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News and Views

Variation in the distribution of a phthalocyanine photosensitizer in naturally occurring tumors of animals

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1. Introduction

The biodistribution and cytotoxicity of photosensitizers have been shown to vary in tumors, not only between classes of photosensitizer [1–5], but also between differently sulfonated forms of the same parent compound [6–14]. For example, the distribution has been reported to be different for related phthalocyanine compounds when viewed in the same tumor model [14,15]. In addition, the local hypoxia which results from early vascular collapse induced by photodynamic therapy (PDT) has been observed to protect tumor cells from the direct effects of photodynamic cytotoxicity in vivo by limiting the oxygen availability for singlet oxygen generation [16], further suggesting that the distribution of a photosensitizer within a tumor can influence its efficiency for that tumor type.

It has been proposed that a variety of tissue factors may be responsible for the preferential uptake and/or retention of hematoporphyrin derivative [17]. It is conceivable that these factors could vary substantially between tumor types. When different tumors were implanted on the chick chorioallantoic membrane (CAM) model, photosensitizer uptake varied with tumor type [18]. Thus the uptake and distribution of a photosensitizer are clearly complex functions which may be influenced by both the chemical properties of the photosensitizer and the physiologic behavior of a specific tumor which may vary between tumor types.

In this report, we evaluate the intratumor distribution of a phthalocyanine preparation in three different, naturally occurring, domestic dog and cat tumors. Our goal was to determine whether the same intratumor biodistribution could be observed in a variety of naturally occurring tumors. Lack of uniformity would highlight

problems in making generalizations or applying information about tumor biodistribution, mechanisms of action or efficiency of a specific photosensitizer based on a limited tumor model. We conclude that it is important to study the photosensitizer biodistribution and efficiency in naturally occurring tumor lines in order to determine whether differences exist between tumor types, and if such can be correlated to clinical response.

2. Experimental details

A chloroaluminum sulfonated phthalocyanine (AlSPc) preparation was used in clinical studies of the response of spontaneously occurring, natural tumors of domestic dogs and cats to PDT. The product was obtained in solution at a concentration of 300 mg ml⁻¹ (Ciba-Geigy Dyestuffs and Chemicals, Basel, Switzerland), lyophilized to a powder, resuspended in Dulbecco's phosphate-buffered saline (1×) (GIBCO, Grand Island, NY) to a concentration of 1 mg ml⁻¹, placed in 6 ml or 10 ml amber vials and stored frozen at -20 °C, in the dark, until needed for patient treatment. High pressure liquid chromatography was used to identify and quantify the phthalocyanine content of the prepared solution, revealing that it is composed primarily of trisulfonated (S₃) and tetrasulfonated (S₄) compounds, with some disulfonated (S₂) compound and a highly sulfonated AlPc compound possibly containing a sulfonate as an axial ligand on the central metal ion.

Veterinary patients received an intravenous injection of the AlSPc preparation at a dosage of 1 mg kg⁻¹ of body weight. After 48 h, samples of tumor tissue were obtained, placed in embedding medium for frozen tissue specimens (OCT Compound, Tissue-Tek-Miles, Elk-

hart, IN) and frozen at -70°C . These tissue specimens were then cut into $6\text{ }\mu\text{m}$ sections, mounted on glass slides and stored at -20°C until examination by both bright field and fluorescence imaging.

An inverted microscope (Axiovert 10, Carl Zeiss, Oberkochen, Germany) was used with $10\times$ and $40\times$ objectives in order to visualize bright field and fluorescence images. A 100 W mercury lamp filtered through an interference filter (365 nm band center, 30 nm full width at half-maximum (FWHM), Carl Zeiss) served as the excitation source. Excitation light was reflected onto the sample using a dichroic filter (FT395, Carl Zeiss) and the emission was isolated with a 615 nm

Fig. 1. Photomicrograph of a hematoxylin and eosin stained section of a feline nasal squamous cell carcinoma. The vascular stroma (vs) surrounds the relatively avascular epithelioid cell masses (e) and covers the surface of the tumor (left). Original magnification, $10\times$.

