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Inactivation of Human Cells Exposed to Fractionated Doses of Low Energy Protons: Relationship between Cell Sensitivity and Recovery Efficiency

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Within the framework of radiation biophysics research in the hadrontherapy field, split-dose studies have been performed on four human cell lines with different radiation sensitivity (SCC25, HF19, H184B5 F5-1 M10, and SQ20B). Low energy protons of about 8 and 20 keV/μm LET and gamma-rays were used to study the relationship between the recovery ratio and the radiation quality. Each cell line was irradiated with two dose values corresponding to survival levels of about 5% and 1%. The same total dose was also delivered in two equal fractions separated by 1.5, 3, and 4.5 hours. A higher maximum recovery ratio was observed for radiosensitive cell lines as compared to radioresistant cells. The recovery potential after split doses was small for slow protons, compared to low-LET radiation. These data show that radiosensitivity may not be related to a deficient recovery, and suggest a possible involvement of inducible repair mechanisms.

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INTRODUCTION

The use of proton beams for radiation therapy has shown a remarkable increase in the past few years, because protons are very attractive for tumour therapy owing to their favorable ballistic properties[1)].

A few years ago, we undertook a collaborative research program focused on an investigation of the biological effectiveness of low-energy protons in four human cell lines, namely tumour epithelial (SQ20B and SCC25) cell lines, and normal epithelial (H184B5 F5-1 M10) and fibroblast (HF19) cell lines. The results were recently published[2)] about dose-response curves for cell inactivation after exposure to gamma-rays and to proton beams. In this paper we report on the effect of gamma-ray and proton dose fractionation on cell inactivation.

It is known from the literature[3)] that cell survival is higher after split doses than following acute exposure, and that repeated irradiation of equal fractionated doses produces an equal cell-killing effect in each irradiation under the assumption of complete repair of the so-called sublethal damage caused by radiation[4,5)]. Indeed, radiotherapy treatments are performed with fractionated doses while assuming that the radiosensitivity to each fraction does not change[6,7)]. However, this assumption is not always experimentally confirmed. Some data show a changing radiosensitivity at each fraction of gamma-rays during radiotherapy treatments[8,9)]. Furthermore, data concerning the biological effects of accelerated heavy ions (He, C and Ne) demonstrate that cells have less recovery from sublethal damage compared to photons in the case of fractionated dose irradiation, and cells irradiated in the region of the spread-out Bragg peak show less recovery than those in the plateau region[10, 11, 12)].

Cell lines derived from different human tumour types can differ in their cellular radiosensitivity[13)]. It has been found that, in some cases, the radiosensitivity measured *in vitro* as the survival fraction at 2 Gy (SF_2) can predict the response to irradiation *in vivo*. The intrinsic radiosensitivity may be dose dependent, at least for certain cell lines. Differences in radiosensitivity between cell types can be explained by two different mechanistic hypotheses. Greater radiosensitivity could result from the incidence of a larger number of lesions per dose unit compared to the average number of most cell lines[14,15)]. Alternatively, a greater radiosensitivity could be due to a deficiency in the lesion repair mechanisms, in terms of the quality and quantity of repairs[16,17)].

In dose fractionated irradiation, it is possible to calculate the recovery ratio (RR) by dividing the surviving fraction from a dose delivered in two or more fractions by the survival after the same dose delivered in one fraction (acute)[18)],

$$RR = S_{split}/S_{acute}. \quad (1)$$

RR measures the amount of sublethal damage repair between the fractions and the possible induction of radioresistance following the first fraction. Several studies have suggested that when repair is completed the RR value may be a resistance index in radiotherapy[19, 20)].

The linear-quadratic (LQ) model, expressed by the relationship

$$S = \exp(-\alpha D - \beta D^2), \quad (2)$$

predicts a continuously bending survival curve. Provided that repair is completed by the time of the second irradiation, and that radiosensitivity does not change following the first dose, the LQ model predicts an RR increasing indefinitely as a function of the dose per fraction according to the formula

$$\ln RR = 2\beta d^2, \quad (3)$$

where d is the dose per fraction in split-dose measurements[21]. From this relationship, β(termed β_{RR} following Peacock et al.[22]) can be calculated as

$$\beta_{RR} = \ln RR/2d^2. \quad (4)$$

It has been observed[15], using the split-dose method to study the recovery capacity of human tumours with different intrinsic radiosensitivity, that most radiosensitive cell lines showed the highest split-dose recovery ratio. In some cases, nevertheless, such as lymphomas[23] and leukaemias[24], tumours very sensitive to radiation, the observed RR values were slightly grater than one.

