Biochemical Aspects of ALA-PDT

Basic mechanisms and optimization for the treatment of Barrett's oesophagus

Petra Hinnen

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Biochemische Aspecten van ALA-PDT

Basale mechanismen en optimalisering voor de behandeling van Barrett slokdarm

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Voor pappa, mamma en Yvonne

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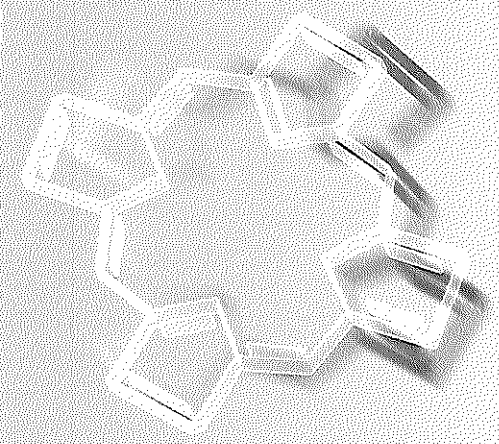

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I. BARRETT'S OESOPHAGUS AND ADENOCARCINOMA OF THE OESOPHAGUS

From the late 1970s, the incidence of adenocarcinoma of the oesophagus has increased more rapidly than that of any other cancer in the western world (Blot *et al.*, 1991). Adenocarcinoma of the oesophagus usually occurs in patients who have Barrett's oesophagus (Cameron *et al.*, 1995). Barrett's oesophagus is a complication of chronic gastro(duodeno)oesophageal reflux (Hamilton, 1985).

The true prevalence of Barrett's oesophagus is unknown. In patients with long-standing gastro-oesophageal reflux disease the prevalence of Barrett's oesophagus is at least 10% (Winters *et al.*, 1987). However, an autopsy study performed by Cameron *et al.* suggested that for every patient discovered during investigation for reflux symptoms there are 20 patients in the general population with undiagnosed Barrett's oesophagus (Cameron *et al.*, 1990). Prospective studies reported a 30-125 times higher risk of developing adenocarcinoma in patients with Barrett's oesophagus than in the general population (Cameron *et al.*, 1985; Drewitz *et al.*, 1997; Hameeteman *et al.*, 1989; Spechler *et al.*, 1984; van der Veen *et al.*, 1989). Barrett's oesophagus is therefore considered to be a relatively common lesion that has a significant malignant potential.

As a consequence of long-term reflux of acid, pepsin and bile, the normal squamous-lined oesophagus is injured (Attwood *et al.*, 1993). Multipotential stem cells in an eroded or ulcerated area may differentiate into three types of metaplastic epithelium; a gastric fundic type, a junctional type and an intestinal type. Barrett's oesophagus is a histological diagnosis. At endoscopy it can be recognized as a deep-red mucosa that contrasts sharply with the pale-pink adjacent squamous epithelium, but the diagnosis is not confirmed until intestinal metaplasia has been revealed by histologic examination (Paull *et al.*, 1976). Intestinal metaplasia is characterized by goblet cells and this particular epithelium carries the highest risk of developing adenocarcinoma (Hameeteman *et al.*, 1989).

Metaplasia in itself is benign, however it has the potential to undergo changes. Barrett's oesophagus can lead to the development of an adenocarcinoma through a multistep process of progression from metaplasia to low-grade dysplasia, high-grade dysplasia and ultimately to invasive cancer (Haggitt, 1994; Hameeteman *et al.*, 1989; Hamilton and Smith, 1987; Miros *et al.*, 1991). The risk of progression of Barrett's oesophagus into an adenocarcinoma seems to be related to the length of the columnar lined segment and the presence of ulcers (Iftikhar *et al.*, 1992; Komorowski *et al.*, 1996). Alcohol consumption and smoking are also associated with the development of adenocarcinoma in Barrett's oesophagus (Gray *et al.*, 1993).

Although the sequence through which an adenocarcinoma develops within Barrett's oesophagus is known, little is known about the natural history of dysplasia in Barrett's oesophagus. Two prospective studies in which patients with high-grade dysplasia were

followed endoscopically for several months reported regression of high-grade dysplasia and state that high-grade dysplasia does not always progress to carcinoma (Levine *et al.*, 1996; Schnell *et al.*, 1996). It is presently not known which patient will develop an adenocarcinoma and at what time interval. Effort has been made to find biochemical markers of neoplastic progression, such as p53 expression, however these markers are under research (Ellis and Loda, 1997; Ireland *et al.*, 1997). In clinical practice, high-grade dysplasia within Barrett's epithelium remains the best clinical predictor of adenocarcinoma.

The treatment of Barrett's oesophagus depends in part on the existence and grade of dysplasia. In most patients reflux symptoms are controlled by anti-reflux therapy, either medical (proton pump inhibitors) or surgical (Nissen fundoplication) (Ortiz *et al.*, 1996; Sampliner, 1994). Although reflux is considered the major metaplasia-inducing factor, in all but one study (Peters *et al.*, 1999) reducing acid exposure of the Barrett's segment was found to influence neither the length nor the grade of dysplasia of Barrett's oesophagus (Ortiz *et al.*, 1996; Sagar *et al.*, 1995; Sampliner, 1994). If surveillance is considered, patients with Barrett's oesophagus without dysplasia may undergo endoscopies every 2 years. Patients with low-grade dysplasia may undergo regular endoscopies every 6 to 12 months. The treatment of patients with high-grade dysplasia in Barrett's oesophagus, however, is still controversial. Some advocate an intensive endoscopic surveillance biopsy protocol (every 3 to 6 months), being convinced that multiple biopsies can differentiate high-grade dysplasia from early cancer (Levine *et al.*, 1993), while others advocate an oesophagectomy as multiple biopsies do not exclude the presence of adenocarcinoma due to sampling errors and difficulties in differentiating between high-grade dysplasia and (early) adenocarcinoma can be difficult (Cameron and Carpenter, 1997; Edwards *et al.*, 1996; Heitmiller *et al.*, 1996; Peters *et al.*, 1994; Rusch *et al.*, 1994). To date, the majority of patients with high-grade dysplasia undergo a prophylactic oesophagectomy. Considering the limited information about the natural history of high-grade dysplasia and lack of clinically useful objective markers for neoplastic progression, it is presently not possible to identify patients who can be followed by endoscopic surveillance and patients who need immediate resection. As a consequence, a large number of patients with high-grade dysplasia undergo a mutilating operation with substantial morbidity and mortality to prevent progression that might in some patients never occur (Ferguson and Naunheim, 1997; Heitmiller *et al.*, 1996; Provenzale *et al.*, 1994).

