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Nucleosomes Indicate the in vitro Radiosensitivity of Irradiated Bronchoepithelial and Lung Cancer Cells

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Key Words
Apoptosis · Cell cultures · Cell death · Irradiation · Lung cancer · Nucleosomes · Radiosensitivity

Abstract
Nucleosomes, which are typical cell death products, are elevated in the serum of cancer patients and are known to rapidly increase during radiotherapy. As both normal and malignant cells are damaged by irradiation, we investigated to which extent both cell types contribute to the release of nucleosomes. We cultured monolayers of normal bronchoepithelial lung cells (BEAS-2B, n = 18) and epithelial lung cancer cells (EPLC, n = 18), exposed them to various radiation doses (0, 10 and 30 Gy) and observed them for 5 days. Culture medium was changed every 24 h. Subsequently, nucleosomes were determined in the supernatant by the Cell Death Detection-ELISA^{plus} (Roche Diagnostics). Additionally, the cell number was estimated after harvesting the cells in a second preparation. After 5 days, the cell number of BEAS-2B cultures in the irradiated groups (10 Gy: median 0.03 × 10^6 cells/culture, range 0.02–0.08 × 10^6 cells/culture; 30 Gy: median 0.08 × 10^6 cells/culture, range 0.02–0.14 × 10^6 cells/culture) decreased significantly (10 Gy: p = 0.005; 30 Gy p = 0.005; Wilcoxon test) compared to the non-irradiated control group (median 4.81 × 10^6 cells/culture, range 1.50–9.54 × 10^6 cells/culture). Consistently, nucleosomes remained low in the supernatant of non-irradiated BEAS-2B. However, at 10 Gy, BEAS-2B showed a considerably increasing release of nucleosomes, with a maximum at 72 h (before irradiation: 0.24 × 10^3 arbitrary units, AU, range 0.13–4.09 × 10^3 AU, and after 72 h: 1.94 × 10^3 AU, range 0.11–5.70 × 10^3 AU). At 30 Gy, the release was even stronger, reaching the maximum earlier (at 48 h, 11.09 × 10^3 AU, range 6.89–18.28 × 10^3 AU). In non-irradiated EPLC, nucleosomes constantly increased slightly. At 10 Gy, we observed a considerably higher release of nucleosomes in EPLC, with a maximum at 72 h (before irradiation: 2.79 × 10^3 AU, range 2.42–3.80 × 10^3 AU, and after 72 h: 7.16 × 10^3 AU, range 4.30–16.20 × 10^3 AU), which was more than 3.5 times higher than in BEAS-2B. At 30 Gy, the maximum (6.22 × 10^3 AU, range 5.13–9.71 × 10^3 AU) was observed already after 24 h. These results indicate that normal bronchoepithelial and malignant lung cancer cells contribute to the release of nucleosomes during irradiation in a dose- and time-dependent manner with cancer cells having a stronger impact at low doses.

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Introduction

Ionizing radiation is established as an essential part of the therapy of many malignant diseases. Similar to chemotherapy, it affects mainly fast proliferating cells and tissues with high turnover, such as tumor cells but also stem cells, lymphocytes and epithelial cells [1]. Therefore radiotherapy protocols aim both to reduce tumor size and to limit potential side effects.

It is well known that radiation induces cell death already at low doses. Although still controversial, apoptosis seems to play an important role in many cell types [2, 3]. However, oncosis and other forms of cell death may contribute to cell loss particularly at high doses [4].

The effects of radiotherapy – single- and double-strand DNA breaks – are mediated directly, by activation of the CD95/Apo1/Fas receptor system and a proteolytic cascade of caspases, or by stimulation of the mitochondria, or by direct damage of the DNA. Often, p53 is upregulated leading to an arrest of the cell cycle and activation of repair systems [5–8]. In case of extended DNA damage or insufficient repair mechanisms, the cells will die by apoptosis or other forms according to the cell type, their energy level and the severity of the lesion [4]. These factors may also influence the delay from the lethal hit to the cell demise.

For the monitoring of the therapeutic efficacy, the quantification of cell death can be applied directly by microscopy or by measurement of cell death products both in tumor tissue and in the circulating blood. The most convenient und less invasive method is the detection of the final products in serum or plasma. If performed during the therapy, it may provide an actual image of the cell death kinetics.