Furthermore, the amount of inducible response appears to be related to the intrinsic radiosensitivity[15,25]. The higher is the radiosensitivity, the higher is the split-dose recovery, and the lower is the inducible response[26].

While fractionation regimens for gamma-rays have been investigated in detail both *in vitro* and *in vivo*, less information is available for protons.

In the present study, doses were delivered in two fractions using low-energy protons of about 8 keV/μm and about 20 keV/μm, and gamma-rays. These data can give information about the relationship between the recovery ratio and the radiation quality.

MATERIALS AND METHODS

Cell culture

Four human cell lines were selected for an inactivation study. Two of them were tumoural, the SCC25 and the SQ20B cell lines, and two normal, the HF19 and the H184B5 F5-1M/10 (hereafter called M10) cell lines. The SCC25 and SQ20B cell lines were derived from human epithelium tumours of the tongue and of the larynx, respectively[27]. They were kindly given to our laboratories by Dr. E. Blakely with the permission of Dr. R. Weichselbaum. The cells were grown in D-MEM: F12 (75:25) supplemented with 0.4 μg/ml hydrocortisone and 20% foetal calf serum. Under these conditions, the plating efficiency (PE) was 40% for SCC25 and 60% for SQ20B, and the doubling time (T_d), evaluated from the growth curve, was 24±2 h for both cell lines. The M10 cell line is a subclone taken from a primary culture of human mammary epithelial cell line H184B5[28] and kindly provided by Dr. T. C. Yang. They grew in -αMEM supplemented with 10% foetal calf serum, and exhibited a PE of 30% and a T_d of 28±2 h. The HF19 cell line is a lung fibroblast cell line from a female foetus[29].

The cells were maintained in a monolayer culture using Eagle's MEM medium plus 10% foetal calf serum. Under these conditions, PE was 20% and T_d was about 24 h.

Irradiations

Irradiations were performed with monoenergetic proton beams, whose LET evaluated at the cell entrance was about 8 keV/μm and about 20 keV/μm. In particular, SSC25, SQ20B and HF19 cells were irradiated at LET values of 7.7 keV/μm and 19.7 keV/μm at the 7 MV Van de Graaff CN accelerator of the INFN Laboratori Nazionali di Legnaro (LNL), Padova. This irradiation facility, its beam dosimetry and the irradiation conditions have been described in detail elsewhere[30]. The cells were plated over specially built stainless steel cylinders of 13 mm diameter, with a 52 μm thick mylar base. The M10 cells were irradiated at LET values of 9.1 keV/μm and 21.4 keV/μm at the 3 MV TTT-3 Tandem accelerator in Naples. This irradiation facility, its beam dosimetry and the irradiation conditions have been described in detail previously[31]. The cells were plated on a 11 mm diameter, 3 μm thick mylar foil sealed with araldite to the bottom of a specially built pyrex cylinder. The performances of the two facilities have been compared[2].

Each cell line was irradiated with two doses, corresponding to about 1% and 5% survival levels. In particular, the doses were 7 Gy and 5 Gy for the SSC25 cells, 17 Gy and 12 Gy for protons and 14 Gy and 10 Gy for gamma-rays for the SQ20B cells, 7 Gy and 5 Gy for protons and 4 Gy and 2 Gy for gamma-rays for the HF19 cells, 9 Gy and 6 Gy for the M10 cells. The same total dose was also delivered in two equal fractions separated by 1.5, 3 and 4.5 hours. The cells were seeded for about 48 hours before the first dose. The cells were irradiated as a monolayer in the exponential phase at room temperature and incubated at 37°C between the two fractions. The same procedure was repeated with gamma-rays from ^{60}Co or ^{137}Cs sources. In all cases, the dose rate was about 1 Gy/min.

Survival evaluation

After irradiation, the cells were trypsinised, diluted and plated at appropriate density. Following a suitable period of incubation, depending on the cell line, colonies were fixed and stained. Colonies with more than 50 cells were considered for determination of the surviving fraction.

