RADIATION RESEARCH **160**, 647–654 (2003)
0033-7587/03 $15.00

Effects of Single-Dose Irradiation on Bronchial Epithelium: A Comparison of BEAS 2B Cell Monolayers, Human Organ Cultures, and Goettinger Minipigs

A. Bergner,[a] J. Stief,[a] S. Holdenrieder,[b] P. Stieber,[b] W. Haimerl,[c] B. Passlick,[d] A. Waldschmidt,[a,e] J. Kummermehr,[e] F. Gamarra[a] and R. M. Huber[a,1]

[a] *Pneumology, Medizinische Klinik-Innenstadt,* [b] *Institute of Clinical Chemistry,* [c] *Department of Radiotherapy, and* [d] *Department of Surgery, Ludwig-Maximilians-University, Munich, Germany; and* [e] *Institute of Radiobiology, GSF—National Research Center for Environment and Health, Neuherberg, Germany*

Bergner, A., Stief, J., Holdenrieder, S., Stieber, P., Haimerl, W., Passlick, B., Waldschmidt, A., Kummermehr, J., Gamarra, F. and Huber, R. M. Effects of Single-Dose Irradiation on Bronchial Epithelium: A Comparison of BEAS 2B Cell Monolayers, Human Organ Cultures, and Goettinger Minipigs. ***Radiat. Res.*** **160, 647–654 (2003).**

To assess the effects of radiation on bronchial epithelium, BEAS 2B cells cultured as monolayers and human bronchial epithelium cultured as organ cultures were exposed to single doses of 0, 10 and 30 Gy. The lactate dehydrogenase in the supernatant of the BEAS 2B cells increased markedly 24 h after irradiation, whereas in the organ cultures only a minor increase was found after 48 h. The nucleosomes in the supernatant of the BEAS 2B cells showed a massive increase in response to irradiation, whereas in the organ cultures no change could be seen. The number of BEAS 2B cells was dramatically diminished after 96 h, whereas in the organ cultures a smaller decrease was observed no earlier than 21 days after irradiation. To assess the effects of brachytherapy in bronchial epithelium *in vivo*, brachytherapy with 30 Gy was performed in Goettinger minipigs, and histological sections of the bronchi were analyzed for morphological alterations and cell numbers. After 2 weeks, only slight cell damage was observable, and after 3 weeks, moderate morphological changes and decreased cell numbers were found. However, after 8 weeks, the epithelium had nearly regained its normal structure. We conclude that the bronchial epithelium has a remarkably high radioresistance and that organ cultures, but not monolayers of BEAS 2B cells, reflect the effects of radiation *in vivo*.

INTRODUCTION

In patients with inoperable lung cancer, brachytherapy is an effective and well-established palliative treatment (*1–3*). In single-application regimens, doses up to 40 Gy are used (*4*), while in fractionated regimens, two to four irradiations of 15–20 Gy each are performed (*3*, *5*). Common side effects include hemoptysis and cough after irradiation (*2*, *5*). Although the dose decreases substantially with distance from the radiation source, irradiation of normal tissue is not entirely avoided. Because of the anatomical structure of the airways, the bronchial epithelium—being close to the radiation source—suffers a high dose during brachytherapy. Surprisingly, the effects of radiation on the bronchial epithelium have been sparsely investigated. In a previous study, our group investigated the acute effects of radiation on organ cultures of human bronchial epithelium and found a remarkably high tolerance of the tissue up to a single dose of 75 Gy (*6*). The present study was aimed at investigating the effects of radiation on bronchial epithelium by comparing three different experimental models. Cells of the immortalized human cell line BEAS 2B represented monolayer cultures, human bronchial epithelium was grown as organ cultures, and Goettinger minipigs served as an *in vivo* model.

To grow organ cultures of human bronchial epithelium, biopsies of large airways or mucosa harvested from surgically removed lungs are cultured on agar-coated dishes immersed in medium. The organ cultures are coated by a differentiated epithelium on a basement membrane consisting of secretory, ciliated and basal cells, and they maintain their structural integrity, including beating cilia, for at least 3 weeks (*7–9*). Organ cultures have been used by our group to study the effects of radiation on normal epithelium (*6*) and to create a co-culture system of bronchial epithelium and cells of lung cancer cell lines to study the kinetics of tumor invasion (*10*) and 5-aminolevulinic acid-induced fluorescence (*11*). Studies of the bronchial epithelium are frequently performed using cell lines like BEAS 2B. The morphology of these cell lines is unlike the morphology found *in vivo*, lacking beating cilia, three-dimensional structure, and hierarchical organization. Data obtained with these cells therefore may not reflect the situation *in vivo*,

[1] Address for correspondence: Pneumology, Medizinische Klinik-Innenstadt, Ziemssenstr. 1, 80336 Munich, Germany; e-mail: huber@medinn.med.uni-muenchen.de.

since cell–cell contact has been found to decrease radiosensitivity in microcolonies (*12, 13*) and multicellular spheroids (*14*). To assess *in vivo* effects, we performed brachytherapy in Goettinger minipigs, mimicking the procedure used to treat humans. With this approach, we aimed at investigating whether BEAS 2B cells as monolayer cultures and organ cultures as three-dimensional systems are similar in their acute radiation response and which model more closely reflects the *in vivo* situation as assessed in minipigs.

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is released rapidly into the cell culture supernatant upon damage of the plasma membrane and has been used widely to quantify cell damage (*15–17*). The advantage of using LDH as a marker for cell injury is that it can be quantified repeatedly in the supernatant over time without disturbing the cell culture itself.

In eukaryotic cells, DNA forms complexes with several proteins known as nucleosomes (for review, see ref. *18*). During cell death, nucleosomes that have been cleaved by endonucleases are released into the serum of patients with benign and malignant diseases (*19–25*), and they can be detected using ELISA test systems (*26*). In patients with malignant diseases, the initiation of radiotherapy resulted in an initial increase in serum nucleosomes that subsequently decreased again. Low levels of nucleosomes in the course of treatment correlated with good clinical outcome (*23*). To test whether this parameter can be used to quantify cell death *in vitro*, we measured the levels of nucleosomes in the supernatants of BEAS 2B cells and organ cultures after irradiation.