Fig. 2. Histologic section of a feline nasal squamous cell carcinoma, 48 h following administration of AISPC, viewed in bright field. The tumor surface is on the left and the tumor vasculature can be seen as open spaces (v) in the supportive stroma surrounding the epithelioid cell masses (e) of the tumor. Original magnification, $10\times$.

Fig. 3. Fluorescence imaging of the histologic section shown in Fig. 2, demonstrating drug localization in the vascular stroma (vs) of the tumor and tumor surface. Original magnification, $10\times$.

Fig. 4. Histologic section of a feline fibrosarcoma, 48 h following administration of AISPC, viewed in bright field. Original magnification, $10\times$.

long-pass filter (Carl Zeiss). All images were recorded using a cooled, slow-scan CCD camera system, with 576×384 pixel and 16 bits per pixel dynamic range (Princeton Instruments, Trenton, NJ), interfaced to a computer. Instrument control and image processing were performed with IPLab software (Signal Analytics Corporation, Vienna, VA). A UniBlitz shutter and driver (model T132, Vincent Associates, Rochester, NY) were used to synchronize the CCD camera with illumination of the specimen by the mercury lamp in order to minimize sample photobleaching.

In order to estimate light distribution, background images were acquired from blank slides with identical parameters. In order to correct for non-uniform illumination, all fluorescence images were normalized

Fig. 5. Fluorescence imaging of the histologic section shown in Fig. 4, demonstrating photosensitizer distribution throughout the tumor without limitation to perivascular areas. Original magnification, 10 $\times$.

Fig. 7. The section viewed in Fig. 6, magnified to 40 $\times$ and viewed in bright field, identifying the structure of one of the cellular whorls typical of hemangiopericytomas.

Fig. 6. Fluorescence imaging of a histologic section of a canine hemangiopericytoma, 48 h following administration of AISPc, showing photosensitizer distribution throughout the tumor with a particular concentration of fluorescence associated with the cellular whorls typical of this tumor type. Original magnification, 10 $\times$.

by the algorithm: normalized fluorescent image = (fluorescence - background)/background. Both fluorescence and background images were corrected for dark noise contributed during the 1 s exposure time.

3. Results and discussion

Tissues from three feline squamous cell carcinomas, one feline fibrosarcoma and three canine hemangiopericytomas revealed different patterns of photosensitizer distribution between the tumor types. In the squamous cell carcinomas, fluorescence was limited to the vascular stroma of the tumor, which comprises the

Fig. 8. Fluorescence imaging of the histologic section, viewed at 40 $\times$ in Fig. 7, showing particularly strong photosensitizer fluorescence within the cellular whorls which characterize hemangiopericytomas.

tumor surface and supportive structure around the epitheloid masses which characterize this tumor (Figs. 1-3). In the fibrosarcoma (Figs. 4 and 5) and the hemangiopericytomas (Figs. 6-8), fluorescence was found to be distributed throughout the tumor tissue with no preference for perivascular areas. In addition, a particular concentration was observed in the cellular whorls which are characteristic of hemangiopericytomas (Fig. 8).

In the feline squamous cell carcinomas where the perivascular concentration is observed, vascular collapse and anoxic tumor necrosis would seem to be the most likely mechanism of post-irradiated tumor necrosis. In contrast, the broad distribution of photosensitizer throughout the tissues of both the feline fibrosarcoma and the canine hemangiopericytomas would suggest

that direct cytotoxicity of tumor cells by activated photosensitizer is a major component of the mechanism of tumor necrosis for this photosensitizer in these two tumor types. In addition, the photosensitizer localization in the whorls of the hemangiopericytomas demonstrates some selective distribution to a tumor structure thought to have vascular origins [19–23].

