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Determination of the maximal tumor/normal skin ratio after HpD or *m*-THPC administration in hairless mouse (SKh-1) by fluorescence spectroscopy—a non-invasive method

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Two major steps in our study on the treatment of skin tumors by photochemotherapy (PCT) were the development of a skin tumor model in hairless mice by chemical carcinogenesis and by the use fluorescence spectroscopy, a semi-quantitative and non-invasive method, to determine the time after i.p. injection of photosensitizer when the tumor/normal skin ratio is the highest. Carcinogenesis provided mice bearing many benign papillomas 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)-chlorin (*m*-THPC) (0.3 mg/kg body weight) were injected, and fluorescence measured at 4, 8, 24, 48, 72 and 96 h after injection. The best tumor/normal skin ratio was 6.2 for HpD and 5.1 for *m*-THPC. The times required to reach these ratios were 48 h for HpD and 72 h for *m*-THPC. Published reports indicate that *m*-THPC gives a much higher tumor/normal skin ratio than HpD. These results must be confirmed by organic extraction. Photodynamic therapy with the same doses of HpD and *m*-THPC used in this pharmacokinetic study must also be carried out to compare the toxicities of the two photosensitizers and to determine which is best for this type of tumor.

Key words: Fluorescence spectroscopy, skin tumors, tumor/normal skin ratio, two-stage carcinogenesis.

Introduction

Photochemotherapy (PCT) is a technique for treating tumors which involves an initial treatment with a photosensitizer followed by irradiation of the tumor with a laser beam. This causes the photosensitizer to produce cytotoxic oxygen species within the tumors. As the photosensitizer is also present in normal skin surrounding the tumor, it is necessary to know how it is distributed between the two tissues.

Most preclinical data on the distribution of photosensitizers in tumors and normal skin have been obtained using animals with transplanted tumors which are not growing in their native connective tissue matrix. However, this situation does not mimic the clinical condition. It is therefore important to evaluate the distribution of photosensitizer within the tumor and normal skin when the tumor is growing in its own connective tissue matrix. The chemical induction of skin tumors provides a suitable system that has been well studied in the mouse.[1] This system has provided the basic concepts of carcinogenesis and has also been used for photochemotherapy.[2]

Studies in our laboratory have been performed on SKH-1 hairless mice and have employed a two-stage process to induce skin tumors. Hairless mice were used to avoid any trauma due to clipping or shaving before the chemical treatment.[3] Hairless and hairy mice seem to react alike to chemical carcinogenesis.[3]

This study was carried out on mice bearing skin tumors to determine the time at which the concentration of photosensitizer in the tumor was greatest, by measuring the tumor/normal skin ratio of photosensitizer after i.p. injection. This time will then be used in future studies to ensure efficient photochemotherapy.[4,5] The photosensitizers used were hematoporphyrin derivative (HpD), currently used in PCT,[6,7] and *m*-tetra(hydroxyphenyl)-chlorin (*m*-

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THPC), a promising new photosensitizer.[8] The distributions of the photosensitizers were monitored by light-induced fluorescence spectroscopy and the tumor/normal skin ratio was measured after systemic injection of the photosensitizer.[9,10]

Fluorescence spectroscopy is a suitable method for monitoring photosensitizer distribution *in vivo* by measuring the fluorescence intensity ratios of tumors and surrounding normal skin at different times after injection. Moreover, for diagnostic purposes, the photosensitizer emission or autofluorescence signal can also be used in diagnosis to distinguish normal from tumor skin. The discrimination is based on the difference of fluorescence intensity and spectral shape between the spectra of these tissues.

Thus, fluorescence spectroscopy may be used to establish a data bank of spectra for normal and malignant tissues. Each organ could then be defined by its autofluorescence spectrum and tumor lesions classified by fluorescence signal amplitude.[10] The tumor spectrum could be compared to those in the spectra bank, to determine if the tumor should be removed without waiting for the pathologist's report on the biopsy that is usually taken in these cases. This is the field of 'optical biopsy', which seems to be an interesting process to identify pathological tissues or some metabolic state.