In this paper, we present data confirming that the bronchial epithelium is remarkably resistant to single-dose irradiation. Organ cultures resemble the *in vivo* radioresistance observed in minipigs very closely, whereas BEAS 2B cells have a significantly different radiation response, being more radiosensitive and showing an earlier onset of damage. Furthermore, we demonstrate that the quantification of nucleosomes in the supernatant of normal tissue is a useful tool to assess radiation damage *in vitro.*

METHODS

Culture Techniques

Cells of the BEAS 2B cell line were kindly provided by Prof. D. A. Gillissen, Pneumology, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany. The cells were grown in multiwell dishes (Falcon, Lincoln Park, NJ) until they were confluent using RPMI 1640 culture medium (Seromed, Heidelberg, Germany) supplemented with 10% newborn calf serum, 2 m*M* L-glutamine and antibiotics (all Life Technologies, Eggenstein, Germany) in a humidified 95% air/5% CO_2 atmosphere.

To harvest tissue for the organ cultures, human bronchial rings were isolated after lung or lobe resection performed in the Asclepios-Fachklinik for Pneumology and Thoracic Surgery, Gauting, Munich. All procedures using human subjects had been approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich. The mucosa was removed from the bronchial rings and cut into pieces ~1–2 mm in diameter. The pieces were placed onto multiwell dishes base-coated with 0.75% agar noble (Difco Laboratories, Detroit, MI) to prevent them from sticking to the dish surface. The agar was dissolved in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, nonessential amino acids and antibiotics (all Life Technologies, Eggenstein, Germany). Supplemented bronchial epithelium cell medium (BEGM, PromoCell, Heidelberg, Germany) was added to each well, and the organ cultures were cultured by floating them in the medium for 14 days to ensure the development of a differentiated epithelium covering the whole surface of the organ culture (*7*).

Irradiation of BEAS 2B and Organ Cultures

Confluent BEAS 2B cells and 14- to 21-day-old organ cultures were irradiated 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 rate 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 140 organ cultures were used in the experiments.

Measurement of Lactate Dehydrogenase (LDH)

Immediately before and at 24-h intervals up to 96 h after irradiation, the supernatant of the BEAS 2B cultures and the organ cultures was withdrawn and transferred to the central laboratory of the Medizinische Klinik-Innenstadt, Ludwig-Maximilians-University, Munich. The concentration of LDH was measured using a Hitachi 917 photometer (Roche Diagnostics, Mannheim, Germany).

Measurement of Nucleosomes

For measuring the concentration of nucleosomes, 200 μl of the supernatant was frozen at −20°C and transferred to the Institute of Clinical Chemistry, Ludwig-Maximilians-University, Munich. The concentration of the nucleosomes was measured using the modified Cell Death Detection^{Plus}-ELISA [Boehringer, Mannheim, Germany; for details see ref. (*26*)].

Measurement of Cell Number

To obtain a single-cell suspension from the organ cultures, the cells were separated enzymatically with 1 mg/ml collagenase and 1.5 mg/ml protease (both Sigma, Deisenhofen, Germany) dissolved in BEGM. Viable cells were identified using trypan blue staining and counted in a Neubauer counting chamber.

Irradiation of Goettinger Minipigs

Nine Goettinger minipigs weighing 45–55 kg and aged 4 to 6 years were used. All procedures were performed in accordance with current animal welfare legislation (Bayerische Tierschutzgesetz) and had been approved by the local regulatory agency (Regierung von Oberbayern). The minipigs were given 0.1 mg/kg Atropin intramuscularly 10 min prior to the intravenous application of 10–20 ml Etomidate (Hypnomidat, Janssen, Neuss, Germany). Inhalation anesthesia was performed using 50–80% N_2O and 1–5% halothane. Brachytherapy was performed using an afterloading technique (Gamma Med II, Sauerwein, Haan, Germany) similar to the clinical protocol used in human oncology with an iridium-192 radiation source. Iridium-192 provided radiation at a high dose rate, although the actual dose rate depended on the age of the radiation source, varying at a distance of 1 cm from the source between 8 Gy/min initially and 4 Gy/min after 72 days. At this time, the iridium-192 had reached its half-life age and was replaced. A single dose of 30 Gy was given to the left and the right main bronchus, and the animals were killed humanely 2 to 8 weeks after irradiation using 10 to 20 ml T61 (Hoechst, Unterschleißheim, Germany). In five animals, bronchoscopy was performed four times each at intervals of 1 to 2 weeks. During bronchos-

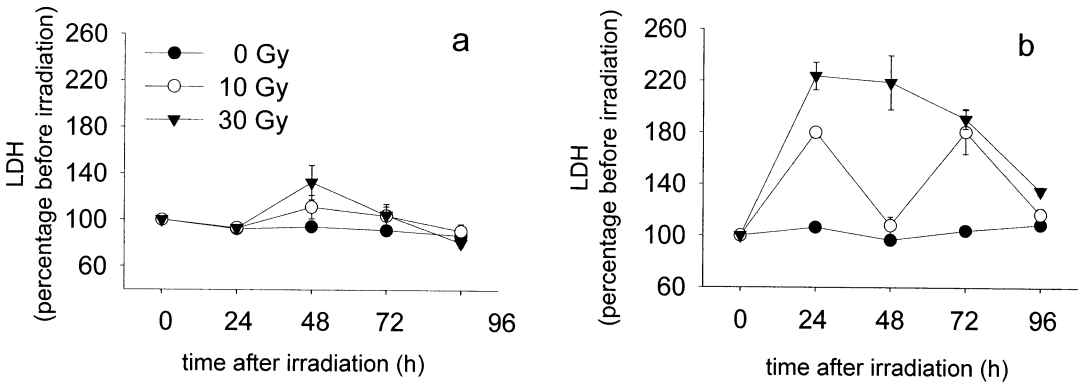

FIG. 1. LDH in the supernatant of organ cultures and BEAS 2B cells after irradiation with 0, 10 and 30 Gy. Panel a: In organ cultures, the LDH increased significantly 48 h after irradiation with 30 Gy ($P < 0.05$). Forty-eight hours after irradiation with 10 Gy, a slight increase in LDH was seen, but it did not reach statistical significance. No increase was found in the sham-irradiated group. Panel b: In BEAS 2B cells, the LDH increased earlier (after 24 h) and more strongly in a dose-dependent manner ($P < 0.01$). In the group that had been exposed to 10 Gy, the LDH decreased after 48 h and increased again after 72 h. In all groups, the LDH had nearly returned to baseline levels after 96 h.

