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The influence of fractionated radiation on proliferation, cell cycle and apoptosis of normal human dermal fibroblasts

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Abstract The aim of the study was to investigate the changes in proliferation rate, cell cycle and apoptosis of normal skin fibroblasts during fractionated irradiation with a fraction dose of 2 Gy. Fibroblasts were irradiated 5 days per week for 12 days using gamma irradiation. Twenty four hours after each fraction, and for three days after finishing experiment the cells were harvested, fixed, and BrdUrd labelling index (BrdUrdLI), cell cycle and level of apoptosis and debris were assessed. It was found that fractionated irradiation caused disturbances in the proliferation rate and the cell cycle. Irradiation caused also constant, statistically significant increase in the number of G2M cells and level of apoptosis and debris, which was observed even during 3 days after irradiation. Data indicate non equal biological effect of each fraction dose. Block at G2/M phase suggests accumulation of sublethal damage and increased radiosensitivity, which was manifested by elevated level of cell death (apoptosis and debris).

Key words fibroblasts • fractionated irradiation • proliferation • radiosensitivity

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Introduction

The mathematical models developed to describe the response of tissue to fractionated irradiation, which are currently used at clinic, take it for granted that every single fraction produces the same biological effect [10, 11]. This approach is based on several assumptions. First of all it is believed that complete repair of sublethal damages occurs in the intervals between each dose fraction and thus each dose contributes equally to the overall effect of treatment. Also variation in radiosensitivity due to oxygenation, cell kinetics, redistribution of the cell in the cell cycle, are not considered in modelling tissue response after radiotherapy. However, it is well known that radiosensitivity of cells in different phases of the cell cycle is not the same and cell cycle disturbances induced by the initial fractions could potentially affect relative radiosensitivity. Recently a number of studies have been published, which do not support the assumption that each fraction produces an equal biological effect [1, 2, 6, 8]. Therefore, the present study was initiated to determine the effect of 10 equal fractions, magnitude of 2 Gy given *in vitro* during 12 days (5 days per week), on proliferation rate, cell cycle and level of apoptosis in normal human skin fibroblasts.

Material and methods

Cell culture

The normal human skin fibroblasts were used in this study. The strain GSH+/+ was derived from skin biopsy

of a healthy 9-year old boy in the Department of Oncology and Pathology, Karolinska Institutet, Sweden [3]. The cells were cultured in a DMEM medium supplemented with 15% foetal calf serum (Biochrom), amphothericine (2.5 μg/ml), gentamycine (10 μg/ml), sodium pyruvate (1 μM/ml, Biochrom), and HEPES (10 μM/ml, Biochrom) in a 5% CO_2 incubator at 37°C. Culture medium was exchanged once a week.

Fractionated irradiation

The experiments were performed three times on 11th, 12th and 13th passages. Exponentially growing cells were trypsinized (1.5 ml of 0.05% trypsin/0.02% EDTA/flask) and seeded into a 25 cm^2 plastic flask at a concentration of 10–30,000 cells per flask in triplicate for each experimental point. Cells were irradiated 5 days per week for 12 days keeping constant interfractional interval equal to 24 hours. All flasks were irradiated at room temperature with 2 Gy of gamma rays using a Theratron 780 E (Theratronics, ^{60}Co, a dose rate of 1.04–1.13 Gy/min, SD ± 3%) and placed immediately in the ASSAB incubator. Twenty four hours after each fraction and for 3 days after finishing of irradiation, 100 μM of BrdUrd was added to flasks designed for cell proliferation analysis. Cells were again placed in the incubator. After 1 hour of incubation at 37°C, the flasks were washed twice with PBS and the cells were trypsinized with 0.05% trypsin and 0.02 % EDTA for 5 min. Following washing with PBS, the cells were fixed in 70% ethanol and prepared for flow cytometric analysis.

