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Photodynamic therapy with haematoporphyrin derivative on mice with solid Ehrlichcarcinomas

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Summary

The tumor-toxic effect of the photosensitizer HpD/Halle activated by laser radiation was demonstrated. Solid Ehrlichcarcinoma was used as model. Investigations into the tumor growth, the HpD-distribution after different kinds of drug administration by means of fluorescence measurements, the phototoxicity and the reduction of tumor masses after HpD application and laser irradiation were carried out.

Introduction

Photodynamic therapy (PDT) and fluorescence diagnosis of tumors is a clinical method which uses photosensitizing dyes (photosensitizers) combined with light and laser irradiation in particular (1).
The photosensitizers cause cytotoxic reactions when activated by light as a result of photooxydation processes. A characteristic fluorescence irradiation is emitted. When the dye accumulates in malignant tissue more than in normal tissue, selective therapy and diagnosis becomes possible. The most commonly used photosensitizer is a mixture of special porphyrines and their aggregates (haematoporphyrin derivative, HpD) (2).
The aim of these investigations was to examine the efficiency of the HpD produced in our own lab. It mostly consists of aggregates. The HpD was applied to tumor-bearing mice. It was subsequently activated by special red laser irradiation. The solid Ehrlichcarcinoma was chosen as the tumor model. The conditions were varied in order to design a model as a prerequisite for applying photochemotherapy in the treatment of humans.

Materials and Methods

Animals

The experiments were performed on male ICRmice with an average weight of 20 g. The animals (VEB Versuchstierproduktion Schönwalde) were fed ad libitum on standard pellets R 13. They had permanent access to water. The temperature in the plastic cages was about 22 °C. Anaesthesia was achieved by an i.m. injection of a 0.05 ml mixture consisting of Rompun® (5 %), Ursotamin® (10 %) and physiological saline (1:3:6).

Tumor

Solid Ehrlichcarcinoma was induced by a s.c. injection of 7 day old Ehrlichascites cells from a donor mouse. 0.125 ml of the cellular suspension was mixed with heparine and saline (cont. 10^6 cells/ml) and given subcutaneously. After 4 days, the tumors could be microscopically observed on the shaved skin surface.

Sensitizer

Haematoporphyrin derivative (HpD) was prepared from bovine blood as described previously (1, 3, 4). A drug with an enriched component of the aggregates, especially DHE-dimers, was obtained by gel filtration chromatography on Sephadex G 25 coarse (Pharmacia Fine Chemicals, Uppsala Sweden). The final solution mixture was adjusted to pH 7.4 and refilled with saline to a concentration of 5 mg/ml.

Laser equipment

Figure 1 shows the absorption spectrum of the used HpD. In the red range only a small absorption peak is displayed (around 620–625 nm). This red and the nearly infrared range is particularly relevant to

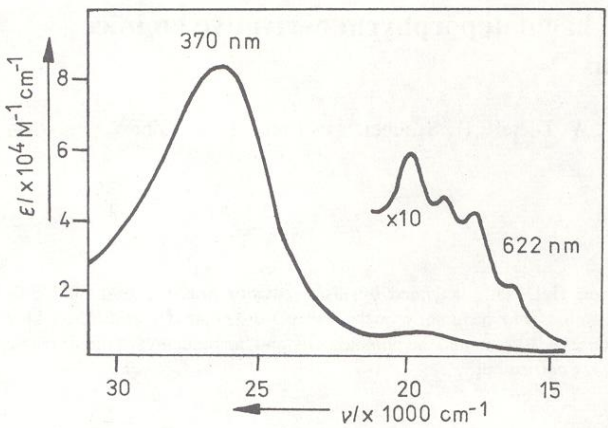

Fig. 1. Absorption spectrum of HpD in PBS ($pH = 7.4$)

