Celecoxib Enhances Radiation Response of Secondary Bone Tumors of a Human Non-Small Cell Lung Cancer via Antiangiogenesis In Vivo

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Purpose: Cyclooxygenase-2 (COX-2) inhibitors mediate a systemic antitumor activity via antiangiogenesis and seem to enhance the response of primary tumors to radiation. Radiosensitizing effects of COX-2 inhibition have not been reported for bone metastases. Therefore, the aim of this study was the investigation of the radiosensitizing effects of the selective COX-2 inhibitor celecoxib in secondary bone tumors of a non-small cell lung carcinoma in vivo.

Materials and Methods: Human A549 lung carcinomas were implanted into a cranial window preparation in male SCID mice (n = 24). Animals were treated with either celecoxib or radiation (7 Gy single photon dose) alone or a combination of celecoxib and radiation, respectively. Untreated animals served as controls. The impact of radiation and COX-2 inhibition on angiogenesis, microcirculation, and tumor growth was analyzed over 28 days by means of intravital microscopy and histological methods.

Results: Monotherapies with radiation as well as celecoxib had significant antitumor effects compared to untreated controls. Both therapies reduced tumor growth and vascularization to a similar extent. The simultaneous administration of celecoxib and radiation further enhanced the antitumor and antiangiogenic effects of single-beam radiation. With the combined treatment approach, tumor vascularization and tumor size were decreased by 57% and 51%, respectively, as compared to monotherapy with radiation.

Conclusion: The combined application of radiation therapy and COX-2 inhibition showed synergistic effects concerning the inhibition of tumor growth and tumor angiogenesis. Therefore, the combination of radiation with COX-2 inhibitor therapy represents a promising approach to improve the therapeutic efficacy of radiotherapy of bone metastases.

Key Words: Angiogenesis · Bone tumor · Cyclooxygenase · Intravital microscopy · Radiation

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Celecoxib steigert die Strahlenempfindlichkeit von sekundären Knochentumoren eines nicht-kleinzelligen humanen Lungenkarzinoms durch Anti-Angiogenese in vivo

Ziel: Cyclooxygenase-(COX-)2-Inhibitoren vermitteln systemisch eine Wirkung gegen Tumoren indem sie die Tumorangiogenese hemmen und das Ansprechen von Primärtumoren auf Bestrahlung zu verbessern scheinen. Über strahlensensibilisierende Effekte einer COX-2-Hemmung bei der Behandlung von Knochenmetastasen wurde bisher nicht berichtet. Das Ziel dieser Studie war daher die Untersuchung der strahlensensibilisierenden Effekte des selektiven COX-2-Inhibitors Celecoxib bei sekundären Knochentumoren eines nicht-kleinzelligen Lungenkarzinoms in vivo.

Material und Methoden: Humane A549 Lungenkarzinome wurden in eine Schädelfensterpräparation bei männlichen SCID-Mäusen (n = 24) implantiert. Die Tiere wurden entweder mit Celecoxib oder Strahlentherapie (7 Gy als Einzeldosis) alleine oder mit einer Kombination aus Celecoxib und Bestrahlung behandelt. Unbehandelte Tiere dienten als Kontrolle. Es wurden der Einfluss der Bestrahlung und der COX-2-Hemmung auf Angiogenese, Mikrozirkulation und Tumorwachstum über einen Zeitraum von 28 Tagen mit Hilfe der Intravitalmikroskopie und histologischer Methoden analysiert.

Ergebnisse: Die Monotherapien in Form einer Einmalbestrahlung mit 7 Gy und einer Behandlung mit Celecoxib zeigten signifikante anti-tumorale Effekte im Vergleich zu unbehandelten Kontrollen. Beide Therapien reduzierten Tumorwachstum und Vasku-

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larisation in ähnlichem Ausmaß. Die simultane Anwendung von Celecoxib und Bestrahlung steigerte die anti-tumoralen und die anti-angiogenen Effekte der Einmalbestrahlung. Mit dem kombinierten Behandlungsansatz wurde die Tumorvaskularisation und die Tumorgröße um 57% beziehungsweise 51% im Vergleich zur Strahlentherapie alleine verringert.

