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Preclinical report

Determination of the maximal carcinoma/normal skin ratio after HpD or *m*-THPC administration in Hairless mice (SKH-1) by fluorescence spectroscopy

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The two major steps in our study on the treatment of skin carcinomas by photochemotherapy (PCT) were the development of a skin tumor model in Hairless mice by a chemical carcinogenesis and the use of fluorescence spectroscopy, a semi-quantitative and non-invasive method, in order to determine the time after i.p. injection of photosensitizer when the tumor/normal skin ratio was the highest. A three-step carcinogenesis protocol provided mice bearing carcinomas and these were used to determine the tumor/normal skin ratios of two photosensitizers by fluorescence spectroscopy. Hematoporphyrin derivative (HpD) (5 mg/kg body weight) and *m*-tetra(hydroxyphenyl) chlorine (*m*-THPC) (0.3 mg/kg body weight) were injected i.p., and fluorescence was measured at 1, 4, 8, 12, 24, 48, 72 and 96 h after injection. The best carcinoma/normal skin ratio would be 3.2 ± 1.4 for HpD and 2.7 ± 2.1 for *m*-THPC, respectively. The delays required to reach these ratios were 72 h for HpD and 24 h for *m*-THPC. These results have to be considered with caution due to the high SEs and they must be confirmed by organic extraction. Photodynamic therapy with the same doses of HpD and *m*-THPC used in this pharmacokinetic study has to be carried out in order to compare the toxicities of the two photosensitizers and to determine which one is the best for this type of tumor. [© 2000 Lippincott Williams & Wilkins.]

Key words: Carcinoma/normal skin ratio, fluorescence spectroscopy, skin carcinomas, three-stage carcinogenesis.

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Introduction

Photochemotherapy (PCT) may be applied to treat skin tumors, which involves an initial treatment with a photosensitizer followed by tumor irradiation with light of a specific wavelength. This causes the photosensitizer to produce cytotoxic oxygen species within the tumors. After a systemic injection, the photosensitizer is located both in normal surrounding skin and in the tumor. Therefore it is necessary to know how it is distributed in these two tissues and also to determine when the photodynamic treatment will be the most efficient.

Most preclinical data on the distribution of the photosensitizers have been obtained using animals with transplanted tumors that grow neither in their native connective tissue matrix nor from their normal epithelium. These s.c. models do not mimic spontaneous human skin tumors. It is therefore interesting to evaluate the photosensitizer distribution inside the tumor and normal skin when the tumor is growing in its own epidermal tissue. Thus, we developed a chemically induced skin tumor model.

The chemical induction of skin tumors provides a suitable system that has been well studied in mice.[1] This system provided the basic concepts of carcinogenesis and was also used for PCT.[2]

The pharmacokinetic study was performed on Hairless SKH-1 mice bearing skin carcinomas in order to determine the time when the tumor/normal skin ratio of photosensitizer was the highest after systemic injection. This time should then be used in further studies to ensure efficient PCT. Even though 5-aminolevulinic acid (ALA) topical application was

usually used for human non-melanoma skin tumor treatment by PCT, in our experiment we have chosen two photosensitizers, hematoporphyrin derivative (HpD) and *m*-tetra(hydroxyphenyl)chlorine (*m*-THPC). HpD is currently used in PCT,[3] and *m*-THPC is a promising second-generation photosensitizer[4] for the treatement of the early bronchial and esophageal cancers.[5]

The distributions of the photosensitizers were monitored by light-induced fluorescence spectroscopy and the tumor/normal skin ratio was measured after i.p. injection of the photosensitizer.[6,7]

Fluorescence spectroscopy is a suitable method for monitoring photosensitizer distribution *in vivo* by measuring the fluorescence intensity of tumors and surrounding normal tissue at different times after photosensitizer administration.