Three to six independent experiments were performed for each cell line. The recovery ratio (RR) with its standard error was then evaluated, for each time interval, as the mean of the RR values of each independent experiment, calculated according to Eq. (1).

RESULTS AND DISCUSSION

The surviving fractions for the four cell lines irradiated with two doses, corresponding to about 5% and 1% survival levels and delivered in two equal fractions separated by 1.5, 3 and 4.5 hours, are reported in Table 1. The survival values at 2 Gy (SF_2) from our previous measurements[2] are reported in Table 2 and show that the radiosensitivities of the four cell

Table 1. Surviving fractions (S) for the different cells lines and radiations. The total dose is split in two equal fractions separated by the time Δt.

Radiation	Cell line	Δt (h)	Total dose (Gy)	S	Total dose (Gy)	S
Gamma-rays	SCC25	0	5.0	0.039 ± 0.004	7.0	0.012 ± 0.001
		1.5		0.066 ± 0.007		0.021 ± 0.002
		3.0		0.101 ± 0.010		0.042 ± 0.004
		4.5		0.106 ± 0.011		0.041 ± 0.004
	HF19	0	2.0	0.042 ± 0.004	4.0	0.011 ± 0.002
		1.5		0.063 ± 0.006		0.014 ± 0.002
		3.0		0.106 ± 0.011		0.027 ± 0.005
		4.5		0.078 ± 0.008		0.027 ± 0.005
	M10	0	6.0	0.043 ± 0.004	9.0	0.015 ± 0.006
		1.5		0.051 ± 0.001		0.012 ± 0.001
		3.0		0.053 ± 0.013		0.018 ± 0.005
		4.5		0.088 ± 0.001		0.020 ± 0.005
	SQ20B	0	10.0	0.062 ± 0.011	13.8	0.014 ± 0.003
		1.5		0.116 ± 0.010		0.031 ± 0.006
		3.0		0.121 ± 0.011		0.026 ± 0.005
		4.5		0.109 ± 0.009		0.035 ± 0.006
Protons (8 keV/μm)	SCC25	0	5.0	0.055 ± 0.007	7.0	0.013 ± 0.001
		1.5		0.078 ± 0.011		0.023 ± 0.001
		3.0		0.120 ± 0.016		0.044 ± 0.001
		4.5		0.093 ± 0.018		0.047 ± 0.001
	HF19	0	5.0	0.056 ± 0.008	7.0	0.032 ± 0.007
		1.5		0.111 ± 0.012		0.040 ± 0.008
		3.0		0.124 ± 0.005		0.046 ± 0.005
		4.5		0.124 ± 0.014		0.044 ± 0.003
	M10	0	6.0	0.059 ± 0.001	9.0	0.007 ± 0.001
		1.5		0.082 ± 0.010		0.007 ± 0.001
		3.0		–		0.011 ± 0.001
		4.5		0.136 ± 0.014		0.014 ± 0.001
	SQ20B	0	12.5	0.051 ± 0.003	17.0	0.050 ± 0.005
		1.5		0.081 ± 0.015		0.069 ± 0.003
		3.0		0.072 ± 0.008		0.078 ± 0.010
		4.5		0.074 ± 0.012		0.072 ± 0.011
Protons (20 keV/μm)	SCC25	0	5.0	0.051 ± 0.004	7.0	0.021 ± 0.001
		1.5		0.078 ± 0.010		0.032 ± 0.003
		3.0		0.126 ± 0.056		0.054 ± 0.009
		4.5		0.129 ± 0.015		0.061 ± 0.009
	HF19	0	5.0	0.046 ± 0.011	7.0	0.026 ± 0.008
		1.5		0.072 ± 0.020		0.042 ± 0.009
		3.0		0.066 ± 0.020		0.053 ± 0.017
		4.5		0.054 ± 0.014		0.044 ± 0.003
	M10	0	6.0	0.073 ± 0.007	9.0	0.060 ± 0.003
		1.5		0.077 ± 0.008		0.048 ± 0.002
		3.0		0.099 ± 0.010		0.063 ± 0.003
		4.5		0.129 ± 0.013		0.080 ± 0.007
	SQ20B	0	12.0	0.073 ± 0.010	17.0	0.056 ± 0.007
		1.5		0.092 ± 0.005		0.084 ± 0.006
		3.0		0.086 ± 0.011		0.092 ± 0.010
		4.5		0.096 ± 0.012		0.086 ± 0.009

Table 2. Surviving fraction at 2 Gy (SF_2) and β values for the different cell lines and radiation qualities for the survival levels of 5% (5%S) and 1% (1%S).