Schlussfolgerung: Die kombinierte Anwendung von Strahlentherapie und COX-2-Hemmung zeigte synergistische Effekte hinsichtlich der Hemmung des Tumorwachstums und der Tumorangiogenese. Somit repräsentiert die Kombination von Strahlentherapie mit einer COX-2-Inhibiton einen vielversprechenden Ansatz, um die therapeutische Effektivität der Bestrahlung von Knochenmetastasen zu verbessern.

Schlüsselwörter: Angiogenese · Knochentumor · Cyclooxygenase · Intravitalmikroskopie · Strahlentherapie

Introduction

Radiotherapy is the standard treatment to achieve local control of bone metastases. As the response rate of tumors to radiation seems to be dependent on the delivered dose, strategies increasing the effective dose of radiation may be crucial to ameliorate the therapeutic efficacy of radiotherapy [16, 22, 25, 27, 45, 50]. Recent strategies to optimize the efficacy of radiation are focused on molecular targets enhancing the radiation sensitivity of malignant tumors [1, 2, 4, 11, 13, 15, 28, 31, 36, 37, 40, 53]. In this respect, the prostaglandin signaling pathway seems to be of particular importance as it has been shown that the modulation of prostaglandin synthesis can ameliorate the response of tumors to radiation [30, 35, 37, 40].

Cyclooxygenase (COX) with its two isoforms COX-1 and COX-2 is the rate limiting enzyme for the synthesis of prostaglandins from free arachidonic acids. COX-1 is constitutively expressed in most normal tissues and is responsible for the production of prostaglandins that mediate regular physiological functions. The inducible isoform COX-2 is usually undetectable in normal tissue and is frequently overexpressed in malignant and inflamed tissues [10, 54]. Elevated levels of COX-2 in tumor cells are associated with resistance to apoptosis [23, 48], tumor angiogenesis [51], and tumor cell invasiveness [8, 9, 49]. It has been shown that the inhibition of COX-2 mediates antitumor activities in various human malignant tissues including prostate, colorectal, breast, and non-small cell lung cancer [3, 14, 24, 34, 41, 42, 44]. In a previous study, it was shown that the selective COX-2 inhibitor celecoxib significantly reduced growth of secondary bone tumors of a non-small cell lung carcinoma. The antitumor effect was mediated by antiangiogenic and proapoptotic mechanisms in bone metastases [20].

Although COX-2 inhibitors were shown to inhibit tumor growth if administered as monotherapeutic agents, several authors provided evidence that the drugs are considerably more effective if combined with a second treatment regimen. In this regard, selective COX-2 inhibitors were recently reported to enhance the response of primary tumors to radiation in vitro and in vivo [30, 32, 33, 35, 37, 40, 52]. On the other hand, the COX-2 inhibitor nimesulide did not increase the radiation response of squamous cell carcinoma cells in vitro [5]. The therapeutic efficacy of a combination of COX-2 inhibition and radiation on secondary bone tumors has not been described so far. We hypothesized that the selective COX-2 inhibitor celecoxib may enhance the radioresponse of secondary bone tumors of a non-small cell lung carcinoma in vivo. The effects of the combined application of celecoxib and radiation were investigated by applying an animal model of bone metastases and intravital microscopy to continuously monitor angiogenesis, vascularization, and growth of secondary bone tumors.

Material and Methods

Animal Model and Cell Lines

Experiments were performed on 24 adult male severe combined immunodeficient mice (SCID, C.B-17/IcrCrl-scid-BR, Charles River Laboratories Inc., Sulzfeld, Germany, 7–8 weeks old, 20–25 g body weight), following institutional guidelines approved by the local animal review board. All surgical procedures were performed in strictly aseptic conditions within a laminar flow unit (Merck Eurolab, Bruchsal, Germany) under deep anesthesia by an intraperitoneal injection of a mixture of ketamine (Ketanest®, 65 mg/kg body weight, Pfizer, Karlsruhe, Germany), xylazine (Rompun®, 13 mg/kg body weight, Bayer, Leverkusen, Germany), and acepromazine (Sedastress®, 2 mg/kg body weight, Medistar, Holzwickede, Germany).

