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The Effectiveness of 20 MeV Protons at Nanosecond Pulse Lengths in Producing Chromosome Aberrations in Human-Hamster Hybrid Cells

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Laser accelerated radiotherapy is a potential cancer treatment with proton and carbon-ion beams that is currently under development. Ultra-fast high-energy laser pulses will accelerate ion beams that deliver their dose to a patient in a "pulsed mode" that is expected to differ from conventional irradiation by increasing the dose delivery rate to a tissue voxel by approximately 8 orders of magnitude. In two independently performed experiments at the ion microprobe SNAKE of the 14 MV Munich tandem accelerator, A_L cells were exposed either to protons with 1-ns pulse durations or to protons applied over 150 ms in continuous irradiation mode. A slightly but consistently lower aberration yield was observed for the pulsed compared to the continuous mode of proton irradiation. This difference was not statistically significant when each aberration type was analyzed separately (*P* values between 0.61 and 0.85 in experiment I and *P* values between 0.32 and 0.64 in experiment II). However, excluding the total aberrations, which were not analyzed as independent radiation-induced effects, the mean ratio of the yields of dicentrics, centric rings and excess acentrics scored together showed (with 95% CI) a significant difference of 0.90 (0.81; 0.98) between the pulsed and the continuous irradiation modes. A similar tendency was also determined for the corresponding RBE values relative to 70 kV X rays. Since the different findings for the comparisons of individual chromosome aberration types and combined comparisons could be explained by different sample sizes with the consequence that the individual comparisons had less statistical power to identify a difference, it can be concluded that 20 MeV protons may be slightly less effective in the pulsed mode.

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INTRODUCTION

Laser accelerated radiotherapy is a potential cancer treatment with proton and/or carbon-ion beams that is currently under development (*1*). In principle, ultra-fast high-energy laser pulses will accelerate ion beams that deliver their dose to a patient in a "pulsed mode" with pulse durations of 1 ns and below (*2*), whereas conventional proton beams from a cyclotron or synchrotron apply the dose within 100 ms ("continuous") into a certain voxel in modern beam scanning proton or ion therapy. Before laser accelerated radiotherapy can be used in the clinic, it is crucial determine whether the ultra-high dose rate makes any difference in terms of biological effects. In particular, comparisons of the relative biological effectiveness (RBE) for single high-intensity pulses of radiations with those for continuous irradiation are needed. Although recent modeling studies (*3, 4*) have shown a possible reduction of reactive oxygen species for ion tracks overlapping in time (i.e., during initial level of DNA damage) in principle, the impact of a pulsed beam on cells or tissues remains unclear.

The effects of such short pulses are the subject of our current studies using pulsed and continuous ion beams at the ion microprobe SNAKE (Superconducting Nanoprobe for Applied nuclear [Kern] physics Experiments) of the 14 MV Munich tandem accelerator. Our recent investigations on the induction of micronuclei, which can be regarded mainly as equivalents of acentric chromatin elements (acentrics), have been aimed at determining the RBE of pulsed and continuous 20 MeV protons relative to 70 kV X rays. The RBE equals the ratio of the absorbed doses of these two radiation qualities that produce the same specified biological effect. Using the micronucleus (MN) test in either HeLa cells (*5*) or keratinocytes of a human tissue model, the so-called 3D human reconstructed skin EpiDermFT™, which preserves the three-dimensional geometric arrangement and communication of cells present in tissues

in vivo (*6*), RBE values of about 1.1 have been determined for both pulsed and continuous 20 MeV protons. However, it should be taken into consideration that in contrast to radiation-induced structural chromosome aberrations, the relevance of radiation-induced micronuclei for quantifying chromosome damage may be limited under the following circumstances. The slope of a dose–response curve for micronuclei can be influenced by the mechanisms of MN formation and MN content, because with increasing numbers of acentrics the relative proportion of acentrics in independent micronuclei may decrease. As a consequence, after irradiation with higher doses or increasing linear energy transfer (LET), individual micronuclei may contain several acentrics, resulting in a significant reduction of the slope of the dose response for micronuclei. Thus, e.g., it was not surprising that the frequencies of micronuclei induced in human lymphocytes by X rays at tube voltages from 50 kV (29.8 keV) to 350 kV (143.5 keV) were not significantly different (*7*), whereas a significant decrease was found for the induction of chromosome aberrations (mainly dicentrics) by X rays at tube voltages from 60 kV (48 keV) to 220 kV (135 keV) (*8*).

The present experiments were designed to avoid such potential saturation effects in data for the analyzed biological end point, which may influence the effectiveness and the reliable evaluation of the RBE for pulsed and continuous 20 MeV protons. Thus the present investigation was carried out to determine the production of chromosome aberrations in monolayers of human-hamster hybrid cells. To determine the RBE for 20 MeV protons for both irradiation modes relative to 70 kV X rays, the investigation focused on radiation-induced aberrations of the chromosome type, i.e., in cells that were irradiated when their chromosomes were under unduplicated conditions (pre-DNA synthesis, G_1 phase of the cell cycle).

MATERIALS AND METHODS

Cell Line

For the present experiments, cells of the human-hamster hybrid (A_L) cell line were used that were kindly provided by Dr. Severin (University of Münster, Germany). This cell line was derived from the CHO-K1 wild-type cell line, a subclone of the parental CHO cell line, which was derived from the ovary of an adult Chinese hamster (Cricetulus griseus). A few years ago, these A_L cells were established in our laboratory to investigate functional or structural chromosome aberrations in monolayer after exposure to non-ionizing or ionizing radiation qualities.