copy, the appearance of the mucosa in the irradiated area of the main bronchi was documented.

Histology

After the minipigs were killed humanely, the lungs were removed and the main bronchi isolated. Bronchial rings 8 mm thick were fixed in 70% ethanol and embedded in paraffin. The paraffin blocks were cut into sections 3 μm thick, and the sections were stained with hematoxylin and eosin (H&E).

Assessment of Cell Number in the Histological Sections

To quantify the cell number in the H&E-stained sections, the circumference of each bronchus was divided into 12 identical segments. In each segment, the number of cells in a sector of the circumference with a length of 60 μm was counted. With this procedure, a representative assessment of the total cell number in each bronchus was accomplished.

Statistics

Values are expressed as means ± SEM except for data on nucleosomes, where, due to a high variability, values are represented as medians ± SD. To analyze the effects of radiation with time, a one-way repeated-measurements analysis of variance (combined with the all pairwise multiple comparison procedure, i.e. Student-Newman-Keuls method) was used. To compare experimental groups, the *t* test or one-way analysis of variance (combined with the Student-Newman-Keuls method) was applied. A $P < 0.05$ was considered statistically significant.

RESULTS

LDH in the Supernatant of BEAS 2B and Organ Cultures after Irradiation

The LDH in the supernatant of BEAS 2B cells and organ cultures was quantified every 24 h up to 96 h after exposure to 0, 10 or 30 Gy. In organ cultures, LDH increased significantly 48 h after irradiation with 30 Gy (27.7 ± 0.7 U/liter = 100% before irradiation compared to 132 ± 15% 48 h after irradiation, mean ± SEM, $n = 20$, $P < 0.05$, Fig. 1a) and subsequently decreased. A similar result was observed after irradiation of organ cultures with 10 Gy, but it did not reach statistical significance (29.2 ± 1.2 U/liter = 100% before irradiation compared to 111 ± 10% 48 h after irradiation, $n = 20$). No increase was found in the sham-irradiated group. In contrast, the concentration of LDH in the supernatant of BEAS 2B cells showed a different pattern in terms of both the magnitude of the increase and its time course (Fig. 1b). Here an increase in LDH was already seen 24 h after irradiation with 10 and 30 Gy. Twenty-four hours after irradiation with 30 Gy, the LDH in BEAS 2B cells increased to 224 ± 11% of the value before treatment (49.8 ± 1.1 U/liter = 100%, $P < 0.01$, $n = 6$). Twenty-four hours after irradiation with 10 Gy, the LDH was found to be 180 ± 4% of the starting value ($P < 0.01$ compared to starting value and 30 Gy, $n = 6$), but it decreased after 48 h only to increase again after 72 h. No increase was found in the sham-irradiated group.

Number of BEAS 2B Cells and Cells in Organ Cultures after Irradiation

Ninety-six hours after irradiation with 0, 10 or 30 Gy, the numbers of cells in BEAS 2B cultures and organ cultures were determined. In organ cultures, no differences could be detected (39.7 ± 5.8 × 10^4 cells/organ culture = 100% for 0 Gy, 102 ± 24% for 10 Gy, and 102 ± 17% for 30 Gy, mean ± SEM, $n = 20$, Fig. 2a). In BEAS 2B cells, the cell number decreased substantially regardless of the radiation dose (50.9 ± 0.5 × 10^5 cells/culture = 100% for 0 Gy, 1.4 ± 0.1% for 10 Gy, and 2.6 ± 0.9% for 30 Gy, $n = 6$, $P < 0.001$). To test for a delayed effect of the radiation in organ cultures, the cell number was also determined after 21 days. After this time, the organ cultures that had been irradiated with 30 Gy showed a decreased cell number compared to the 10-Gy and the sham-irradiated groups (17.4 ± 4.5 × 10^4 cells/organ culture = 100% for