Typical products of cell death are nucleosomes – complexes which are formed from a core particle of several histone components and DNA on the outside [9, 10]. During cell death, endonucleases preferentially bind to the easy accessible linking sites between the nucleosomes and cut the chromatin into multiple mono- and oligonucleosomes [11, 12]. In cases of enhanced cell death, e.g. during radiotherapy, they are also released into the circulation and can be detected in elevated amounts in serum or plasma.

Former studies on serial measurements of nucleosomes in the serum of cancer patients during radiotherapy revealed a steep increase in the nucleosomal number 1–3 days after the start of treatment followed by a decline that showed interindividual differences in the time delay and the rate of decrease. Patients with a rapid and complete decline in the nucleosome number were found to have an efficient reduction in the tumor volume; those with constantly high values or a delayed and incomplete decrease were correlated with a poor response to therapy and progressive disease [13].

As cells of various origin are degraded in (or also outside) the radiation field, it was highly interesting to explore to which extent dying normal and tumor cells contribute to the quantity of circulating nucleosomes and whether the increase in nucleosomes during radiotherapy reflects the extent of tumor cell apoptosis.

Addressing these questions, we investigated in vitro the sensitivity of both cell types to radiation by exposing normal epithelial cells and squamous lung cancer cells to different doses of ionizing radiation. After this single hit, the number of nucleosomes in the supernatant of the cell cultures was quantified daily for 5 days in order to gain insight in the dose- and time-dependent cell death processes after irradiation.

Materials and Methods

Culture Techniques

BEAS-2B is an immortalised human bronchial epithelium cell line and was kindly provided by Prof. D.A. Gillissen, Department of Pneumology, University of Bonn, Germany. The EPLC 32M1 is an adherent-growing non-small cell lung cancer line that was derived from a human squamous-cell carcinoma [14] and was kindly provided by Dr. G. Jaques, University of Marburg, Germany.

Both cell lines were grown until they were confluent in multi-well dishes (Falcon, Lincoln Park, N.J., USA) using RPMI 1640 culture medium (Seromed, Heidelberg, Germany), supplemented with 10% new born calf serum, 2 m*M L*-glutamine and antibiotics (all Lifetechnologies, Eggenstein, Germany) in a humidified 5% CO_2 atmosphere.

Irradiation of BEAS-2B and EPLC

Confluent BEAS-2B and EPLC were irradiated on a single occasion with 0, 10 or 30 Gy in the Department of Radiotherapy, Ludwig-Maximilians-University Munich, using the linear accelerator Sli 15 (Elekta, Hamburg, Germany) at a high dose of 4 Gy/min. The sham-irradiated group (0 Gy) was subjected to the same procedure without irradiation. A total of 18 BEAS-2B cultures and 18 EPLC were used in the experiments.

Measurement of Nucleosomes

Immediately before and at 24-hour intervals (up to 96 h after irradiation), the supernatants of the BEAS-2B and EPLC cultures were changed, aliquoted, frozen at –20 °C and transferred to the Institute of Clinical Chemistry of the Ludwig-Maximilians-University. The concentration of the nucleosomes was measured using a modified version of the Cell Death Detection^{plus}-ELISA (Roche Diagnostics, Germany) [15].

The Cell Death Detection^{plus}-ELISA is based on a quantitative sandwich enzyme immunoassay principle: monoclonal mouse anti-

bodies directed against DNA (ss- and ds-DNA) and histones (H1, H2A, H2B, H3 and H4) detect specifically mono- and oligonucleosomes.

The supernatant was placed in a streptavidin-coated microtiter plate and incubated with a mixture of anti-histone-biotin, anti-DNA-peroxidase and incubation buffer (1% BSA, 0.5% Tween, 1 m*M* EDTA in PBS) for 2 h. The antibodies bound to the histone and DNA component of the nucleosomes, respectively, and fixed the immunocomplexes to the microtiter plate by streptavidin-biotin interaction. After the incubation period, unbound antibodies were removed by a washing step. The retained peroxidase-linked complexes were incubated with 2,2′-azino-di(3-ethylbenzthiazolin-sulfonate). The substrate reacted with the peroxidase resulting in color development proportional to the amount of nucleosomes captured in the antibody-sandwich. Quantification of the nucleosomes was performed by photometrical determination of the absorbance at 405 nm against substrate solution as blank (reference wavelength 492 nm).

Measurement of Cell Number

BEAS-2B cells were grown in a second culture, and cells were harvested daily at the time the supernatant was changed in the first culture. Viable cells were identified using a trypan blue staining and counted in a Neubauer counting chamber.