Materials and methods

Chemicals

HpD was produced in the laboratory[11] and *m*-THPC was obtained from Scotia Pharmaceuticals (Guildford, UK). 7,12-Dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma (France). Acetone (Prolabo, France) was used as solvent.

Induction of benign papillomas on mice

Female hairless (SKH-1) mice (7–9 weeks old) were obtained from Charles River (USA) and kept in plastic cages at 25°C with a 12 h light/dark cycle. They were given a standard diet and water *ad libitum*.

Skin tumors were induced chemically by a standard two-stage initiation–promotion protocol. Each mouse was given three topical applications of 25.6 μg (100 nmol) DMBA in 0.1 ml acetone, one to each of three sites on the back skin delimited by tatooing. The mice were given, starting 1 week later, twice weekly topical administrations of 3.1 μg (5 nmol) TPA in 0.1 ml acetone to the initiated sites for 24 weeks. Control mice received only acetone for all the initiation and promotion steps. All (100%) of the mice developed tumors after 17 weeks of treatment and about 26% had 11–15 tumors per mouse. These tumors had diameters of 1–7 mm after 24 weeks of promotion. The promotion step had to be continued for over 20 weeks. Regression is negligible when TPA treatment is stopped in mice bearing papillomas.[12]

Some of the tumor-bearing mice used for pharmacokinetic studies were sacrificed for histopathological evaluation of the tumors. The tumors were found to be benign papillomas.

Pharmacokinetic study

Six mice bearing at least three tumors 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).[13,14] The mice were their own controls, as fluorescence measurements were made on the tumors and the normal skin before injection of the photosensitizers. Then three measurements of the fluorescence of tumors and normal skin were made three times at intervals after injection (4, 8, 24, 48, 72 and 96 h). Each photosensitizer was tested on three mice.

The spectrofluorimeter consisted of a CP 200 spectrograph (Jobin Yvon), with an optical multichannel analyzer (OMA) managed by Spectramax software, and three optical fiber probes, one for excitation, one for the collection of fluorescence emission and the last for backscattered excitating light power measurement.[9,10] The excitation light (410 nm) was provided by a 300 W Xenon lamp through a 9 nm bandpass interferential filter. The *in vivo* fluorescence intensities of *m*-THPC (652 nm) and HpD (630 nm) in tumors and normal skin were plotted against the time before injection. All the measured spectra were normalized to the autofluorescence spectra (at 520 nm) obtained after drug injection.