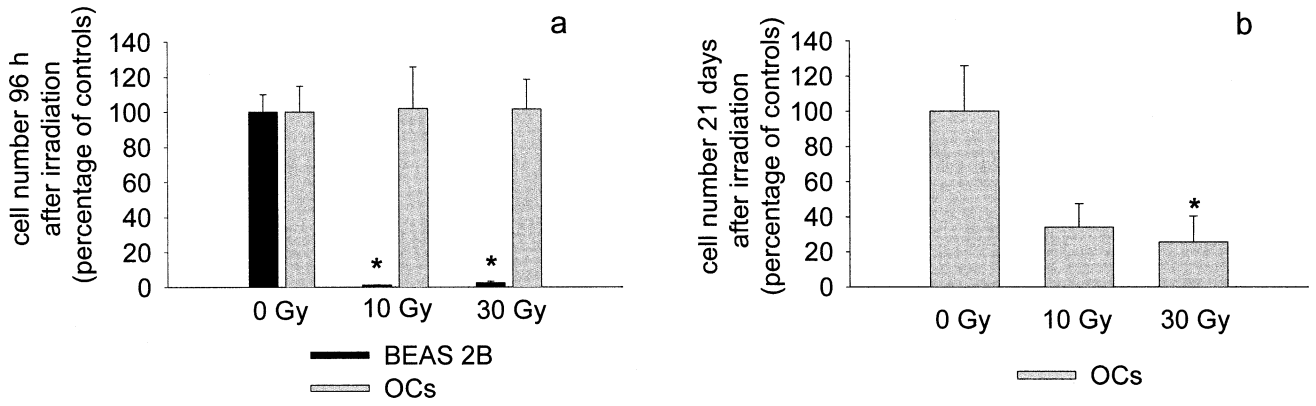

FIG. 2. Cell number in BEAS 2B cultures and organ cultures after irradiation. Panel a: Ninety-six hours after irradiation, the BEAS 2B cells and cells in organ cultures were harvested and the number of cells in each culture was determined. In organ cultures, no differences could be detected between the groups (gray columns, $n = 20$). In BEAS 2B cells, the number of cells decreased dramatically after irradiation (black columns, $n = 6$, $^*P < 0.001$ compared to 0 Gy). Panel b: To test for a delayed effect of radiation in organ cultures, the cell number was determined 21 days after irradiation. After this time, the group that had been irradiated with 30 Gy showed a decreased cell number compared to the 10-Gy- and the sham-irradiated groups ($n = 20$, $^*P < 0.05$ compared to 10 Gy and 0 Gy).

0 Gy, 34 ± 13% for 10 Gy and 25 ± 15% for 30 Gy, $n = 20$, $P < 0.05$ compared to 10 Gy and 0 Gy, Fig. 2b).

Nucleosomes in the Supernatant of BEAS 2B Cells and Organ Cultures after Irradiation

The supernatant of BEAS 2B cells and organ cultures was changed every 24 h, and the concentration of nucleosomes was measured up to 96 h after irradiation. In organ cultures, no differences between the groups that had been irradiated with 0, 10 or 30 Gy could be detected [starting values 65 ± 53 arbitrary units (AU) for 0 Gy, 79 ± 47 AU for 10 Gy, and 77 ± 68 AU for 30 Gy, median ± SD, $n = 20$, Fig. 3a]. In contrast, the nucleosomes in the supernatant of BEAS 2B cells increased dramatically after irradiation with 30 Gy (287 ± 989 AU = 100% immediately before irradiation compared to 2532 ± 730% 24 h and 3869 ± 1601% 48 h after irradiation, $n = 6$, $P < 0.001$, Fig. 3b) and then decreased to almost the starting level after 96 h. After irradiation with 10 Gy, the nucleosomes increased slowly to a maximum after 72 h, although this maximum was less pronounced compared to that seen after 30 Gy (244 ± 1561 AU = 100% immediately before irradiation compared to 793 ± 773% after 72 h).

Bronchoscopy in Minipigs after Irradiation

After brachytherapy with a single dose of 30 Gy, bronchoscopy was performed in five minipigs four times each at intervals of 1 to 2 weeks. Two weeks after irradiation, the mucosa in the area where the main bronchi had been irradiated showed only minor local irritations. After 3–4 weeks, the mucosa displayed noticeable changes consisting of marked reddening, edematous swelling, and clearly observable blood vessels showing a circular arrangement. Six to 8 weeks after irradiation, the mucosa had returned to a nearly normal appearance.

Morphology of the Bronchial Epithelium in Minipigs after Irradiation with 30 Gy

After the minipigs were killed, the main bronchi were isolated and sections 3 μm thick were sliced and stained with H&E. Without irradiation, the bronchial mucosa of the

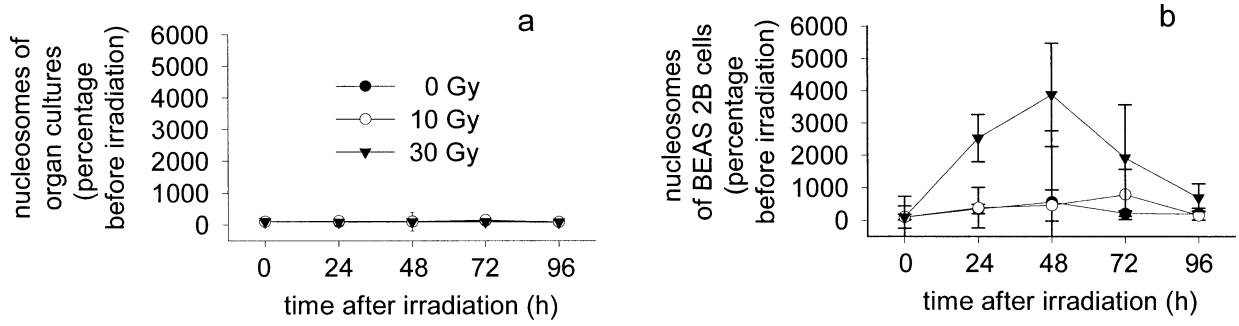

FIG. 3. Nucleosomes in the supernatant of organ cultures and BEAS 2B cells after irradiation. The nucleosomes in the supernatant of BEAS 2B cells and organ cultures were quantified up to 96 h after irradiation with 0, 10 and 30 Gy. Panel a: In organ cultures, no differences between the groups could be detected. Panel b: In the supernatant of BEAS 2B cells that had been irradiated with 30 Gy, the nucleosomes increased considerably after 24 h and 48 h and subsequently decreased to almost the starting levels ($P < 0.001$). Changes after 10 Gy were less pronounced compared to 30 Gy.