This technique has already been used to determine the fluorescence distribution of 5-ALA-induced protoporphyrin IX (Pp IX) and *m*-THPC in human and animal carcinomas. These pharmacokinetic studies have been mainly carried out on early bronchial and esophageal carcinoma, and colorectal, mammary, skin, liver or bladder carcinomas.[8–12]

Materials and methods

Chemicals

HpD was made in the laboratory[13] and *m*-THPC was obtained from Scotia Pharmaceuticals (Guildford, UK). 7,12-Dimethylbenz[*a*]anthracene (DMBA), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and 4-nitroquinoline-*N*-oxide (4-NQO) were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Acetone (Prolabo, Fontenay-sous-Bois, France) was used as solvent.

Induction of carcinomas on mice

Female Hairless (SKH-1) mice (7–9 weeks old) were obtained from Charles River (St-Aubin-les-Elbeuf, France) and kept in plastic cages at 25°C with a 12 h light/darkness cycle. They were given a standard diet and water *ad libitum*.

Skin carcinomas were induced chemically by a three-stage initiation–promotion–conversion protocol. Each mouse was given three topical applications of 25.6 mg (100 nmol) DMBA in 0.1 ml acetone, one to each of three sites on the back skin delimited by tatooing. Starting 1 week later, the mice were given twice weekly topical administrations of 3.1 mg (5 nmol) TPA in 0.1 ml acetone to the initiated sites for 20 weeks. Finally, for 3 months the initiated and promoted sites were given twice weekly applications of 125 μg (657 nmol) 4-NQO. Control mice received only acetone for all the tumor induction steps.

The tumor-bearing mice used for pharmacokinetic studies were sacrificed at the end of the experiment for histopathological evaluation of the tumors. They were found to be epidermal carcinomas.

Pharmacokinetic study

Six mice bearing carcinomas large enough to receive the sensor were used to determine the distributions of *m*-THPC and HpD in the tumors and normal skin. HpD was dissolved in physiological serum and *m*-THPC was dissolved in ethanol:polyethyleneglycol 400:water (20:30:50 v/v). Each mouse was given an i.p. injection of HpD (5 mg/kg body weight) or *m*-THPC (0.3 mg/kg body weight).[14,15] The mice were their own controls, as fluorescence measurements were performed on the tumors and the normal skin before injection of the photosensitizer. Then three measurements of the fluorescence of tumors and normal skin were carried out at intervals after injection (1, 4, 8, 12, 24, 48, 72 and 96 h). Each photosensitizer was tested on three mice and all fluorescence measurements were performed with an excitation wavelength of 410 nm.

The spectrofluorimeter consisted of a CP 200 spectrograph (Jobin Yvon, Longjumeau, France), with an optical multichannel analyzer (OMA) managed by Spectramax software, and three optical fiber probes, one for excitation, one for emission and one to measure backscattered power measuring.[6,16] The excitation light (410 nm) was provided by a 300 W xenon lamp through a bandpass filter. The *in vivo* fluorescence intensities of *m*-THPC (652 nm) and HpD (630 nm) in carcinomas and normal skin were plotted against the time after injection. All the measured spectra were normalized according to the flavin autofluorescence peak (at 520 nm) obtained before drug injection.

Results

Intraperitoneal injection of SKH-1 Hairless mice bearing chemically induced carcinomas with HpD or *m*-THPC resulted in the distribution of the drugs in the skin and tumors (Figure 1). The highest carcinoma/normal skin ratio was 3.2 ± 1.4 for HpD (Figure 1A) 72 h after injection and 2.7 ± 2.1 for *m*-THPC (Figure 1B) 24 h after injection. The ratio reaches a first maximum only a few hours after the photosensitizer i.p. injection, and would be followed by a decrease with most of the HpD and *m*-THPC

located in normal skin. This phenomenon was previously observed in mice bearing chemically induced papillomas.[7]