The human lung carcinoma cell line A 549 was obtained from the German Cancer Research Institute (Heidelberg, Germany). Tumor cells (1×10^7/ml) were injected subcutaneously into the left flank of each donor mouse and grown to a volume of 0.5 to 1.0 cm^3. After sacrificing the donor mouse, the tumor was excised, cut into small pieces (volume 0.5–1.0 mm^3) in Dulbecos Modified Eagle's Medium (DMEM,) at 4 °C and implanted into the recipient mouse in the following manner:

Surgical preparation of the cranial window was performed as described in detail elsewhere [21]. In brief, an oval cavity of approximately 2.0 mm × 1.0 mm × 0.5 mm was milled into the calvaria, eliminating parts of the external tabula of the calvaria including the spongious bone underneath. Then one small piece (approx. 0.5–1.0 mm^3) of the human non-small cell lung carcinoma A549 was implanted into the cavity. To prevent dehydration or mechanical damage to the tumors, the preparation was sealed with a glass cover slip and bone cement.

Radiation Therapy and COX-2 Inhibitor Treatment

Radiotherapy was delivered on day 15 after tumor implantation with a single dose of 7 Gray (Gy) γ-radiation to the cranium using a Co-60 source (Siemens, Gammatron, Erlangen, Germany).

The selective COX-2 inhibitor celecoxib was a generous gift of Pharmacia, Inc. (St. Louis, MO, USA). Celecoxib was dissolved in a carboxymethylcellulose (CMC)-based vehicle at 5 mg celecoxib/ml vehicle. Each animal was treated once daily by s.c. injection of 30 mg/kg body weight celecoxib (celecoxib, n = 6) or the equivalent amount of the (CMC)-based vehicle alone (control, n = 6). Animals that received radiation on day 15 were either treated with the vehicle (radiation, n = 6) or with celecoxib (celecoxib + radiation, n = 6) under the conditions described above. Treatments started on day 8 after tumor implantation and were continued until termination of experiments on day 28 after tumor implantation.

Intravital Microscopy

Within the first week after tumor implantation, mice were observed daily under epi-illumination with a stereotactic microscope (Leica $MZ7_5$, Leica, Germany) employing a 5- to 40-fold magnification. At 24-hour intervals, the first appearance of newly formed blood vessels entering the implanted tumor tissue and the onset of perfusion in these vessels were determined. Intravital fluorescence videomicroscopy was performed using an epi-illumination fluorescence microscope unit (Leica, Germany) equipped with a 4×(EF 4/0.12, Leitz, Wetzlar, Germany) and 40x (Zeiss Achroplan 40×/0.75 w, Carl Zeiss, Germany) objective on days 7, 14, 21, and 28 after tumor implantation. For offline analysis, regions of interest were recorded on videotapes using a S-VHS videocassette recorder (AG-7350, Panasonic, Japan) at a rate of 50 frames/s and a digital camera (Kappa CF 8/1, Kappa Opto-electronics, Germany).

Using an adequate fluorescence filter set for green light (bandpass 515–560 nm), the intravenous injection of fluorescein isothiocyanate (FITC)-labeled dextran (Sigma, St. Louis, MO, FITC-Dextran, FD 2000S, molecular weight 2,000,000; 0.1 ml of a 5% solution in 0.9% NaCl as a plasma marker) enabled the observation of the tumor microcirculation.

Offline Analysis of Tumor Growth and Microhemodynamics

Tumor growth was determined offline by measuring its two-dimensional (2D) surface area in mm^2 from standardized digital photographs of the cranial window preparation at 10-fold magnification on days 7, 14, 21, and 28 after tumor implantation using a computer-based analysis program (AnalySIS® V3.0, Soft Imaging System, Münster, Germany).