BrdUrd labelling index

Cells fixed in 70% ethanol were digested into nuclei at 37°C with 0.4 mg/ml pepsin (Sigma Chemicals) in 0.1 M HCl for 20 min. The nuclei suspension was filtered through a 35 μm nylon mesh and centrifuged at 2000 rpm for 5 min. The pellet was resuspended in 2 M HCl for 12 min to partially denaturate the DNA. After two washes in phosphate-buffered saline (PBS), the pellet was incubated in PBS containing 0.5% normal goat serum (NGS) (Sigma Chemicals), 0.5% Tween 20 (Sigma Chemicals) and a mouse-derived anti-BrdUrd monoclonal antibody (DAKO). After 1 hour, the nuclei suspension was washed in PBS and suspended in PBS/NGS/Tween containing goat anti-mouse IgG FITC conjugate (DAKO) for 1 hour. After two further washes in PBS, the suspension was stained for total DNA with PBS containing 10 μg/ml propidium iodide (PI).

Flow-cytometric data analysis

The stained preparations were analysed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA, USA). Doublets and clumps were excluded from the stained preparations by gating on a bivariate distribution of the red peak *vs*. area signal. Excitation of the FITC-labelled cells and the DNA-associated PI was accomplished with an argon-ion laser tuned to 488 nm and operated using 15 mW in the standard FACScan configuration. At least 20,000 events were collected in each specimen. A threshold for the debris (smallest DNA fragments) was set at 15. The BrdUrdLI was calculated as the percentage of nuclei which incorporated BrdUrd. The amount of cells at cell cycle phases as well as level of apoptosis and debris were calculated using the ModFit LT programme for the cell cycle analysis (ModFit LT, Verify, Software House, Inc., USA). Apoptotic cells were identified as the object with a fractional DNA content which was not less than 20% of the 2n DNA content. Cell death was calculated as a sum of apoptosis and debris.

Statistical analysis

The statistical analysis of data was performed with the use of T-test for independent samples (STATISTICA v. 5, 1997). The level of significance was set at $P < 0.05$.

Results

Control samples (unirradiated) were collected for the first three days of the experiments. All investigated parameters in the control group were assessed in the same manner as samples after irradiation. Control values were assumed as 100% and all experimental values were expressed as the percentage of control. Exposition of fibroblasts to fractionated irradiation caused changes in all the examined parameters. BrdUrdLI decreased after a single 2 Gy dose below 40% of control value (Fig. 1a). After the second fraction, BrdUrdLI dropped to 10% and then until the 5th day, an increase in the percentage of BrdUrd labelling cells could be observed. However, during the next few days proliferation rate was decreasing, reaching 30% level on the 10th day. After finishing the irradiation, BrdUrdLI started to increase, but even after the 3rd day it did not reach the control value. A similar pattern was noticed in case of S-phase fraction (Fig. 1b). On the 2nd and 3rd days after irradiation, the percentage of cells in S-phase dropped to about 30% of control value and this decrease was statistically significant ($P < 0.05$). Later, on the 4th day, the value of this parameter increased to almost 90% of control value and during the next two days again a transient decrease in the number of SPF (S-phase fraction) cells was observed (40% of control value). After finishing the irradiation, the percentage of SPF cells started to increase. The changes in G0/G1 cells during fractionated irradiation were not so profound as in other investigated cell cycle phases. After the first 4 fractions, transient accumulation of G0/G1 cells of about 10% above control value was observed (Fig. 1c). Since the 5th day from the beginning to the end of irradiation the means of G0/G1 cell were close to control. However, the numbers decreased during the three days after finishing the experiment. From the beginning of fractionated irradiation, the mean number of G2/M cells increased significantly to about 250% of control value at the end of the experiment (Fig. 1d). This