Chromosome Number of the A_L Cell Line

Prior to the radiation experiments, the chromosome number of non-exposed A_L cells was examined. To characterize our A_L cells, the intercellular distribution of chromosomes was analyzed in a total of 400 metaphases with preserved cytoplasm. Since there was no substantial variation between the results from three replicates, the data were pooled. The chromosome analysis showed a modal number of 21 chromosomes (85% of analyzed cells), whereas 15% of the analyzed cells had a loss or gain of chromosomes. A karyotype containing 21 chromosomes is demonstrated in Fig. 1.

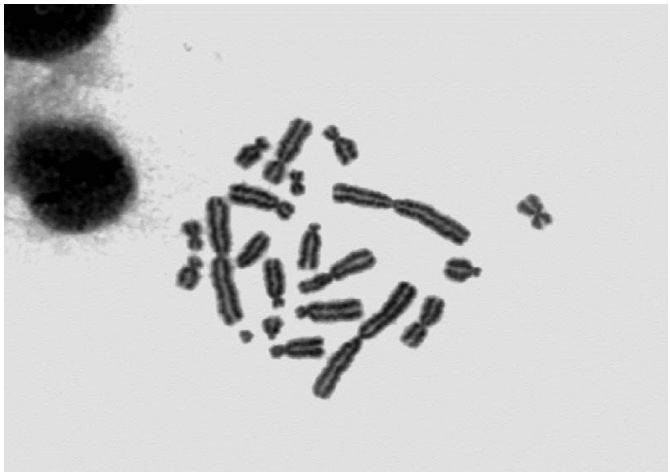

FIG. 1. Example of A_L cell (human hamster hybrid cell line) with the modal number of 21 chromosomes.

Cell Culture

For each experiment of simultaneous exposure of cells to protons or X rays, a frozen aliquot from the same stock culture of A_L cells was thawed and seeded in culture with exponentially growing unsynchronized cells into special irradiation containers that had been developed specifically for use at the ion microprobe SNAKE of the 14 MV Munich tandem accelerator. A detailed description of the construction of these containers for cell irradiation has been reported previously (*2, 9*). These cell containers are built by stretching and clamping a Mylar foil between two stainless steel plates. To irradiate all cells, the total cell growth area was limited to 0.36 cm^2 by stainless steel barriers. About 30,000 A_L cells were seeded in RPMI-1640 medium supplemented with 16% fetal calf serum, 100 U/ml penicillin and 50 μg/ml streptomycin to obtain subconfluent cultures. The A_L cell monolayers were maintained in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Under these growth conditions, the cells had a doubling time of 18 h.

Irradiation Conditions

The layout and methods for irradiation of monolayer of cells have been reported in detail (*5*); a brief description is given here. About 24 h before irradiation experiments, cells were trypsinized, carefully resuspended in fresh medium, and grown as monolayer cultures on a microstructured Mylar foil pretreated with Cell-TAK (BD Bioscience) in stainless steel cell chambers designed as described. Shortly before irradiation, about half of the culture medium was removed and the irradiation containers were tightly closed by clamping another Mylar foil and fixing a third metal plate. During irradiation at room temperature, the cell chamber is placed vertically in the focal plane of the ion microbeam; i.e., the cells are not covered by medium, but residual medium in a reservoir ensures a saturated atmosphere. Typically, the total time required for sample preparation and irradiation, i.e. the time outside of the CO_2 incubator, is less than 15 min, including about 12 min in the upright position. Under these conditions, we did not observe enhanced numbers of chromosome aberrations in unirradiated cells compared to samples that remained in the incubator.

To demonstrate reproducibility as well as intertest variability, two independent experiments I and II for exposure of A_L cells to 20 MeV

protons or simultaneously to 70 kV X rays as the reference radiation were carried out. In the proton experiments I and II during separate beam times, three replicates were exposed to 3.6 Gy at either continuous or pulsed proton irradiation mode. A pulsed proton beam with 25 MeV (2.21 keV/μm) was used in experiment I and with 23 MeV (2.37 keV/μm) in experiment II. A continuous proton beam with 20 MeV (2.65 keV/μm) was used in experiment I and with 23 MeV (2.37 keV/μm) in experiment II. These small differences in energy have been taken into account when calculating the actual dose in each experiment.

Cell Irradiation with Pulsed and Continuous Proton Beams

A pulsed proton beam with up to 10^5 protons per pulse focused into a spot of approximately 100×100 μm^2 was prepared for cell and tissue irradiation experiments using the ion microprobe SNAKE at the Munich tandem accelerator. The pulsed beam preparation has been described in detail (*2*). By scanning the beam in a regular pattern, a nearly homogeneous dose distribution was obtained, where 50–60% of the total dose was delivered by one single proton pulse, with small additional doses from adjacent beam pulses. The homogeneity of the dose distribution was controlled by a YAG scintillator viewed by a CCD camera or radiochromic films. During irradiation, the container with the cells attached to the carrier foil was mounted directly behind the beam exit nozzle. Thus the protons had to cross the vacuum exit window, 7.5 μm polyimide foil (Kapton), an air gap of approximately 30 μm, and the cell carrier foil, 6 μm Mylar. According to a SRIM simulation (*10*), the cumulative energy loss of 20 MeV protons until they reach the cells is 0.11 MeV, resulting in a slightly increased dose-averaged LET in water of 2.661 keV/μm at the target in comparison to 2.648 keV/μm for 20 MeV protons.

