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Morphometric analysis of bromodeoxyuridine distribution and cell density in the rat Dunning prostate tumor R3327-AT1 following treatment with radiation and/or hyperthermia

P. Peschke[1], V. Klein[2], G. Wolber[2], E. Friedrich[3] and E.W. Hahn[4]

[1]Department of Radiation Oncology, German Cancer Research Center, Heidelberg, Germany, [2]Department of Biophysics and Medical Radiation Physics, German Cancer Research Center, Heidelberg, Germany, [3]Department of Biology, University of Koblenz-Landau, Landau, Germany and [4]Department of Radiology, UT, Southwestern Medical Center, Dallas, USA

Summary. To monitor cellular response to single doses of radiation (RT) and/or local tumor hyperthermia (LTH) proliferation kinetics were determined in the anaplastic prostate adenocarcinoma R3327-AT1 grown in Copenhagen rats. Tumor-bearing animals were injected i.v. with a bolus of bromodeoxyuridine (BrdUrd), and at defined times after treatment the tumors were surgically removed, fixed and embedded in paraffin. BrdUrd incorporated into the DNA of S-Phase nuclei was detected on 4-6 μm-thick tissue sections using a monoclonal anti-BrdUrd antibody followed by streptavidin-biotin and alkaline phosphatase as a reporter system. Cell nuclei were stained with the fluorescence dye DAPI (Diaminophenylindole). Morphometric analysis was performed using a computer-assisted Leitz-TAS/plus system. Depending on tumor size, up to 18,000 nuclei were routinely analyzed. Untreated tumors of standardized size (8-10 mm) exhibited a BrdUrd-labeling index (LI) of (6.9±1.6)%. In general, the LI was higher in the periphery than in the center, being more pronounced in larger tumors. After 6 Gy γ-rays, the mean LI decreased to 1.8% (24 h) and rose afterwards to 5.4% by 168 h. Following LTH (43.5 °C, 35 min water bath), the mean LI rapidly decreased to 2% (8 h), rose to 9.8% (48 h), and plateaued at 6% after 168 h. A combined treatment consisting of irradiation (6 Gy) followed by LTH yielded smallest LI (2.4±0.18%) and lowest cell density (111±0.6 nuclei per field) by 168 h. The morphometric procedure was reliable and reproducible and can be used to characterize and compare the effects of different therapies on cell kinetics. Of particular value is that these analyses are done on an intact tissue architecture and hence enable a better interpretation of flow cytometric results of treatment-induced alterations within different topohistological regions in solid tumors. Moreover, the technique provides the basis for 3D reconstruction of the cellular activity and heterogeneity of experimental neoplasms.

Key words: Radiation, Hyperthermia, Bromodeoxyuridine, Immunohistochemistry

Offprint requests to: Dr. Peter Peschke, Deutsches Krebsforschungszentrum, Forschungsschwerpunkt Radiologie, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Fax:+ 6221-422572. e mail: P.Peschke@DKFZ-Heidelberg.de

Introduction

There is great interest in understanding how the radiation therapist can handle problems such as "accelerated repopulation" which is assumed to occur during radiation treatment. This recruitment of clonogenic tumor cells has been shown to take place during radiation treatment in animal tumors (Withers, 1988; Trott and Kummermehr, 1991) and has been thought to be an important factor contributing to failure of local control. To this end, experimental fractionation schemes are being examined to overcome this repopulation phenomena and at the same time retain or even improve the therapeutic ratio (Withers and Thames, 1988; Begg et al., 1991; Baumann et al., 1994). However, in preclinical studies some fractionation schemes are difficult to investigate fully because the experimental tumor actually repopulates faster than can be coped with by the irradiation schedule (Hahn et al., 1976). To study the structural changes following ionizing radiation and/or hyperthermia we have selected the Dunning prostate tumor system R3327 which is considered to be a useful model of human prostate cancer (Isaacs and Coffey, 1983). Flow cytometric analysis revealed that the anaplastic tumor subline R3327-AT1 has a labeling index (LI) of 7.5±0.5%, an S-

phase duration (Ts) of 8 h, a potential tumor doubling time (Tpot) of 4.7 days and a cell loss factor (θ) of 15% (Lohr et al., 1993). Previous studies have demonstrated that tumor growth of the Dunning tumor subline R3327-AT1 can be effectively controlled by ionizing irradiation alone (Thorndyke et al., 1985; Hahn et al., 1989; Rao et al., 1991; Henke et al., 1996), as well as by using hyperthermia in combination with radiation (Gottlieb et al., 1988; Peschke et al., 1996).