A non-invasive endoscopic ablative therapy could be an alternative to surgical resection, in patients with low or high-grade dysplasia and also in patients with a high surgical risk or for those who are averse to surgery. The conversion of intestinal metaplasia into normal squamous epithelium to abolish the risk of adenocarcinoma is an appealing concept. Endoscopic ablative therapies that are currently under investigation are photodynamic therapy (Barr *et al.*, 1996; Overholt *et al.*, 1999), multipolar electrocoagulation (Sharma *et al.*,

1999), argon plasma beam coagulation (van Laethem *et al.*, 1998), KTP laser coagulation (Barham *et al.*, 1997), Nd:Yag laser coagulation (Luman *et al.*, 1996) and endoscopic mucosal resection (Ell *et al.*, 2000) all in combination with acid suppression by proton pump inhibitors to allow squamous regeneration in a neutral environment (Berenson *et al.*, 1993).
We have been interested in the possibilities of using 5-aminolaevulinic acid in photodynamic therapy (ALA-PDT) of Barrett's oesophagus. This thesis presents five studies that focus on the basic mechanisms of ALA-PDT with the aim of improving therapy.

II. PHOTODYNAMIC THERAPY

a. history

Photodynamic therapy (PDT) is an experimental treatment for localized premalignant and malignant tumours. PDT is based on the accumulation of photosensitizers in these tissues after intravenous or oral administration. Subsequent illumination with a specific wavelength induces a photochemical reaction between light and the photosensitizing substance. With a combination of photosensitizer dose, time interval between administration and illumination and light distribution, it may be possible to destroy tumours without damaging normal adjacent tissue.
The principle of light activation of photosensitive substances is not new. In 1898, McCall-Anderson discovered the photosensitizing ability of porphyrin molecules as his patients with skin photosenstivity were found to have haematoporphyrin (Hp) in their pigmented urine (McCall-Anderson, 1898). Early in the 19th century Raab reported the killing of protozoa after the photochemical interaction between light and acridine orange (Raab, 1900). In 1903, Von Tappeiner and Jesionek treated skin carcinomas by topical application of eosin and illumination (Tappeiner von and Jesionek, 1903). Meyer-Betz injected himself with Hp and described the resulting skin photosensitivity with severe skin edema and erythema lasting for more than two months (Meyer-Betz, 1913). In 1940, Hp was discovered to have tumour-localizing properties. Lipson *et al.* (Lipson *et al.*, 1961) discovered that a derivative of Hp had even better tumour localizing properties than Hp itself and haematoporphyrin derivative (HpD) was used in most studies until Dougherty *et al.* isolated the active fraction of HpD, porfimer sodium (commercially known as Photofrin®) in 1984 (Dougherty *et al.*, 1984). To date Photofrin® is the only drug approved for clinical application of PDT.
In 1956, Berlin *et al.* were the first to administer 5-aminolaevulinic acid (ALA) to animals and humans (to study haem and billirubin synthesis). Volunteers were subjected to light and skin photosensitivity lasting for 2 days was described (Berlin *et al.*, 1956). The first clinical applications of ALA-PDT were studied by Kennedy *et al.* in 1990. They applied

ALA to skin tumours and successfully treated them with light (Kennedy *et al.*, 1990). A large number of studies have been performed in the 1980's and 1990's investigating the pharmacokinetics of ALA and ALA-induced protoporphyrin IX (PPIX) in tumour cell models, animals and humans ((Peng *et al.*, 1997; Peng *et al.*, 1997), reviews).
To date, many different premalignant and malignant tumours have been treated with PDT, including skin cancer (Fritsch *et al.*, 1998), bladder cancer (Kriegmair *et al.*, 1996; Riesenberg *et al.*, 1996), lung cancer (Kato, 1998), brain tumours (Popovic *et al.*, 1995), gynaecological tumours (Gannon and Brown, 1999) and tumours in the gastro-intestinal tract (Gossner *et al.*, 1998; Mlkvy *et al.*, 1995; Tan *et al.*, 1999). Another potential application of PDT is intra-operative PDT after tumour resection (Abulafi *et al.*, 1997; van Hillegersberg *et al.*, 1995).

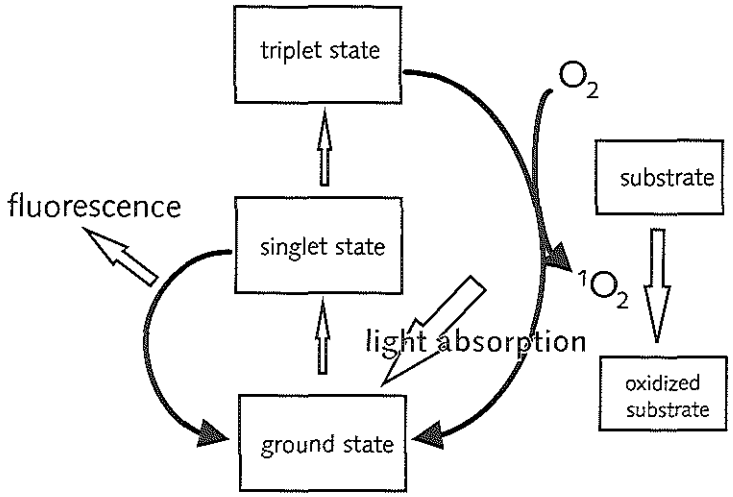

FIGURE 1 *Photochemical reaction type II, O_2 = oxygen, $^1O_2$ = singlet oxygen*