The functional microvessel density (FVD, mm/mm^2) was determined as the length of all perfused microvessels within a tumor in relation to its 2D surface area [19–21]. FVD was quantified using a computer-based image analysis program (CapImage®, Engineering Office Dr. Zeintl, Heidelberg, Germany).

Histopathologic Assessment

All mice were sacrificed on day 28 after tumor implantation and the tumors were immediately excised along with the surrounding tissue of the calvaria and the brain for further histopathologic investigation. Tissue samples were processed for paraffin embedding. Three-µm serial sections were stained with hematoxylin–eosin (H&E).

Statistics

All numerical data are presented as median with 25% and 75% quartiles. Using the software program SigmaStat® for Windows (Version 2.03, SPSS, Chicago, IL), data were analyzed statistically with ANOVA on ranks. The Student–Newman–Keuls method was applied for multiple comparison procedures. Differences were considered significant at $p < 0.05$.

Results

Tumor Growth

As shown in Figure 1, 7 days after tumor implantation tumor size was identical in all groups. Afterwards, the tumor dimensions increased in all groups until day 28. Celecoxib and radiation therapy alone resulted in similar growth behaviors of the tumors. At the end of the experiments the tumor size was significantly reduced in both groups as compared to controls. However, the 2D tumor size significantly increased until day 28 after tumor implantation as compared to day 7 (prior to initiation of celecoxib/vehicle treatment)

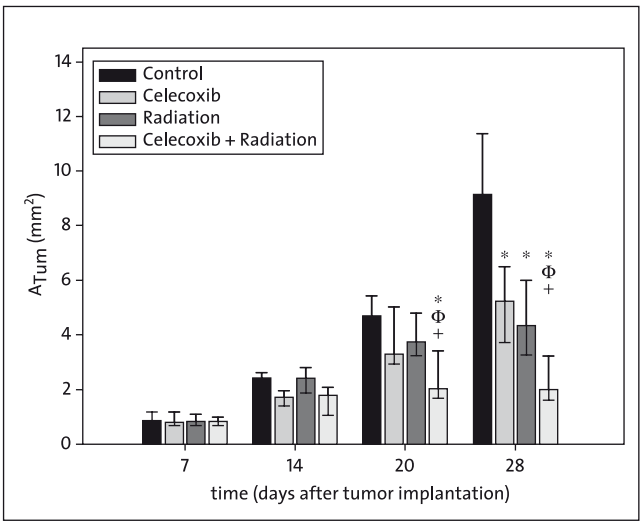

Figure 1. Growth of secondary A549 lung carcinomas was analyzed for 28 days by intravital microscopy. The graph depicts the two-dimensional tumor surface (mm^2) of the tumors with different treatments on days 7, 14, 21, and 28 after tumor implantation. Median values are represented together with the 25% and the 75% quartiles (n = 6 for each group and time-point). ANOVA, Student–Newman–Keuls method, $^*p < 0.05$ vs. control, $^{\Phi}p < 0.05$ vs. radiation, $^+p < 0.05$ vs. celecoxib.

Abbildung 1. Das Wachstum des sekundären Lungenkarzinoms A549 wurde mit Hilfe der Intravitalmikroskopie 28 Tage lang analysiert. Die Grafik zeigt die zweidimensionale Oberfläche (A_{Tum} in mm^2) der Tumoren während unterschiedlicher Behandlungen an den Tagen 7, 14, 21 und 28 nach Tumorimplantation. Dargestellt sind die Mediane mit ihren jeweiligen 25% und 75% Quartilen (n = 6 für jede Gruppe sowie jeden Zeitpunkt). ANOVA, Student–Newman–Keuls-Methode, $^*p < 0{,}05$ vs. Kontrolle, $^{\Phi}p < 0{,}05$ vs. Bestrahlung, $^+p < 0{,}05$ vs. Celecoxib.

and 14 (prior to single beam irradiation). The combination of radiation with celecoxib resulted in a significant reduction of tumor size on day 28 as compared to controls and the monotherapies. Furthermore, the combination of celecoxib and radiation induced a growth arrest of the tumors after the delivery of irradiation.