Statistics

The values in the text are expressed as medians and ranges. The increases and decreases in the time-dependent kinetic investigations are calculated between the several medians in percent changes. The Wilcoxon test was used (1) to compare the experimental groups (control, and 10 and 30 Gy), (2) to compare the values during irradiation with the pre-irradiation value and (3) to compare the values of two specific groups (BEAS-2B and EPLC) which were treated with the same doses (10 and 30 Gy). A p value <0.05 was considered statistically significant.

The values in the figures are presented as medians, and additionally as 25th and 75th percentiles (bars).

Results

During the observation period, the cell number of non-irradiated BEAS-2B increased slightly (median initially: 1.44×10^6 cells/culture, range $1.07–1.85 \times 10^6$ cells/culture; after 120 h: $4.81 \pm 3.18 \times 10^6$ cells/culture, range $1.50–9.54 \times 10^6$ cells/culture; increase of 235%). In BEAS-2B irradiated with 10 Gy, the cell number decreased rapidly, reaching very low levels already after 72 h (0.27×10^6 cells/culture, range $0.05–0.39 \times 10^6$ cells/culture) corresponding with a decrease of 81% ($p = 0.031$). In BEAS-2B irradiated with 30 Gy, the reduction in the cell number started already after 24 h, and after 72 h it reached 0.19×10^6 cells/culture (range $0.09–0.31 \times 10^6$ cells/culture) corresponding to a decrease of 87% ($p = 0.031$). The decline in the values continued during the following days. The lowest numbers were obtained after 120 h (10 Gy: 0.03×10^6 cells/culture, range $0.02–0.08 \times 10^6$ cells/culture, decrease of 98%; 30 Gy: 0.08×10^6 cells/culture, range $0.02–0.14 \times 10^6$ cells/culture, decrease of 95%), being highly significant (10 Gy: $p = 0.005$, 30 Gy: $p = 0.005$) compared with the non-irradiated control group (4.81×10^6 cells/culture, range $1.50–9.54 \times 10^6$ cells/culture) (fig. 1).

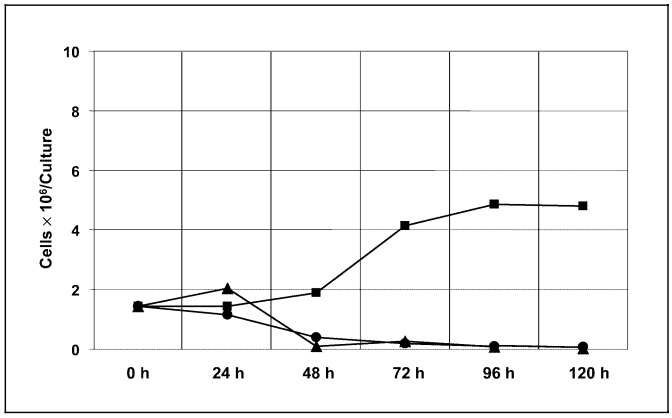
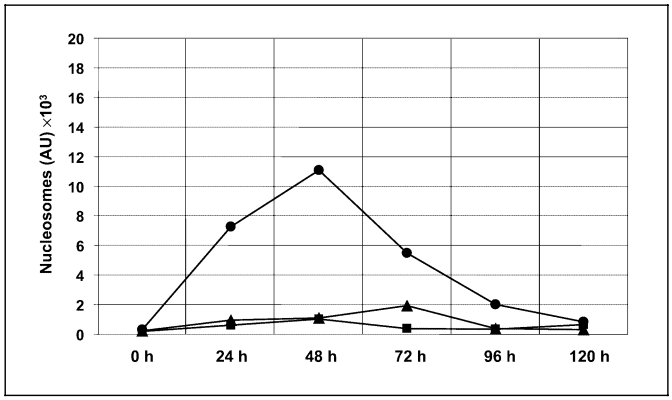


Fig. 1. Kinetics of the cell number of normal BEAS-2B (medians) without irradiation (■) (control group) and after irradiation with 10 Gy (▲) and 30 Gy (●). Cells were harvested before irradiation (0 h), and 24, 48, 72, 96 and 120 h after single-dose irradiation.