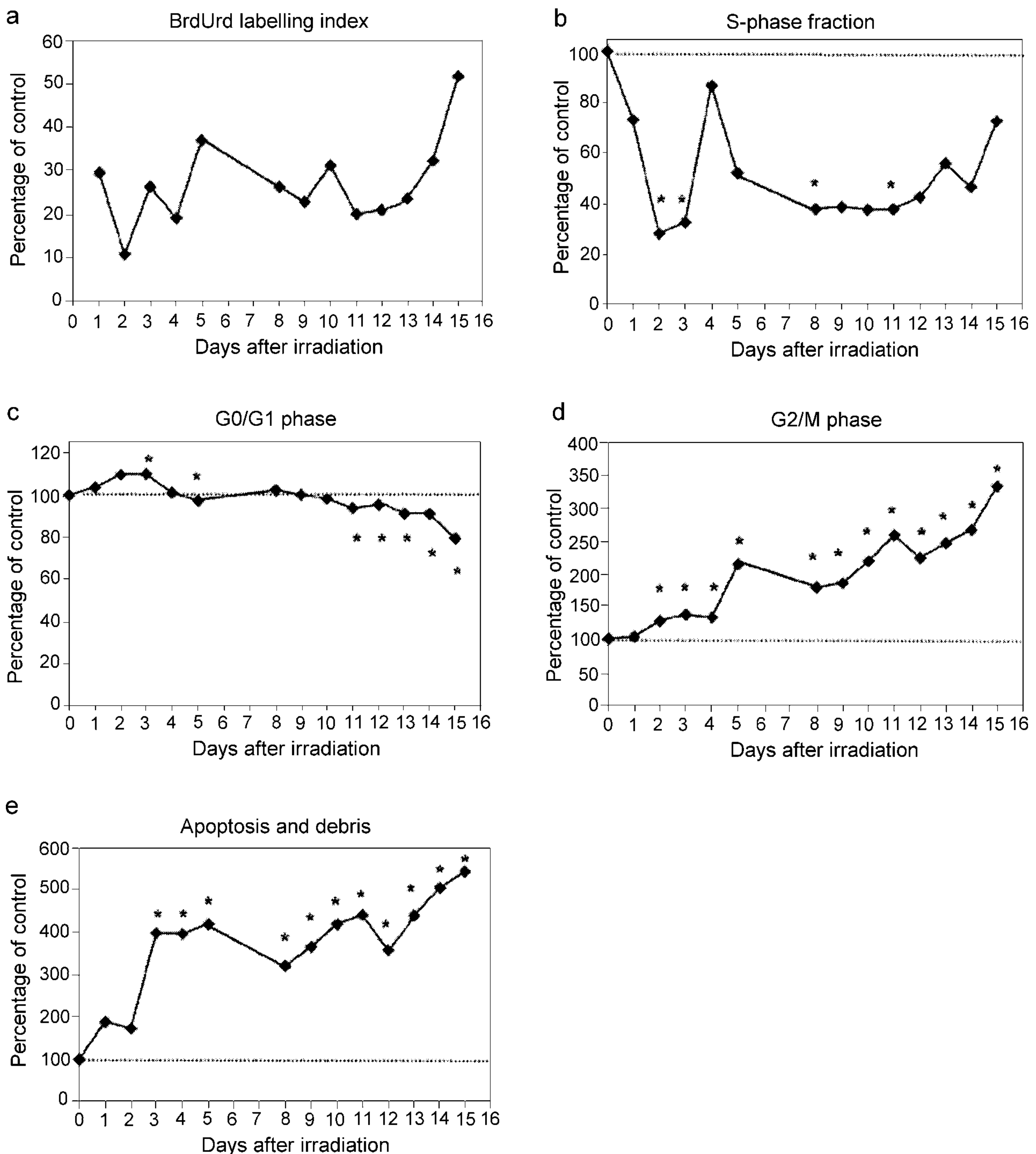

Fig. 1. Changes in the BrdUrdLI (a), cell cycle distribution (b,c,d) and level of apoptosis and debris (e) of normal human skin fibroblasts during daily irradiation with 2 Gy (total dose 20 Gy). Experimental points are the means of three experiments. An asterisk indicates the statistically significant ($P < 0.05$) differences between the investigated group and the group after the first dose of 2 Gy.

increase was still observed even during 3 days after finishing the irradiation. Debris are small DNA fragments and can represent late stages of apoptosis, therefore they were included in the analysis. Level of debris and apoptosis were analysed together. On the 4th day of the experiment a statistically significant increase of the level of apoptosis and debris to about 400% of control value was observed (Fig. 1e). Transient decrease of their number was seen on the 8th day, followed by a constant increase of apoptosis and debris lasting to the end of the experiment and even 3 days later.