Radiation	Cell Line	SF_2	β (Gy^{-2})	$\beta_{RR(5\%S)}$ (Gy^{-2})	$\beta_{RR(1\%S)}$ (Gy^{-2})
Gamma−rays	SCC25	0.28 ± 0.03	0.031 ± 0.012	0.080 ± 0.008	0.052 ± 0.004
	HF19	0.28 ± 0.01	–	0.074 ± 0.008	0.041 ± 0.006
	M10	0.35 ± 0.01	–	0.040 ± 0.004	0.011 ± 0.015
	SQ20B	0.72 ± 0.03	0.017 ± 0.002	0.010 ± 0.002	0.007 ± 0.001
Protons (8 keV/μm)	SCC25	0.30 ± 0.08	0.092 ± 0.036	0.070 ± 0.003	0.054 ± 0.001
	HF19	0.34 ± 0.01	–	0.083 ± 0.017	0.041 ± 0.017
	M10	0.37 ± 0.02	–	0.047 ± 0.006	0.018 ± 0.001
	SQ20B	0.71 ± 0.08	0.011 ± 0.012	0.006 ± 0.003	0.004 ± 0.001
Protons (20 keV/μm)	SCC25	0.18 ± 0.02	–	0.074 ± 0.004	0.044 ± 0.003
	HF19	0.34 ± 0.01	–	0.032 ± 0.010	0.025 ± 0.021
	M10	0.37 ± 0.01	–	0.032 ± 0.006	0.008 ± 0.001
	SQ20B	0.63 ± 0.05	0.004 ± 0.007	0.005 ± 0.003	0.003 ± 0.001

lines are different.

In Figs. 1A and 1B we report, for the two survival levels, the recovery ratios as a function of the time between doses for all cell lines and radiation types. As can be seen, RR increased with time and seemed complete after the first three hours, except for M10 cell line, when irradiated with gamma-rays at a dose corresponding to 5% survival.

The highest value of the RRs measured at different time intervals is chosen as the maximum recovery ratio (RR_{max}). In Figs. 2A and 2B we report, for the two survival levels, RR_{max} as a function of the cell radiosensitivity (SF_2; survival fraction to 2 Gy gamma-rays). It can be seen that the RR_{max} values ranged between 2.0 and 2.7 for gamma-rays, 1.6 and 2.8 for 8 keV/μm protons, 1.5 and 2.5 for 20 keV/μm protons, in the case of a 5% survival level. Concerning the 1% survival level, the RR_{max} ranges were, respectively, 1.6–3.5, 1.7–3.8, and 1.4–2.9.

Overall, a smoothly decreasing trend for RR_{max} was observed as the radiosensitivity decreased (gamma-rays SF_2 increases) for both survival levels. This trend was more evident in the case of a 1% survival level, and indicates that the cell lines, showing the highest radiosensitivity (independently of their tumour or normal origin), showed a greater recovery in comparison to the cell lines characterized by the highest radioresistance. This is in agreement with the results reported by the authors for other cell lines[22, 26].

If we consider the relationship $\ln(RR) = 2\beta_{RR}d^2$, coming from the hypothesis that the survival curves can be described by a linear quadratic function, we can calculate $\beta_{RR,}$ which can be considered to be a measure for repair. In Table 2 we report on the β_{RR} values for the two survival levels calculated from the RR_{max} values according to Eq. (4), together with the β values derived from the survival curves[2] and the SF_2 as measured in our previous work[2]. When significant, β and β_{RR} are different, according to Peacock et al.[22] for gamma-rays. Comparing β and β_{RR}, it should be pointed out that these experiments were performed with asynchronous

