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DISTRIBUTION OF HYPOXIA AND PROLIFERATION ASSOCIATED MARKERS IN SPONTANEOUS CANINE TUMORS

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The therapeutic response of malignant tumors depends on a number of factors associated with tumor microenvironments including the possibility that these microenvironments change during treatment. Two factors, tumor hypoxia and cell proliferation, have been examined in spontaneous canine tumors undergoing multifraction radiation therapy. The approach utilizes immunohistochemical analyses of hypoxia (CCI-103F) and proliferation associated (PCNA) antigens in biopsy samples taken before and after 5 daily fractions of 3 Gy (total dose 15 Gy). The tissue samples were formalin-fixed and paraffin-embedded for the immunohistochemical study. Immunostaining of the sections for PCNA and hypoxia marker reveals little or no overlap when the analysis is made prior to irradiation. An increased degree of overlap seems to occur after 15 Gy but the situation is complicated by a change towards more diffuse PCNA immunostaining in the cells of the irradiated tissues.

The radiation response of tumor cells in vivo is dependent on both microenvironmental and genetic factors. Hypoxia arising from deficiencies in tumor microcirculation has received much attention (1) and has stimulated the development of methods for detecting and measuring tumor hypoxia (2) including the immunochemical hypoxia marker approach (3). Among its advantages, the immunohistochemical method allows for a comparison of hypoxia with other physiological parameters on a single tissue sample without the use of radiotracers (4).

For canine tumors prior to treatment the percentage of tumor cells labeled with the hypoxia marker, CCI-103F, was found to range from 0 to 87% with a mean value of 12.7% as measured by morphometry on immunostained tissue sections (5). This can be compared to hypoxic fractions ranging from 0 to 50% with a mean around 15% for many animal tumors (6). From a microenvironmental point of view, hypoxic cells in canine tumors appear in zones distant to blood vessels and in regions devoid of mitotic figures. On a macroscopic scale, the distribution of hypoxia was uniformly distributed in both peripheral and central regions of the tumors (5). This significantly increases the likelihood of obtaining representative tissue samples by tumor biopsy.

While tumor oxygen status prior to radiation treatment can be predictive of radiation response (e.g., 7), changes in oxygen status during fractionated radiation treatment might be important in some cases. With this in mind, hypoxia marker binding was monitored in spontaneous canine tumors undergoing fractionated radiation therapy (8). For the majority of tumors (8 out of 13) there is little or no change in binding intensity of the marker after 15 Gy as measured by enzyme linked immunosorbent assay (ELISA). In one tumor there was a clear decrease, in one tumor a decrease of marginal significance and in three tumors, a clear increase in the intensity of hypoxia marker binding ((8); Thrall et al., 1994 unpublished).

In the present study, immunohistochemical analysis of tissue sections from the same group of tumors has been performed. One of the purposes of the present study was to compare immunohistological and ELISA results. A

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second purpose was to investigate the relationship that hypoxia might have to other physiological factors which impact on radiation response. Hypoxia and proliferation, for example, are closely linked in the natural history of tumors as cells proliferating near blood vessels push other cells beyond the diffusion distance of oxygen and nutrients. It is possible that hypoxia is merely an indicator of an agressively expanding tumor which fails treatment for reasons other than hypoxia.

Anti-PCNA monoclonal antibodies have been widely used as markers of cell proliferation in formalin-fixed tumor tissue sections. While the PCNA method for the identification of S-phase cells is somewhat controversial (9) and, compared to BUdR techniques, tends to overestimate growth fractions in paraffin-embedded tissue sections (10), it does identify those cells which have had a recent history of proliferation.

Material and Methods

Hypoxia and proliferation markers. The hypoxia marker, CCI-103F, and rabbit polyclonal antibodies which detect CCI-103F adducts in hypoxic tissue have been reported previously [3–5, 8]. A commercially available mouse monoclonal antibody, which cross-reacts with PCNA from a variety of mammalian species, was used to detect the proliferation-associated marker (Clone PC10; DAKO Corporation, Carpinteria, CA).