Angiogenesis and Tumor Vascularization

First, newly formed vessels were observed within 6 days after tumor implantation in all tumors followed by a rapid onset of perfusion in these vessels within another 24 hours. Intravital microscopy showed that the angiogenic sprouting originated from vessels located within the surrounding bone.

As shown in Figure 2, functional vessel density significantly increased between days 7 and 14 in all treatment groups. In controls, FVD then remained on the same level during the rest of the investigation. The therapeutic efficacy of celecoxib and radiation as monotherapies on tumor vascularization was similar. Both therapy regimes resulted in significantly smaller FVDs on day 28 as compared to controls. Between

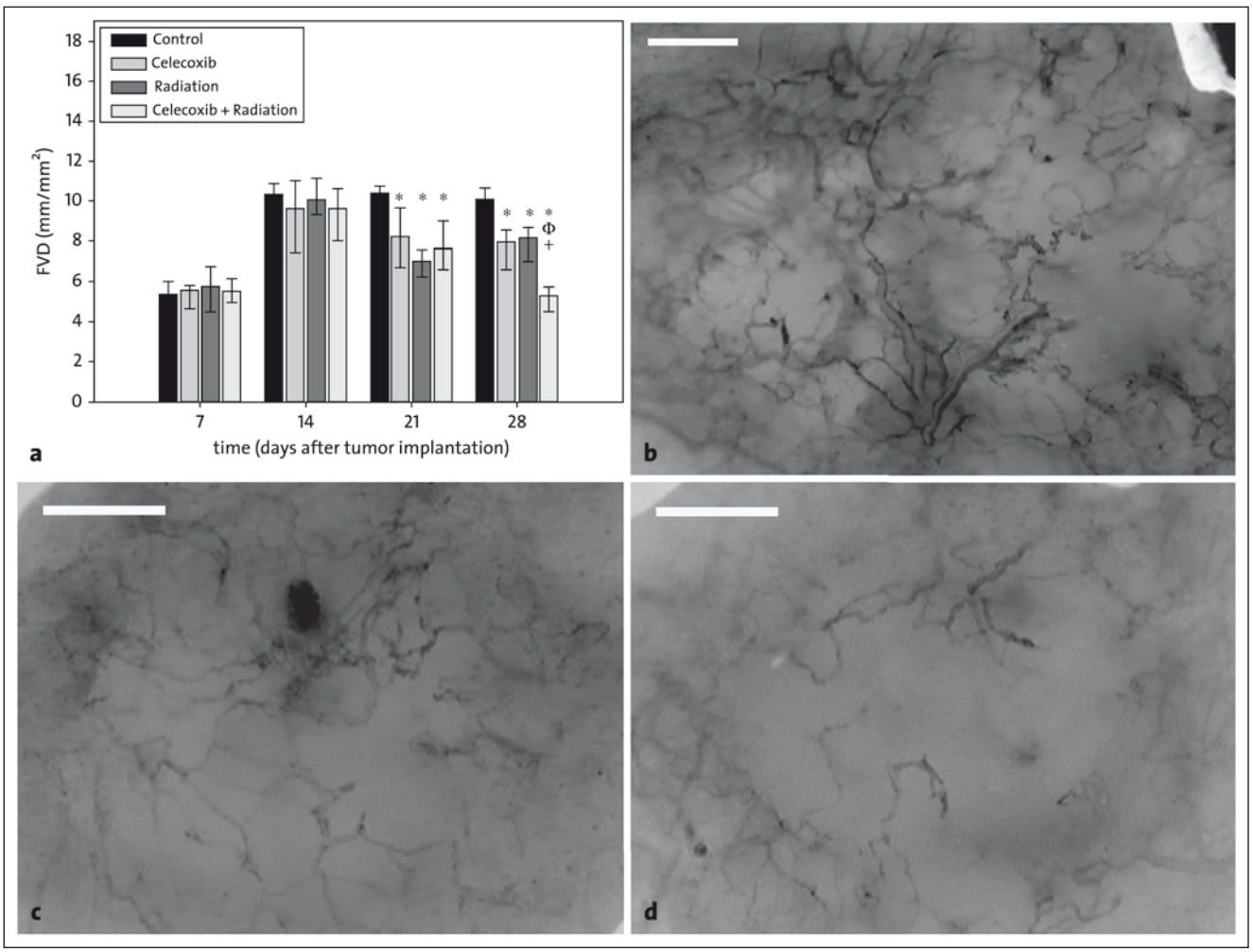

Figure 2a to 2d. Vascularization of A549 tumor xenografts was analyzed for 28 days by intravital microscopy. (**a**) The graph depicts the functional vessel densities (FVD) of the tumors with different treatments on days 7, 14, 21, and 28 after tumor implantation. Median values are represented together with the 25% and the 75% quartiles (n = 6 for each group and time-point). ANOVA, Student–Newman–Keuls–method, *p < 0.05 vs. control, $^{\Phi}$p < 0.05 vs. radiation, $^{+}$p < 0.05 vs. celecoxib. Representative photographs of A549 tumor xenografts from intravital microscopy imaging of the tumor vascularization on day 28 after tumor implantation; control (**b**), radiation (**c**), celecoxib + radiation (**d**); scale bars represent 500 µm.

Abbildung 2a bis 2d. Die Vaskularisierung des Tumor-Xenotransplantates A549 wurde mit Hilfe der Intravitalmikroskopie 28 Tage lang analysiert. (**a**) Die Grafik zeigt die funktionelle Gefäßdichte (FVD in mm/mm²) der Tumoren während unterschiedlicher Behandlungen an den Tagen 7, 14, 21 und 28 nach