The delivered energy dose *ED* can be calculated from

$$ED = \frac{N}{A}\frac{LET}{\rho}, \tag{1}$$

where *LET* is the linear energy transfer, ρ the target density (assuming water as a tissue equivalent), and *N*/*A* the number *N* of particles that impinge on the beam size area *A*. For dose control the beam size *A* of the beam spot could be observed by the CsI scintillator (*2*). The totally irradiated area was well controlled with much better than 1% accuracy when scanning the beam by setting hundreds of individual beam spots side by side. The number of protons *N* per pulse is given by

$$N = I(e \cdot r)^{-1}, \tag{2}$$

where *e* is the unity charge. *N* was measured by the electric beam current *I* for beam pulses repeating at a repetition rate $r = 5/128$ MHz, where 5 MHz is the base frequency of the pulsing system and the pulse frequency was reduced by a factor of $2^7 = 128$. The systematic error of the beam current measurement through leakage currents and instrumental errors was estimated to be less than 5%. The largest error in continuous irradiation mode stemmed from beam fluctuations between current measurements at the Faraday cup situated in front of the target position and from fluctuations of the single pulse intensity in pulsed irradiation mode, respectively. The mean error of a single shot dose was estimated to be of the order of $\pm$ 10% but should be averaged for the many pulses applied on one cell dish and by doing three independent irradiations for each experiment. In both proton experiments the pulse length in "pulsed mode" was 1.0 ns.

The same setup was used for preparation of the pulsed and the continuous beam for proton irradiation. Thus potential systematic errors were compensated by direct comparison of results from the pulsed and the continuous beam experiments. In particular, errors in the electrical beam current measurement by leakage currents and instrumental errors were compensated. A quadratic $A = 2.0 \times 2.0$-mm^2 beam spot was prepared with the installed micro slit system and the homogeneity was visualized and controlled on a CsI scintillator (*2*). The delivered dose was adjusted by the time *t* that the cells needed to be irradiated for the requested dose of 3.6 Gy according to Eq. (1) by using the measured Faraday cup current *I*; i.e.

$$N = (I \cdot t) \cdot e^{-1}. \tag{3}$$

In continuous mode, cell samples were irradiated for $t = 150$ ms. Such an irradiation time is typically used to irradiate a certain voxel of a tumor in common treatment plans of raster scanned beams.

X Irradiation for Dose–Response Curve

To obtain dose–response relationships for chromosome aberrations for reference, A_L cells were exposed to 70 kV X rays (Philips RT100; Philips Medical Systems, Eindhoven, The Netherlands) in the dose range up to 4 Gy (experiment I) as well as up to 8 Gy (experiment II) using a dose rate of approximately 1 Gy per min (10 mA, 2.0 mm Al, HVL = 1.9 Al) and a source–cell distance of 30 cm to a field of 20 cm $\times$ 20 cm.

Postirradiation Cell Culture and Chromosome Analysis

Immediately after irradiation the lid was removed again, and irradiated A_L cells were trypsinized and reseeded in 4 ml RPMI-1640 medium supplemented with 20% fetal calf serum and antibiotics (penicillin/streptomycin) in a 6-well plate and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The trypsinizing and reseeding was necessary because the total cell growth area was limited to 0.36 cm^2 by stainless steel barriers that were removed prior to irradiation and not used again. In experiment I, colcemid at a final concentration of 0.03 μg ml^{-1} was added to the cultures 6 h after incubation, after which they were incubated for a further 18 h. In the presence of colcemid during this incubation period, a synchronization of the cycling A_L cells that were at the G_1 phase of the cell cycle during irradiation could be achieved, whereas A_L cells irradiated at the S and G_2 phases could not be arrested by colcemid at the metaphase because these cells had already finished their first cell cycle after irradiation. In experiment II, colcemid at the higher concentration of 0.1 μg ml^{-1} was present in the cultures during the last 4 h of a total incubation period of 20 h. The colcemid technique of experiment II was standardized in our previous experiments to identify the presence of the single human chromosome of A_L cells in first metaphases by uniformly stained sister chromatids (unpublished data) using a combination of FISH (fluorescence *in situ* hybridization) painting and harlequin staining for cell cycle-controlled chromosome analysis (*11*). Subsequently, the cultures were centrifuged, and the supernatant was removed and replaced by a hypotonic potassium chloride solution (0.075 *M*) at 37°C for 10 min. All subsequent steps were carried out at room temperature. The cells were fixed, i.e., the culture medium was removed and the cell pellet resuspended in a mixture of three parts of methanol and one part of glacial acetic acid. Aliquots of fixative were added drop by drop on individual slides. After air drying, the cells were stained with 2% acetic Orcein for 10 min. With this technique, the cytoplasm of the cells remained well preserved and chromosome loss due to preparation procedures was avoided.