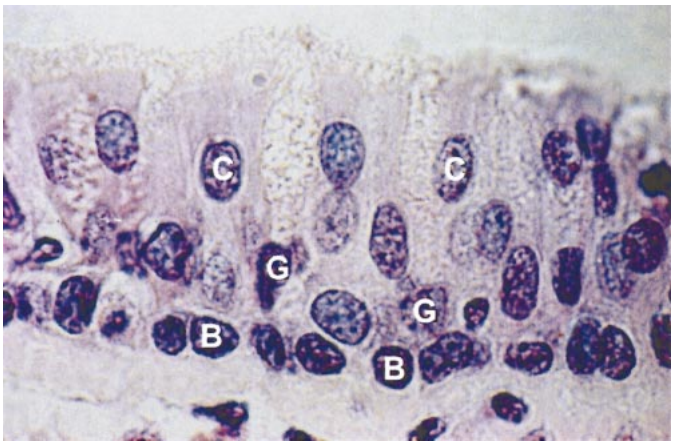

FIG. 4. Appearance of the normal bronchial epithelium of Goettinger minipigs. The main bronchi of minipigs were isolated and sections 3 μm thick were sliced and stained with H&E. The bronchial mucosa consisted of a ciliated pseudostratified columnar epithelium containing basal (B), ciliated (C) and goblet cells (G). Original magnification: 2160×.

minipigs consisted of a ciliated pseudostratified columnar epithelium containing basal, ciliated and goblet cells (Figs. 4 and 5a). Two weeks after irradiation with 30 Gy, only subtle changes such as incipient cell depletion and a slight irregularity in the basal layer were observed (Fig. 5b). After 3 weeks, the cell density was noticeably diminished, and cytomorphological abnormalities such as nuclear and cytoplasmic enlargement developed in the basal layer (Fig. 5c). Four to 5 weeks after irradiation, the cell density in the epithelium appeared to be minimal and the basal layer consisted of mono- and polynuclear giant cells (Fig. 5d). Beginning with week 6, regeneration of the epithelium was noticeable, and regrowth of normal-appearing basal cells could be seen (Fig. 5e). At week 8, the epithelium had regained a nearly normal architecture with a cell density that was indistinguishable from that of nonirradiated bronchi (Fig. 5f).

Number of Cells in the Bronchial Epithelium in Minipigs after Irradiation

After irradiation with 30 Gy, the number of cells in the bronchial epithelium in the H&E-stained sections was determined with respect to time. After irradiation, the cell number declined and reached a minimum after 4 weeks with ~60% of the cell number seen in nonirradiated bronchi (Fig. 6). Subsequently, the cell number increased again and reached a value after 8 weeks that was similar to that before irradiation (547 ± 16 cells/mm epithelium in nonirradiated bronchi = 100%, 65 ± 4% at 3 weeks and 93 ± 3% at 8 weeks after irradiation, $n = 1–4$).

DISCUSSION

In the present study, we investigated the acute effects of single-dose radiation on bronchial epithelium using three different experimental models. For *in vitro* experiments, BEAS 2B cells as a monolayer system and organ cultures as a three-dimensional culture system were used, while Goettinger minipigs served as an *in vivo* model. Irradiating BEAS 2B cells with 10 or 30 Gy resulted in extensive damage, since the cell number 96 h after irradiation was decreased to ~2% of the control value. The cells were irradiated when they were confluent, and thus cell–cell contact occurred during the experiments. In organ cultures, where the three-dimensional and hierarchical structure of the epithelium is preserved (*7–9*), a remarkable radioresistance was found even after irradiation with 30 Gy. No effect on the cell number was observed after 96 h, and a decrease in cell number was seen only after 21 days. Using nucleosomes as a marker for cell death, no effect was found in organ cultures regardless of radiation dose, but nucleosomes increased dramatically for monolayers of BEAS 2B cells after irradiation with 10 and 30 Gy. Similarly, the effects on LDH seen for BEAS 2B cells were clearly stronger than those for organ cultures. Furthermore, the time course of the increase in LDH was different. Twenty-four hours after irradiation of BEAS 2B cell monolayers, but only 48 h after irradiation of organ cultures, an increase in LDH was observed. These data clearly indicate that BEAS 2B cells in monolayers differ considerably in their acute radiation response from three-dimensional organ cultures. From these observations we conclude that data obtained in monolayer cultures may not properly reflect the situation *in vivo* and that interpretation of their significance in relation to three-dimensional, hierarchically structured tissues can be complex.

In a previous study (*6*), we demonstrated that the morphology of histological sections of organ cultures was unchanged 18 days after irradiation up to 75 Gy. This is consistent with the findings in this study, since both bronchoscopy and the morphology of the histological sections revealed substantial alterations of the airways in minipigs no earlier than 3 to 4 weeks after irradiation. Similarly, the cell number in the bronchial epithelium of minipigs decreased very slowly to reach a minimum 4 weeks after irradiation; this resembled the slow decline in the cell number in organ cultures. In contrast, the number of BEAS 2B cells was dramatically reduced 96 h after irradiation. We therefore conclude that not only are organ cultures much more radioresistant than BEAS 2B cells, but this higher radioresistance also reflects the *in vivo* situation very closely. Consequently, we believe that, for investigating effects of radiotherapy *in vitro*, the significance of organ cultures has been underestimated.

The bystander effect describes the ability of cells to affect the radiosensitivity of other cells not directly hit by radiation or not necessarily susceptible to it [for reviews, see refs. (*27–29*)]. This may be caused by a diffusible factor released into the supernatant by the irradiated cells or may be mediated via gap junctions requiring close cell–cell contact. Increased and decreased radiosensitivity has been reported, but the mechanisms involved are largely unknown.

