Bacteriochlorin a, a New Photosensitizer in Photodynamic Therapy

In Vivo Results

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The photosensitizing properties of bacteriochlorin a (BCA), a nontoxic derivative of bacteriochlorophyll a, were investigated in vivo. BCA has an absorption band at a wavelength at which tissue penetration is optimal (760 nm), and it shows preferential tumor retention in Greene melanoma implanted in the anterior chamber of rabbit eyes. A dose of 20 mg/kg BCA was administered IV at 4–7 mm tumor diameter; 24 hr later the tumor was irradiated with near-infrared light (30 min, 760 nm, 150–280 J/cm²). On the day after the irradiation it appeared that tumor growth had stopped: fluorescence angiography showed nonperfusion of the tumor. Histopathology after enucleation showed subtotal tumor necrosis with occasionally small clusters of viable cells around a blood vessel and at the tumor periphery. Neither BCA nor light alone had any effect on the eye or melanoma.

Photodynamic therapy (PDT) is based on the selective retention of photosensitizers in neoplastic malignant tumors and the subsequent destruction of these tumors upon irradiation with light of an appropriate wavelength. Tumor death is believed to be due to the generation of singlet oxygen (type II reaction). Some studies¹ indicate that upon irradiation of porphyrins, the triplet-excited sensitizer generates a small amount of hydroxyl radicals (type I reaction), which may contribute to photodamage, especially in tissues with a high level of ascorbate, such as the eye.²

The photosensitizer used most often in laboratory and clinical experiments is hematoporphyrin derivative (HpD) or its purified component Photofrin II™. PDT using HpD has been applied with more or less favorable results to human malignant tumors such as skin, head, neck, bladder, gynecologic, breast, brain and eye tumors.³ (See also references cited in Reference 3.)

Because of the restricted physical and chemical properties of Photofrin II, considerable work is currently underway for development of new photosensitizers.⁴ Ideally, a new photosensitizer should possess the following properties: 1) a major absorption peak at a wavelength where tissue penetration is optimal; 2) preferential tumor tissue retention; 3) minimal dark toxicity; 4) a high quantum yield for the generation of type I or type II photochemical reactions; and 5) chemical purity to obtain reproducible results.

Bacteriochlorin a (BCA), a derivative of bacteriochlorophyll a, has proven to be a very effective photosensitizer, both for the oxidation of model compounds and for the killing of murine L929 fibroblasts in vitro.⁵ In vivo it shows preferential tumor retention in Greene melanoma implanted in the anterior chamber of rabbit eyes.⁶ BCA has an absorption maximum at 760 nm with a high molar absorption coefficient of 32,000 M⁻¹·cm⁻¹. The molar absorption coefficient of HpD at 632 nm, the wavelength used for the excitation of the sensitizer, is 1170 M⁻¹·cm⁻¹. At 760 nm light transmission through tissue is about ten times higher than at 632 nm.⁷

The current study is the first report on the photosensitizing effectiveness in vivo of BCA using amelanotic Greene melanoma implanted in the anterior chamber of rabbit eyes as tumor model. The amelanotic Greene melanoma is a malignant tumor that grows rapidly in the anterior chamber of the rabbit eye and therefore provides a useful model for studying treatment responsiveness.

Materials and Methods

Preparation of BCA

Bacteriochlorophyll a was obtained by extraction from the photosynthetic anaerobic bacterium Rho-
*dopirillum rubrum* and purified according to the method of Omata and Murata. Bacteriochlorophyll a was obtained by saponifying bacteriochlorophyll a as described by Oster et al. The central magnesium ion of bacteriochlorophyll a was removed with 50 mM sodium acetate (pH 4.5), and the BCA formed was then extracted with ethylacetate. Ethylacetate was evaporated under reduced pressure, and the BCA was freeze-dried overnight. Until used it was stored in the dark at −20°C under nitrogen. Throughout the extraction and modification procedures care was taken to work as far as possible in the dark at 4°C.

To prepare a solution suitable for IV injection, freeze-dried BCA was dissolved in dimethyl sulfoxide (DMSO) (J. T. Baker Chemicals, Deventer, Holland) and was diluted with sterile 0.9% phosphate-buffered saline (PBS), after which freshly obtained rabbit plasma was added. Optimal results were obtained with a solution with final concentrations of 20% DMSO, 20% plasma, and 60% PBS. For each experiment the solution was freshly prepared and used within 30 min.