b. photochemical reactions
ALA-PDT is based on the intracellular accumulation of ALA-induced protoporphyrin IX (PPIX), a photosensitizing substance that can produce tissue destruction after absorbing light of an appropriate wavelength. The cytotoxic effect of PDT is the result of photo-oxidative reactions. The first step of these reactions is absorption of light energy by PPIX (ground state) to produce an excited state (singlet state) (***FIGURE 1***, after (van Hillegersberg *et al.*, 1994)). From this singlet state the photosensitizer can decay back to the ground state and emit light in the form of fluorescence. For the photodynamic effect, however, the

photosensitizer should undergo intersystem crossing to the excited triplet state of the molecule. Two competing reactions of the excited sensitizer can occur (Foote, 1991). Triplet state molecules can (a) react directly either with substrate or solvent by hydrogen atom or electron transfer to form radicals and radical ions, which after interaction with oxygen can produce oxygenated products (Type I reaction), or, (b) it can transfer its energy to oxygen directly to form highly reactive singlet oxygen (oxygen radicals, $^1O_2$, Type II reaction). The two reaction types can occur simultaneously but singlet oxygen is probably the most important cytotoxic agent responsible for the destruction of tissue (Weishaupt *et al.*, 1976). The singlet oxygen reacts in turn with amino acids, unsaturated fatty acids and nucleic acids at its site of generation, because of the limited diffusion length and short lifetime of these radicals (Moan, 1990). This results in damage to cell function and structure. From the above it is evident that PDT requires the presence of three components for its action, namely: a photosensitizer, light and oxygen.

c. photosensitizer

An ideal photosensitizer (Bonnett and Berenbaum, 1989) should:

(1) have a low systemic toxicity,
(2) have minimal dark toxicity,
(3) preferentially localize in (pre)malignant cells,
(4) be rapidly cleared from normal tissue,
(5) absorb wavelengths in the red or nearby infrared region of the light spectrum as tissue penetration is deeper than in the blue region,
(6) have a high triplet state quantum yield, e.g. high probability of triplet state formation per absorbed light energy quantum and
(7) be a chemically well-defined stable substance.

To date no such photosensitizer has been found. So far, the clinically most frequently studied photosensitizer for the treatment of Barrett's oesophagus is porfimer sodium (Photofrin®) (Overholt *et al.*, 1999). Photofrin® is a mixture of non-metallic oligomeric porphyrins linked mainly through ether bounds and only meets criteria 2 and 5 of the ideal photosensitizer criteria (Dougherty *et al.*, 1984). A major disadvantage of Photofrin® is the limited selectivity with regard to uptake and retention by tumour versus normal cells (Orenstein *et al.*, 1996). All tissues accumulate porphyrins to some extent, however, tissues remote from light are safe from light-induced damage. Skin cells also accumulate porphyrins and a serious side effect of Photofrin® is continued light photosensitivity of exposed skin for several weeks or months after treatment (Dougherty *et al.*, 1990). Another drawback of this photosensitizer, in particular for the treatment of a superficial lesion like Barrett's oesophagus, is stricture formation in up to 34% of the patients due to deep oesophageal wall damage (Overholt *et al.*, 1999).

In this thesis 5-aminolaevulinic acid (ALA) is studied. The difference with Photofrin® is that the photosensitizer - protoporphyrin IX (PPIX)- is synthesized *in situ* from ALA. ALA is a naturally occurring intermediate in the haem biosynthetic pathway. Production of PPIX will be discussed in the next section of this chapter. Several advantages (meeting criteria 1,2,4,5,7 of the ideal photosensitizer criteria) using ALA-induced PPIX photosensitization have been reported.

First, PPIX is rapidly eliminated from the body and hence the risk of skin photosensitivity is limited to 1-2 days (Berlin *et al.*, 1956; Kennedy and Pottier, 1992).

Secondly, in several studies it has been found that epithelial surfaces as epidermis, buccal mucosa, gastro-intestinal tract mucosa, respiratory tract mucosa, vaginal mucosa and glands or organs in continuity with such surfaces such as liver, sebaceous- and salivary glands may accumulate PPIX. However, tissues of mesodermal origin like muscle, connective tissue, cartilage did not show significant PPIX accumulation after ALA administration (Bedwell *et al.*, 1992; Kennedy and Pottier, 1992; Loh *et al.*, 1993; Loh *et al.*, 1993; Peng *et al.*, 1997). ALA-PDT treatment of Barrett's oesophagus would cause superficial damage to the oesophageal wall with much less risk of stricture formation (Gossner *et al.*, 1998).

Thirdly, ALA appears to offer better selectivity in terms of photosensitizer accumulation between tumour and normal tissues. Several *in vivo* animal studies have shown semi-selective ALA-induced PPIX accumulation in tumours compared to normal tissue (Bedwell *et al.*, 1992; Berlin *et al.*, 1956; Regula *et al.*, 1995; Regula *et al.*, 1994; van Hillegersberg *et al.*, 1995). However in human studies this has not been convincingly demonstrated and this is still a controversial matter (Barr *et al.*, 1996; Tan *et al.*, 1999).

Lastly, ALA can be given orally as well as applied locally (in creams, to treat skin lesions), providing an acceptable and convenient route of administration, which may be an advantage when treating patients on an outpatient basis (Gossner *et al.*, 1998; Loh *et al.*, 1993).

d. excitation light

As an optimal interaction between the photosensitizer and light is one of the premisses for an effect of PDT, it is important that light of a specific wavelength is used. The choice of the wavelength is determined by the absorption spectrum of the photosensitizer. Porphyrins are known for their strong absorption of UV light (the Søret band). The absorption capacity of 4 additional absorption bands (between 500 nm to 650 nm) is less intense (Bonnett and Berenbaum, 1989). As the penetration of light in tissues increases at higher wavelengths (***FIGURE 2***) (Wilson *et al.*, 1985), the weakest absorption band at about 630 nm is currently used for most clinical applications of ALA-PDT (Barr *et al.*, 1996; Grant *et al.*, 1993; Regula *et al.*, 1995). By increasing the intensity in this wavelength the photodamage can be achieved. Currently a dye laser pumped by a KTP laser (532 nm, Potassium Titanium Phosphate) is most commonly used. Advantages of the use of laser light for PDT are the well defined

wavelengths that makes it possible to match with the excitation wavelength of the photosensitizer and the possibility of coupling laser light into optical fibers.
Depending on the area to be treated, various forms of light delivery systems have been constructed. For use in the oesophagus cylindrical light diffusers in a centering balloon system are used (Gossner *et al.*, 1999; Overholt *et al.*, 1996).