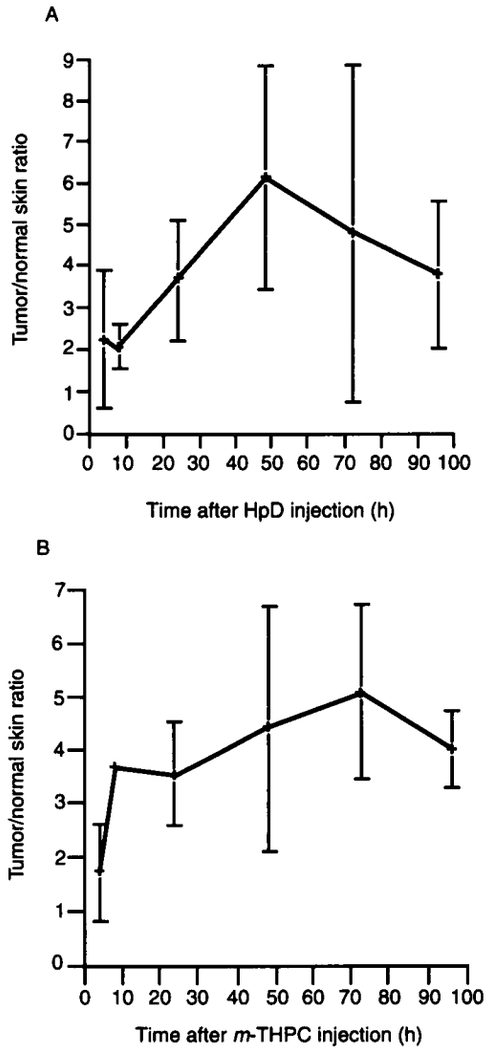

Figure 1. Time course for changes in the tumor/normal skin ratio after i.p. injection of hairless mouse (SKh-1) with HpD (A) or *m*-THPC (B).

Results

The i.p. injection of SKH-1 hairless mice bearing chemically induced tumors with HpD or *m*-THPC resulted in the distribution of the drugs in the skin and tumors (Figure 1). The highest tumor/normal skin ratio that could give the most efficient photochemotherapy was 6.2 for HpD (Figure 1A) at 48 h after injection and 5.1 (Figure 1B) for *m*-THPC at 72 h after injection. A few hours after injection, the ratios of both photosensitizers reached a first optimum, 2.2 for HpD and 3.7 for *m*-THPC, which was followed by a small decrease. Then, there was a regular increase until a second maximum, the highest tumor/normal skin ratio. This type of curve has

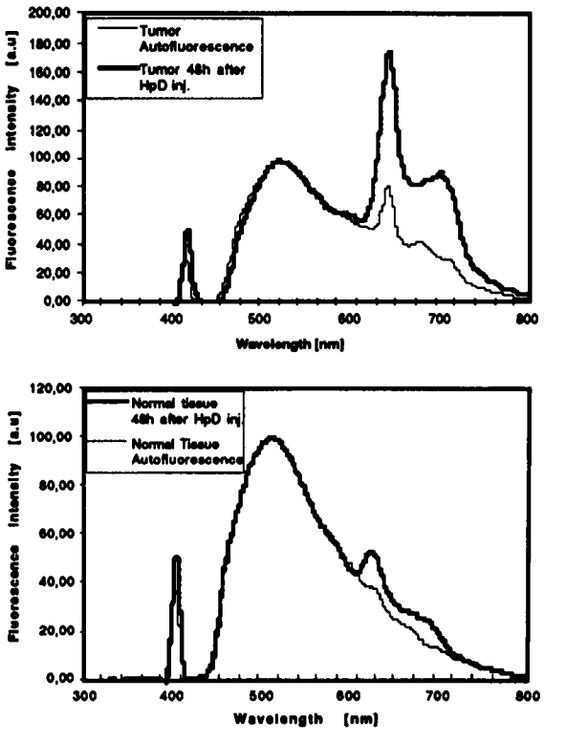

Figure 2. Spectra of the tumor (A) and the normal skin (B) before and 48 h after HpD injection.

also been reported by others[15,16] and could be due to the enterohepatic cycle. Figures 2 and 3 show the spectra of *m*-THPC and HpD in the tumors and normal skin at the best time for irradiation.

As it is difficult to measure repetively exactly the same spot at different times significant variations are observed with the same animal. Moreover, each value of ratio is the mean of nine measurements (three per mouse) more or less close. This error can be due to the difference between animals and tumors even if they are very similar. Furthermore, one cannot exclude the influence of a temporal variation of the optical parameters of the tissue.[16]

For the two photosensitizers, the comparison between the autofluorescence spectrum of the tumor and the spectrum at the time at which the ratio is highest, showed a difference in fluorescence intensity at the main emission peak of each photosensitizers, 630 nm for HpD and 652 nm for *m*-THPC. This difference in fluorescence intensity was directly related to the concentration of photosensitizer in the tumor. 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