Tumorimplantation. Dargestellt sind die Mediane mit ihren jeweiligen 25% und 75% Quartilen (n = 6 für jede Gruppe sowie jeden Zeitpunkt). ANOVA, Student-Newman-Keuls-Methode, *p < 0,05 vs. Kontrolle, $^{\Phi}$p < 0,05 vs. Bestrahlung, $^{+}$p < 0,05 vs. Celecoxib. Intravitalmikroskopisch gewonnene repräsentative Fotografien der Vaskularisierung des Tumor-Xenotransplantates A549 am Tag 28 nach Tumorimplantation. von Kontrolle (**b**), Bestrahlung (**c**), Celecoxib + Bestrahlung (**d**); die Balken repräsentieren jeweils 500 µm.

days 14 and 28, FVD decreased in both groups. In tumors treated with celecoxib, the FVD decreased constantly (day 14: 9.6 mm/mm^2 (8.0/10.5), day 28: 7.9 mm/mm^2 (6.6/8.4), $p < 0.05$). In irradiated tumors, FVD strongly decreased between days 14 and 21 after tumor implantation (10.0 mm/mm^2 (9.4/11.1) vs. 7.0 mm/mm^2 (6.5/7.3), $p < 0.05$) and increased again between days 21 and 28 after tumor implantation (7.0 mm/mm^2 (6.5/7.3) vs. 8.1 mm/mm^2 (7.2/8.6), $p < 0.05$). However, at the end of the experiments, FVD was statistically greater in both groups as compared to the baseline levels on day 7 after tumor implantation. The combination of celecoxib and radiation induced a significant decrease of FVD from day 14 to day 28 (day 14 after tumor implantation: 9.6 mm/mm^2 (8.0/10.6), day 28 after tumor implantation: 5.3 mm/mm^2 (4.9/5.7), $p < 0.001$). Direct comparison of celecoxib + radiation with the monotherapy regimes showed that the combination of radiation with celecoxib was superior to both monotherapies in terms of the ability to reduce the final functional vessel density on day 28 after tumor implantation. In contrast to the monotherapies, the simultaneous treatment with celecoxib and radiation achieved a reduction of the peak FVD down to the baseline levels measured on day 7 after tumor implantation.