On the technological level, semiquantitative characterization of proliferation activity has developed from scoring mitototic cells in histological slices and ^{3}H-thymidine labeling to immunohistological techniques which allow the detection of BrdUrd incorporated specifically into DNA during S-phase (Gratzner, 1982) as well as the determination of cell cycle-specific proteins. These advanced techniques, which have been reviewed recently (Ritter, 1993; Iatropoulos and Williams, 1996), facilitated the study of cell kinetics in malignant cell populations before and after treatment (Tenforde et al., 1990; Pollack et al., 1995; Zatterstrom et al., 1995). In parallel, the development of multiparametric flow cytometric techniques, which allow a simultaneous characterization of ploidy and cell cycle progression, have become an interesting tool to elucidate pretherapeutic cell kinetic parameters (Begg et al., 1985). In contrast, it has been shown that the analysis of posttreatment effects is more difficult, due to the complex conditions in vivo which are known to result in heterogeneous tumor response. With respect to these difficulties we have established an immunohistological technique which has proven useful in determining the spatial distribution of S-phase cells in solid tumors. This is of special importance for heterogeneously growing tumors, which differ regionally in vasculature, metabolism, host infiltrates and extracellular matrix components as well as in further parameters which might influence the phenotype of otherwise identical cells. Monitoring the effects of a localized treatment modality such as radiation and/or hyperthermia on the basis of an intact tissue might lead to a better understanding of the complex patterns of response in solid tumors. Here we summarize our results using the above mentioned morphometric image analysis system to determine the topohistological distribution of proliferating cells after radiation and/or hyperthermia in the anaplastic Dunning prostate tumor subline R3327-AT1.

Materials and methods

Chemicals and drugs

BrdUrd, Diaminophenylindole, Naphtol-As-BI-phosphate, and Fast-Red were purchased from Sigma (Germany). The monoclonal antibody against BrdUrd is a product of Becton Dickinson (Germany). Biotinylated donkey-anti mouse antibodies and streptavidin-conjugated alkaline phosphatase were obtained from Dianova (Germany).

Animal and tumor system

Fresh cubic pieces (~8mm^3) of first passage tumor tissue of the R3327-AT1 subline of the Dunning prostate tumor (originally obtained from J.T. Isaacs, Johns Hopkins University, Baltimore MD) were transplanted s.c. into the distal right thigh of anesthetized young male adult (ca. 200 g) Copenhagen rats (Wiga, USA). Treatments were applied under anesthesia 10-15 days after implantation when the tumors reached a mean diameter of 8-9 mm i.e. a volume of 250 to 350 mm^3. The rats were first lightly immobilized with Ketavet (125 mg/kg) and then deeply anesthetized with a mixture of halothane (0.5 Vol%) and a 1:2 mixture of oxygen and nitrous oxide.

Experimental design

BrdUrd dose optimization

To define an optimal concentration range for the labeling index, tumor-bearing animals (R3327-AT1, s.c., Tpot ~ 4.7 d) were injected i.v. with a bolus of 2.5, 5, 10, 20, 50, 100, and 200 mg BrdUrd. Animals (n = 3 per datapoint) were sacrificed 4 hours later. The tumors were surgically removed, fixed and embedded in paraffin.

Labeling kinetics

To find the best time for measuring, the topohistological BrdUrd distribution in the solid tumors (n = 3) was studied by using a BrdUrd concentration optimized by the above described concentration studies and by scoring the labeling index of tumors at 2, 4, 24, 48, 96, and 192 hours after injection.

Radiation

Single photon doses of 6 Gy were given at a dose rate of approximately 0.5 Gy/min (Cobalt 60, Siemens Gammatron S). A field size of 10.6x8.6 cm allowed 6 animals to be irradiated at a time. The proximal margin of the tumor was positioned inside the 95% isodose line while the bodies of the animals were located outside the treatment field. The hind legs were protected by a lead block.

Hyperthermia

Local tumor hyperthermia treatments (LTH) were given for 30 min + 5 min equilibration time at 43.5 °C, using a circulating constant temperature water bath (±0.1 °C). The body of the anesthetized rat was positioned on a plastic jig with holes cut out, through which the tumor-bearing leg was projected and restrained in the water bath below by tying a toe with thread and anchoring the taut thread. During heating, intratumoral and rectal temperatures were monitored with thermocouples.

Radiation and hyperthermia

For the combined treatment modality LTH was started within 5-10 minutes after RT. The results of three independent experiments were plotted for monitoring posttreatment effects. Sham-treated tumors of similar starting volume served as controls. Animals were maintained according to the guidelines for laboratory animals established by the German Government.