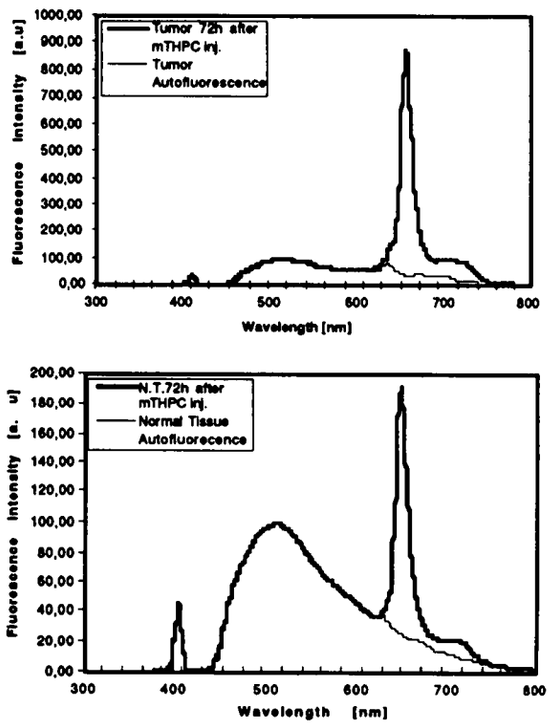

Figure 3. Spectra of the tumor (A) and the normal skin (B) before and 72 h after *m*-THPC injection.

a peak at 635 nm, which was probably due to endogenous porphyrins. This peak was also present in the autofluoroscence spectra of the normal tissue, but had a much lower intensity.

Discussion and conclusion

In this model, 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 between individuals. As a result, the experiment required fewer mice. Fluorescence spectroscopy also seems to be a suitable method for distinguishing between normal and tumor skin, by measuring the autofluorescence spectra.

We have used this technique to determine the maximal tumor/normal skin ratios for two photosensitizers, HpD and *m*-THPC. The results indicate that HpD produces a slightly higher tumor/normal skin ratio than *m*-THPC in a shorter time. The HpD tumor/normal skin ratio is 1.5-fold higher than that reported by Mukhtar[15] for SENCAR mice bearing chemically induced skin tumors, although the dose of photosensitizer injected i.p. and the carcinogenesis processes were the same. However, the HpD tumor/normal skin ratio found in most reports (2–4) is consistent with ours.[8,17]

For m-THPC, we have obtained a similar ratio to the one reported by Glanzmann *et al.*[16] with a shift of 24 h, 3.5 (at 24 h) for our tumors and 3.6 (at 0 h) for squamous cell carcinomas induced on the hamster cheek pouch mucosa by the application of DMBA, although they used a 2-fold lower *m*-THPC dose (0.15 mg/kg body weight). This shift could be related to the different administration ways, i.p. injection for us and i.v. for Glanzmann. Using a similar *m*-THPC dose (0.3 mg/kg body weight), photosensitizer administration mode (i.p.) and technique of fluorescence measurement (fluorescence spectroscopy), Rezzoug *et al.*[9,18] showed in s.c. HT-29 tumors an identical time (72 h) for the maximal ratio that was 2.5-fold lower than ours. A lower vascularization of the s.c. tumors could explain this reduced ratio. However, some clinical studies[19,20] in man report ratios of about 14–15 for malignant mesothelioma or squamous cell carcinoma, the *m*-THPC dose used being 0.3 mg/kg body weight. Concerning the time between injection and irradiation, it is usually 12–72 h. The slightly better tumor/normal skin ratio for HpD is not significant compared to *m*-THPC.

However, we cannot conclude that HpD is a better photosensitizer than *m*-THPC in spite of its good tumor/skin ratio. Although the injected dose of *m*-THPC was about 15 times smaller than that of HpD, it gave a tumor/normal skin ratio nearly equal to the HpD ratio. This may be because *m*-THPC has a greater fluorescence yield than HpD (the quantum yield of fluorescence is 139-fold higher for *m*-THPC than for HpD) or a higher tropism for tumor cells. It is also possible that, even with a smaller tumor/normal skin ratio, *m*-THPC could give rise to more radical species and consequently cause more necrosis of the tumor after irradiation. Vonarx-Coinsmann *et al.*[21] indicate that *m*-THPC gives a greater quantum yield of $^1O_2$ than HpD.