These results of fluorescence spectroscopy concerning the value and the exact localization of the maximal ratios should be considered with caution due to the importance of the SEs. The high SEs obtained for carcinoma/normal skin ratios can be explained by the difficulty in putting the sensor exactly at the same tumor or normal skin site for each measurement. Moreover, tissue fluorescence measurements at a given time can vary for each animal but also for the same animal depending on the sensor localization on the tissue. Mice of the same species can react differently to photosensitizer injection. On the other hand, they may not present the same porphyrin endogenous concentration in their tissues, which explains a possible variability of autofluorescence measurements. Moreover, the tissue structure is not identical from one mouse to another even if there is a similarity between the three mice and optical parameters of the tissue could vary during the experiment.[17]

However, in spite of these problems we have noticed that for each photosensizer the three mice showed a similar carcinoma/normal skin ratio curve with two maxima even if they are not located at the same times for each mouse (Figure 2).

Moreover, a preliminary pharmacokinetic study carried out by a chemical extraction method in a papilloma skin model after HpD injection also showed the same type of curve for ratios than the one yielded by fluorescence spectroscopy with, however, three maximal tumor/normal skin ratios located 12, 48 and 72 h after HpD injection.

Figures 3 and 4 show the spectra of *m*-THPC and HpD in the carcinomas and normal skin at the best time for irradiation.

For the two photosensitizers, the comparison of autofluorescence spectra before injection and the spectra at the best time for treatment showed a difference in fluorescence intensity at the main emission peak of each phosensitizer, 630 nm for HpD and 652 nm for *m*-THPC. This difference in fluorescence intensity was particularly marked for tumors and was directly related to the concentration of photosensitizer in the tissues. Photobleaching can be neglected in this experiment because the acquisition time was short (0.5 s) and the excitation power was low (1 mW). The autofluorescence spectra in tumors had a peak at 635 nm due to endogenous porphyrins. This peak was also present in the autofluorescence spectra of the normal tissue, but at a much lower intensity.

Discussion

Fluorescence spectroscopy is a particularly attractive technique because it is non-invasive. Hence, mice can be used as their own controls and they can be monitored throughout the experiment to avoid differences. As a result, the experiment required fewer mice.

The results seem to indicate that HpD gives a slightly higher carcinoma/normal skin ratio than

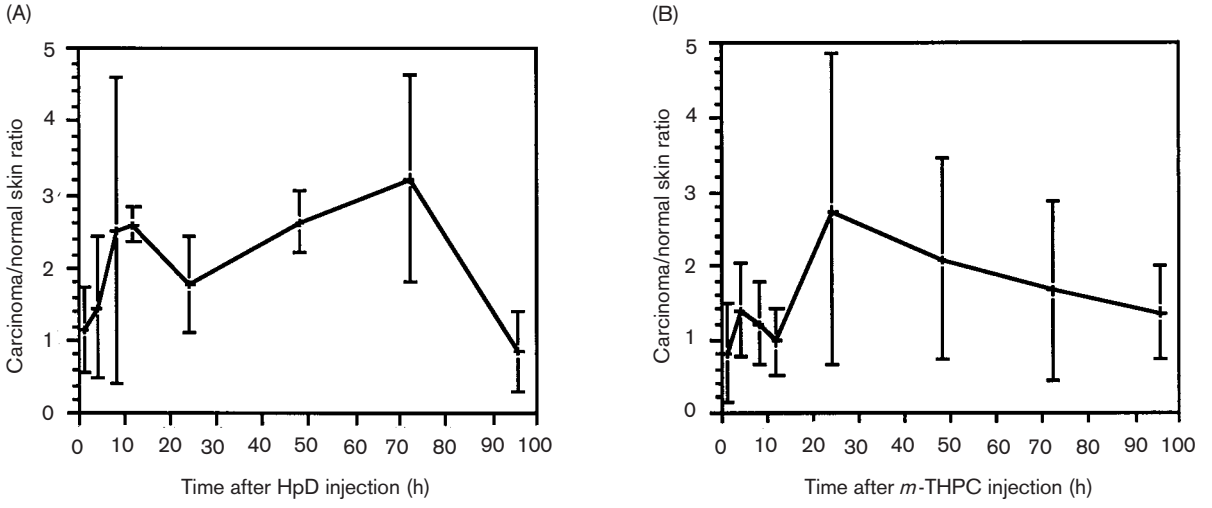

Figure 1. Time course for changes in the carcinoma/normal skin ratio after i.p. injection of Hairless mice (SKH-1) with HpD (A) or *m*-THPC (B) (*n*= 9 for each time).

