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Photodynamically induced inactivation of propionibacterium acnes using the photosensitizer methylene blue and red light

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Key words: Propionibacterium acnes – Acne vulgaris – Photodynamic therapy – Porphyrins – Methylene Blue – Red light

Summary: Irradiation of Propionibacterium acnes with visible light results in oxygen-dependent photodestruction processes based on the photodynamic activity of endogeneous porphyrins. The additional uptake of externally applicated photosensitizers such as Methylene Blue, increases the photoinduced inactivation rate. Methylene Blue absorbs in the spectral region between 500 and 700 nm. The irradiation of Methylene Blue incubated bacterial colonies with red light of a krypton ion laser (647 nm and 676 nm) increases the rate of inactivation by a factor of ten in comparison with 407 nm irradiation (main absorbance of porphyrins) of unincubated bacteria. The use of red light irradiation allows the treatment of superficial as well as more deeply lying skin tissue. It should be possible to treat acne vulgaris with red light in combination with a topical administration of photosensitizers. Modifications in the fluorescence characteristics of the bacteria during the photodynamic therapy may be used for monitoring the therapeutic effect.

Durch Methylenblau und rotes Licht induzierte photodynamische Inaktivierung von Propionibacterium acnes

Schlüsselwörter: Propionibacterium acnes – Acne vulgaris – Photodynamische Therapie – Methylenblau – Rotlicht

Zusammenfassung: Die Bestrahluung von Propionibacteria acnes mit sichtbarem Licht induziert sauerstoff-abhängige Photodestruktionsprozesse, die auf der photodynamischen Aktivität endogener Porphyrine basieren. Die zusätzliche Aufnahme von extern applizierten Photosensibilisatoren wie z. B. Methylenblau erhöht die photoinduzierte Inaktivierungsrate. Methylenblau absorbiert im Spektralbereich zwischen 500 und 700 nm. Die Bestrahlung von Methylenblau-inkubierten Bakterienkolonien mit der roten Strahlung eines Kryptonionen-Lasers (647 nm und 676 nm) erhöht die Inaktivierungsrate um den Faktor 10 im Vergleich zur 407 nm-Strahlung (Hauptabsorptionsgebiet der Porphyrine) der nicht inkubierten Bakterien. Die Verwendung von Strahlung im roten Spektralbereich erlaubt die Behandlung von sowohl oberflächlichen, als auch tieferliegendem Hautgewebe. Es sollte daher möglich sein, Acne vulgaris mittels Rotlicht-Bestrahlung und topischer Applikation von Photosensibilisatoren zu behandeln. Modifikationen im Fluoreszenzverhalten der Bakterien während der Photodynamischen Therapie können für eine Beobachtung des therapeutischen Effekts genutzt werden.

Photosensitizers induce cytotoxic reactions during light irradiation. After light absorption the sensitizer molecules get into the metastable triplet state (intersystem crossing) followed by a reaction with molecular oxygen. Energy transfer (phototoxydation type II) or charge transfer (photooxydation type I) may occur resulting in the formation of singlet oxygen or reactive radicals. These species damage the surrounding biomolecules [1].

The skin bacterium Propionibacterium acnes involved in the pathogenesis of acne vulgaris produces porphyrins, e. g. coproporphyrin III and protoporphyrin IX [2]. Porphyrins are well-known photosensitizers used in the photochemotherapy of tumors [3]. They absorb in the visible spectral region, especially around 400 nm. Meffert [4] treated acne vulgaris using a lamp emmitting blue light. However, the optical penetration depth of violet radiation in biological tissue is less than 1 mm. The range of high tissue transmission is in the red and infrared region (optical penetration depth: 3 to 8 mm [5]. Porphyrins have only a small absorbance in this region.