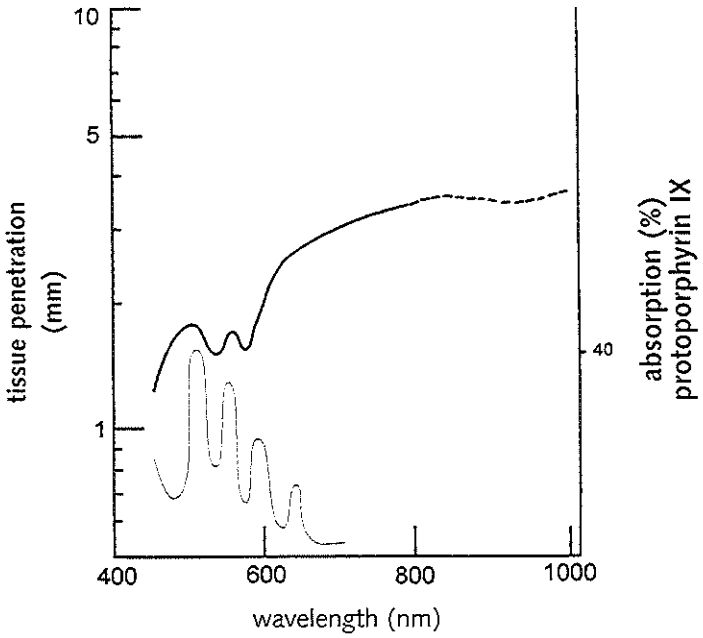

FIGURE 2 Absorption spectrum of PPIX (thin line) and the tissue penetration of various wavelengths. Arbitrary absorption units (%), 100% at 400 nm.

III. HAEM BIOSYNTHESIS

a. without exogenous ALA administration
Haem biosynthesis is essential for every cell and requires eight molecules of glycine and eight molecules of succinyl CoA for each molecule of haem (***FIGURE 3***). Haem is the prosthetic group for a number of haemoproteins. These include for instance myoglobin, haemoglobin, mitochondrial cytochromes, microsomal P450, catalase and peroxidase. There

are eight enzymes involved in the synthesis of haem.
The first reaction of the haem biosynthetic pathway, which is the rate-limiting step, is the condensation of glycine and succinyl CoA to form ALA, catalysed by the mitochondrial enzyme ALA-synthase (ALA-S). ALA-S appears to have one of the most rapid turn-over rates of the proteins in mitochondria that works far below its K_m (Druyan *et al.*, 1969). The short half-life of ALA-S is a suitable property for its regulatory role in haem formation. Control of the rate of synthesis of this enzyme by haem itself is believed to be the major mode of regulation of haem biosynthesis (feedback inhibition). Haem can directly inhibit ALA-S as well as the transcription, translation and transport of the enzyme into mitochondria (Hayashi *et al.*, 1972). The next step in haem biosynthesis is catalyzed by the cytosolic enzyme ALA-dehydratase, which catalyzes the condensation of two molecules of ALA with a loss of two molecules of water to form the monopyrrole porphobilinogen (PBG). Subsequently, the cytosolic enzyme porphobilinogen deaminase (PBG-D) joins four molecules of PBG in a head-to-tail fashion to generate a linear tetrapyrrole. In the absence of the enzyme uroporphyrinogen III synthase (URO-S), this product is released from PBG-D as hydroxymethylbilane, which then cyclizes to form uroporphyrinogen I. In the presence of URO-S as well as PBG-D, the hydroxymethylbilane is rapidly transformed into uroporphyrinogen III (Levin, 1968). Uroporphyrinogen decarboxylase, also localized in the cytosol, catalyses the sequential removal of the four carboxyl groups of the acetic acid side chains in uroporphyrinogen to yield coproporphyrinogen (Granick and Mauzerall, 1958). Coproporphyrinogen oxidase, which is present in the intermembrane space of mitochondria removes the carboxyl group and the two hydrogens from the propionic acid groups of two pyrrole rings to form vinyl groups (Grandchamp *et al.*, 1978). The reaction thus yields a divinyl compound, protoporphyrinogen IX. This molecule is readily oxidized into protoporphyrin IX (PPIX) by protoporphyrinogen IX oxidase, an enzyme located in the inner mitochondrial membrane (Feirrera *et al.*, 1988). The final step of haem biosynthesis is the insertion of the reduced form of iron (Fe^{2+}) into PPIX. This reaction is catalyzed by ferrochelatase (FC), which is located in the inner mitochondrial membrane (Jones and Jones, 1969). In addition to Fe^{2+}, Zn^{2+} can also be incorporated in PPIX by FC, a possibility used *in vitro* for the determination of the activity of ferrochelatase.

b. after ALA administration
Exogenous ALA bypasses the rate-limiting step catalysed by ALA-S and the feedback inhibition by haem on this particular enzyme. PBG-D then becomes the rate-limiting enzyme in haem formation (Bishop and Desnick, 1982). Excess ALA overloads the system and induces accumulation of photosensitive PPIX molecules. All cells are more or less able to accumulate PPIX from ALA (Sardesai *et al.*, 1964).