**Experimental Procedures**

All experiments adhered to the ARVO Resolution on the Use of Animals in Research.

Ten New Zealand white rabbits of either sex weighing 1.5–3.5 kg were used.

Greene amelanotic hamster melanoma was maintained by serial passage in the anterior chamber of rabbit eyes. Prior to implantation the animals received IM hypnorn™ (10 mg/ml fluanison and 0.315 mg/ml fentanyl citrate; Janssen Pharmaceutica, Beerse, Belgium) for general relaxation; corneal anesthesia was obtained by instillation of 0.4% Oxybuprocaine eye drops. A piece approximately 1 mm³ in size of viable tumor tissue was implanted transcorneally onto the iris of both eyes. After 7–8 days the tumors had reached a diameter of 4–7 mm, were well vascularized, and were suitable for photoradiation. BCA was given IV in a single dose of 20 mg/kg. After injection the animals were kept in a dark room.

Twenty-four hr after BCA administration the tumor in the right eye was irradiated with a 1600-W Xenon lamp (Zeiss, Germany) with appropriate filter combinations (Fig. 1). Filter combination C was used in the first eight experiments. To improve the spectral power distribution for PDT it was replaced by combination B in the subsequent experiments. Light intensity was measured with a EG&G model 450 photometer equipped with a multiprobe 550/2 with a sensitive area of 1 cm², a flat filter, and three neutral density filters (Melles Griot 03FNG057). In the experiments the light irradiance (740–770 nm) ranged from 55 to 125 mW/cm²; the total dose ranged from 150 to 280 J/cm².

The temperature, measured with a thermistor near the tumor against the cornea in the irradiated area, never exceeded 29°C. Sedation prior to and during irradiation was the same as for tumor implantation. One rabbit receiving BCA but no illumination and one rabbit receiving illumination but no BCA served as controls.

The PDT-induced effects were evaluated by slit-lamp examination of the eye and by fluorescein angiography immediately and 24 hr after PDT, just before sacrifice of the animal and subsequent enucleation.

Follow-up after irradiation was restricted to 24 hr because of the presence of the untreated tumor in the other (left) eye, which served as an internal control throughout the experiment. However, one may assume that after PDT an effective photosensitizer induces more than 90% necrosis in a tumor within 1 day. In one experiment only, in which implantation in the contralateral eye did not succeed, the observation period was extended 144 hr. Histopathologic examination was performed on all eyes.

**Results**

**Biomicroscopy and Fluorescein Angiography**

Immediately after PDT, slit-lamp examination revealed that the tumor had become pale, with some enlarged dark vessels and with large hemorrhages (Fig. 2) in the tumor, some of which extended into the anterior chamber. Fluorescein angiography showed nonperfusion of the tumor vessels and profuse leakage of fluorescein (Fig. 3) around the tumor. No corneal opacity was observed. Twenty-four hr after PDT, fluorescein angiography did not show any reopening of blood vessels in the pale and shrunken
Histopathology

Histopathologic examination 24 hr after PDT showed the tumors to be almost completely necrotic and hemorrhagic (Fig. 4). Viable cells were present only around some blood vessels; the red blood cells in these vessels appeared to be normal. Some viable tissue also was present at the tumor periphery. Tumor blood vessels in the necrotic areas generally were severely damaged, with disorganized vessel walls or loss
of endothelial cells; if the walls were preserved, the lumen was tightly packed with swollen red blood cells.

In the experiment in which the tumor was followed for 6 days, histopathology showed a large centrally located necrotic area with hemorrhages. This necrotic area was surrounded by apparent vital tumor tissue with substantial spontaneous necrosis. In the retina a minor local edema was observed.

In two experiments hemorrhagic keratitis had developed, in one accompanied by a local minor cataract of the lens. One eye showed local hyperemia of the choroid and a slight retinal edema together with a remarkable number of polymorphonuclears in the vitreous on the optic nerve head.