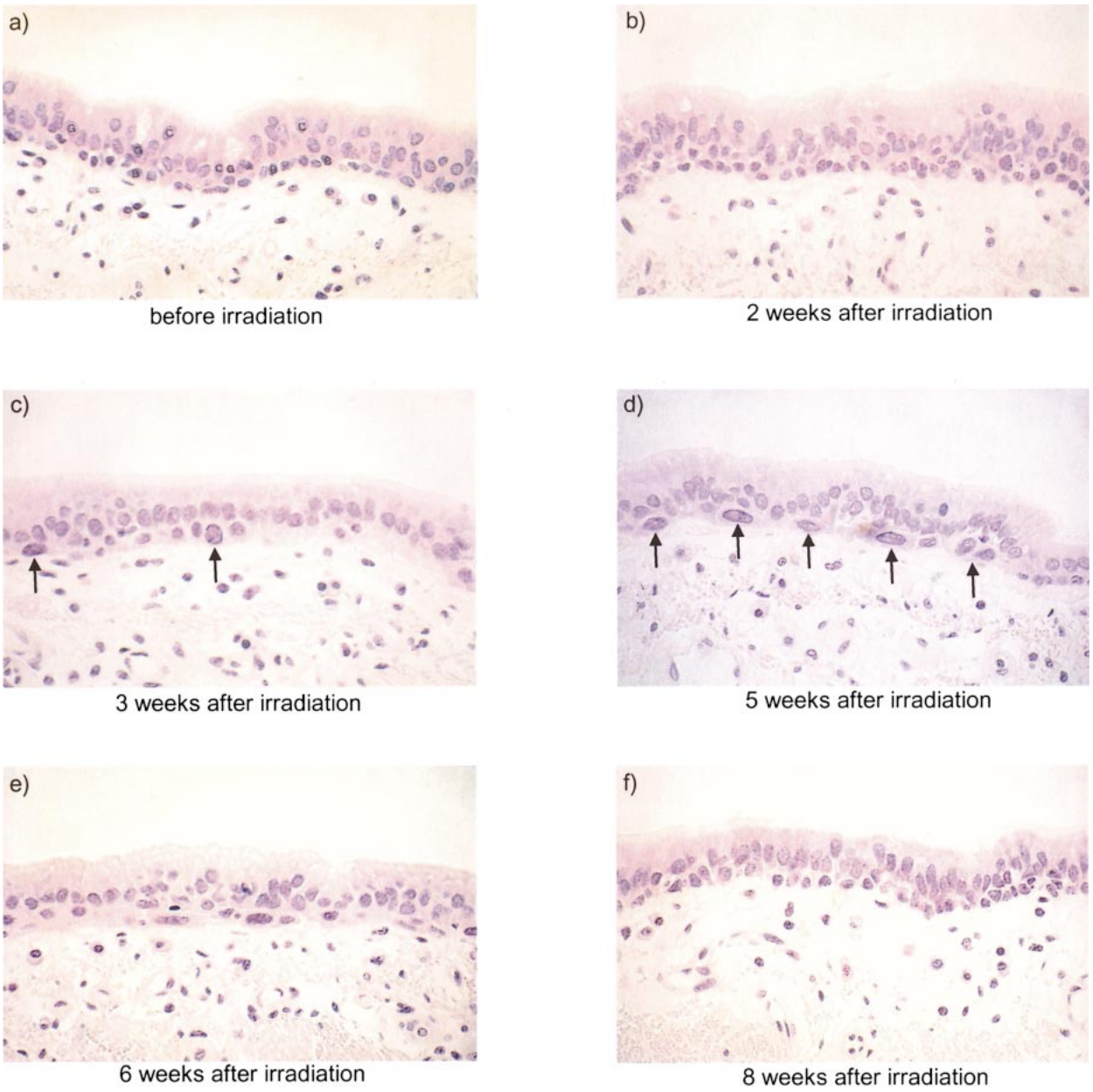

FIG. 5. Morphology of the bronchial epithelium in minipigs after irradiation with 30 Gy. After isolation of the main bronchi, sections 3 μm thick were sliced and stained with H&E. The time after irradiation is indicated under each panel. Original magnification: 860×. Panel a: Normal appearance of the bronchial epithelium without irradiation. Panel b: Two weeks after irradiation, slight irregularities in the basal layer were observable. Panel c: After 3 weeks, the cell density was noticeably diminished, and cytomorphological abnormalities such as nuclear and cytoplasmic enlargement developed in the basal layer (arrows). Panel d: Five weeks after irradiation, the basal layer constituted of mono- and polynuclear giant cells (arrows). Panel e: After 6 weeks, regeneration of the epithelium began and regrowth of normal-appearing basal cells could be seen. Panel f: Eight weeks after irradiation, the epithelium had regained a nearly normal architecture.

Ballarini and colleagues pointed out that if the contact is poor, factors released into the culture medium appear to be important, whereas if the cells are in close contact, gap junctions appear to play a major role (*27*). Chandna *et al.* found that human glioma cells irradiated as microcolonies with doses up to 2 Gy showed increased radioresistance compared to single cells (*12*). Similarly, Moussa and coworkers reported that irradiation of cells of head and neck carcinoma cell lines grown as microcolonies also resulted in enhanced radioresistance (*13*). Using cells of the human squamous carcinoma cell line CaSki, Kwok and Sutherland found that the cells were more radioresistant when they were grown in multicellular spheroids 30 to 70 μm in diameter than in monolayer cultures (*14*). In our study, the BEAS 2B cells were confluent when they were irradiated, and therefore cell–cell contact was present, but in organ cultures the three-dimensional structure of the bronchial epithelium was preserved. Mothersill states that very few pri-

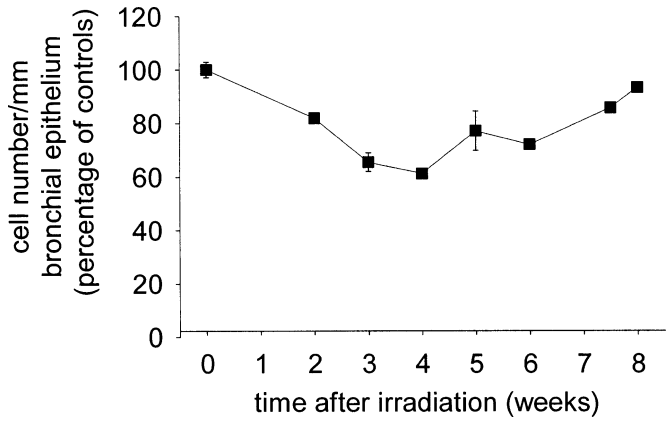

FIG. 6. Cell number in the bronchial epithelium in minipigs after irradiation. In H&E-stained sections of the main bronchi, the number of cells in the epithelium was determined with respect to time. After irradiation, the cell number slowly declined to reach a minimum of ~60% after 4 weeks. Subsequently, the number increased, and after 8 weeks it was similar to that in nonirradiated bronchi (n = 1–4).

mary culture techniques have been applied in radiobiology (*30*), and Djordjevic points out in a comprehensive review that we do not have adequate systems to study cellular interactions in an *in situ*-like environment (*28*). We believe that organ cultures constitute a culture system that preserves the properties of the situation *in vivo*, and in the present study we have shown that the effects of radiation on organ cultures very closely resemble those seen *in vivo.*

Cell–cell contact could influence radiosensitivity by gap junctional reciprocity, cell shape-mediated changes in repair-related gene expression, and alterations in chromatin packing [for review, see ref. (*31*)]. However, an evaluation of the mechanisms underlying the phenomenon was beyond the scope of the present study and will be addressed in future investigations.