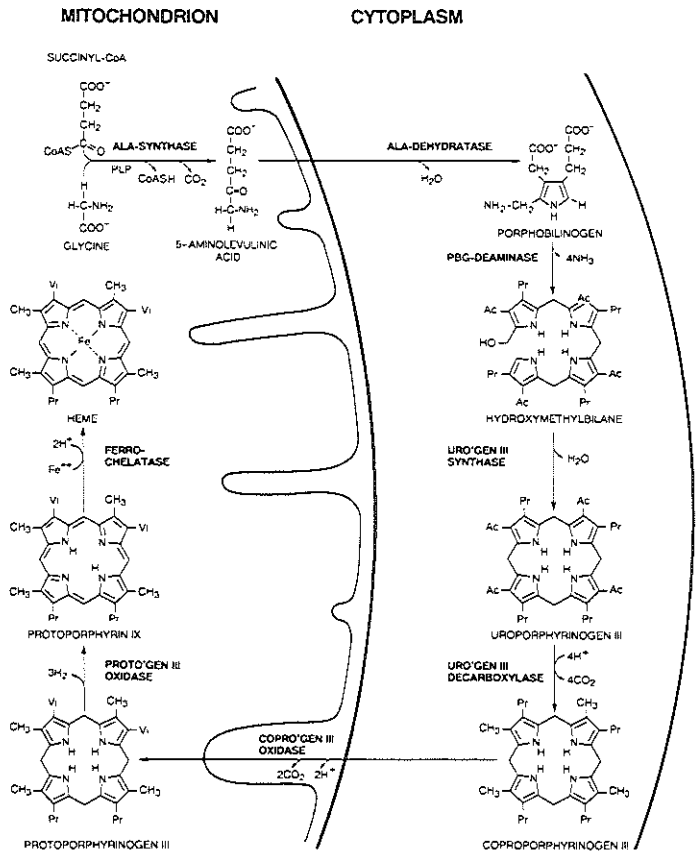

FIGURE 3 *The heme biosynthetic pathway.*
PLP, pyridoxal-5-phospate; ALA, 5-Aminolevulinic Acid; PBG, Porphobilinogen; URO'gen, Uroporphyrinogen; COPRO'gen, Coproporphyrinogen; PROTO'gen, Protoporphyrinogen; Ac, Acetate; Pr, Propionate; Vi, Vinyl
(by courtesy of Dr S.S. Bottemley)

Several groups, reviewed by Peng *et al.*, have shown that porphyrins accumulate in neoplastic tissue after the administration of ALA (Peng *et al.*, 1997). In general this is thought to occur when PBG-D activity is increased and FC activity is decreased. Increased PBG-D activity relative to normal tissue has indeed consistently been found in tumours (el-Sharabasy *et al.*, 1992; Navone *et al.*, 1990; Schoenfeld *et al.*, 1988) as well as in rapidly dividing cells,

e.g. regenerating liver cells (Schoenfeld *et al.*, 1988; Schoenfeld *et al.*, 1987) suggesting that this phenomenon might be common in situations of increased cell replication. In contrast with the consistent studies concerning the activity of PBG-D, there seems to be a difference in FC activity among different tumour types (Dailey and Smith, 1984; el-Sharabasy *et al.*, 1992; Rubino and Rasetti, 1966; Smith, 1987; van Hillegersberg *et al.*, 1992). Dailey and Smith found a decreased FC activity in the Morris hepatoma model, however they also pointed out that some porphyrins can act as inhibitors of FC (Dailey and Smith, 1984). Smith found decreased FC activities in human skin tumours but also in normal skin tissue compared to those in rat liver mitochondria (Smith, 1987). El-Sharabasy determined the FC activity in whole blood samples of children and adults with acute lymphoblastic leukaemia (ALL), non-Hodgkin's lymphoma (NHL) or Hodgkin's disease (HD) and found lowered activities of FC in patients with ALL, slightly decreased activities and increased activities of FC in blood of patients with NHL and HD, respectively, compared to healthy control groups (el-Sharabasy *et al.*, 1992). Compared to liver, which is one of the main haem-synthesizing tissues, most tissues have low FC activities (Webber *et al.*, 1997). The FC activity was found to be decreased in a colon carcinoma liver metastasis model (van Hillegersberg *et al.*, 1992). From the above it is clear that the activities of PBG-D and FC are altered in (pre)malignant tissues depending on the type of tissue. These findings provided the biological rationale for the clinical use of ALA-PDT.

IV. PHOTOBIOLOGY OF ALA-PDT

The reaction of singlet oxygen with target biomolecules is generally considered as the principal initiating pathway leading to photodynamic damage (Moan and Berg, 1991; Weishaupt *et al.*, 1976). Singlet oxygen diffuses only about 20 nm in its lifetime (Moan, 1990). Consequently, cellular structures close to a high photosensitizer and a high oxygen concentration will be preferentially damaged. Hypoxia significantly reduces the synthesis of PPIX (Wyld *et al.*, 1998) and together with the reduced yield of singlet oxygen, hypoxia diminishes the ultimate ALA-PDT effect (Georgakoudi *et al.*, 1999).
In most cell lines, animal models and human studies, PPIX is the main metabolite of ALA accumulating at its site of production in the mitochondria (Ackroyd *et al.*, 1999; Iinuma *et al.*, 1994; Loh *et al.*, 1993; van den Boogert *et al.*, 1999). Because PPIX is formed in mitochondria and because the range of action of oxygen radicals is very short, mitochondrial damage is most likely to be the major target in the cytotoxicity of ALA-PDT (Iinuma *et al.*, 1994; Liang *et al.*, 1998). Other cell compartments are also damaged by ALA-PDT e.g. the endoplasmatic reticulum and the nuclear envelope (Malik and Lugaci, 1987). Increased levels of intracellular calcium and sodium as well as cellular loss of potassium have been

observed after ALA-PDT (Schoenfeld *et al.*, 1994).
The mechanism of PDT-induced cell death initially appeared to have the characteristics of necrosis (Henderson and Dougherty, 1992), but an apoptotic mechanism is also likely as specific mitochondrial damage might trigger apoptosis. Apoptosis is often described as "programmed cell death" whereby a sequence of events results in DNA and cellular fragmentation avoiding the inflammatory effects which result from necrosis (membrane leakage, release of lysosomes) (Kessel and Luo, 1998; Noodt *et al.*, 1996; Webber *et al.*, 1996).
Direct cytotoxicity has been shown to be insufficient to explain the effects of ALA-PDT. Cells from murine tumours remaining *in situ* after PDT undergo necrosis, whereas those explanted immediately after PDT remain viable *in vitro* (Henderson *et al.*, 1985). This suggests that local tissue factors, for instance an effect on microcirculation (oxygen supply) (Leveckis *et al.*, 1995; Roberts *et al.*, 1994) and/or the presence of neutrophils may play a role as well (de Vree *et al.*, 1996).