Immunohistochemical techniques

At specific times (4, 8, 24, 48, 72, and 168 h) after the end of treatment the tumors were surgically removed, fixed with Ethanol/Acetic acid (1 vol%) and embedded in paraffin. BrdUrd incorporated into the DNA of S-phase nuclei was detected according to the following brief description: 4-6 μm-thick tissue sections were placed on silan-coated slides and rehydrated using a standard procedure. After DNA denaturation with 1M HCl (30 min, 37 °C) and blocking of unspecific binding sites with rat normal serum (15 min) a monoclonal anti-BrdUrd antibody (1:30, 30 min) was added. As reporter system we used a biotinylated rat-anti-mouse-IgG (1:50, 30 min) followed by streptavidin-biotin-conjugated alkaline phosphatase (1:100, 30 min). The slides were put into the staining solution for 8 min (80 mg Fast Red and 20 mg Naphthol-AS-Bi-Phosphate in 80 ml 0.2M Tris buffer, pH 7.4) and counterstained with DAPI (Diaminophenylindole) in order to determine total cell number.

Image analysis

Morphometric analyses were performed with a computer-assisted Leitz-TAS/plus system. For each slide, two-directional columns of approximately 50-70 fields (~100 to 180 tumor cells/field) were counted (Fig.1). From the number of cells analyzed for BrdUrd-uptake by light microscopy (Fast Red substrate) and for total cell number as determined by fluorescence microscopy (DAPI) the labeling index (LI) for each tumor was calculated.

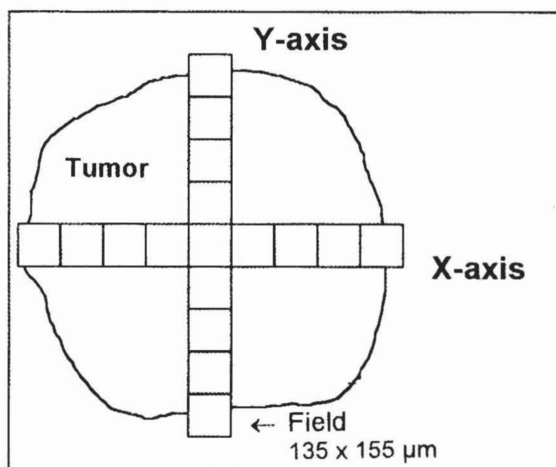


Fig. 1. 50 to 70 pictures, 135 x 155 μm each, were assessed in two orthogonal colums along the maximal radii of each tumor slice.

Biostatistics

Therapeutic experiments were performed in triplicate. Results are expressed as the mean ± standard error of the mean of three experiments. For the evaluation of differences between treatment groups the unpaired T-test as well as the Duncan test of the analysis of variance was used. The level of 0.05 was considered as significant.

Results

Topohistological distribution of labeled cells in control tumors

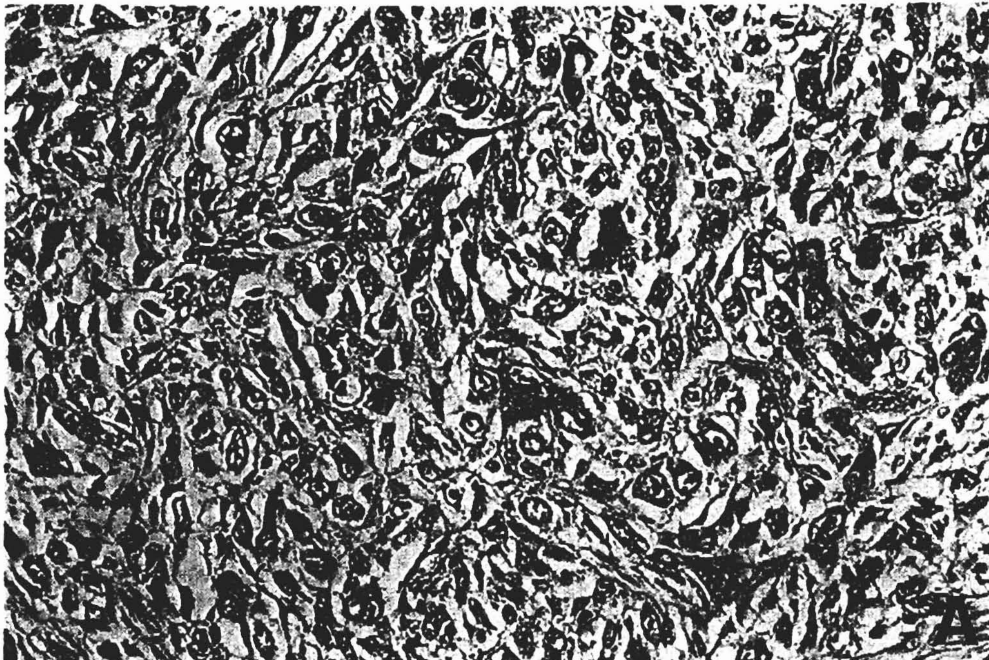
As a baseline, the reproducibility, accuracy and sensitivity of the BrdUrd-labeling technique were determined. Image analysis was performed for each solid tumor scanning up to 120 fields of 135 x 155 μm each. For that purpose up to 20 000 nuclei were analyzed in a tissue slice of about 2.5 mm^2 removed from the area of the largest tumor diameter. Morphologically, the subline R3327-AT1 consisted of sheets with cigar-shaped anaplastic tumor cells intermingled with stromal cells. Depending on size, the number of BrdUrd-positive tumor cells decreased from peripheral to the more central tumor regions (Fig. 2), the difference being more pronounced with larger tumors (Results not shown). In the sham-treated prostate adenocarcinoma 141±16 nuclei per field were analyzed leading to a mean LI of 6.9±1.6%. While cell number was well correlated with tumor size LI was not dependent on volume as summarized in Fig. 3.