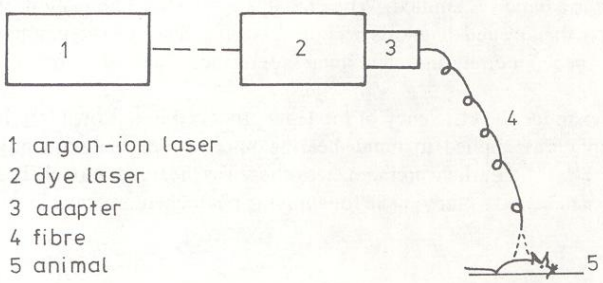

Fig. 2. Arrangement of laser equipment

photodynamic therapy since it shows that a high tissue penetration is attained there. One can obtain a red shift of so-called Q bands (absorption bands in the spectral range 450–700 nm) of HpD when the photosensitizer accumulates in the tissue. Hence, the most efficient wavelength also for the treatment of more deeply lying tumors is about 630 nm. In this experimental arrangement, a helium-neon laser (HNA 188, CZ Jena, 632.8 nm, P = 60 mW) or an argon-ion laser pumped dye laser (ILA 120, CZ Jena/FSL 100 ZWG Academy of Sciences of the GDR, Berlin) was used. The active medium used for the dye laser was rhodamine 6G and kresyle violet or DCM, as well (P = 300 mW, 630 nm). The laser beam was coupled to a 200 micrometer single optical fibre. The end illuminated a region of about 1 cm^2, including the tumor (Fig. 2).

For fluorescence investigations, UV radiation of the same argon-ion laser (about 50 mW, 364 nm) was

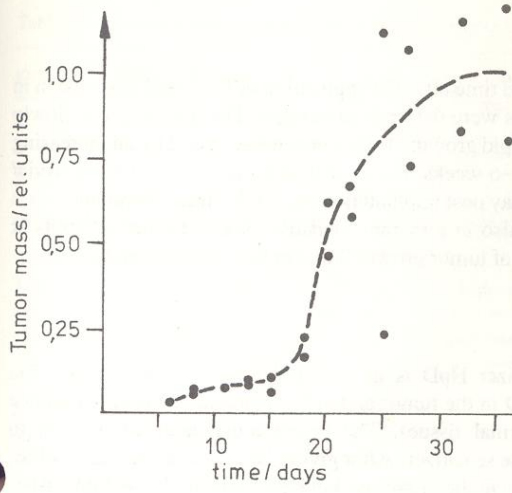

Fig. 3. Tumor growth in dependence on the period after application of EAC-cell suspension

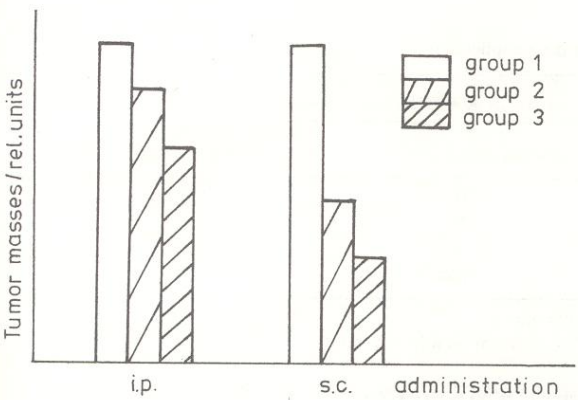

Fig. 4. Tumor masses after drug administration and laser irradiation. group 1: control animals; group 2: animals with drug application but without irradiation; group 3: drug and light application

used. The laser was linked to a special device which produced a background-free excitation irradiation (5) and a fluorescence detector system consisting of a fibre, a monocromator, a multiplier, and a lock-in device.