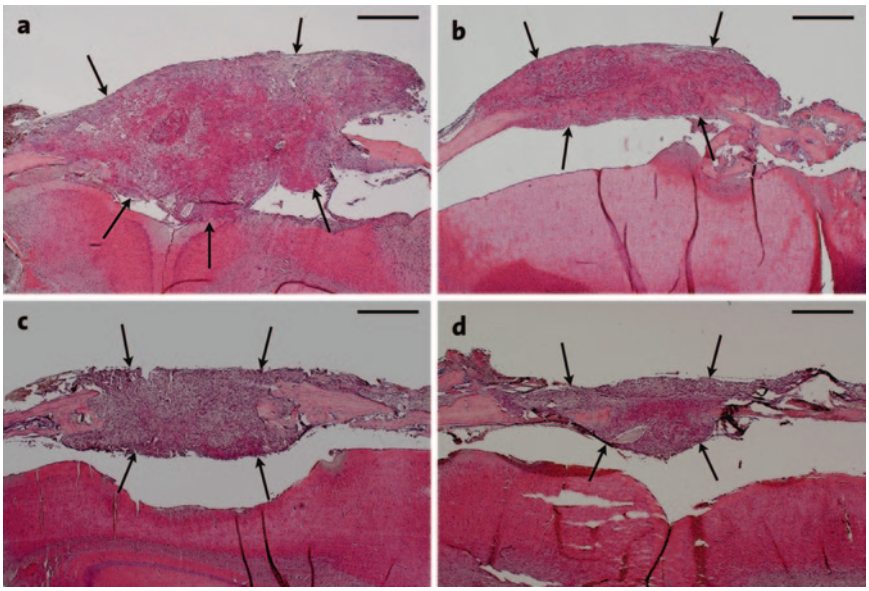

Figure 3a to 3d. Light micrographs of representative 4-µm thick vertical sections through cranial defects, 28 days after tumor implantation. The sections were stained with hematoxylin–eosin. Scale bars represent 500 µm. Tumor borders are marked by arrows. (**a**) Control. A549 lung carcinoma. Large tumor formations were found in untreated secondary bone tumors of A549 lung carcinomas. Tumors treated with either celecoxib (**b**) or radiation (**c**) were similar in size and were found to be markedly smaller than untreated tumors. Tumors treated with radiation and celecoxib (**d**) exhibited the smallest tumor size at 28 days after tumor implantation.

Abbildung 3a bis 3d. Lichtmikroskopisch gewonnene repräsentative Fotografien von histologischen 4 µm dicken vertikalen Schnitten auf Höhe des Defektes in der Schädeldecke mit den Tumor-Xenotransplantaten A549 am Tag 28 nach Tumorimplantation. Die Schnitte wurden mit Hämatoxylin-Eosin gefärbt. Die Balken repräsentieren jeweils 500 µm. Die Tumorgrenzen sind mit Pfeilen markiert. (**a**) Kontrolle. Lungenkarzinom A549. In den Schnitten mit den unbehandelten sekundären Lungenkarzinomen A549 wurden große Tumorformationen nachgewiesen. Die Tumoren, die entweder mit Celecoxib (**b**) oder der Bestrahlung (**c**) behandelt wurden, waren in ihrer Größe ähnlich, aber insgesamt deutlich kleiner als die unbehandelten Tumoren. Die Tumoren, die sowohl mit der Bestrahlung als auch mit Celecoxib behandelt wurden (**d**), waren am Tag 28 nach Tumorimplantation insgesamt betrachtet die kleinsten.

Histology

Representative H&E stained tissue sections of the control group and the experimental groups are shown in Figure 3. Typical signs of a malignant tumor growth were observed in controls. In accordance with in vivo findings, the volume of untreated secondary lung carcinomas increased significantly compared to the small pieces (volume 0.5–1.0 mm^3) that were initially implanted (Figure 3a). The implanted tumor tissue developed to a large tumor formation that had grown above, below, and into the calvaria. Histology revealed extensive infiltration and resorption of the adjoining bone as signs for typical growth behavior of malignant tumors. However, infiltration into the underlying brain was not observed. Compared to controls, the volume of A 549 tumors in all treatment groups was found to be markedly smaller (Figure 3b to 3d). Consistent with the in vivo findings, the combination of celecoxib and radiation showed markedly smaller tumor volumes as compared to the monotherapies with celecoxib or radiation alone.