The aim of this paper was to investigate the photodynamic action of external applicated photosensiti-

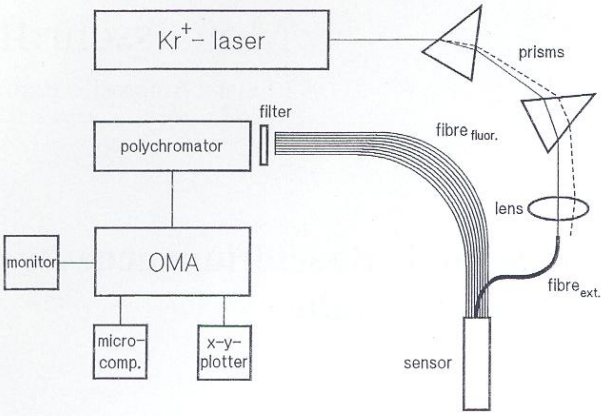

Fig. 1 Experimental set-up for the phototreatment and the measurement of fluorescence spectra

zers, which absorb strongly in the region of high tissue transmission. In this case we used Methylene Blue as a dye of superior photodynamic activity [6-8].

Materials and methods

Experimental set-up

Fig. 1 shows the set-up using a krypton ion laser (Spectra Physics) as light source. The radiation is coupled into a 600 µm light fiber which is positioned in the center of a fiber bundle. The peripheral fibers work as a sensor for the emitted fluorescence light. Fluorescence spectra were recorded using a polychromator and an optical multichannel analyzer. For more information see [2].

Samples

Cultures of Propionibacterium acnes were kindly supplied by Berit Kjeldstad, Trondheim, Norway. The cultures grown on Blood Agar Base no 2. OXOID, pH 6,5 (B_2) under dark conditions at 37° C in anaerobic jars (Gas Pack System, OXOID) for 5 days. Irradiation and fluorescence measurements were caried out on 20 colonies on agar plates under normal aerobic conditions. The irradiation area ob about 0.2 cm^2 was greater than the colony area. Aqueous Methylene Blue solution (10 mM) was added as a layer of 2 mm thickness above the agar with the bacterial colonies. The solution was removed after an incubation time of 20 minutes. The plates were then washed with isotonic NaCl solution.

Calculation

For determination of the colony forming units after irradiation, the colonies were removed from the agar plate, added to an anaerobic medium (RCM, OXOID) and diluted in steps by a factor of ten down to 10^{-8}. 100 µl of the appropriate solution were spread on

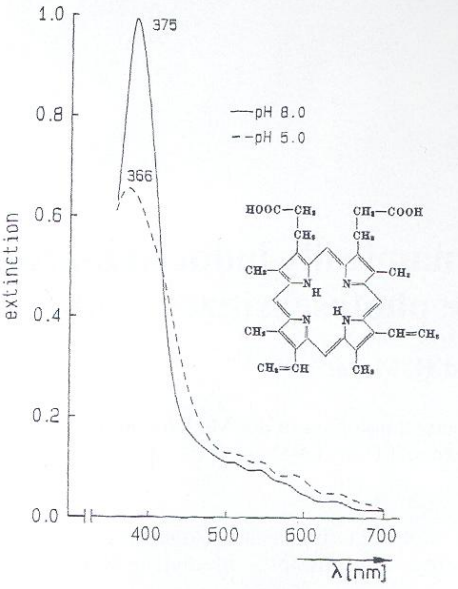

Fig. 2 Absorption spectrum of protoporphyrin IX in buffer solution at pH 8.0 and pH 5.0, concentration 10 g/ml. Protoporphyrin in aqueous solution consists of aggregates (main absorbance around 365 nm) and fluorescent monomers (main absorbance around 400 nm)

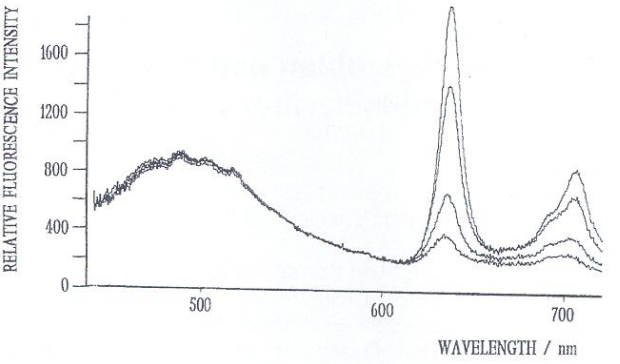

Fig. 3 Autofluorescence spectrum of Propionibacterium acnes and its photoinduced modification during 407 nm-irradiation with light doses of 0, 0.2, 1.0 and 4.0 J/cm^2

the surface of B_2-agar plate and incubated in anaerobic jars over 48 hours. The grown colonies were counted.