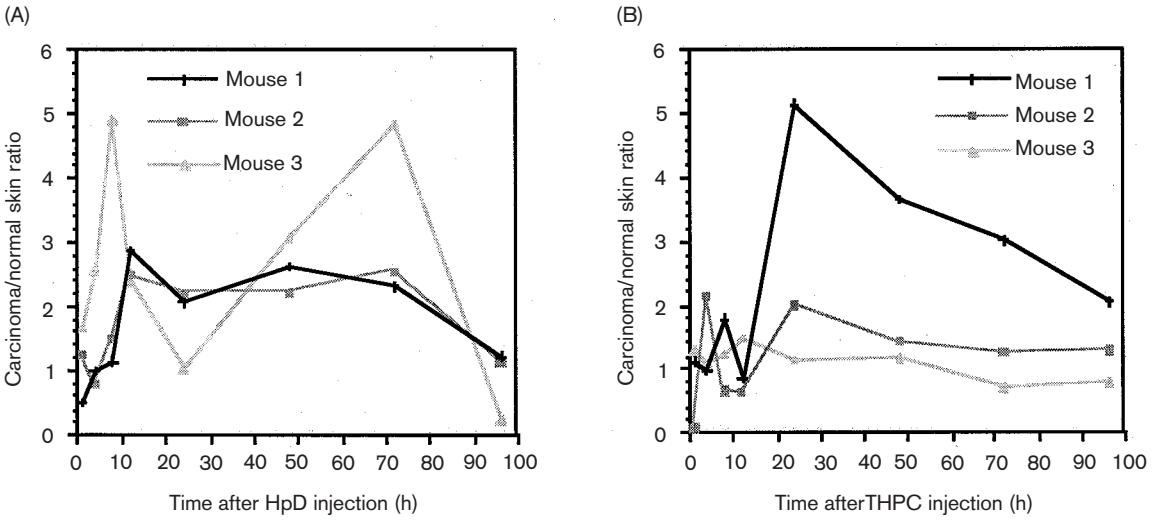

Figure 2. Time course for changes in the carcinoma/normal skin ratio after i.p. injection of three Hairless mice (SKH-1) with HpD (A) or *m*-THPC (B).