Treatment

24 hours before starting the PDT treatment, the mice received an i.m. or s.c. injection (around the tumor mass) of HpD. The period of light exposure was 30 min. Some experiments were performed at 24 hour intervals (see Tab. III and IV). The animals were divided into three groups. They were kept under the same conditions. The first group consisted of controls (no drugs, no irradiation), the second one received the drug only without laser irradiation. In former experiments no detectable influence of laser radiation alone was recorded ($\lambda = 600-700$ nm, $P = 100-200$ mW/cm^2). For evaluation the mice were sacrificed on the 10th day (experiment with i.p. application of HpD, see: Results) or 15th day (s.c. application) after their last treatment. The tumor was removed and its weight was determined.

Results

Tumor growth

Figure 3 displays the dependency of tumor mass and time after the application of EAC-cell suspension in the group of untreated animals. Two tumorweights were determined per day. The tumors grow slowly within the first 13–15 d, followed by a period of rapid growth. They were characterized by an increasing amount of auto-necrotic tumorous tissue. After 4–6 weeks, the animals died from the large, necrotic/ulcerated tumors. The treatment started on the 5th day post implantationem. At this time the tumors could be clearly detected macroscopically. They were also in a therapy-sensitive phase. Earlier attempts at treatment which were started in a progressed stage of tumor growth failed in this experimental model.

Fluorescence investigations

The prerequisite for the efficiency of the sensitizer HpD is its accumulation in tumor tissue. The measurements were aimed at the detection of HpD in the tumor and at the gradient of the fluorescence signal between tumor and its environment (normal tissue). The fluorescence intensity is said to correspond roughly to the tissue concentration of the sensitizer. After giving HpD i.v. or i.p., the visible, typical, reddish fluorescence was found exclusively in the tumor marking the limits of the malign tissue. When applying the sensitizer s.c., the concentration gradient between tumorous and normal tissue was not so clearly expressed. HpD did not accumulate within the tumor when applied superficially. In order to quantify our observations intensity measurements of the fluorescence were carried out (5 animals per group) (see Tab. I).

Table I Fluorescence signal obtained 24 h after drug application

administration	signal	gradient
	(arbitrary units)	
i.v.	50 ± 10	10
i.p.	45 ± 10	8
s.c.	$100^* \pm 15$	5*
superficial	~5	signal too weak

superficial: HpD was painted on the skin above the tumor
gradient: signal (tumor)/signal (0.5 cm away), *: dependent on injection point

Table II Toxic rate (%) dependent on light energy (c = 50 mg/kg BW)

group	HpD	IR (min)	n	Lethal rate after treatment (%)			
				24 h	48 h	72 h	10 days
1	–	–	10	0	0	0	0
2	*	–	10	0	0	0	0
3	*	30	9	70	90	100	100
4	*	10	15	30	67	67	67

IR: irradiation, n: number of animals

Photodynamic therapy

At the beginning, toxic experiments on tumor-bearing animals by i.p. administration of undiluted HpD solution (50 mg/kg BW) and laser irradiation (λ = 633 nm, P = 50 mW) were carried out (See Tab. II). After the administration of HpD alone no visible effects were observed. Both the drug administration and laser irradiation show the typical response of a photosensitizer. A strong dependence on the applied light dose was observed. Subsequently, we used a drug dose of 10 mg/kg BW in the following PDT treatments.

Table III shows the results of photodynamic therapy after i.p. administration and Table IV those after s.c.

Table III Tumor masses after PDT (i. p. administration, 2 irradiations, P = 100 mW)

group	HpD	IR (min)	n	Average tumor mass (mg)	υ (mg)
1	–	–	11	524	192
2	*	–	7	453	193
3	*	30	10	358	239

IR: irradiation, n: number of animals, υ = standard deviation

Table IV Tumor masses after PDT (s.c. administration, 3 irradiations, P = 100 mW)

group	HpD	IR (min)	n	Average tumor mass (mg)	υ (mg)
1	–	–	7	1,160	504
2	*	–	4	608	214
3	*	30	10	393	164

IR: irradiation, n: number of animals, υ = standard deviation

Table V Macroscopical and histological effect of the PDT
(30 min exposure, 24 h after s.c. application/5 mg/kg BW HpD)