BrdUrd dose optimization

To obtain the optimal concentration of BrdUrd the animals of standardized weight were injected with 2.5-200 mg BrdUrd followed by a fixed circulation time (4 h). The LI generated by a dose of 2.5 mg BrdUrd was significantly ($p \leq 0.05$) lower than the labeling indices seen for the doses ranging from 5 through to 50 mg/animal with LI's of 5.2% to 6.9%, respectively. The highest LI of 8.8% was observed for 100 mg BrdUrd, which was not significantly different from the LI of 8.4% at the 200 mg dose (Fig. 4). In the concentration range applied the number of nuclei per field did not change. Similarly, no side effects were noted in the animals even with the highest amount of drug concentration. Hence, for all studies monitoring therapeutic response on the cellular level we used a bolus injection of 20 mg/animal (= 100 mg/kg).

Time kinetics

The best time to surgically remove tumor tissue was determined by injecting a defined BrdUrd concentration (20 mg/animal) and varying the time of drug circulation between 2-192 hours. An LI of 6.9% was achieved 4 hours post injection which continually increased to a significantly higher value ($p \leq 0.05$) of 10.5% at 24 h The maximal peak value of BrdUrd-labeled cells (11.5%) was found 48 h after bolus injection. Further prolongation of drug exposure significantly reduced the amount of BrdUrd-positive cells (Fig. 5). No consistent changes in the number of nuclei per field were observed in the untreated AT1 tumor during the complete observation period.



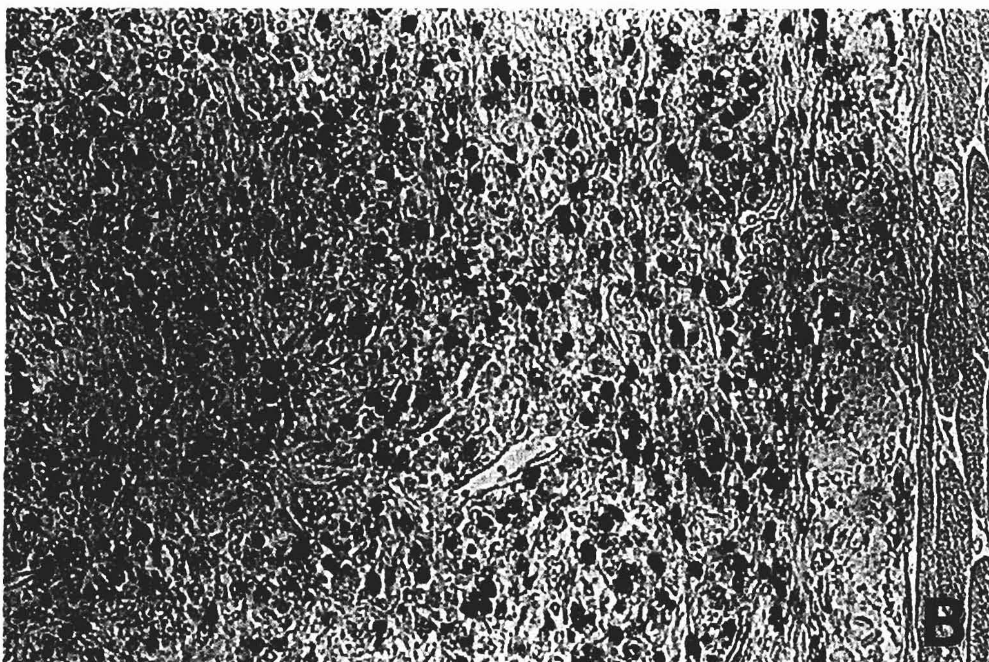


Fig. 2. Dunning prostate tumor subline R3327-AT1.
A. Hematoxylin-Eosin. **B.** BrdUrd incorporated into the DNA of S-phase nuclei is stained using a monoclonal anti-BrdUrd antibody with a streptavidin-biotin system and alkaline phosphatase as detection system. x 400