Also, nucleosomes increased constantly and slightly in the supernatant of non-irradiated BEAS-2B (initially: 0.18×10^3 arbitrary units (AU), range $0.07–0.47 \times 10^3$ AU; after 120 h: 0.63×10^3 AU, range $0.44–2.17 \times 10^3$ AU; increase of 246%). However, after irradiation with 10 Gy, BEAS-2B increased rapidly reaching a maximum at 72 h (before irradiation: 0.24×10^3 AU, range $0.13–4.09 \times 10^3$ AU; after 72 h: 1.94×10^3 AU, range $0.11–5.70 \times 10^3$ AU; increase of 693%; $p = 0.219$) and after 120 h almost dropped to values before treatment (0.30×10^3 AU, range $0.07–0.55 \times 10^3$ AU; decrease of 537%). At 30 Gy, the release was even stronger, with an earlier peak already after 48 h (before irradiation: 0.29×10^3 AU, range $0.14–2.68 \times 10^3$ AU; after 48 h: 11.09×10^3 AU, range $6.89–18.28 \times 10^3$ AU; increase of 3,763%; $p = 0.031$) followed by a decline to low levels after 120 h (0.82×10^3 AU, range $0.24–1.36 \times 10^3$ AU; decrease of 1,245%). For all time points investigated immediately after irradiation (24–96 h), the differences between the sham group and the group irradiated with 30 Gy were highly significant ($p < 0.05$) (fig. 2).

In non-irradiated EPLC, the levels of nucleosomes increased constantly (initially: 2.48×10^3 AU, range $1.58–4.83 \times 10^3$ AU; after 96 h: 5.14×10^3 AU, range $2.80–11.82 \times 10^3$ AU; increase of 107%). In EPLC irra-



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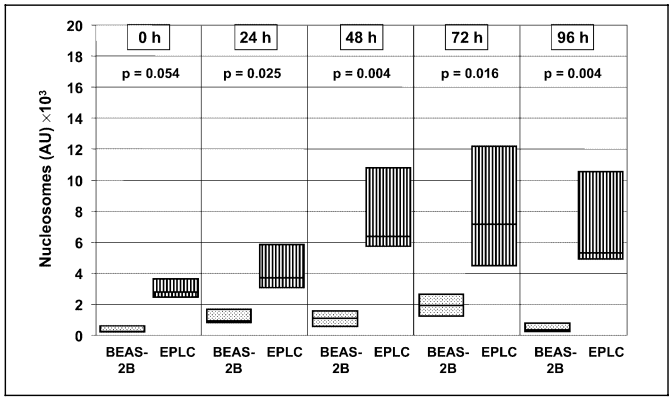

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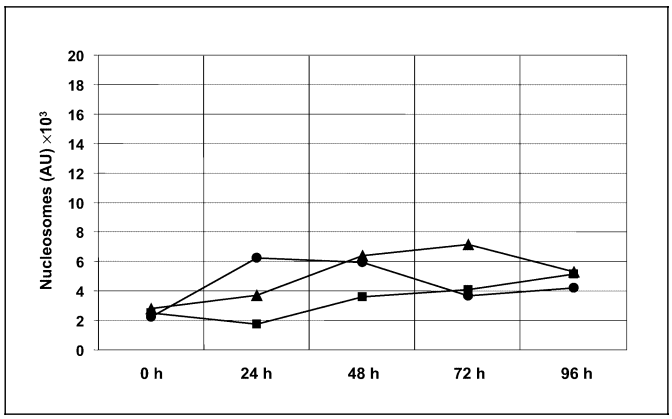

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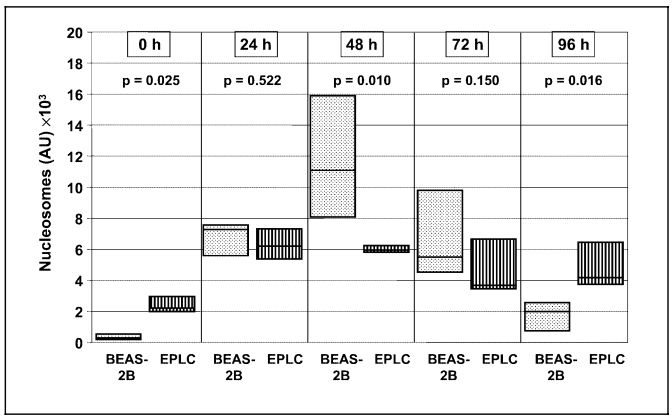

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Fig. 2. Release of nucleosomes after irradiation of normal BEAS-2B (medians) without irradiation (■) (control group) and after irradiation with 10 Gy (▲) and 30 Gy (●). Nucleosomes were measured in the culture supernatant before irradiation (0 h), and 24, 48, 72, 96 and 120 h after single-dose irradiation.