V. CLINICAL STUDIES

In 1996, Barr *et al.* were the first to treat five patients with Barrett's oesophagus and high-grade dysplasia with ALA-PDT and acid suppression (Barr *et al.*, 1996). Four to 6 hours after the oral administration of 60 mg/kg ALA dissolved in fruit juice, the Barrett's segment was illuminated by laser light of 630 nm. High-grade dysplasia was eradicated in all patients, without recurrence after 26-44 months of endoscopic and histological follow-up. In two cases, non-dysplastic Barrett's epithelium was found underneath regenerative squamous mucosa.
Gossner and colleagues, with a design similar to Barr *et al.*, treated 32 patients (10 high-grade dysplasia, 22 early cancer) with ALA-PDT (Gossner *et al.*, 1998). High-grade dysplasia and thin mucosal cancer (≤ 2mm, 77% of all early cancers) was eradicated in all patients at a mean follow-up of 10 months. In two patients non-dysplastic Barrett's epithelium was detected underneath the newly formed squamous epithelium. Fifteen patients experienced transient nausea up to 6 hours after ALA administration and in 21 of 32 patients transient increases (3 days) in transaminase levels were documented. In both studies, no strictures or photosensitivity of the skin were reported.
Biddlestone *et al.* described the histopathology of the treated Barrett's oesophagus after ALA-PDT in 5 patients (Biddlestone *et al.*, 1998). Three of these cases had already been reported by Barr *et al.* in 1996. The importance of their study is that they found Barrett's mucosa underneath the regenerated normal squamous epithelium in all 5 cases. In two cases this glandular mucosa was dysplastic. The existence of Barrett's epithelium underneath

the newly formed squamous epithelium is also called pseudoregression of Barrett's mucosa and has also been described after other techniques of endoscopic ablation of Barrett's mucosa (Berenson *et al.*, 1993). The importance of this finding is not yet clear, one can speculate that the glandular mucosa is protected by the overlying normal squamous epithelium from the acid environment, with a reduction in inflammation and reactive cell proliferation resulting in a reduced neoplastic risk. However, long-term follow-up studies are required to investigate the behaviour of such concealed glandular mucosa.
From the above it is apparent that ALA-PDT has the capacity of producing mucosal ablation of Barrett's mucosa harbouring dysplasia or early adenocarcinoma. However, ALA-PDT needs to be improved and this requires more preclinical studies. In addition, the completeness and duration of response need to be prospectively evaluated before PDT becomes an accepted modality in the management of this disease.

VI. PRE-CLINICAL STUDIES FOR FURTHER OPTIMIZATION OF ALA-PDT

For many years, studies have been performed in cell lines, animal models and humans to find ways to optimize the effects of ALA-PDT. The three components required for the action of PDT (photosensitizer, oxygen and light) have been the subject of research for this matter. In this section relevant studies performed during the past few years will be reviewed.
The intracellular concentration of ALA-induced PPIX (van den Boogert *et al.*, 1999) and the intracellular localisation of PPIX at the time of PDT (Iinuma *et al.*, 1994; Liang *et al.*, 1998) seem to have an impact on the results of ALA-PDT. As a consequence, timing of PDT after ALA administration is important as PPIX initially accumulates in mitochondria but at longer time intervals it will diffuse and localize at less crucial cellular sites and/or will be converted into haem by that time (Steinbach *et al.*, 1995).
Insight was gained in the possibilities of increasing the intracellular concentration of PPIX and of achieving the same concentration of PPIX with lower doses of ALA. The latter could be achieved by intravenous injection of ALA, studied in an animal model (Loh *et al.*, 1993). It was also shown that the same amount of PPIX could be induced in carcinoma cells for concentrations 30-150 fold lower of ALA-esters than in the presence of non-esterified ALA (Gaullier *et al.*, 1997). Chelation of iron by for instance desferrioxamine has been shown to induce higher levels of intracellular PPIX through the inhibition of ferrochelatase, the enzyme responsible for conversion of PPIX into haem by the insertion of iron into PPIX (Berg *et al.*, 1996; Curnow *et al.*, 1998; Tan *et al.*, 1997).
From other pre-clinical studies it can be learned that optimal oxygenation of the target tissue has to be one of the priorities at the time of PDT. These studies have shown that

hypoxia significantly reduces the synthesis of PPIX (Wyld et al., 1998) and together with the reduced yield of singlet oxygen, hypoxia diminishes the ultimate ALA-PDT effect (Georgakoudi et al., 1999).
The effect of adjuvant therapies such as pre-treatment with adriamycine before ALA-PDT (Casas *et al.*, 1997) or concomitant laser-induced hyperthermia (Liu *et al.*, 1997) have been studied in mice bearing a transplantable mammary adenocarcinoma and rat liver tumours respectively and were shown to be synergistic to the effect of ALA-PDT.
The wavelength (Szeimies *et al.*, 1995), total light dose (Bays *et al.*, 1997) and the application techniques of light (Gossner *et al.*, 1999; Overholt *et al.*, 1996) have been studied in relation to the PDT effect. Of especial interest is the illumination schedule used for PDT. It seems, at least in cell lines and animal models, that light fractionation has a significant positive effect on the amount of cell death. Light fractionation schemes seem to improve the ultimate effect, probably through recovery of vascular constriction during the dark period and as a result re-oxygenation of the tissues (Curnow *et al.*, 1999). This subject is discussed in more detail in chapter 8, the general discussion of this thesis.

