 14 human carcinomas of the lung had a range of 2.2 to 8.9 days (36), thus consistent with the in vitro doubling time of the cell lines of the present study. Inherent radiosensitivity might hypothetically serve as a monitor for the total dose needed for cure. Fertile & Malaise (1) found a correlation between SF2 in vitro and radiocurability in vivo. Extrapolations from this correlation line indicate that tumours with SF2 = 0.3 need 50 Gy and tumours with SF2 = 0.4 need 60 Gy for cure. Since 90% of the SCLC cell lines of the classic type had an SF2 below 0.40 (Table 3), this means that 60 Gy ought to be sufficient to cure the majority of these tumours locally. This is consistent with certain clinical data (7, 8).

The model of Fertile & Malaise applied to the median SF2 of the NSCLC cell lines, which was 0.56, indicated that approximately 85 Gy were needed for local cure of 95% of these tumours, a dose much too high to apply in practice. Only 3/12 (25%) of the NSCLC tumour cell lines had an SF2 below 0.40 and would thus have been curable with 60 Gy. The high inherent radioresistancy in most NSCLC tumours seems to be a plausible explanation for the poor treatment results obtained with irradiation.

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