It would be interesting to compare the same values of tumor/normal skin ratio for the two photosensitizers when obtained by organic extraction. A photodynamic study will be carried out with the same doses of HpD and *m*-THPC used in this pharmacokinetic study to compare their type of toxicity towards this skin tumor, and to determine how to the maximal tumor necrosis in the shortest time. The results will take into consideration the fact that *m*-THPC irradiation is performed at 650 nm.

This wavelength gives a better $^1O_2$ quantum yield and a better transmission in living tissues than 630 nm generally used for HpD.

Some clinical trials have been carried out to treat different types of skin tumors mainly with HpD[22,23] and δ-aminolaevulinic acid[24–26] as photosensitizers.

Photochemotherapy with new topical forms of HpD and *m*-THPC should be used to treat baso- or spinocellular carcinomas, as these are the most frequently encountered human skin tumors. We therefore plan to use a carcinogen to obtain carcinomas in addition to the induction protocol described in this paper.

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References

1. Yuspa SH, Dlugosz AA. Cutaneous carcinogenesis: natural and experimental. In: Goldsmith LA, ed. *Physiology, biochemistry and molecular biology of the skin*, 2nd edn. Oxford: Oxford University Press 1991: 1365–402.
2. Agarwald R, Athar M, Elmets CA, Bickers DR, Mukhtar H. Photodynamic therapy of chemically- and ultraviolet B radiation-induced murine skin papillomas by chloroaluminium phtalocyanine tetrasulfonate. *Photochem Photobiol* 1992; **56**: 43–50.
3. Iversen OH. Hairless mouse skin in two-stage chemical carcinogenesis. *Virchows Arch B Cell Pathol* 1982; **38**: 263–72.
4. Spinelli P, Dal Fante M. Photodynamic therapy of solid tumors. *Semin Hematol* 1992; **29**: 142–54.
5. Dougherty TJ, Marcus SL. Photodynamic therapy. *Eur J Cancer* 1992; **28A**: 1734–42.
6. Kessel D. Hematoporphyrin and HpD: photophysics, photochemistry and phototherapy. *Photochem Photobiol* 1984; **39**: 851–9.
7. Dougherty TJ. Photosensitizers: therapy and detection of malignant tumors. *Photochem Photobiol* 1987; **45**: 879–89.
8. Lofgren LA, Ronn AM, Abramson AL, *et al.* Photodynamic therapy using *m*-tetra-(hydroxypheny)chlorin. *Arch Otolaryngol Head Neck Surg* 1994; **120**: 1355–62.
9. Guilleman F, A'Amar O, Rezzoug H, *et al.* Optical instrumentation suitable for a real time dosimetry during photodynamic therapy. *Prog Biomed Optics, Proc Optoelectronic Research and Technologies* 1995; **2627**: 92–99.
10. A'Amar O, Lignon D, Begorre H, *et al.* Autofluorescence spectroscopy of normal and malignant tissues: both *in-vivo* and *ex-vivo* measurements in the upper aero-digestive tract and lung tissues. *Prog Biomed Optics, Proc Advances in Laser and Light Spectroscopy to Diagnose Cancer and Other Diseases III: Optical Biopsy* 1996; **2679**: accepted.
11. Azzi V, Notter D, Merlin JL, *et al.* Composition and spectral characteristics, cytotoxicity and quantum yield of singlet oxygen formation of haematoporphyrin derivative preparation. In: Spinelli P, Dalfante M, Marchesini R, eds. *Photodynamic therapy and biomedical lasers*. Amsterdam: Excerpta Medica 1992: 715–9.