VII. AIMS AND OUTLINE OF THIS THESIS

ALA-PDT has physical, biochemical and clinical aspects. The aim of this thesis was to study the haem biosynthetic pathway before and after ALA administration and to find tools to manipulate haem synthesis in order to optimize ALA-PDT for patients with Barrett's oesophagus and superficial adenocarcinomas of the oesophagus. In this thesis, the activities of PBG-D and FC in different tissues are the main theme, as these activities probably play an important role in determining the rate of PPIX synthesis and its accumulation after ALA administration.
The first question addressed was whether relative activities of PBG-D and FC were different in human Barrett's oesophagus, adenocarcinoma, normal squamous epithelium and normal gastric mucosa **(Chapter 2)** and whether the ratio between the two enzymes predicts the accumulation of PPIX **(Chapter 4)**.
Prior to starting a clinical study in patients undergoing oesophageal resection, we asked ourself a second question concerning the light from operating lights which could be damaging to other tissues besides the target tissue in patients given photosensitizing agents. This question was whether the use of acrylate yellow filters on operating lights could have a protective effect in terms of a reduction of unwanted tissue damage. This effect was studied in a human hepatocellular cell line and in pigs, used as a pre-clinical model **(Chapter 3)**.
As timing of illumination is one of the important factors in the ultimate PDT effect, the third question addressed was whether the pharmacokinetics of ALA and PPIX were different

during the first hours after ALA ingestion in normal oesophageal epithelium and Barrett's epithelium **(Chapter 5)**.
The fourth question was whether ALA-PDT affected the relative activity of the two enzymes. This question is of potential importance for improving PDT, as a relative suppression of FC induced by ALA-PDT followed by a second exposure to ALA could lead to a greater accumulation of the photosensitizer PPIX in the illuminated tissue. This was studied in lymphoblastoid cell lines and described in **Chapter 6**.
Whether a two-phase illumination scheme (light fractionation) in ALA-PDT was indeed more effective in terms of increased PPIX accumulation and the damaging effect of PDT than only one illumination was the fifth question investigated in lymphoblastoid cell lines and reported in **Chapter 7.**

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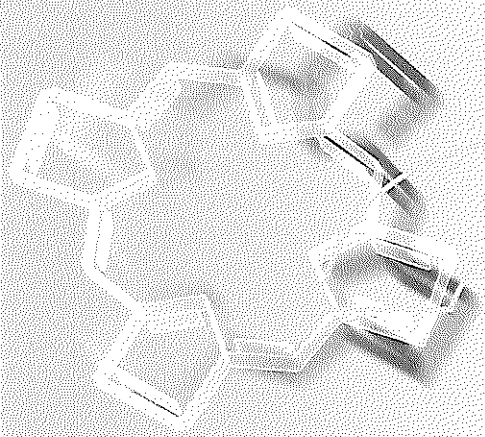
Biochemical basis of ALA-induced PPIX accumulation: a study in patients with (pre)malignant lesions of the oesophagus.



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Summary

Administration of 5-aminolaevulinic acid (ALA) leads to porphyrin accumulation in malignant and pre-malignant tissues, and ALA is used as a prodrug in photodynamic therapy (PDT). To understand the mechanism of porphyrin accumulation after the administration of ALA and to investigate whether ALA-induced protoporphyrin IX might be a suitable photosensitizer in Barrett's oesophagus and adenocarcinoma, we determined the activities of porphobilinogen deaminase (PBG-D) and ferrochelatase (FC) in various malignant and pre-malignant as well as in normal tissues of the human oesophagus. A PDT power index for ALA-induced porphyrin accumulation, the ratio of PBG-D to FC normalised for the normal squamous epithelium of the oesophagus, was calculated to evaluate inter-tissue variation in the ability to accumulate porphyrins. In malignant and pre-malignant tissue a twofold increased PBG-D activity and a marginally increased FC activity was seen compared to normal squamous epithelium. A significant increased PDT power index in Barrett's epithelium and adenocarcinoma was found. Our results suggest that, after the administration of ALA, porphyrins will accumulate in a greater amount in Barrett's epithelium and adenocarcinoma of the oesophagus due to an imbalance between PBG-D and FC activities. The PDT power index here defined might be an useful indicative parameter to predict the susceptibility of these tissues to ALA-PDT.

Introduction

Heme biosynthesis (***FIGURE 1***) is essential to every cell and requires 8 molecules of glycine and 8 molecules of succinyl CoA for each molecule of heme. The first intermediate is 5-aminolaevulinic acid (ALA); two molecules of ALA are converted to porphobilinogen, which is metabolized to porphyrinogen intermediates by porphobilinogen deaminase (PBG-D). The last step is the incorporation of iron into protoporphyrin IX (PPIX), catalysed by ferrochelatase (FC). The synthesis of ALA is the rate-limiting step. If exogenous ALA is provided, then other enzymes become rate-limiting in heme formation.
Some cancer cells have been found to have an increased activity of PBG-D (el-Sharabasy *et al.*, 1992; Navone *et al.*, 1990,1991; Rubino & Rasetti, 1966; Schoenfeld *et al.*, 1988a) and in most studies these cells have been found to have a decreased activity of FC (Dailey & Smith, 1984; el-Sharabasy *et al.*, 1992; Rubino & Rasetti, 1966; Smith, 1987; Van Hillegersberg *et al.*, 1992). For such cells administration of ALA will lead to the accumulation of porphyrins, especially PPIX (Anderson *et al.*, 1981). This provides a biological rationale for the clinical use of ALA photodynamic therapy (ALA-PDT).