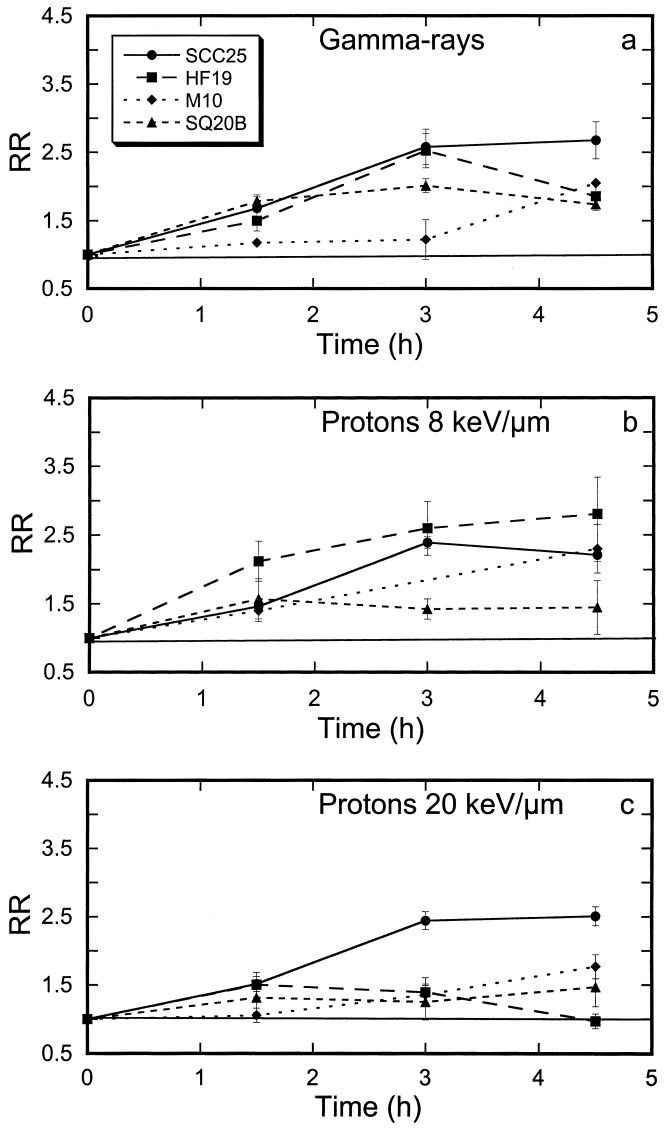

Fig. 1A. Recovery ratio (RR) for the 5% survival level as a function of time between doses for the cell lines and radiation type: (a) gamma-rays, (b) 8 keV/μm protons and (c) 20 keV/μm protons. The horizontal solid line shows level 1 for RR.

cultures. Redistribution in the cell-cycle between doses may complicate the interpretation. However, the doubling time of the cell lines used in these experiments exceeded 24 h, whereas the time interval between fractions was below 5 h. Therefore, redistribution does not appear to be a major problem in these experiments.

In Fig. 3 we report, for the two survival levels, the β_{RR} values as a function of the cell