Both culture conditions ensured that structural aberrations of the chromosome type could be analyzed exclusively in metaphases of the first cell cycle after radiation exposure. Applying this chromosome analysis was necessary because previous findings in synchronized CHO cells indicated that cells irradiated in early S phase were two to three times more sensitive to cytogenetic damage than cells exposed in G_1 (*12, 13*). Chromosome preparation was carried out according to a standardized laboratory procedure originally described for human lymphocytes (*14, 15*). All slides were coded. Only complete cell nuclei containing the modal number of 21 chromosomes were analyzed for structural chromosome aberrations, i.e. dicentrics, centric rings, excess acentric fragments and total aberrations. Excess acentric

TABLE 1
Frequency of Structural Chromosome Aberrations and Intercellular Distribution of Dicentrics in A_L Cells after Exposure to 70 kV X Rays during the DNA Pre-synthesis (G_0/G_1) Phase of the Cell Cycle

Dose (Gy)	Cells scored	Chromosome aberrations per cell				Intercellular distribution of dicentrics					
		Dic	R_c	Ace	Total	0	1	2	3	σ^2/y	*U* value
0	3000	0.0007	0	0.0023	0.003	2998	2			1.00	−0.02
Irradiation experiment I											
0.5	900	0.006	0.004	0.016	0.026	895	5			1.00	−0.10
1	600	0.018	0.010	0.042	0.070	589	9	1		1.17	3.00
2	500	0.052	0.034	0.124	0.210	476	22	2		1.10	1.68
4	400	0.118	0.078	0.263	0.459	367	40	2	1	1.10	1.40
Irradiation experiment II											
0.5	1000	0.004	0.003	0.018	0.025	996	4			1.00	−0.08
1	800	0.015	0.013	0.044	0.072	788	12			0.99	−0.31
2	600	0.047	0.022	0.112	0.181	574	24	2		1.09	1.59
4	400	0.100	0.063	0.213	0.376	363	34	3		1.05	0.75
8	200	0.480	0.295	0.850	1.625	125	56	17	2	1.01	0.04

Notes. Data are from independently performed experiments I and II. Dic, dicentrics; R_c, centric rings; Ace, excess acentrics; Total, total aberrations; σ^2/y, dispersion ratio; *u* value, test quantity.

fragments were recorded as all fragments minus one per dicentric or centric ring assuming complete exchange processes. Thus total aberration data equal total acentrics, i.e. excess acentrics plus each acentric fragment associated with dicentrics and centric rings.

Statistics

A null hypothesis that the observed difference between the mean frequencies of dicentrics, centric rings, excess acentric fragments and total aberrations from three replicates (±SE) for pulsed and continuous 20 MeV proton exposure are not different was tested using *z*-test statistics. Since the conditions for the applications of the normal probability distribution hold for these data (i.e., both sample sizes are much larger than 100 cells and the products of sample sizes and proportions are greater than five), this statistical procedure could be applied. A difference at a two-sided *P* value <0.05 was considered statistically significant.

A Wilcoxon matched-pairs signed-ranks test (*16*) was carried out for the yields of dicentrics, centric rings or excess acentric fragments because an elevated frequency of any of these structural chromosome aberrations is considered to be a sensitive and characteristic bioindicator of radiation exposure. The test was used to verify the null hypothesis H_0 that no difference (two-tailed, at 5% significance) between the RBE of 20 MeV protons for pulsed and continuous irradiation modes ($T_{critical} < T_{calculated}$) could be observed.

RESULTS

Chromosome Aberration Data for Cell Exposure to X Rays and Protons

Table 1 presents the basic data from experiments I and II on the specific classes of structural aberrations of the chromosome type in A_L cells after exposure to 70 kV X rays together with the corresponding background frequencies. The background frequencies were obtained from a total of 3,000 cells that were pooled from the unexposed (control) cell samples analyzed in experiments I and II. With increasing dose, the yield of chromosome aberrations increased in the four specific classes of aberration types. Since the measured dicentric yields are generally used as the most characteristic biological end point of radiation-induced chromosome aberrations (*14, 15*), the distribution of these aberrations among cells is also given in Table 1. The coefficients, σ^2/y (variance/mean), approximate unity if the intercellular distribution of the aberrations follows a Poisson distribution. A value of the test quantity *u* in excess of 1.96 indicates overdispersion at the 5% level of significance (*17*). At 1.0 Gy in experiment I, the intercellular distribution of dicentrics was slightly overdispersed compared to Poisson, whereas at the other eight doses there was no evidence for deviation from regular dispersion, as seen from the dispersion coefficients. In fact, there is no recognizable trend of overdispersion with the X-ray dose.

For comparison with our earlier MN data obtained after exposure of cells to 3.0 Gy of 20 MeV protons (*5, 6*), a similar high dose was used in the present investigation. The dose of 3.6 Gy of pulsed and continuous 20 MeV protons was used because this dose is beyond the ratio of the linear and quadratic coefficients (α/β) of the reference radiation, where the quadratic component of the dose response dominates. Table 2 presents the basic data for the specific classes of structural chromosome aberrations in A_L cells induced by 3.6 Gy of 20 MeV protons, which were determined in the independently performed experiments I and II with three cell samples each. In both experiments the same batches of cells were used as in the accompanying X-ray experiments. In both pulsed and continuous irradiation modes, the intercellular distribution of the dicentric data showed regular dispersion in all replicates of both experiments. Since there were no statistically significant differences between the three single cell monolayer replicates, the pooled data are also given as mean values ± SE for three replicates. A slight but consistently lower