1. day:	expressed, circumscribed edema, pale skin, greyish, starting necroses
3.–5. day:	recurrent edema, clear peritumorous inflammation, formation of a haemorrhagic scab above the tumor, progressing necroses
10.–15. day:	slight accompanying inflammation, hardly any vital tumor tissue
after 20. day:	expulsion of the haemorrhagic scab, a new increase of tumor mass and tumor necrosis (complete), respectively, in single cases

application (see Fig. 4. too). The mice which had received s.c. injections were killed on the 15th day after the last laser treatment because they suffering from strong edema and inflammation before. The group with i.p. administration also suffered edema, inflammation and slight erythema but these alterations of the irradiated area disappeared in the week following exposure. These effects may be explained by the higher HpD concentration in the tumor tissue after s.c. application.

In order to prove whether the reductions of tumor masses were also based on hyperthermic effects (6) the temperature during the treatment was measured by means of thermocouples. A temperature increase of less than 5 K was obtained. During the irradiation a change in colouring (greyish) of the tumor area was noticed. Tumorfree parts of the exposed area didn't show this reversible effect.

Treatment with HpD alone without any laser irradiation reduced the tumor mass (group 2). These results are in agreement with investigations on other photosensitizers (7, 8).

Photodynamic therapy with laser radiation increases the effect of tumor mass reduction compared to untreated tumors.

Statistics: Statistically significant differences in the experiment with s.c. administration were shown by the Analysis of Variance ($F = 13.54 > F_{Tab} = 5.78/0.01$) and by the nonparametric u-test (Mann-Whitney statistic, $u = 3.5 < U_{Tab} = 5/0.001$, comparison between groups 3 and 1). For the experiment with i.p. administration the Student's t-test was used. There was a significant difference ($P = 0.10$) between group 1 and 3 but the difference in tumor mass between group 2 and 3 was not significant.

Histological investigations

Some of the treated tumors were examined histologically by LM. Necrosis and cell destructions were observed. However, only some tumors were completely destroyed. In Table V macroscopical and histological effects are listed.

Discussion

The tumor-toxic effect of the photosensitizer HpD used with laser irradiation (by means of special dye and gas-ion lasers) on solid Ehrlichcarcinomas was demonstrated. The tumor mass reduction could be verified significantly in the treated animals. A cytotoxic effect of HpD alone (i.p. and s.c. application) without any laser irradiation was found to be in accordance with other studies (7, 8). The laser irradiation increases the cytotoxic effect depending on light dose and wavelength.

Light of the wavelength 632 nm was used for its high tissue penetration. In this case the temperature rise is negligible. Earlier investigations that we have conducted using the green light of an argon-ion beam (514 nm and 488 nm) and of a copper-vapour laser (510.6 nm) also led to a significant reduction of the tumor mass (as reported e.g. by Bellnier et al. too (9)), but there was also a strong hyperthermic effect (caused by the absorption of blood and HpD) which added to the photooxidation reactions.

During the PDT experiments a reversible change in the colour of the irradiated tumor was noticed. This effect may be partially due to circulatory stasis within the stroma of the tumor. The accumulation of the fluorescing compounds of HpD within the tumor and surrounding tissue was determined by fluorescence technique. Assuming that the fluorescence signal corresponds to the concentration of HpD, a ratio between tumor and normal tissue can be calculated. Our maximum values of about 10:1 (24 h after i.p. administration) were established by other authors (2).

The HpD concentration after s.c. application (around the tumor mass) inside the tumor is diffusion-dependent, but higher than that of i.p. administration. Because of this a greater cytotoxic effect by the use of the photosensitizer alone can be expected. However, in this case the small difference between the concentration of the dye in the tumor and its surrounding tissue has negative effects on the selectivity of the therapeutic strategy.

The essential efficacy of our self-made HpD linked to laser irradiation of appropriate wavelength in photodynamic therapy was proven in these animal experiments.

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