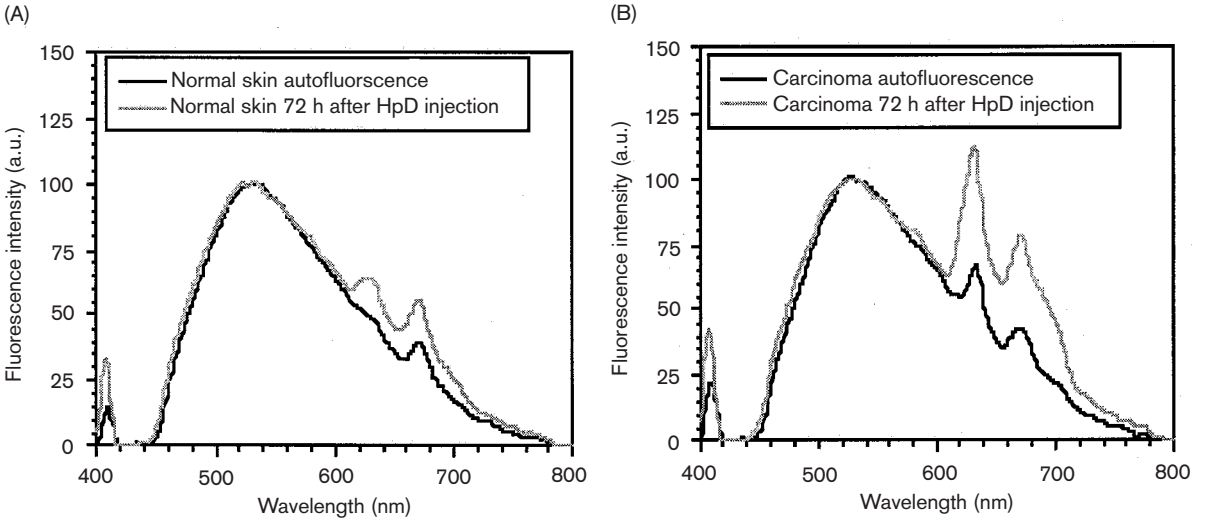

Figure 3. Spectra of the normal skin (A) and the carcinoma (B) before and 72 h after HpD injection, normalized according to the flavin peak at 520 nm.

m-THPC over a longer time (72 h instead of 24 h). Indeed, the maximal HpD and *m*-THPC carcinoma/normal skin ratios were 3.2 ± 1.4 and 2.7 ± 2.1, respectively. These ratios were approximatively 2-fold lower than those obtained in Hairless mice bearing chemically induced skin papillomas with the same photosensitizers.[7] This could be explained either by a difference of affinity between hyperplastic and neoplastic cells for the photosensitizer or by different tumor vascularization. Tumors chemically induced are growing in their own epidermal tissue. Neovascularization appears to ensure the increased tumor oxygen and nutriment requirements.[18] Despite papillomas, carcinoma development leads nearly systematically to an ulceration with a tissue central necrosis. Consequently, blood vessels irrigate only the tumor boards. Thus, ulcerated invasive carcinomas that were used in our HpD and *m*-THPC biodistribution experiments are tumors with blood vessels partially destroyed during necrosis. The vascularization of ulcerated carcinomas being reduced, the photosensitizer supply would be also diminished and could explain the difference in the ratio observed between carcinomas and papillomas.

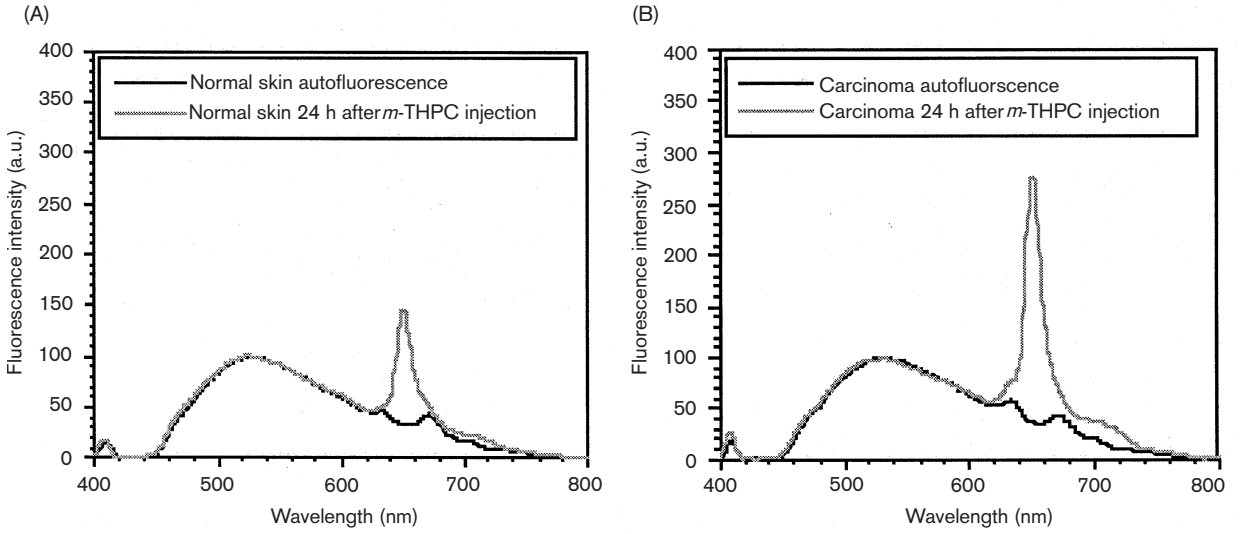

Figure 4. Spectra of the normal skin (A) and the carcinoma (B) before and 24 h after *m*-THPC injection, normalized according to the flavin peak at 520 nm.