TABLE 2
Frequencies of Dicentrics (Dic), Centric Rings (R_c), Excess Acentrics (Ace) and Total Aberrations (Total) as Well as the Intercellular Distribution of Dicentrics in A_L Cells after Exposure to 3.6 Gy of 20 MeV Protons at Pulsed (P) or Continuous (C) Irradiation Modes (IM)

IM	Cells scored	Chromosome aberrations per cell				Intercellular distribution of dicentrics					
		Dic	R_c	Ace	Total	0	1	2	3	σ^2/y	U value
Irradiation experiment I											
P	206	0.073	0.034	0.175	0.282	192	13	1		1.07	0.69
P	166	0.090	0.060	0.175	0.325	151	15			0.92	−0.80
P	155	0.077	0.032	0.167	0.276	143	12			0.97	−0.29
C	192	0.073	0.021	0.135	0.229	179	12	1		1.07	0.75
C	306	0.111	0.052	0.275	0.438	274	30	2		1.01	0.14
C	210	0.062	0.062	0.181	0.305	198	11	1		1.10	1.02
$\sum$P	527	0.080 ± 0.009	0.042 ± 0.016	0.173 ± 0.005	0.295 ± 0.025	486	40	1		0.97	−0.49
$\sum$C	708	0.086 ± 0.026	0.047 ± 0,021	0.209 ± 0,071	0.342 ± 0.106	651	53	4		1.05	0.87
Comparison of $\sum$P and $\sum$C		$z = 0.22$ $P = 0.83$	$z = 0.19$ $P = 0.85$	$z = 0.51$ $P = 0.61$	$z = 0.43$ $P = 0.67$						
Irradiation experiment II											
P	318	0.082	0.035	0.179	0.296	295	20	3		1.15	1.95
P	400	0.068	0.033	0.150	0.251	375	24	0	1	0.08	1.12
P	375	0.072	0.045	0.157	0.274	349	25	1		1.00	0.07
C	361	0.078	0.053	0.169	0.300	335	24	2		1.07	0.93
C	343	0.079	0.035	0.181	0.295	318	23	2		1.07	0.97
C	400	0.088	0.043	0.170	0.301	368	29	3		1.09	1.24
$\sum$P	1093	0.073 ± 0.007	0.038 ± 0.006	0.164 ± 0.015	0.275 ± 0.023	1019	69	4	1	1.10	2.42
$\sum$C	1104	0.082 ± 0.006	0.043 ± 0.009	0.173 ± 0.007	0.298 ± 0.003	1021	76	7		1.08	1.77
Comparison of $\sum$P and $\sum$C		$z = 0.98$ $P = 0.33$	$z = 0.46$ $P = 0.64$	$z = 0.54$ $P = 0.59$	$z = 0.99$ $P = 0.32$						

Notes. Data are from independent experiments I and II, each with three replicates. The comparison of $\sum$P and $\sum$C was carried out by using z-test statistics.

aberration yield was observed for the pulsed mode compared to the continuous mode of proton irradiation. This difference was not statistically significant when each aberration type was analyzed separately (P values between 0.61 and 0.85 in experiment I and P values between 0.32 and 0.64 in experiment II, Table 2). However, excluding the total aberrations, which were not analyzed as independent radiation-induced effects, the mean ratio of the yields of dicentrics, centric rings and excess acentrics scored together showed (with 95% CI) a significant difference of 0.90 (0.81; 0.98) between the pulsed and the continuous irradiation modes. This finding was confirmed by the Wilcoxon test showing that the null hypothesis of no difference (two-tailed, at 5% significance, $T_{critical} < T_{calculated}$) can be rejected, because $T_{critical} = 1$ and $T_{calculated} = 0$.

Evaluation of the RBE of Pulsed and Continuous 20 MeV Protons

A weighted least-squares approximation was used to fit the data for the 70 kV X-ray-induced dicentrics, centric rings, excess acentrics and total aberrations with the linear-quadratic function,

$$y = c + \alpha D + \beta D^2, \quad (4)$$

where c is given by the corresponding control (background) values. Reciprocal variances of the mean (total number of cells analyzed, n, divided by variance, σ^2) were used as weights. In Table 3, the linear and quadratic coefficients (α and β) of the dose–response curves for dicentrics, centric rings, excess acentrics and total aberrations are given for doses up to 4 Gy in experiment I and for doses up to 4 Gy and up to 8 Gy in experiment II. The corresponding dose–response curves for the four classes of chromosome aberrations induced by doses up to 4 Gy are presented in Fig. 2.

The RBE for 20 MeV protons for both irradiation modes was calculated using the dose of the reference radiation, 70 kV X rays, that produced a response equal to 3.6 Gy of protons (Table 3). In proton experiment I (Table 2), the pooled frequencies of dicentrics of 0.080 ± 0.009 (pulsed irradiation mode) and 0.086 ± 0.026 (continuous irradiation mode) correspond to the values obtained for 3.02 and 3.17 Gy of X rays, resulting in RBE values of 0.84 ± 0.06 and 0.88 ± 0.17, respectively. In proton experiment II (Table 2), the pooled frequencies of dicentrics of 0.073 ± 0.007 (pulsed irradiation mode) and 0.082 ± 0.006 (continuous irradiation mode) correspond to the values obtained for 3.12 and 3.36 Gy of X rays using the dose–response relationship up to 4 Gy, resulting in RBE values of 0.87 ± 0.05 and 0.93 ± 0.04, respectively. Using the dose–response curve up to 8 Gy, the pooled frequencies of dicentrics correspond to