Controls

In the two untreated controls (namely, one animal which was given an injection with BCA without subsequent irradiation, and one animal of which one eye was irradiated without a prior injection of BCA), no
effect on the tumor was observed by biomicroscopy, fluorescein angiography, or histopathology. The tumor retained its aspect as a pink, well-vascularized structure with an unchanged growth pattern. Microscopic examination of the tumors showed viable melanoma cells with round-to-ovoid nuclei and many mitoses.

Lumina of the blood vessels were partly filled with normal red blood cells and were bordered with intact endothelial cells. Occasionally a few spontaneous necrotic cells could be observed, which is not an unusual finding in this fast-growing tumor.

In all rabbits no toxic effects of BCA but the PDT-induced effects could be found.

Discussion

The results show clearly that PDT with the new photosensitizer BCA is effective in the treatment of Greene melanoma. Subtotal tumor necrosis was ob-
tained 24 hr after irradiation with near infrared light of 760 nm. The light-induced damage was largely restricted to the irradiated area, indicating that BCA-PDT can be considered for use as localized treatment. Cessation of the blood flow in the tumor immediately after PDT was found by fluorescein angiography. Vascular damage was also evident by large hemorrhages in the tumor and vascular leakage of fluorescein. One day after BCA-PDT, histopathology showed almost total necrosis of the tumor and extensive vascular damage.

Precaution must be taken to aim the light beam at the tumor only. It was found in BCA distribution experiments that the cornea, 24 hr after administration of the sensitizer, also contains BCA. This could be the cause of the hemorrhagic keratitis that was observed in two experiments. In one experiment we observed local hyperemia together with a minor retinal edema. However, it has been reported that bacterial pigments upon irradiation with light of the proper wavelength are readily photodestructed. This photobleaching effect can be considered an advantage because of the differences in BCA levels in the tumor and in the surrounding tissues. These differences allow the destruction of the small amounts of photosensitizer in the surrounding tissues by the photobleaching process before photodamage can occur, and at the same time allow destruction of the tumor, which contains a much higher BCA concentration. Also, the gradual destruction during therapy may lead to a continually increasing depth of penetration into the tumor. Along the same line of reasoning, this photobleaching process could reduce the time of skin photosensitivity, which often is an unwanted side effect of PDT with the currently used Photofrin II.

Using HpD and the same tumor model, Franken et al found immediate cessation of blood flow after PDT. One day later histopathology showed vascular damage and subtotal tumor necrosis. Tse et al demonstrated the cytotoxic effects of HpD-PDT in patients with intraocular and orbital malignant melanoma.

Occasional survival of tumor cells around a functional blood vessel in an otherwise completely necrotic area can be explained by local insufficient light exposure, lack of oxygen, or too low a concentration of BCA. Insufficient light exposure is unlikely, since the area of viable cells is surrounded in all directions by a field of necrosis. Lack of oxygen as the cause of failure of photodynamic treatment also is improbable, since relatively high oxygen concentrations may be expected near intact blood vessels. Too low a concentration of BCA caused by perivascular wash-out of the pigment or by an heterogeneous distribution of BCA as a result of the rapid growth of Greene melanoma may explain the observed cell survival. These very localized low concentrations cannot be amended by increasing the administered dose of BCA, since the increased dose will also increase the amount of BCA in tumor surrounding tissues and thus probably give rise to unacceptable side effects of the treatment.

The rather high dose of BCA (20 mg/kg), together with a light dose of up to 280 J/cm², were chosen for these first experiments in order to obtain effects sufficiently evident to establish the applicability of BCA as a photosensitizer for destruction of intraocular tumors. Experiments are currently in progress to establish optimal doses of light and photosensitizer for this use. Also, it is imperative to document in vivo the penetration depth of the PDT effect at the longer wavelength of BCA (760 nm) as compared to the shorter wavelength of 632 nm of Photofrin II, the current clinical standard.

We conclude from these experiments that BCA has the following properties: 1) nontoxicity (for the rabbit); 2) a high molar absorption coefficient at 760 nm, the wavelength at which the penetration of light in tissue is optimal; 3) excellent photosensitizing properties in vitro; and 4) good tumor localizing and tumor destructive characteristics in vivo.

**Key words:** bacteriochlorin a, eye, photodynamic therapy, photosensitizer, tumor

**Acknowledgments**

The authors are indebted to Mr. E. R. Barthen and Miss Joke Ouwehand for technical assistance.

**References**