The importance of the hierarchical structure of the epithelium is underlined by the fact that changes in cell morphology caused by the radiation occurred primarily in the basal layer of the bronchial epithelium of the minipigs. Damage in the stem cell compartment, which is most susceptible to radiation (*32*), will lead to a decreased cell number after a time when the lack of compensation for losses of mature differentiated cells becomes manifest. This process most likely contributed to the delayed responses in organ cultures and in the bronchial epithelium of the minipigs, whereas in BEAS 2B cells no hierarchical structure existed.

Twenty-four hours after irradiation with 10 Gy, the LDH increased in BEAS 2B cells before returning to the starting level after 48 h. After 72 h the LDH increased again. In the group that had been irradiated with 30 Gy, the LDH stayed elevated but then decreased slowly after 96 h. A possible explanation for this phenomenon would be that irradiation with 10 Gy induced apoptosis in radiosensitive cells within the first 24 h followed by a wave of mitotic cell death, which led to a second LDH peak after 72 h (*32*). Irradiation with 30 Gy could have caused more severe damage that led to a continuous elevation in LDH. However, the significance of this phenomenon is still somewhat unclear, and in future studies further experiments to address this point will be performed.

In the blood of patients with malignant tumors, acute inflammation or autoimmune diseases, nucleosomes appear in elevated concentrations; this has been attributed to increased rates of cell death (*19–25*). In patients with malignant diseases who are undergoing radiotherapy, the initiation of therapy caused an increase in serum nucleosomes that subsequently decreased to or below starting levels. Low levels of nucleosomes in the course of treatment correlated with good clinical outcome (*23*). In the present study, we measured the levels of nucleosomes in the supernatant of BEAS 2B cells and organ cultures after irradiation to test whether this parameter can be used to quantify cell death *in vitro* in non-malignant tissue. In BEAS 2B cells, we found that the levels of the nucleosomes in the supernatant increased dramatically to ~3800% of the starting level 48 h after irradiation with 30 Gy. In organ cultures, no difference between the experimental groups could be seen, and this difference in radiosensitivity correlated with the results obtained for cell number and LDH. We therefore conclude that nucleosomes can be used to assess radiation-induced cell death *in vitro.* Future studies are planned to validate this promising approach.

In summary, we have shown that the bronchial epithelium is remarkably resistant to radiation doses that are routinely used in clinical brachytherapy. Organ cultures resemble the acute *in vivo* radioresponsiveness very closely, whereas BEAS 2B cells cultured as monolayers show a radiosensitivity that does not reflect the situation *in vivo.* Furthermore, we have demonstrated that the quantification of nucleosomes in the supernatant of tissue cultures may be useful to assess radiation damage *in vitro.*

ACKNOWLEDGMENTS

This work was supported by the Wilhelm Sander-Foundation grant 2000.077.1 to A. Bergner and R. M. Huber. Data presented in this paper are part of the doctoral theses of J. Stief and A. Waldschmidt.

Received: February 13, 2003; accepted: July 15, 2003

REFERENCES

1. S. W. Gollins, P. A. Burt, P. V. Barber and R. Stout, High dose rate intraluminal radiotherapy for carcinoma of the bronchus: Outcome of treatment of 406 patients. *Radiother. Oncol.* **33**, 31–40 (1994).
2. R. M. Huber, R. Fischer, H. Hautmann, B. Pollinger, T. Wendt, D. Muller-Wening and K. Haussinger, Palliative endobronchial brachytherapy for central lung tumors. A prospective, randomized comparison of two fractionation schedules. *Chest* **107**, 463–470 (1995).
3. R. M. Huber, R. Fischer, H. Hautmann, B. Pollinger, K. Haussinger and T. Wendt, Does additional brachytherapy improve the effect of external irradiation? A prospective, randomized study in central lung tumors. *Int. J. Radiat. Oncol. Biol. Phys.* **38**, 533–540 (1997).
4. R. Stout, Endobronchial brachytherapy. The Manchester experience

using single dose HDR-Micro selection technique. In *Lung Cancer. Frontiers in Science and Treatment* (G. Motta, Ed.), pp. 295–300. Grafica LP, Genoa, Italy, 1994.