TABLE 3
Dose–Yield Coefficients α ± SEM and β ± SEM for Different Yields of Chromosome Aberrations (CA), i.e. Dicentrics (Dic) Centric Rings (R_c) Excess Acentrics (Ace) and Total Aberrations (Total), in A_L Cells after Exposure to 70 kV X Rays as Reference

Exp.	CA	Dose range (Gy)	Linear coefficient α (10^{-2} Gy^{-1})	Quadratic coefficient β (10^{-2} Gy^{-2})	RBE (± SD) of 20 MeV protons (corresponding X-ray dose, Gy) (P)	(C)	Comparison of the RBE for (P) and (C) *z* value	*P* value
I	Dic	0–4	1.190 ± 0.423	0.476 ± 0.165	0.84 ± 0.06 (3.02)	0.88 ± 0.17 (3.17)	0.22	0.82
I	R_c	0–4	0.787 ± 0.233	0.309 ± 0.091	0.73 ± 0.17 (2.61)	0.78 ± 0.26 (2.82)	0.16	0.87
I	Ace	0–4	3.138 ± 1.003	0.937 ± 0.379	0.81 ± 0.02 (2.91)	0.92 ± 0.22 (3.31)	0.50	0.62
I	Total	0–4	5.163 ± 1.598	1.724 ± 0.612	0.80 ± 0.18 (2.88)	0.84 ± 0.19 (3.04)	0.15	0.88
II	Dic	0–4	0.940 ± 0.455	0.442 ± 0.189	0.87 ± 0.05 (3.12)	0.93 ± 0.04 (3.36)	0.94	0.35
II	R_c	0–4	0.720 ± 0.250	0.209 ± 0.100	0.79 ± 0.09 (2.86)	0.86 ± 0.06 (3.11)	0.65	0.52
II	Ace	0–4	3.817 ± 0.804	0.432 ± 0.301	0.87 ± 0.06 (3.13)	0.91 ± 0.03 (3.27)	0.60	0.55
II	Total	0–4	5.668 ± 1.371	1.036 ± 0.527	0.85 ± 0.05 (3.06)	0.91 ± 0.01 (3.27)	1.18	0.24
II	Dic	0–8	0.625 ± 0.354	0.629 ± 0.101	0.81 ± 0.05 (2.93)	0.87 ± 0.03 (3.13)	1.03	0.30
II	R_c	0–8	0.461 ± 0.252	0.365 ± 0.071	0.73 ± 0.07 (2.64)	0.79 ± 0.10 (2.94)	0.49	0.62
II	Ace	0–8	2.985 ± 0.729	0.873 ± 0.181	0.81 ± 0.05 (2.92)	0.84 ± 0.01 (3.03)	0.59	0.56
II	Total	0–8	4.195 ± 1.296	1.849 ± 0.339	0.79 ± 0.04 (2.86)	0.84 ± 0.01 (3.03)	1.21	0.23

Notes. The RBE (± SD) of pulsed (P) and continuous (C) 20 MeV protons is calculated for the mean aberration yield of three cell samples each exposed to 3.6 Gy in independent experiments I and II. The comparison of the RBE for (P) and (C) irradiation mode was calculated by using *z*-test statistics.