12. Aldaz CM, Conti CJ, Chen A, Bianchi A, Walker SB, Digiovanni J. Promoter independence as a feature of most skin papillomas in SENCAR mice. *Cancer Res* 1991; **51**: 1045–50.
13. Mukhtar H, Agarwald R, Athar M, Lewen RL, Elmets CA, Bickers DR. Photodynamic therapy of murine skin tumors using Photofrin®-II. *Photodermatol Photoimmunol Photomed* 1991; **8**: 169–75.
14. Wang Q, Altermatt HJ, Ris HB, *et al.* Determination of 5,10,15,20-tetra-(*m*-hydroxyphenyl)chlorin in tissues by high performance liquid chromatography. *Biomed Chromatogr* 1993; **7**: 155–7.
15. Morlet L, Vonarx-Coinsmann V, Lenz P, *et al.* Correlation between *meta*(tetrahydroxyphenyl)chlorin (*m*-THPC) biodistribution and photodynamic effects in mice. *J Photochem Photobiol B: Biology* 1995; **28**: 25–32.
16. Glanzmann T, Theumann JF, Braichotte D, *et al.* Pharmacokinetics of meso-(tetrahydroxyphenyl)chlorin (*m*-THPC) studied by fluorescence spectroscopy on early cancer of the cheek pouch mucosa of Golden Syrian hamsters. *Prog Biomed Optics, Proc Optical Biopsy and Fluorescence Spectroscopy and Imaging* 1994; **2324**: 89–96.
17. Ma L, Moan J, Berg K. Evaluation of a new photosensitizer, meso-tetra-hydroxyphenyl-chlorin, for use in photodynamic therapy: a comparison of its photobiological properties with those of two other photosensitizers. *Int J Cancer* 1994; **57**: 883–8.
18. Rezzoug H, A'Amar O, Barberi-Heyob M, *et al. In vivo* photodynamic therapy with meso-tetra(*m*-hydroxyphenyl)chlorin (*m*-THPC): influence of light intensity and optimization of photodynamic efficiency. *Prog Biomed Optics, Proc Photochemotherapy: PDT and other modalities* 1996; **2924**, in press.
19. Ris HB, Altermatt HJ, Inderbitzi R, *et al.* Photodynamic therapy with chlorins for diffuse malignant mesothelioma: initial clinical results. *Br J Cancer* 1991; **64**: 1116–20.
20. Braichotte D, Savary JF, Glanzmann T, *et al.* Clinical pharmacokinetic studies of tetra(meta-hydroxyphenyl)-chlorin in squamous cell carcinoma by fluorescence spectroscopy at 2 wavelengths. *Int J Cancer* 1995; **63**: 198–204.
21. Vonarx-Coinsmann V, Foultier MT, Cempel N, *et al. In vitro* and *in vivo* photodynamic effects of a new photosensitizer: tetra (*m*-hydroxyphenyl)chlorin. *Lasers Med Sci* 1994; **9**: 173–81.
22. Lin CW. Photodynamic therapy of malignant tumors-Recent developments. *Cancer Cells* 1991; **3**: 437–44.
23. Rosenthal DI, Glatstein E. Clinical applications of photodynamic therapy. *Ann Med* 1994; **26**: 405–9.

24. Cairnduff F, Stringer MR, Hudson EJ, *et al.* Superficial photodynamic therapy with topical 5-aminolaevulinic acid for superficial primary and secondary skin cancer. *Br J Cancer* 1994; **69**: 605–8.
25. Peng Q, Warloe T, Moan J, *et al.* Distribution of 5-aminolevulinic acid-induced porphyrins in nodulo-ulcerative basal cell carcinoma. *Photochem Photobiol* 1994; **62**: 906–13.
26. Calzavara-Pinton PG. Repetitive photodynamic therapy with topical δ-aminolaevulinic acid as an appropriate approach to the routine treatment of superficial non-melanoma skin tumours. *J Photochem Photobiol B: Biology* 1995; **29**: 53–7.

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