Hypoxia marker administration. CCI-103F dissolved in 0.9% saline at 1.5 g/l was filter sterilized and administered to the dogs as an intravenous infusion via the cephalic vein during 24 h. The nominal whole body dose of CCI-103F was 40 mg/kg or 118 μM. No detectable cytotoxicity was observed in cells incubated in tissue culture under anoxia for two hours at concentrations up to 200 μM (Chou & Raleigh, unpublished) and it was assumed that CCI-103F at a maximum whole body concentration of 118 μM is not toxic to hypoxic cells in vivo. CCI-103F was given 24 h prior to the first and the sixth irradiation in a fractionated course of radiation treatment. Although analysis of hypoxia is theoretically possible at times sooner than 24 h after CCI-103F injection, the 24-h time point was convenient with respect to clearance of unbound CCI-103F from circulation and to biopsy sampling at a time when the dogs were under anaesthesia for their radiation treatment.

Canine tumor biopsy and irradiation. Dogs were treated with fractionated radiation therapy comprised of 16 or 19 daily (Monday through Friday) fractions of 3.0 Gy to a total of 48 or 57 Gy. Cobalt-60 gamma rays were delivered by means of an isocentric, AECL Theratron 780 irradiator. The prescribed dose was the minimum tumor dose as determined by computerized treatment planning (Multidata Treatment Planning Computer). The dogs were under general anesthesia for approximately 20 min during which time biopsy and irradiation procedures were performed. Anesthesia was induced and maintained with isoflurane. The fractional inspired oxygen value during irradiation was 100%. Immediately prior to irradiation, paired (and in some cases triplicate) biopsies of 50 mg were taken under aseptic conditions by means of a 14 guage Tru-Cut needle or 4 mm diameter trephine punch biopsy from different regions of the tumor. Biopsies were taken immediately before the first irradiation (0 Gy) and immediately before the sixth irradiation at which time (5×3 Gy =) 15 Gy had been delivered to the tumor. The choice of 15 Gy was arbitrary and not tied to specific expectations with respect to changes in tumor hypoxia at such a dose. Practically speaking, it was the earliest time that the decay of the hypoxia marker signal from the first injection allowed for a second interrogation (8).

Tissue processing and immunostaining. The tumor biopsy samples were fixed promptly in 10% neutral phosphate-buffered formalin, processed into paraffin blocks and sectioned onto slides at a thickness of 4–6 microns for subsequent analysis. Slides were deparaffinized and rehydrated through an alcohol series to water immediately prior to immunostaining. For those slides to be stained for hypoxia marker, the sections were treated briefly with 0.01% protease immediately before immunostaining in order to enhance availability of the hypoxia marker antigen.

Slides of contiguous sections were subjected to immunostaining with either hypoxia or PCNA antibody markers. The anti-CCI-103F antiserum was used at a dilution of 1:50 in phosphate buffered saline (PBS) and the PCNA antibody was used at a dilution of 1:100 in PBS. The markers were detected by means of biotin-streptavidin-peroxidase indirect immunostaining. The presence of CCI-103F adducts in the tissues was revealed by means of 3-amino-9-ethylcarbazole (AEC) enzyme substrate chromogen (red) and the PCNA antigens were revealed by means of 3,3′-diaminobenzidine (DAB) chromogen (brown). A light counterstain of Mayer's hematoxylin was then applied followed by coverslipping with glycerol gelatin. Appropriate controls for both markers were used as described previously (4).

Image collection. The tumor chosen to illustrate immunostaining patterns (Figure) was a basal cell carcinoma which appeared as a round, broad-based mass in the caudal aspect of the right metacarpal region of a 15.27 kg dog. In all cases including this basal cell tumor, four, contiguous, 4 micron thick sections were selected at intervals of 100 microns along the major axis of each biopsy sample taken. For the representative images shown in the Figure, two contiguous sections from each of two biopsies of the tumor were immunostained for hypoxia and proliferation markers. One biopsy sample was taken before irradiation (Figure, A and B) and the second was taken from a different region of the tumor after 15 Gy (Figure, C and D). Images were selected and digitized by means of an