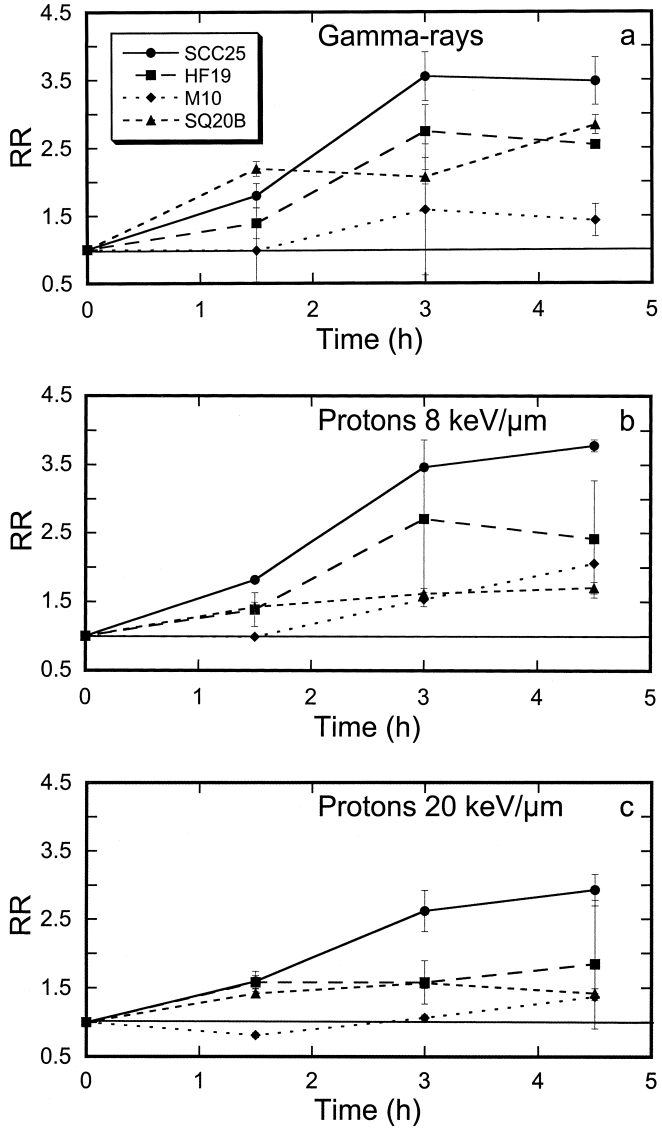

Fig. 1B. Recovery ratio (RR) for the 1% survival level as a function of time between doses for the cell lines and radiation type: (a) gamma-rays, (b) 8 keV/μm protons and (c) 20 keV/μm protons. The horizontal solid line shows level 1 for RR.

radiosensitivity for 2 Gy gamma-rays. An inverse correlation between β_{RR} and the gamma-ray SF_2 for each cell line is also evident. This suggests, in agreement with Peacock et al.[22], that the radiosensitivity may not be related to a deficient recovery.

In Fig. 4 β_{RR} is plotted as a function of LET for the two survival levels (5% and 1%). For the SQ20B cell line, the most radioresistant-ones, β_{RR} decreases with LET for both survival

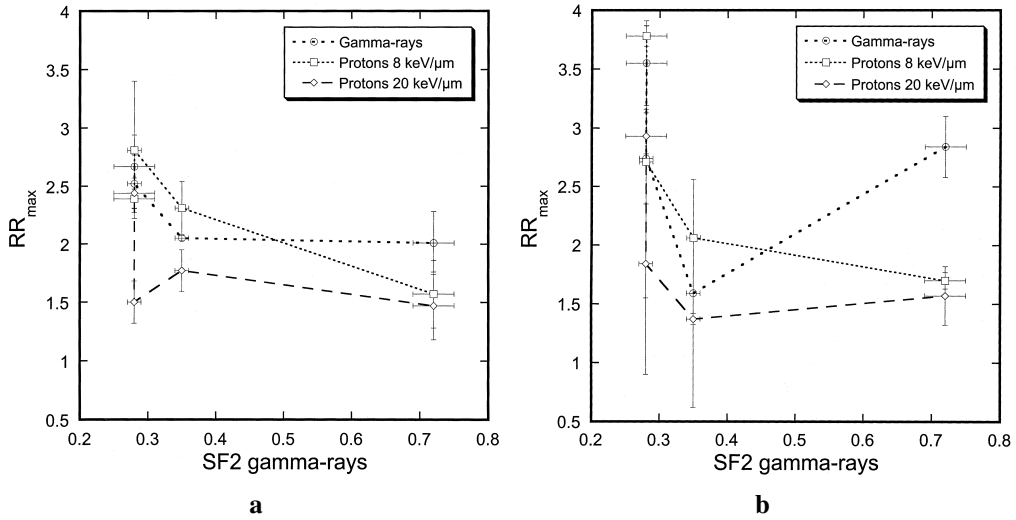

Fig. 2A. Maximum recovery ratio (RR_{max}) for the 5% survival level as a function of the cell radiosensitivity for 2 Gy of gamma-rays (SF_2).

Fig. 2B. Maximum recovery ratio (RR_{max}) for the 1% survival level as a function of the cell radiosensitivity for 2 Gy of gamma-rays (SF_2).