Results

First of all, the photoinactivation of Propionibacterium acnes was investigated using the endogeneous porphyrins of these microorganisms as photosensitizers. Fig. 2 shows the absorption spectrum of protoporphyrin with a strong absorption band around 400 nm (Soret band). A krypton ion laser was used at its violet line (407) both in the inactivation studies and in fluorescence excitation. Therefore, on-line measurements on the fluorescence behaviour of the bacteria during

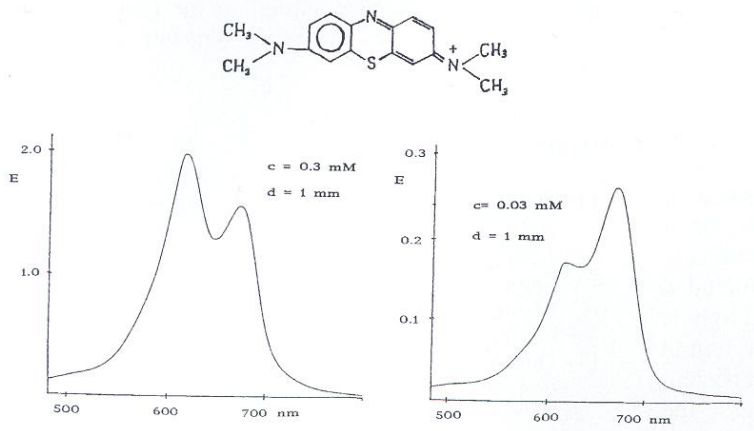

Fig. 4 Absorption spectrum of Methylene Blue in an aqueous solution (30 μM) which consists of a mixture of dimers (612 nm) and monomers (667 nm)

the photodynamic treatment could be caried out.

Autofluorescence spectra in dependence of the irradiation time are depicted in Fig. 3. The broad fluorescence band with a maximum around 480 nm is typical for the coenzyme NADH, the fluorescence in the red spectral region is attached to protoporphyrin IX. During irradiation the fluorescence intensity in the red spectral region decreases with increasing light doses due to a photobleaching process. It is remarkable, that the bleaching rate differs for various emission wavelengths. The fluorescence behaviour around 670 nm is different from that of the main fluorescence peaks at 635 nm and 700 nm. No decrease of the fluorescence intersity at 670 nm occurs for low light doses (up to 0.2 J/cm^2), but can be obtained during further light irradiation. This behaviour may be explained by the formation of fluorescent photoproducts, so-called photoprotoporphyrins, followed by photobleaching of these molecules [9].

The irradiation of the bacteria cultures was stopped when the integral fluorescence decreased to 10 % of the initial value before treatment. That was at a light doses of about 4 J/cm^2. The determination of the colony forming units after the irradiation showed a photodynamically induced inactivation of about 90 % of the cells.

In a second series the photodestruction of Propionibacterium acnes incubated with the photosensitzer Methylene Blue was studied. The absorption properties of an aqueous Methylene Blue solution, which consists of monomors and dimers with different absorption maxima, is shown in Fig. 4. The photosensitizer absorbs in the red spectral region which is of interest for the non-thermal phototreatment of biological tissue because of high light penetration depth. Methylene Blue incubated bacteria were irradiated with the same laser supplied with different resonator mirrors emitting radiation at 647 nm and 676 nm. In an experiment before, the influence of red light on unincubated bacteria and the dark toxicity of Methylene Blue was investigated. Due to the low absorbance of the porphyrins in the red region (see Fig. 2) irradiation of unincubated bacteria resulted in a small inactivation