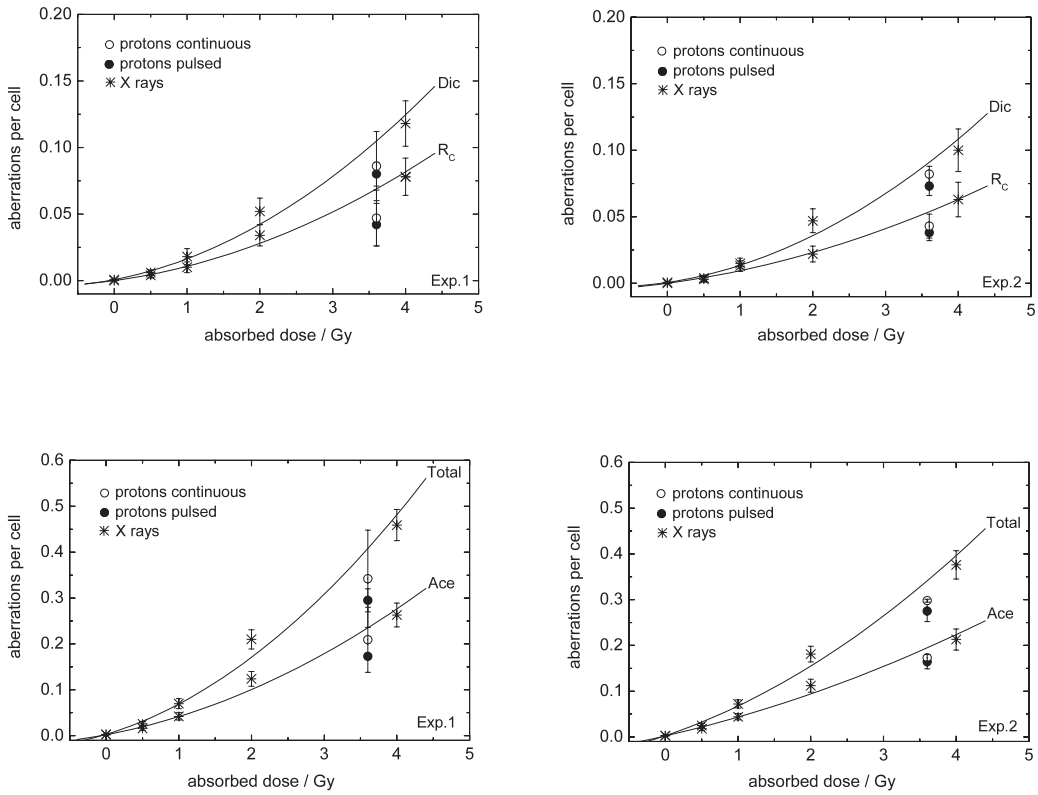

FIG. 2. Linear-quadratic dose–response curves for 70 kV X-ray-induced dicentrics (Dic) and centric rings (R_c) (upper panel) as well as excess acentrics (Ace) and total aberrations (Total) (lower panel) obtained in two independent experiments (Exp. I and Exp. II). The corresponding mean values of these aberration classes each determined from three single probes of exposure to 3.6 Gy of 20 MeV protons in pulsed and continuous irradiation modes in Experiments I and II are also shown. Standard errors of the mean are indicated by vertical bars.

the values obtained for 2.93 and 3.13 Gy of X rays, resulting in RBE values of 0.81 ± 0.05 and 0.87 ± 0.03, respectively. In Table 3, the corresponding RBE values for pulsed and continuous 20 MeV protons were also given for the production of centric rings, excess acentrics and total aberrations, respectively. Using the *z*-test statistics, we found that the RBE obtained for each aberration type did not show any significant difference between the pulsed and continuous irradiation modes (RBE values from 0.73 ± 0.17 to 0.87 ± 0.06 and from 0.78 ± 0.26 to 0.92 ± 0.22, respectively; *P* values between 0.62 and 0.88 in experiment I and *P* values between 0.23 and 0.62 in experiment II). However, when dicentrics, centric rings and excess acentrics were analyzed together, the mean ratio of the RBE showed a significant difference of 0.93 (0.88; 0.99) between the pulsed and continuous irradiation modes. This significant difference was confirmed by the Wilcoxon test showing that the null hypothesis of no difference (two-tailed, at 5% significance, $T_{critical} < T_{calculated}$) can be rejected.

DISCUSSION

Previous Studies Analyzing Chromosome Aberrations

The investigation of chromosome aberrations in cells or tissues after exposure to ultra-high dose rates of low-LET radiation dates back more than four decades due to the option of using pulsed radiations mainly from linear accelerators and betatrons. In general, these earlier radiobiological experiments were carried out because ultra-high dose-rate machines were of some concern to radiotherapists with regard to the theory of radiochemical consumption of oxygen. According to this theory, the radiochemical consumption of oxygen at sufficiently high doses would make cells radiobiologically hypoxic and therefore radioresistant. A few experiments have been described in which chromosome aberrations were induced by ultra-high dose rates of low-LET radiation. For example, there was evidence of a reduction in dicentric yield from 15% to 10% for exposure of human lymphocytes to 2 Gy X rays delivered in a 2-ns pulse relative to a conventional dose rate of 1 Gy/min (*18*). However, in an additional experiment from the same laboratory [cited in ref. (*19*)] the aberration yield was independent of the exposure rate, although a slightly wider range of dose rates was examined. In a further study (*19*), no significant difference in the induction of chromosome aberrations in human lymphocytes could be observed by 15 MeV electrons delivered in microsecond pulses and at conventional dose rate (1 Gy/min). These conflicting results for the induction of chromosome aberrations could be due to a lack of cell cycle-controlled analysis as well as to the very low number of cells analyzed.

Over the following three decades, a number of chromosome aberration studies were performed with different proton energies using a continuous irradiation mode. However, as noted by Edwards (*20*) and the ICRP (*21*), most of these experiments were carried out without corresponding data for a reference radiation obtained under the same experimental conditions. Based on a compilation of the linear-quadratic dose–response curves of dicentrics determined in three studies (*22–24*), Edwards (*20*) noted that for proton energies above 8 MeV, the linear coefficients α agree very well with those obtained for hard X rays (200–250 kV). Using this X-ray quality as reference, RBE values for these proton energies of about 1 can be derived. In addition, the intercellular distribution of dicentrics given in the three reports do not show any deviation from regular dispersion, as seen from the dispersion coefficients, σ^2/y. Compared with these extensive results on the induction of chromosome aberrations by protons or generally by particle irradiation in continuous irradiation mode, relatively little information has been published on the induction of chromosome aberrations obtained in pulsed irradiation mode. Since clinically relevant biological damage is rather reflected by chromosomal damages, especially the formation of a dicentric chromosome involving an interaction between DNA double-strand breaks of two chromosomes has to be investigated in cells after exposure to particle pulses of nanosecond durations. Up to now, the knowledge of chromosome aberrations in cells or tissues after irradiation under conditions where the full dose at one irradiation site is deposited by a few pulses less than 1 ns in duration is still very limited.