These observations suggest that the biodistribution of a photosensitizer may vary substantially between naturally occurring tumor types. While all of the samples examined were obtained at the same time point following photosensitizer administration (48 h), it cannot be determined from these limited observations whether the difference observed between tumors is a reflection of a different time needed in each tumor type to follow the same distribution pattern or, as we prefer to believe, represents true differences in the ultimate localization of the photosensitizer. In either case it is clear that the intratumor localization of a photosensitizer can vary between tumor types.

We do not know whether the phenomenon reported here is shared by other photosensitizers, differs widely between various tumors or species or has any bearing on the clinical response. However, our observations in this limited number of cases suggest that making generalizations or extrapolating information about the biodistribution of a photosensitizer from one tumor model to another, based on an assumption of the uniformity of behavior between tumor types, may be inappropriate. When photosensitizer localization within a tumor is found to vary between tumor types, studies which correlate the intratumor biodistribution profile with short- and long-term clinical response could provide information which would help to determine whether these variations affect clinical efficiency.

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Immobilization of molecules, membranes and cells for modern optical and non-optical microscopy by photo-cross-linking

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The limitations in the resolving power of classic optical microscopies dictated by diffraction optics have been circumvented by omitting lenses (near-field optical scanning methodology) or using other scanning probe modalities. These provide the means for detecting even atomic structures without the handicaps of preparative artifacts and non-physiological conditions prevailing in electron microscopies.

The scanning probe techniques include scanning force microscopy (SFM) [1], scanning tunneling microscopy (STM), electrochemical SFM and STM, and magnetic and lateral force microscopy, which enable one to visualize atoms or molecules without tedious sample preparation. The common element is a raster scanning of the sample, with the third-dimension data reflecting the particular physical property specific for the type of interaction between the sensor and the sample (height, force, conductivity etc.). Data acquisition is followed by on-line computerized data and image processing. In SFM, the raster scanning of a very fine tip over the surface of any material can map its surface at an atomic or molecular resolution depending on the sharpness of the tip and the flatness of the surface. The scanning is generally effected with a piezoelectric drive generating Ångstrom level displacements of the sample or the scanning tip, relative to each other.

One of the serious requirements of such analytical approaches for the surface in question is that it be stable in space during data acquisition. This condition is fulfilled easily in the case of metal surfaces, integrated circuits (chips) or similar objects. However, the investigation of soft biological materials, molecules, membranes and particularly whole (living) cells requires immobilization without significant distortion of structure. In the absence of such immobilization, the sample may be displaced or destroyed during its interaction with the scanning tip.

The simplest way to achieve the required static condition is to carry out measurements on dried samples. Apart from the non-physiological nature of a dried sample, the ever-present hydration layer at the surface of the sample, which is highly dependent upon environmental humidity, may disturb the accurate data collection. The possibility for scanning probe detection of biological materials in liquid (mostly aqueous) phase became possible very recently; yet an almost unsurmountable problem persists with respect to the physical displacement of the sample.

As an important milestone in the development of atomic force microscopy, Karrasch et al. [2,3] recently published a method for covalently binding biological samples to solid (glass) supports for scanning probe (or other) microscopy in buffer solution. Coverslips were chemically modified with a photoactivable cross-linker, *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS), and biological samples such as viruses, molecules or membrane pieces were squeezed onto derivatized coverslips and then covalently cross-linked to the glass surface by irradiation with UV light. The samples prepared this way could be repeatedly scanned in the scanning force microscope without loss of image quality, while identical but not immobilized samples were pushed away by the stylus. A strong squeezing of the sample and the derivatized glass surface was mandatory during irradiation for immobilization, presumably in order to bring the hydrophobic ANB-NOS into close proximity of the generally hydrophilic biological material.

Immobilizing biological samples for analytical purposes is not new. Silanization [4], and surfaces coated with polylysine [5], lectin (mostly concanavalin [6] or even antibody [7] have already been applied for such purposes. The advent of SFMs brought about a new challenge, because of the relatively strong intermolecular interaction between scanning tips and the sample material. Karrasch et al. [2,3] combined two methods for