Radiation

Mean size of tumor diameter in all treatment groups was 8.4±1.5 mm. In the R3327-AT1 Dunning prostate tumors, LI's were significantly ($p \leq 0.0001$) reduced by a single dose of 6 Gy (Fig. 6a). The reduction in LI below the control value of 6.4±0.7%, which was found throughout the complete observation period was most pronounced at 24 h through to 72 h with LI values of 1.8±0.2% and 2.8±0.2%, respectively. Similarly, the number of nuclei per field was reduced ($p \leq 0.003$) following irradiation. The decrease in cell number reached a nadir by 72 h and 168 h where values of 108±7.1 and 113±6.4 nuclei per field were obtained compared to a mean of 151±5.2 for the controls ($p \leq 0.05$).

Hyperthermia

One hyperthermia treatment (43.5 °C, 35 min) significantly ($p \leq 0.004$) altered the LI in situ as measured by bromine uptake in AT1 tumor cells. The process was accompanied by a decrease in cell density with a nadir at 24 h followed by a steady state level for the rest of the observation time (Fig. 6b). Compared to radiation, the drop in LI occurred more quickly, with a minimum at 8 h. With ongoing time, bromine uptake significantly ($p \leq 0.05$) increased over control values by 48 h after treatment followed by a significant ($p \leq 0.05$) decrease at 72 h and a return to baseline levels by 168 h.

Radiation + Hyperthermia

A combined treatment consisting of irradiation (6 Gy) immediately followed by LTH significantly ($p \leq 0.05$) reduced the LI from 5.9±0.4% for controls to 2.6±0.6% and 2.4±0.6% at 8 h and 24 h, respectively (Fig. 6c). The LI returned to nearly control values by 72 h but again decreased to 2.4±0.18% by 168 h ($p \leq 0.05$). Interestingly, the number of nuclei per field steadily decreased after the combined treatment modality from a control value of 144±5.1 to 81±10.8 and 87±6.1 at 24 h and 48 h, respectively ($p \leq 0.05$). By 72 h the number of nuclei per field increased to near control values 123±3.9 but subsequently decreased significantly at 168 h to a value of 111±6.8 ($p \leq 0.05$).

Discussion

Immunohistochemical detection of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdUrd), which is incorporated by S-phase cells, represents a convenient

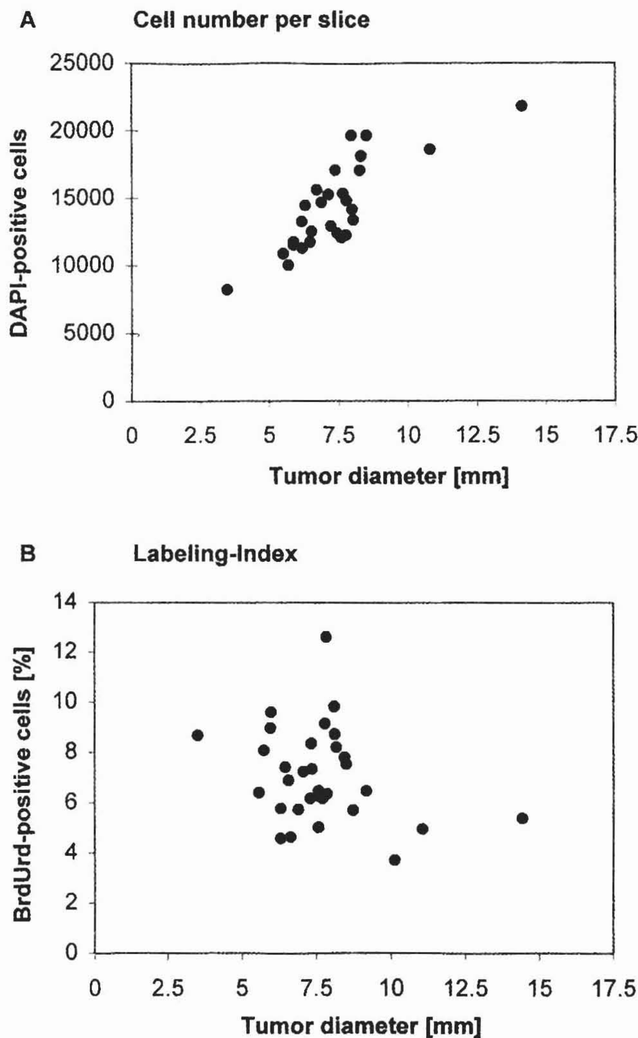


Fig. 3. Correlation of cell number **(A)** and labeling index **(B)** with tumor diameter in the untreated Dunning prostate carcinoma R3327-AT1.