Currently, pharmacokinetic studies are being carried out on animal malignant tumor models such as squamo-cellular or subcutaneous carcinomas. Glanzmann *et al.*[17] reported a *m*-THPC biodistribution study by fluorescence spectroscopy on a chemically induced early squamous cell carcinoma of the Syrian hamster cheek pouch. The maximal carcinoma/normal skin ratio was located, as for our study, 24 h after *m*-THPC i.v.injection and was about 1.5-fold higher than ours. Moreover, in our experiment, the first maximum occurs 4 h after injection instead of 1 h for Glanzmann—this shift being probably due to the way the photosensitizer was administered. Among s.c. carcinoma models, Rezzoug *et al.* reported a pharmacokinetic study by fluorescence spectroscopy with *m*-THPC (0.3 mg/kg body weight) injected i.p. in Swiss nude mice bearing HT29 s.c.tumors. The maximal tumor/normal skin ratio was 2.0 ± 0.8 and was located 72 h after injection (unplublished data). This ratio was very close to our value obtained with chemically induced carcinomas (2.7 ± 2.1). Some similar values (2–4) were previously reported in the literature by teams working in mice bearing s.c. carcinomas (mammary, bladder or colon carcinoma) and receiving i.v. or i.p. injections of high HpD or *m*-THPC doses.[19–23] Moreover, the maximal tumor/normal skin was generally located between 24 and 72 h for the two photosensitizers.

However, in human the maximal tumor/normal skin ratio can be higher. Indeed, this ratio was 15 for some *m*-THPC pharmacokinetic studies in patients bearing malignant mesothelioma or mouth squamo-cellular carcinoma.[24,25] On the other hand, with *m*-THPC as photosensitizer, Ronn *et al.*[26] obtained tumor/normal skin ratios for patients suffering from different organ carcinomas (prostate, larynx and pharynx) equivalent to the ones corresponding to experiments achieved in s.c. carcinoma animal models. Thus, it seems that for a given species, the tumor selectivity is independent of both carcinomas used and injected photosensitizer doses. Moreover, in similar experimental conditions, individuals often react differently, which explains the presence of high SEs.

Conclusion

Whatever tumor type (papilloma or carcinoma), the differences in the tumor/normal skin ratio between HpD and *m*-THPC are not significant. Consequently, we cannot conclude that HpD is a better photosensitizer than *m*-THPC. Indeed, with a smaller tumor/normal skin ratio *m*-THPC could give more radical species and, consequently, cause more necrosis of the tumor after irradiation. Vornax-Coinsmann *et al.*[27] report that *m*-THPC supplies a greater quantum yield of singlet oxygen than HpD. Thus, these pharmacokinetic results have to be confirmed by an organic extraction method and then a PDT trial should be carried out with the same experimental conditions used in the biodistribution studies to compare the toxicity of HpD and *m*-THPC towards papillomas and carcinomas, and to determine if the maximal tumor/normal skin ratio effectively gives the optimal tumor necrosis. Some authors reported that PDT treatment

would be more efficient a few hours after photosensitizer injection. Indeed, it would be wise to associate the vascular and cellular effects by treating tumors when the plasma concentration of photosensitizer is still high and its tumor concentration important, even if it does not correspond to the maximal tumor/normal skin ratio.[28–31]

In the future, we plan to develop an intradermis carcinoma model in Hairless mice that will allow us to obtain cutaneous tumors in a shorter time (a few weeks instead of 8 months for chemically induced carcinomas). Moreover, it will be interesting to use this syngenic tumor model for PDT and pharmacokinetic studies after systemic or topical injection as this molecule is already used to treat human baso or spinocellular carcinomas.

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