Comparison with Earlier Investigations

Although recent modeling studies using the biophysical Monte Carlo code PARTRAC (*3, 4*) have principally shown a possible reduction of reactive oxygen species for ion tracks overlapping in time (i.e., during initial level of DNA damage), the impact of a pulsed beam on cells or tissues remains unclear. In the present study, the effectiveness of pulsed and continuous 20 MeV proton irradiation modes was determined by analyzing different classes of aberrations in A_L cells. In experiments I and II, the pooled data for dicentrics, centric rings, excess acentrics and total aberrations obtained for both irradiation modes did not differ significantly from one another (*P* values between 0.32 and 0.85, Table 2). However, the mean ratio of the yields of dicentrics, centric rings and excess acentrics scored together showed a significant difference of 0.90 (0.81; 0.98) between the pulsed and the continuous irradiation modes, with slightly less effectiveness for pulsed mode. A likely explanation as to why the combined comparison is statistically significant while the individual comparisons

are not is simply that the individual comparisons are inevitably based on smaller numbers. In addition, there is no evidence that systematic errors in dosimetry had an influence on this result, because the same setup was used for preparation of the pulsed and the continuous beam for proton irradiation. Thus many sytematic errors could be compensated by direct comparison of findings from the pulsed and non-pulsed beam experiments. In particular, the errors in the electrical beam current measurement by leakage currents and instrumental errors were compensated. Thus it can be stated that 20 MeV protons produce slightly less effectiveness in the pulsed mode.

Using the linear-quadratic dose–response relationships for the X-ray data, RBE values from 0.73 ± 0.17 to 0.87 ± 0.05 and from 0.78 ± 0.26 to 0.93 ± 0.04 were evaluated for pulsed and continuous 20 MeV protons, respectively, analyzing each specific type of chromosome aberration (Table 3). While these RBE values did not show any significant differences between the irradiation modes (P values between 0.62 and 0.88 in experiment I and P values between 0.23 and 0.62 in experiment II), the ratio of the RBE of pulsed and continuous 20 MeV protons in producing dicentrics, centric rings and excess acentrics scored together showed a significant difference of 0.93 (0.88; 0.99), with slightly less effectiveness for the pulsed mode.

These present findings can only be compared with our recent results obtained by analyzing micronuclei in HeLa cells with a modal number of 62 chromosomes (*5*) as well as in keratinocytes of an EpiDermFT^{TM} 3D reconstructed human skin tissue with 46 chromosomes (*6*). In both irradiation experiments with 20 MeV protons, the mean ratio of the MN yields did not show any significant difference between the pulsed and continuous irradiation modes, i.e. 1.04 (0.90; 1.18) in HeLa cells and 0.93 (0.37; 1.48) in keratinocytes. This result was consistent with the corresponding RBE values of pulsed and continuous 20 MeV protons relative to 70 kV X rays, which did not differ significantly from one another (z-test statistics, $P > 0.05$). Thus, in principle, it can be stated that analyzing only one specified aberration type in a cell induced by 20 MeV protons, i.e. micronuclei, dicentrics, centric rings or excess acentrics, no significant differences between pulsed and continuous irradiation modes could be observed, even if different cell types have been used. However, this finding does not mean that the comparisons of individual chromosomal alterations are inconsistent with the combined comparison but simply that the sample size is smaller with the consequence that the analysis has less statistical power to reveal a difference. In addition, it should be taken into consideration that micronuclei, which can be regarded mainly as equivalents of acentrics, are analyzed in the first interphase postirradiation (daughter cell), whereas acentrics are already scored in the first metaphase postirradiation (parent cell). Therefore, it cannot be excluded that the observed MN data may be influenced by a disparity in the ratio of micronuclei per daughter cell and acentric fragments per parent cell, which would be expected based on theoretical considerations described by Savage (*25*). Furthermore, the slope of the dose–response curve for micronuclei determined for 70 kV X rays as reference can be influenced by the mechanisms of MN formation and MN content, because the relative proportion of acentrics in independent micronuclei may decrease with increasing numbers of acentrics. In the present study, an influence can also be seen in the slopes of the dose–response curves for 70 kV X rays obtained for the specified aberration types regarding the different dose ranges analyzed up to 4 and 8 Gy. There are clear differences in the linear coefficients (smaller for the 8-Gy fit) and the quadratic coefficients β (larger for the 8-Gy fit) of the two fitted X-ray curves (Table 3). In general, since the fitted coefficients depend on where the data are truncated, the present findings indicate that linear-quadratic may generally not be a good fit in the dose range up to 8 Gy. Such an assumption seems to be justified because the fitted curve for 4 Gy greatly underestimates the actual aberration yield determined for 8 Gy.

In summary, from our cell and tissue experiments so far, there is evidence for a different radiobiology with the pulsed irradiation mode that would characterize protons generated from an advanced laser technology. Since our proton pulses have been focused to beam spot of approximately 100 × 100 μm^2, the same ion density could be achieved as expected by the laser-driven pulses for radiotherapy of malignant tumors in the future. To evaluate the potential of such high-intensity ion pulses *in vivo*, further radiobiological experiments should be performed. For example, since both analyzed biological end points (chromosome aberrations and micronuclei) are thought to develop from pairwise interaction of DNA DSBs initially formed close in space and/or time, they can in principle be used to investigate not only the temporal but also the spatial aspect of the irradiation mode. In our laboratory, relevant experiments are in progress to investigate the influence of different spot application modes on the effectiveness of 20 MeV protons in producing micronuclei and chromosome aberrations. Using an additional irradiation experiment with 55 MeV carbon ions, the findings for the low-LET 20 MeV protons can also be compared with a high-LET radiation quality.

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