Discussion

Consistent with previous findings, radiation as well as celecoxib showed distinct antivascular properties in the present study [3, 7, 14, 17, 24, 26, 34, 38, 39, 41, 42, 47, 49, 55]. Radiotherapy and COX-2 inhibition induced a decrease of tumor vascularization and an inhibition of tumor growth, without significant differences between the two treatment regimes. The decrease in functional vessel density and the inhibition of tumor growth proceeded simultaneously indicating that the antitumor effects of radiotherapy and celecoxib treatment can be attributed – at least in part – to antiangiogenic mechanisms. However, both monotherapy regimes were not effective enough to stop tumor progression, although tumor angiogenesis was successfully suppressed. Interestingly, our time-course data showed that radiation induced a strong decrease of tumor vascularization within the first 7 days after single dose irradiation with 7 Gy. Subsequently however, the functional vessel density partially recovered between day 21 and day 28 after tumor

implantation, which may explain why radiotherapy alone did not effectively stop tumor growth.

Based on findings that the modulation of prostaglandin synthesis can ameliorate the response of malignant primary tumors to radiation [30, 35, 37, 40], we hypothesized that combining radiation with COX-2 inhibition may increase the radioresponse of secondary bone tumors. Previous studies showed that the continuous administration of different COX-2 inhibitors prior to single beam radiation of human tumor xenografts demonstrated to potentate the tumor response to radiation in vivo, while the normal tissue was not sensitized to radiation [18, 30, 33]. A recent study demonstrated that elevated levels of COX-2 correlate with reduced patient survival after radiation therapy [12]. This observation indicates that COX-2 and/or its downstream eicosanoid products may protect tumor cells from radiation damage. The radioprotective capacity of COX-2 has been further supported by the finding that radiation dose-dependently induced the expression of COX-2 in PC-3 tumor cells in vitro [43].

The present study demonstrated that, in contrast to the monotherapies with either radiation or celecoxib, the combination of both regimes was capable to halt tumor progression effectively within the observation time period. There was no tumor progression observed following radiation, if the mice bearing the tumors were additionally treated with celecoxib. The profound inhibition of tumor progression was accompanied by a sustained regression of tumor feeding blood vessels. In comparison to the reduction of tumor size and functional vessel density achieved with radiotherapy only, treatment with radiation and celecoxib reduced tumor vascularization and tumor size by another 57% and 51%, respectively. These results are consistent with the enhanced inhibition of capillary sprouting from rat aortic rings by combined administration of radiation and the COX-2 inhibitor rofecoxib [7]. Davis et al. [6] suggested that COX-2 derived prostaglandins are important survival factors for malignant tumors and their vasculature after the initial radiation damage. The inhibition of these survival factors with celecoxib enhanced the vascular damage induced by radiation in vivo as demonstrated by increased microvessel permeability of the vasculature of Col26 murine colon cancer. In accordance with the studies by Dicker et al. [7] and Davies et al. [6], the present data suggest that celecoxib enhanced the radioresponse of secondary bone tumors by inhibiting angiogenesis within the tumor xenografts. Together the data add to the growing rationale of combining radiotherapy of tumors with specific signaling inhibitors including PDGF and VEGF [29, 46].

Although more experiments with additional tumor cell lines and different modalities of treatment, such as fractionated doses of radiation, should be performed to confirm the present data in a wider field, it is concluded that the simultaneous administration of celecoxib and radiation seems to be a rationale to enhance the therapeutic potential of local radiotherapy of bone metastases. Due to the intrinsic antitumor properties of celecoxib, this regime offers the advantage to ameliorate the radioresponse of bone metastases locally and accomplish a systemic tumor therapy in nonirradiated regions concomitantly.

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