Fig. 3. Release of nucleosomes after irradiation of EPLC (medians) without irradiation (■) (control group) and after irradiation with 10 Gy (▲) and 30 Gy (●). Nucleosomes were measured in the culture supernatant before irradiation (0 h), and 24, 48, 72 and 96 h after single-dose irradiation.

Fig. 4. Release of nucleosomes after irradiation of BEAS-2B (▨) and EPLC (▥) with 10 Gy. Nucleosomes were measured in the culture supernatant before irradiation (0 h), and 24, 48, 72 and 96 h after single-dose irradiation. Medians, and 25th and 75th percentiles are shown. p values indicating differences between the groups were calculated by Wilcoxon test.

Fig. 5. Release of nucleosomes after irradiation of BEAS-2B (▨) and EPLC (▥) with 30 Gy. Nucleosomes were measured in the culture supernatant before irradiation (0 h), and 24, 48, 72 and 96 h after single-dose irradiation. Medians, and 25th and 75th percentiles are shown. p values indicating differences between the groups were calculated by Wilcoxon test.

diated with 10 Gy, we observed a considerably higher release of nucleosomes with the maximum again at 72 h (before irradiation: 2.79 × 10^3 AU, range 2.42–3.80 × 10^3 AU; after 72 h: 7.16 × 10^3 AU, range 4.30–16.20 × 10^3 AU; increase of 157%; p = 0.031). After 96 h, the values were comparable with the non-irradiated group (5.31 × 10^3 AU, range 4.71–13.76 × 10^3 AU). In EPLC treated with 30 Gy, the maximum (6.22 × 10^3 AU, range 5.13–9.71 × 10^3 AU; increase of 180%; p = 0.031) was observed already after 24 h followed by a slow decline (after 96 h: 4.19 × 10^3 AU, range 3.29–9.27 × 10^3 AU) (fig. 3).

Comparing BEAS-2B and EPLC which were irradiated with 10 Gy, EPLC started before therapy from a higher level (EPLC: 2.79 × 10^3 AU, range 2.42–3.80 × 10^3 AU; BEAS-2B: 0.24 × 10^3 AU, range 0.13–4.09 × 10^3 AU; p = 0.054) and exhibited a stronger increase. After 72 h, EPLC levels (7.16 × 10^3 AU, range 4.30–16.20 × 10^3 AU) were more than 3.5 times higher than BEAS-2B levels (1.94 × 10^3 AU, range 0.11–5.70 × 10^3 AU; p = 0.016). The subsequent decline after 96 h was less pronounced in EPLC than in BEAS-2B (EPLC: 5.31 × 10^3 AU, range 4.71–13.76 × 10^3 AU; BEAS-2B: 0.37 × 10^3 AU, range 0.22–1.04 × 10^3 AU; p = 0.004) (fig. 4).

After irradiation with 30 Gy, both cell lines showed a rapid and strong increase. The release of nucleosomes of BEAS-2B (with its maximum after 48 h) was significantly higher than that of EPLC (BEAS-2B: 11.09 × 10^3 AU, range 6.89–18.28 × 10^3 AU; EPLC: 5.95 × 10^3 AU, range 4.31–8.35 × 10^3 AU; p = 0.010). However, nucleosome values from EPLC reached their peak already after 24 h. Once again, the following decrease in the values was more pronounced for BEAS-2B than for EPLC after 96 h (BEA-2B: 1.99 × 10^3 AU, range 0.72–3.93 × 10^3 AU; EPLC: 4.19 × 10^3 AU, range 3.29–9.27 × 10^3 AU; p = 0.016) (fig. 5).

Discussion

The induction of cell death in malignant cells is the basis of the antitumor effect of ionizing radiation. Besides cancer cells, normal epithelial cells, lymphocytes and stem cells are also damaged and killed leading potentially to complications during therapy [1]. In order to reduce tumor size and to limit side effects, several protocols have been developed optimizing dosage and frequency of radiotherapy. The monitoring of the response to radiotherapy is mainly performed by imaging techniques. However, for early information and for serial measurements, serum biomarkers indicating the extent of cell death would be valuable for predicting response to therapy and treatment monitoring. In a recent study in 16 patients with various cancers, we described that the kinetics of nucleosomes, during the initial phase of radiotherapy, correlated with the clinical outcome that was estimated by imaging techniques later on [13]. As these nucleosomes could derive from various origins, we investigated here in vitro the dose- and time-dependent release of nucleosomes in normal and malignant bronchoepithelial cells of the lung in order to estimate the contribution of both cell types to the nucleosome values obtained.