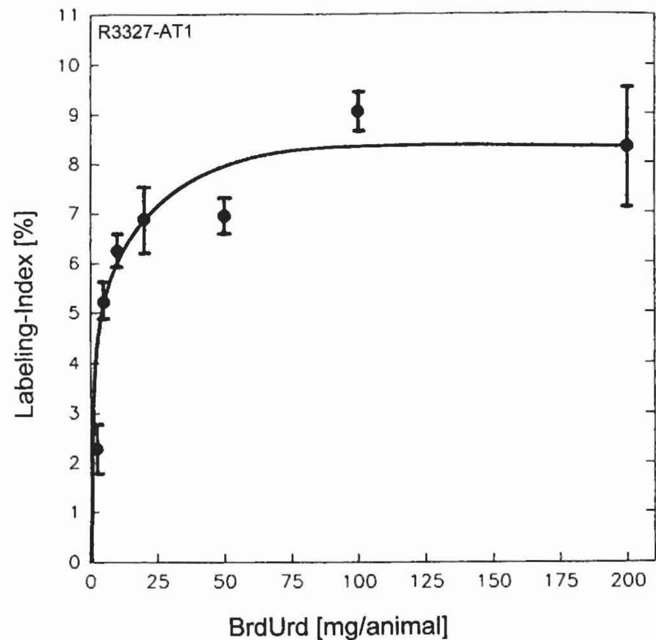


Fig. 4. Efficiency of labeling following various concentrations of BrdUrd (2.5-200 mg BrdUrd/animal) in the Dunning prostate carcinoma R3327-AT1 after a circulation time of 4 h.

technique to study the proliferation of cells in experimental tumors during normal growth and following therapy. We and others (Ramsay et al., 1988; Zatterstrom et al., 1995; Dörr et al., 1996) feel that this procedure is reliable and can be used to analyze and compare specific regions of tumors for cellular activity and integrity. Of outmost value is that these analyses are carried out on an intact tissue architecture which is particularly important after treatment-induced cell loss (discrimination of debris in flow cytometry). Furthermore, three-dimensional reconstruction of the cellular activity and heterogeneity of tumors after treatment is possible. As would be expected the concentration of BrdUrd uptake in the AT1 tumor cells, measured as the number of stained cells was highly dependent on the concentration of BrdUrd injected. The optimal condition for BrdUrd staining was evaluated to be 100 mg BrdUrd per kg followed by an incubation period of four hours. This concentration dissolved in isotonic NaCl, is far lower than that needed to produce any cytotoxic effects. If necessary, the concentration can be increased, especially if shorter periods following treatment are intended to be analyzed in experimental tumors or proliferative active normal tissues (Kikuyama et al., 1987; Tenforde et al., 1990; Zolzer et al., 1994;

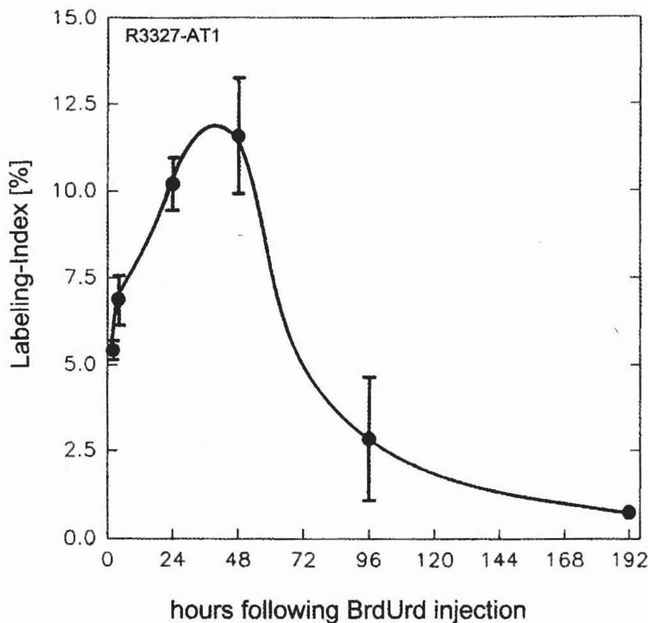


Fig. 5. Labeling-index in dependence of the duration of BrdUrd circulation time (2-192 h) in the Dunning prostate carcinoma R3327-AT1. A fixed dose of 20 mgBrdUrd/animal (100 mg/kg) was injected.