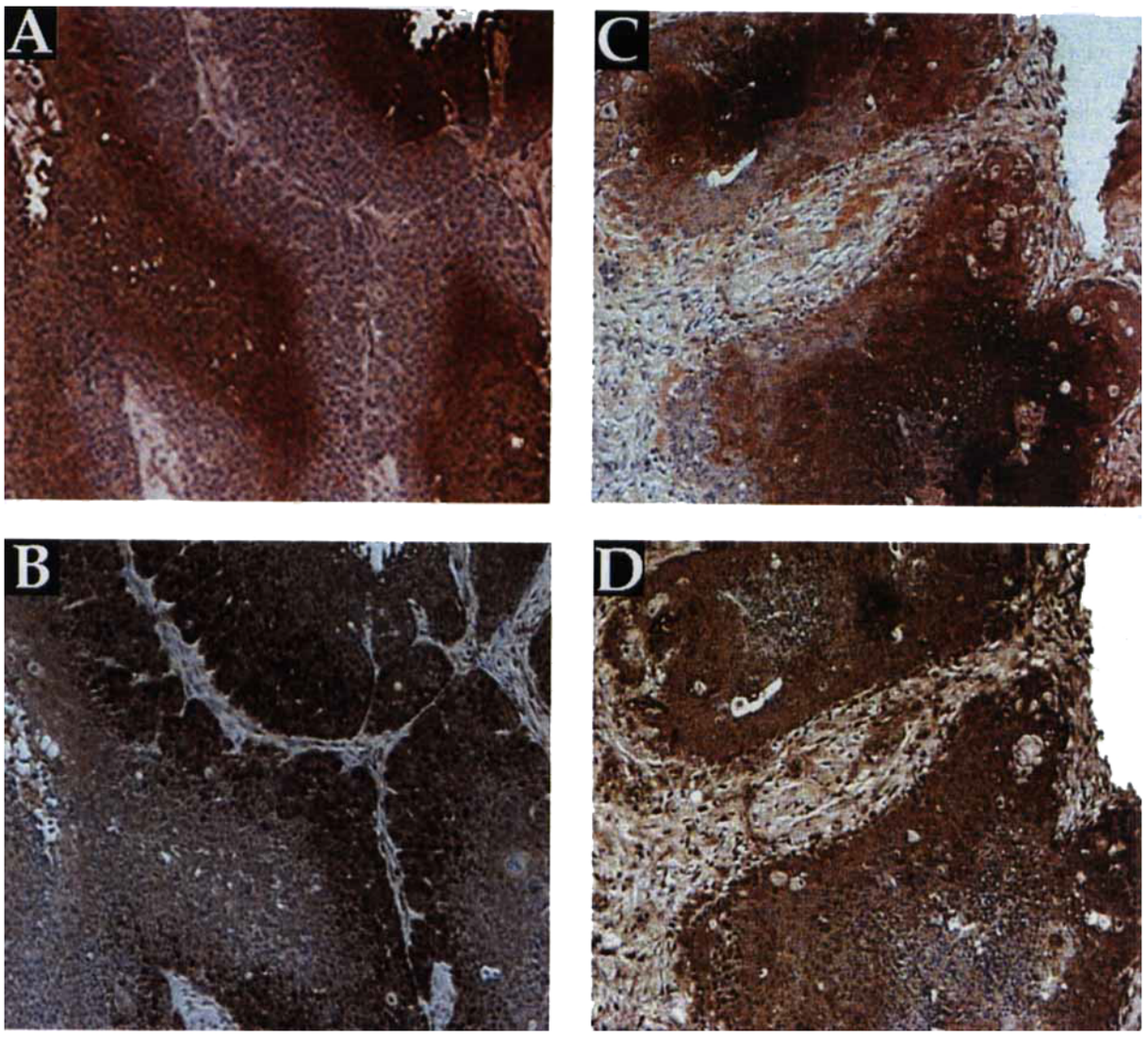

Fig. Immunostaining of formalin-fixed tissue sections from a canine basal cell carcinoma for PCNA (brown) and hypoxia marker (red). A) and B) are 4 mm contiguous tissue sections prior to irradiation. C and D are 4 mm contiguous sections from a different location in the tumor after 5 × 3 Gy irradiation.

image analysis system comprised of a Zeiss Axiovert 35 inverted microscope and Universal Imaging Corp. Image-1/AT Processing and Analysis System incorporating a color camera, high resolution RGB color monitor, workstation with 486/33 microprocessor, monitor and MS DOS version 5.0 software. The images were prepared for laser printer reproduction by means of Adobe Photoshop version 2.5 software and a Macintosh IIsi computer.

Results

A qualitative picture of the relationship of hypoxia and proliferation associated markers is shown in the Figure.