The effective damage of the cells by irradiation was shown by the rapid and complete reduction in the cell number in BEAS-2B irradiated with 10 and 30 Gy. In contrast, non-irradiated BEAS-2B exhibited a constantly increasing cell number. In EPLC the cell number was quantified only 96 h after irradiation, which was comparable to the results of BEAS-2B (data not shown).

It is likely that the increasing amount of untreated cells is not only due to proliferation but also to cell death processes. Strong evidence for this hypothesis is given by the values of nucleosomes which reflect the extent of cell death. They increased in parallel and in a comparable magnitude with the cell number in BEAS-2B. A similar phenomenon was observed in EPLC, but nucleosome values started initially from a higher level. This indicates a higher spontaneous turnover in malignant cells and correlates with in vivo findings of elevated spontaneous nucleosome values in the serum of patients with malignant diseases [13, 16]. Even if the cell number is decreasing rapidly in irradiated cell lines, a mixture of degradation and proliferation might still occur only with a stronger prevalence of cell death.

Nucleosomes increased in both BEAS-2B and EPLC during irradiation in a dose- and time-dependent manner. As nucleosomes are intracellular components released during cell death, their quantity reflects the number of cells dying spontaneously or after lethal damage by irradiation. Generally, the cell killing effect of radiation was observed in both BEAS-2B and EPLC as a considerable increase in nucleosomes after treatment with 10 and with 30 Gy.

A dosage of 30 Gy resulted in an early (after 24 to 48 h) and high maximum of nucleosomes. Because of the strong damage, many cells are probably killed immediately after exposure to irradiation and are not able to activate any cellular rescuing mechanisms. At 10 Gy, the maximum was observed later and less pronounced compared with the higher dosage in BEAS-2B. In this case, cells might still have the possibility to arrest their cell cycle and enable the repair of DNA damages. Only if they cannot be compensated appropriately, these cells will be determined to undergo cell death later on [17]. This hypothesis is supported by the well-known upregulation of p53 after radiation-induced DNA damages. p53 is involved in cell cycle arrest as well as in the regulation of apoptosis. In case of extended damage, or dysfunctional or insufficient repair systems, p53 induces apoptotic cell death by activation of BAX, BAD, and the mitochondrial release of cytochrome C [5–8]. Since tumor cells frequently exhibit a high spontaneous instability of DNA and their repair mechanisms are often dysfunctional due to specific mutations, they are likely to present a damaged DNA which cannot be eliminated adequately [17, 18]. This explains the high spontaneous rate of cell death in malignant cells as well as their high sensitivity to radio- and chemotherapy. Consistently, in EPLC we found a strong effect of irradiation already at 10 Gy, which could not be enhanced by 30 Gy.

Comparing BEAS-2B and EPLC, the maximum obtained after irradiation with 10 Gy was significantly higher in EPLC, underlining the high sensitivity of tumor cells to irradiation. After irradiation with 30 Gy, both cell lines demonstrated a rapid and strong increase, with BEAS-2B

reaching higher maximum levels. Subsequently, the values dropped more significantly in BEAS-2B than in EPLC probably caused by the higher rate of spontaneous proliferation and cell death in EPLC.

The massive induction of apoptosis in HeLa Hep2 cells has been described earlier in an immunohistochemical study by Mirzaie-Joniani et al. [3]. Up to 60% of the cells irradiated with 5 and 15 Gy displayed apoptotic characteristics detected by TUNEL and M30 Cyto-DEATH technology. Doses below 2 Gy did not have any effect. The highest induction rate was seen after treatment with 5 Gy. Higher doses did not significantly raise apoptotic rates. The values increased already after 24 h and reached the peak after 168 h [3]. Unfortunately, the values between 72 h and 168 h after irradiation were not investigated.

In accordance with this study, we confirm that techniques measuring cell death characteristics are useful tools for the estimation of in vitro radiosensitivity in cell lines. Further, we demonstrated that nucleosomes reflect the extent of cell death induced by irradiation. Our results indicate that normal bronchoepithelial and malignant lung cancer cells contribute to the release of nucleosomes during irradiation in a dose- and time-dependent manner. At low doses, cancer cells had a clearly higher effect on the release of nucleosomes.

In vivo, the situation during radiotherapy is more complex: Normal and malignant cells integrated in tissue might react differently to irradiation possibly in relation to the linkage with adjacent cells. Therefore, further studies in whole organ cultures consisting of tumor cells, fibroblasts and normal bronchoepithelial cells might reveal new insights in tissue radiosensitivity [19, 20].

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