Discussion

Progression of the mammalian cells through the cell cycle is a tightly regulated process. Progression of the cell to the next cycle is dependent on successful completion of the previous cell cycle event. The failure to complete a previous cell cycle event can lead to the delay until the previous cell cycle event is completed. In mammalian cells, induced by radiation sublethal damage, for example, unrejoined DNA breaks, might be a signal for G2-phase delay. In our study we have

observed a constant accumulation of cells in G2/M phase and an increased level of apoptosis and debris during fractionated irradiation, which was still seen for 3 days after finishing of irradiation. The block of cells in G2/M phase suggests accumulation of sublethal damages and hence increased radiosensitivity manifested by a higher level of apoptosis and debris. Therefore, assumption of full repair of sublethal damage and recovery within 24 h break between fractions in fractionated radiotherapy is not valid. The data contradict the assumptions of equal effect per fraction. Similar effect was observed by others authors. Aruga *et al.* [1] observed a change in the cell cycle distribution of SQ-5 cells during daily 2 Gy irradiations. The authors showed prominent G2/M accumulation from the 7th day and increased radiosensitivity (assessed by surviving fraction at 2 Gy) from the 10th day of irradiation. The increase of radiosensitivity with irradiation (accumulated dose) was also reported by Durand [2]. The author performed experiments with V79-171 cell line grown as spheroids and showed that the cell ability to accumulate or/and repair of sublethal damage decreased with time of irradiation and accumulated dose what was the consequence of increased radiosensitivity. Also experiments performed by Scott *et al.* [8] did not support the assumption of equal biological effect of each dose. Results obtained using three prostate cancer cell lines indicate that cell survival after one dose of 2 Gy cannot be used to predict response after 5 daily fractions with 2 Gy. However, the data published so far are not consistent. Suzuki *et al.* [9] investigated cell survival after fractionated irradiation of four human tumour cell lines and one normal fibroblast line. In tumour cell lines, at each successive dose the survival was exponentially decreasing, but when normal human fibroblasts were exposed to daily irradiation cell survival curve after the 3rd dose started to bend upward. This suggests that the assumption of equal cell killing rate by each dose may be not applicable to all cell lines. Schwartz *et al.* [7] when examining 10 human tumour cell lines observed large cell line-dependent variations in the length of the G2-phase block. There was highly significant inverse correlation between the length of G2-phase delay after exposure and the frequency of induced unrejoined chromosome breaks suggesting the failure to repair damage due to premature exit from G2 phase. However, the authors did not show any significant relationship between the length of G2-phase delay and inherent radiation sensitivity, suggesting that unrejoined chromosome breaks probably represent only a minor fraction of induced lethal lesions in normal repair proficient mammalian cells. Also experiments performed on animal models are not consistent. The data obtained by Joiner *et al*. [4] state that there is no indication of unequal effect per fraction in mouse skin irradiated with 2.5 Gy doses given 2 to 20 times (with an 8 h interval). On the other hand, Rezvani *et al.* [6] showed contradictory results on rat skin. Daily irradiation of rats with a 2 Gy dose gave different results than values predicted by the model assuming equal effect per fraction. Also Pabst [5] reported unequal effect per fraction based on mouse oral mucosa.

The until now published data of the biological effect of 2 Gy fraction are still not consistent. However, some published data should affect the current thinking about dose fraction relationship because unequal biological effect of each fraction was indicated. Therefore, new mathematical models for prediction of unequal effect of fractionated radiotherapy should be developed.

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