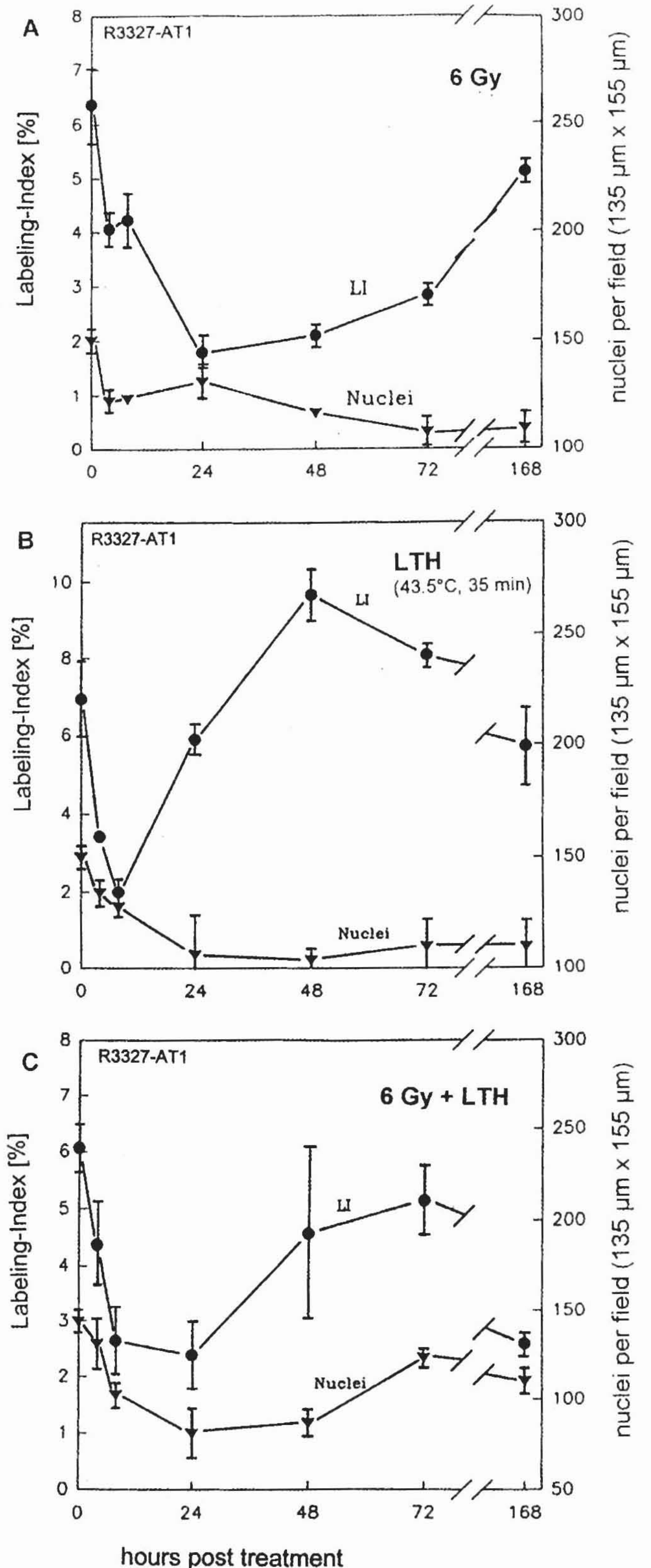


Fig. 6. Kinetics of labeling-Index (LI) and cell density (nuclei per field) assessed by image analysis in the Dunning prostate carcinoma R3327-AT1 0 to 168 h after **(A)** a single dose irradiation with 6 Gy, **(B)** local tumor hyperthermia (LTH) at 43.5 °C for 35 min, and **(C)** a combined treatment with irradiation (6 Gy) and LTH (43.5 °C, 35 min).

Zatterstrom et al., 1995; Dörr et al., 1996). The technique has already been adopted for clinical application. BrdUrd-labeled cells were detected in biopsy specimens or smears of various origin by indirect immunostaining (Khan et al., 1988; Fukuda et al., 1990; Hirano et al., 1991; Miller et al., 1991; Ito et al., 1992).

Our morphological analysis indicates that absolute cell numbers correlated well with tumor volume in the R3327-AT1 subline. The mean LI of 6.9±1.6% found for the anaplastic AT1 lies within the range of human prostatic neoplasms which generally have LI's around 2% for the differentiated type while in the course of dedifferentiation LI rises to 10% and more (Scrivner et al., 1991). Unlike most fast growing rodent tumors, no central necrotic regions have been observed in untreated tumors up to a diameter of 15 mm. Interestingly, the morphometric analysis indicates that the LI is higher at the periphery than in the center of the tumor. Our measurements clearly show that within this cohort of tumors the distribution pattern of BrdUrd-labeled cells was not dependent on volume and that one central slice through the tumor was representative for the determination of LI (Fig 3).