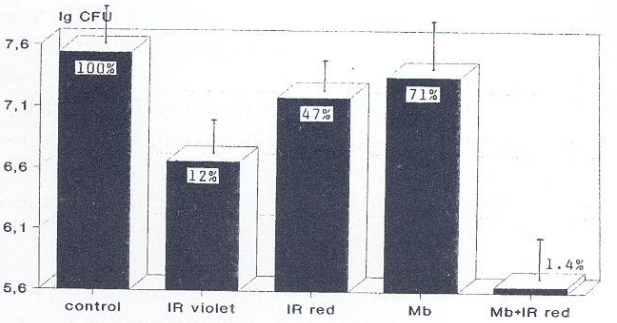

Fig. 5 Survival fraction of Propionibacterium acnes after irradiation and control groups.
Colony forming units are shown in a logarithmic scale.
Mb: Methylene Blue, IR: irradiation (4 J/cm^2)

effect. No significant inactivation was obtained for the group of Methylene Blue administration without any light treatment.

However, the rate of photodestruction of Methylene Blue incubated bacteria irradiation with red light was about 99 %. The average values and standard deviations of the colony forming units after different treatment procedures are depicted in Fig. 5 in a logarithmic scale.

In order to control the influence of red light irradiation on porphyrin fluorescence, in one experiment the mirrors were changed to 407 nm radiation before and after treatment. Photobleaching of the endogeneous prophyrins could be detected. This effect can be explained by a low absorbance of the porphyrins at 647 nm and by an influence of singlet oxygen and/or radicals on the porphyrins formed as a result of the photodynamic activity of Methylene Blue.

Discussion

The porphyrins produced by the bacteria Propionibacterium acnes are photodynamically active. Light irradiation results in a self-destruction of the cells. Due to the absorption properties of these molecules, the inactivation rate is moderate for irradiation with red light (Fig. 4 and [10]) and significally higher for irradiation

with blue light. Therefore, a photodynamic therapy of acne vulgaris using short-wavelength photoradiation and the endogeneous porphyrins as photosensitizers without any additional drugs, should be possible. This may explain the improvement of this skin disease after phototreatment with blue light by Meffert et al. [4].

Due to the fact, that violet light is also absorbed by other pigments in the skin, e. g. hemoglobin and melanin, the penetration depth is limited to approximately 1 mm. In order to treat deeper placed bacteria included in follicles (length of some mm), the use of red light is more desirable. Protoporphyrin absorbs in the region of about 630 mm, but the molar absorption coefficient is about 10^{-3} M^{-1} cm^{-1} only. In order to increase the efficiency of the light treatment, photosensitizers with a stronger absorbance in the spectral region of high tissue penetration were added. The uptake of Methylene Blue and long wavelength red radiation increases the photoinactivation rate rapidly.

Besides the cytotoxic photodynamic effect photoinduced changes of the spectral behaviour of the endogeneous porphyrins were observed due to a photobleaching process and a process of photoproduct formation [9]. Thus, irradiation of the porphyrins of the bacteria induces

a) a radiative relaxation process (fluorescence),
b) cytotoxic reactions via an energy- and/or charge transfer process,
c) a photodestruction process which results in photochemical changes of the porphyrins (see Fig. 3).

Thus, the measurement of variations in the fluorescence behaviour gives information about the formation of cytotoxic species.

These in vitro results demonstrate that it should be possible to treat acne vulgaris with red light in combination with a topical administration of well-known photosensitizers with a high yield of singlet oxygen formation and a high absorbance in the red and near infrared region like tetraphenylporphins [11], phthalocyanins [12], benzoporphyrins [13] or the thiazin dye Methylene Blue. The modification of the fluorescence spectrum during therapy may be used for monitoring the therapeutic effect. Of course, the effeciency of the treatment will be dependent on the concentration of oxygen and of the photosensitizer within the follicle lumen. Further investigations on the penetration of the photosensitizer into pilosebaceaus follicles are necessary.

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