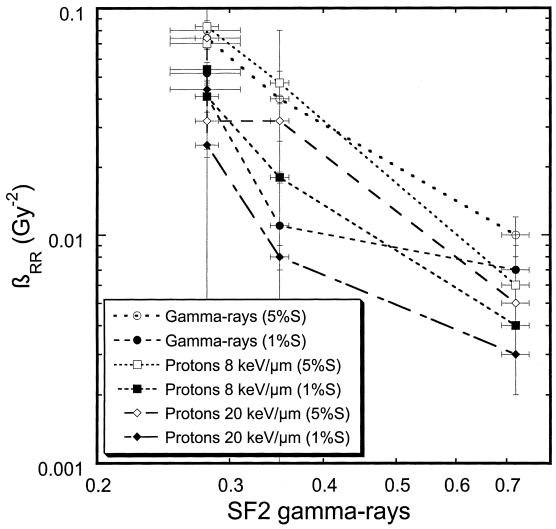

Fig. 3. β_{RR} for the two survival levels of 5% (5%S) and 1% (1%S) calculated from the RR_{max} values as a function of the cell radiosensitivity for 2 Gy gamma-rays.

levels, while for M10 cells, after an initial increase with LET, it decreases for 20 keV/μm protons. Although the other cells did not show any great differences between the gamma-rays and an 8 keV/μm proton beam, the β_{RR} values concerning protons of 20 keV/μm are in general

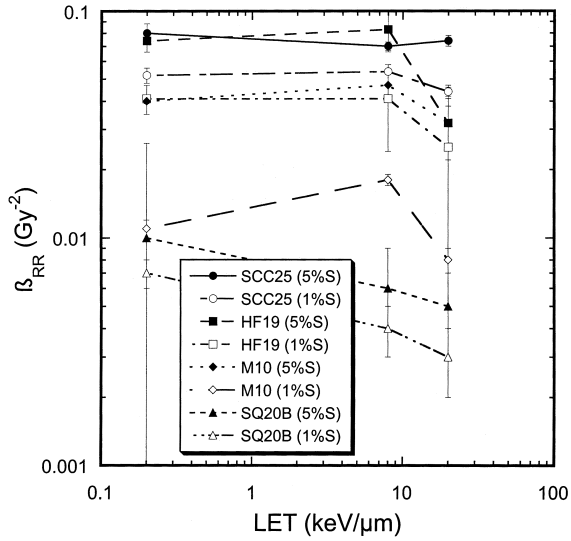

Fig. 4. β_{RR} for the two survival levels of 5% (5%S) and 1% (1%S) as a function of the LET of the different radiations.

lower compared to the others, according to the fact that radiation damage repair is less efficient as LET increases.

A possible explanation for differential radiosensitivity could be a cell line-dependent inducible repair response. Indeed, Lambin et al.[25] showed that hypersensitivity at low X-ray doses is observed mostly in radioresistant cell lines, and suggested that radioresistance is induced by low-doses of radiation. Therefore, the amount of inducible radioresistance might explain, at least in part, the observed differences in radiosensitivity at high doses. In this hypothesis, our data indicate that the radiation quality affects the inducible repair response, since RR_{max} tends to decrease as LET increases. The nature of inducible radioresistance in human cells is still unknown. It has been shown that the heat-shock protein 70[32] and nitric oxide[33] affect the cellular radiosensitivity. Interestingly, nitric oxide secreted by human tumour cells exposed to heavy ions induces radioresistance in recipient cells[34]. These data suggest that radioresistance induced by charged particles could be modulated by cellular processes.

CONCLUSIONS

Inactivation measurements performed on normal and tumour cell lines suggest that recovery following split doses of low-energy protons increases with the time between fractions, and seems complete after the first three hours.

The measured maximum recovery ratio slightly decreased with the radiosensitivity both at the 1% and 5% survival levels. This trend is more evident for the 1% survival level, where

the most radiosensitive cell lines show a greater recovery in comparison to the most radioresistant ones.

Furthermore, the lowest RR_{max} value was observed for 20 keV/μm protons, indicating that the recovery potential after split doses was relatively small for slow protons.

The dependence of RR_{max} on the radiation quality already reported for heavy ions[35, 36] was observed here with low-energy protons for the first time.

Finally, the cell radiosensitivity and the corresponding β_{RR} values confirm that the intrinsic radiosensitivity may not be related to a deficient recovery. A possible explanation for differential radiosensitivity might be inducible radioresistance after the first dose fraction.

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