5. M. Taulelle, B. Chauvet, P. Vincent, C. Felix-Faure, B. Buciarelli, R. Garcia, Y. Brewer and F. Reboul, High dose rate endobronchial brachytherapy: Results and complications in 189 patients. *Eur. Respir. J.* **11**, 162–168 (1998).
6. D. Kotsianos, D. Bach, F. Gamarra, W. Haimerl, R. Knuchel, S. M. Lang, K. J. Pfeifer and R. M. Huber, High-dose-rate brachytherapy: Dose escalation in three-dimensional miniorgans of the human bronchial wall. *Int. J. Radiat. Oncol. Biol. Phys.* **46**, 1267–1273 (2000).
7. R. Bals, F. Gamarra, A. Kaps, S. Grundler, R. M. Huber and U. Welsch, Secretory cell types and cell proliferation of human bronchial epithelial cells in an organ-culture system. *Cell Tissue Res.* **293**, 573–577 (1998).
8. L. Fjellbirkeland, R. Bjerkvig, S. K. Steinsvag and O. D. Laerum, Nonadhesive stationary organ culture of human bronchial mucosa. *Am. J. Respir. Cell Mol. Biol.* **15**, 197–206 (1996).
9. S. K. Steinsvag, M. Strand, O. Berg, M. Miaguchi and J. Olofsson, Human respiratory mucosa in a nonadhesive stationary organ culture system. *Laryngoscope* **101**, 1323–1331 (1991).
10. S. E. Al-Batran, S. T. Astner, M. Supthut, F. Gamarra, K. Brueckner, U. Welsch, R. Knuechel and R. M. Huber, Three-dimensional *in vitro* cocultivation of lung carcinoma cells with human bronchial organ culture as a model for bronchial carcinoma. *Am. J. Respir. Cell Mol. Biol.* **21**, 200–208 (1999).
11. F. Gamarra, S. Wagner, S. Al Batran, I. Maier, M. Castro, H. Hautmann, A. Bergner, R. Baumgartner and R. M. Huber, Kinetics of 5-aminolevulinic acid-induced fluorescence in organ cultures of bronchial epithelium and tumor. *Respiration* **69**, 445–450 (2002).
12. S. Chandna, B. S. Dwarakanath, D. Khaitan, T. L. Mathew and V. Jain, Low-dose radiation hypersensitivity in human tumor cell lines: Effects of cell–cell contact and nutritional deprivation. *Radiat. Res.* **157**, 516–525 (2002).
13. H. Moussa, S. A. Mitchell, R. Grenman and M. C. Joiner, Cell–cell contact increases radioresistance in head and neck carcinoma cell lines. *Int. J. Radiat. Biol.* **76**, 1245–1253 (2000).
14. T. T. Kwok and R. M. Sutherland, The influence of cell–cell contact on radiosensitivity of human squamous carcinoma cells. *Radiat. Res.* **126**, 52–57 (1991).
15. J. L. Goergen, A. Marc and J. M. Engasser, Determination of cell lysis and death kinetics in continuous hybridoma cultures from the measurement of lactate dehydrogenase release. *Cytotechnology* **11**, 189–195 (1993).
16. G. C. Jagetia and S. K. Adiga, Influence of various concentrations of Taxol on cell survival, micronuclei induction, and LDH activity in cultured V79 cells. *Cancer Lett.* **96**, 195–200 (1995).
17. A. J. Racher, D. Looby and J. B. Griffiths, Use of lactate dehydrogenase release to assess changes in culture viability. *Cytotechnology* **3**, 301–307 (1990).
18. S. Holdenrieder, P. Stieber, H. Bodenmuller, M. Busch, J. Von Pawel, A. Schalhorn, D. Nagel and D. Seidel, Circulating nucleosomes in serum. *Ann. NY Acad. Sci.* **945**, 93–102 (2001).
19. Z. Amoura, J. C. Piette, H. Chabre, P. Cacoub, T. Papo, B. Wechsler, J. F. Bach and S. Koutouzov, Circulating plasma levels of nucleosomes in patients with systemic lupus erythematosus: Correlation with serum antinucleosome antibody titers and absence of clear association with disease activity. *Arthritis Rheum.* **40**, 2217–2225 (1997).
20. P. Anker, Quantitative aspects of plasma/serum DNA in cancer patients. *Ann. NY Acad. Sci.* **906**, 5–7 (2000).
21. W. Emlen, J. Niebur and R. Kadera, Accelerated *in vitro* apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J. Immunol.* **152**, 3685–3692 (1994).
22. G. J. Fournie, J. P. Courtin, F. Laval, J. J. Chale, J. P. Pourrat, M. C. Pujazon, D. Lauque and P. Carles, Plasma DNA as a marker of cancerous cell death. Investigations in patients suffering from lung cancer and in nude mice bearing human tumours. *Cancer Lett.* **91**, 221–227 (1995).
23. S. Holdenrieder, P. Stieber, H. Bodenmuller, M. Busch, G. Fertig, H. Furst, A. Schalhorn, N. Schmeller, M. Untch and D. Seidel, Nucleosomes in serum of patients with benign and malignant diseases. *Int. J. Cancer* **95**, 114–120 (2001).
24. S. Holdenrieder, P. Stieber, T. Forg, M. Kuhl, L. Schulz, M. Busch, A. Schalhorn and D. Seidel, Apoptosis in serum of patients with solid tumours. *Anticancer Res.* **19**, 2721–2724 (1999).
25. K. Kuroi, C. Tanaka and M. Toi, Plasma nucleosome levels in node-negative breast cancer patients. *Breast Cancer* **6**, 361–364 (1999).
26. S. Holdenrieder, P. Stieber, H. Bodenmuller, G. Fertig, H. Furst, N. Schmeller, M. Untch and D. Seidel, Nucleosomes in serum as a marker for cell death. *Clin. Chem. Lab. Med.* **39**, 596–605 (2001).
27. F. Ballarini, M. Biaggi, A. Ottolenghi and O. Sapora, Cellular communication and bystander effects: A critical review for modelling low-dose radiation action. *Mutat. Res.* **501**, 1–12 (2002).
28. B. Djordjevic, Bystander effects: A concept in need of clarification. *Bioessays* **22**, 286–290 (2000).
29. C. Mothersill and C. Seymour, Radiation-induced bystander effects: Past history and future directions. *Radiat. Res.* **155**, 759–767 (2001).
30. C. Mothersill, Development of primary tissue culture techniques for use in radiobiology. *Radiat. Res.* **150** (Suppl.), S121–S125 (1998).
31. P. L. Olive and R. E. Durand, Drug and radiation resistance in spheroids: Cell contact and kinetics. *Cancer Metastasis Rev.* **13**, 121–138 (1994).
32. E. J. Hall, Dose–response relationships for normal tissue. In *Radiobiology for the Radiologist*, 4th ed., pp. 45–74. J. B. Lippincott, Philadelphia, 1994.