While most of the rodent tumor models are rather heat sensitive (Moorthy et al., 1984; Gottlieb et al., 1988; Jones et al., 1989; Van Geel et al., 1994) the Dunning AT1 subline showed prominent heat resistance (Peschke et al., 1996). Following treatment, small foci of necrosis occasionally did appear, located preferentially in the more central tumor regions. Yet, the small size of these areas of cell inactivation (100-300 μm) did not influence image analysis. The lower cell density after treatment is partly due to the occurrence of edema which disappeared within two days. The rapid decrease in mean LI after a single treatment with LTH supports the idea that heat induces cell-cycle perturbations and produces a delay in the exit of cells from the G1-phase. Indeed, flow cytometric BrdUrd-pulse-chase studies in exponentially growing asynchronous Chinese hamster ovary (CHO) cells indicated both a delay in the exit of cells from the G1 compartment and a G2 accumulation following hyperthermia (Rice et al., 1984; Mackey and Dewey, 1989; Ormerod et al., 1992; Higashikubo et al., 1993). For the in vivo situation an induction of mitotic synchrony by intermittent hyperthermia was reported for the Ehrlich ascites carcinoma (Sapozink et al., 1973). For the Dunning R3327-AT1 subline the release from the heat-induced G1 arrest reached a maximum at 48 hours, when an elevated LI value of 10% was assessed. Detailed analysis of defined topohistological regions showed that the LI in the central region remained rather low, while it dramatically increased in the periphery compared to non-treated tumors. These results emphasize the benefit of the procedure in such that cell function parameters, such as proliferation activity, can be analyzed in a two- or a three-dimensional pattern in coordination with structural parameters within localized regions which is of special relevance for the cellular assessment of treatment response.

Irradiation of normal eukaryotic cells results in delayed progression through the G1, G2, and S phases of the cell cycle; in the latter case only after high doses of ionizing radiation. While regulation of G1 arrest is modified by the p53 tumor suppressor gene product, the G2 delay has been observed in virtually all eukaryotic cells examined in response to irradiation (Bernhard et al., 1995).

A slow but steady decrease in cell numbers occurred after treatment with a single dose of 6 Gy. Occasionally, host immune cells were observed in irradiated tumors infiltrating from the tumor periphery into focal areas of necrosis. Nevertheless, the amount of these cells was much smaller than reported for a solid rat rhabdomyosarcoma (Jung et al., 1990; Tenforde, 1990) where more than 80% of the total tumor cell population was determined to be host cells by 4 to 8 days after irradiation. In parallel to the continuous decrease in cell density LI first decreased with a minimum value at 24 h, presumably due to a radiation-induced cell cycle delay. Similar results have been obtained by flow cytometric studies with experimental tumors where irradiation enhanced the fraction of labeled G2-phase cells due to a radiation-induced G2 block within 24 h (Nusse et al., 1985; Skog and Tribukait, 1985; Zatterstrom et al., 1995). The onset of cell cycle progression is indicated by a permanent increase of LI to nearly control values between 24 h and 168 h. Cell density remained significantly below the control level during the complete posttherapeutic observation time indicating that the process of radiation-induced repopulation, as reported previously (Hermans and Barendsen, 1969; Denekamp and Thomlinson, 1971; Szczepanski and Trott, 1975; Ramsey et al., 1988), is not prominent in the subline R3327-AT1 under the given experimental conditions.

The efficacy of a combined application of irradiation and heat could be documented in the R3327-AT1 even after a single dose as low as 6 Gy. While the kinetics of LI following treatment revealed a sigmoid-shaped curve, the reduction of cell density was prompt supporting the idea that cell inactivation might be higher than with heat or irradiation alone. The slight increase in nuclei per field around 72 h might either represent the cellular alterations influenced by edema or mirror the proliferative response of cells doomed to die after irradiation. The complex alterations following treatment with irradiation and/or hyperthermia as studied by means of two-parameter flow cytometry in human melanoma cell cultures (Zolzer et al., 1994) exhibited a more pronounced S-phase delay after hyperthermia alone or thermoradiotherapy than with irradiation alone. Similarly, growth delay studies have documented the marked response of the Dunning prostate tumor subline R3327-AT1 to thermoradiotherapy with thermal enhancement ratios (TER's) of 1.6 and 1.4 for percutaneous single doses (Peschke et al., 1998) and continuous low dose irradiation, respectively (Peschke et al., 1996).

To better understand treatment-induced alterations at

the cellular level, parallel studies have been performed to determine DNA synthesis more quantitatively, assessing the cellular uptake of BrdUrd by Neutron activation analysis (Hahn et al., in preparation). In addition, the modes of cell death (necrosis vs. apoptosis) induced by a thermoradiotherapy in experimental tumors are presently under investigation using flow cytometric procedures.

In conclusion, we found the described morphometric procedure to be reliable and reproducible. Specific regions of tumors can be characterized for a defined functional parameter within an intact tissue architecture. This is particularly important for the analysis of treatment response (Brugal, 1995). Furthermore, the technique provides the basis for three-dimensional reconstruction of the cellular proliferative activity and morphological heterogeneity of solid tumors.

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