Contiguous 4 micron sections are well-registered at low magnification both before and after 15 Gy. These images are representative of the many sections derived from this basal cell carcinoma and for sections from other tumors under investigation. Qualitatively, the hypoxic marker and PCNA immunostaining are localized in different microenvironments. The PCNA marker is close to the blood vessels and generally associated with cell nuclei. The hypoxia marker is distant to blood vessels and bound to cell cytoplasm. Immunostaining intensity for the hypoxia marker varied noticeably among cells in the hypoxic regions. Generally speaking, hypoxic zones in the sections show little evidence of proliferation during the 24 h period of the hypoxia labeling experiment.

Immunostaining for both PCNA and hypoxia marker binding occurs in the tumors after 15 Gy but the relationship between the markers appears to have changed in the

sense that there appears to be a greater degree of overlap. The staining for the PCNA antigen is more diffusely distributed in the cells and not as strongly associated with cell nuclei after 15 Gy.

Discussion

Prior to irradiation, PCNA and hypoxia marker immunostaining is consistent with earlier observations that hypoxic regions of dog tumors are devoid of mitotic figures (5). Both observations support the view that hypoxic tumor cells proliferate slowly or not at all and that PCNA is not expressed in resting cells (11). Prior to irradiation, a small degree of overlap between PCNA and hypoxia marker immunostaining appears to occur along the edges of hypoxia marker labeled regions. This is in contrast to multicell spheroids cultured in air-saturated media where no overlap between actively proliferating cells and hypoxic cells is seen. For example, 3H-thymidine-labeled, proliferating cells are located in the first 3–5 cell layers of spheroids while 3H-misonidazole-labeled, hypoxic cells generally appear at a depth of 10–12 cell layers (12). This result may indicate (A.J. Franko, private communication, 1994) that the oxygen tension which produces quiescence is significantly higher than the c. 10 mmHg pO_2 associated with sharply increasing hypoxia marker binding (13). Another possibility is that proliferating cells migrate into non-proliferating regions in a time frame that is shorter than that required for the loss of PCNA. The half-life of PCNA in cells is c. 20 h (14) and the rate of cell migration in solid tumors could be 1 to 2 μm/h (15) and so it is possible that the migration of PCNA positive cells into quiescent regions occurs during the 24 h time frame of the experiment.

There appears to be more overlap between proliferating and hypoxic regions after 15 Gy, but the analysis is complicated somewhat by the diffuseness of the PCNA labeling in the cells of irradiated tissue. It has been reported that PCNA is tightly bound to nuclear replicon clusters in S-phase cells resulting in distinct granular staining patterns. More diffuse nucleoplasmic immunostaining is reported for non-S-phase cells (16). It may be that radiation has induced cell cycle perturbations which change the relative distribution of S-phase and non-S-phase cells in the tumor. It has been reported that PCNA analysis as a measure of growth fraction may not be valid for tumor cells exposed to anticancer drugs (10) and a similar limitation may apply to irradiated cells.

The intensity of immunostaining for hypoxia marker varies on a microscopic scale in the tumor sections (Figure). Variations in binding on a microscopic scale have been reported recently for radioactively-labeled misonidazole as well (A.J. Franko and M.B. Parliament, Proceedings of the 42nd Annual Meeting of the Radiation Research Society, Nashville, TN, April 29-May 4, 1994). In principle, variations in binding intensity are to be expected in a spontaneous tumor comprised of cells of heterogeneous genotype, phenotype and degree of maturation. The intratumoral variation in binding intensity should not affect the usefulness of the hypoxia marker approach as long as the primary determinant of binding is oxygen concentration and volume averaged intensities of binding (e.g., ELISA, ^{19}F MRS, ^{18}F PET, ^{125}I SPECT) are not used to estimate the absolute extent of hypoxia.

It has been suggested that squamous cell carcinomas are more likely to produce corded structures than basal cell carcinomas (17) but it is clear that corded patterns associated with chronic hypoxia can exist in basal cell carcinomas as well (Figure). The conclusion that basal cell carcinomas may not be good candidates for hypoxia directed therapies (17) does not seem warranted.

In summary, PCNA and hypoxia marker antigens are in two distinct microenvironments in the canine tumors prior to irradiation. Following irradiation, immunostaining for the hypoxia marker is clearly evident as expected from earlier ELISA studies. There appears to be a degree of overlap between cells immunostaining for both antigens but PCNA immunostaining appears to be more diffuse in the cells of irradiated tumors.

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