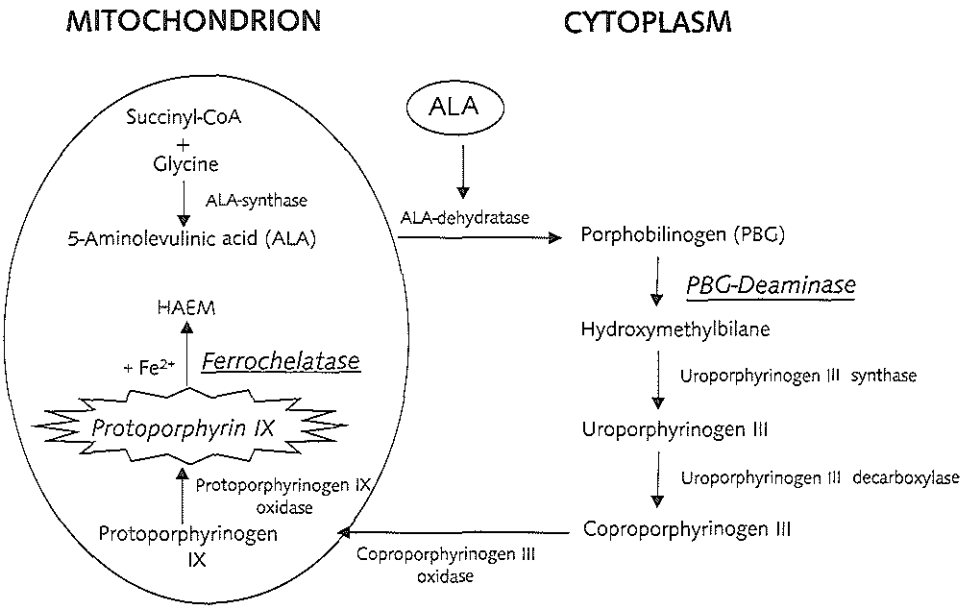

FIGURE 1 Haem biosynthetic pathway

Barrett's (columnar-lined) oesophagus results from long-term gastroesophageal reflux (Mossberg, 1966). It is of clinical importance because of its malignant potential. Barrett's oesophagus can lead to the development of adenocarcinoma through a multistep process of progression from metaplasia, to low grade dysplasia, high grade dysplasia and ultimately to invasive cancer (Hameeteman *et al.*, 1989; Hamilton & Smith, 1987). High grade dysplasia in Barrett's oesophagus presents a difficult management problem. Options include endoscopic surveillance and/or esophagectomy (Cameron, 1997; Clark *et al.*, 1996; Levine *et al.*, 1993). A new non-surgical management option involves eradicating the dysplastic epithelium and columnar mucosa by PDT. In contrast to other photosensitisers, many of which localise in the microvasculature of all tissue layers of hollow organs, ALA induces much higher levels of PPIX in the mucosa compared to submucosa or muscularis mucosae (Loh *et al.*, 1993). ALA-PDT has been used to treat high grade dysplasia in Barrett's oesophagus, resulting in necrosis of dysplastic mucosa with regeneration of normal squamous mucosa (Barr *et al.*, 1996; Gossner *et al.*, 1995; Regula *et al.*, 1995).

To optimize ALA-PDT for Barrett's oesophagus and early carcinoma knowledge of the mechanism of porphyrin accumulation in these tissues is required. We determined the activities of PBG-D and FC in normal tissue as well as in malignant and pre-malignant tis-

sue of the human oesophagus. These two enzymes play an important role after the administration of ALA; PBG-D is in many cells the rate-limiting enzyme when exogenous ALA is administered and FC is the enzyme directly responsible for the conversion of PPIX to heme. We propose the use of a *PDT power index* for the inter-tissue variation in the ability to accumulate PPIX, in order to create a parameter which might indicate the susceptibility of tissues to ALA-PDT.

Materials and methods

TISSUE SAMPLES

Between August 1996 and February 1997 tissue was obtained from 27 patients (16 males and 11 women) undergoing an esophageal resection. The mean age was 61 years (43-81 years). Nine patients had a squamous cell carcinoma, 18 had an adenocarcinoma of the distal oesophagus and in 9 of these patients Barrett's epithelium was present. Samples from histologically proven Barrett's mucosa, squamous cell carcinoma and adenocarcinoma as well as samples from normal gastric mucosa and normal squamous epithelium were taken immediately after the resection. In some instances, samples could not be taken from Barrett's mucosa. Tissue samples were embedded in formalin, sectioned, and stained with hematoxylin and eosin. The grade of tumour differentiation was described as well as the grade of dysplasia in Barrett's mucosa. Barrett's mucosa was classified as indefinite, low grade dysplasia (LGD) and high grade dysplasia (HGD). In addition adjacent tissue samples were frozen (-70°C) until the moment of biochemical analysis. All determinations were performed in duplicate within 6 weeks of resection. Control experiments showed no change in activities in samples stored at -70°C for this time. This temperature was found to be essential; when stored at -20°C, FC activity decreased within a few days, whereas the PBG-D activity remained stable for weeks.

CHEMICALS

The following reagents were obtained from Porphyrin Products (Logan, UT, USA): PPIX disodium salt, zinc PPIX and porphobilinogen (PBG). Coproporphyrin, Triton X -100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris-HCL was purchased from Boehringer Mannheim (Mannheim, Germany) and other chemicals were purchased from Merck (Darmstadt, Germany).

FERROCHELATASE AND PORPHOBILINOGEN DEAMINASE ASSAYS

FC activity was measured by a modification of the method of Li *et al.* as described previously (Li *et al.*, 1987; Van Hillegersberg *et al.*, 1992). PBG-D measurements were performed by a modification of the method of Wilson *et al.* (Wilson *et al.*, 1986). Tissue samples, kept on ice, were homogenised in water (1:5, wt/wt) using a Potter Elvehjem homogenizer (Kontess Glass Co., Vineland, NJ, USA). An aliquot of 50 µl of a 200 mol/L solution of PBG in 0.1 mol/L Tris-HCL buffer, pH 8.0, were added to 50 µl of the homogenate. This mixture was incubated for one hour at 37 °C. The reaction was stopped by adding 600 µl of Tris-HCl buffer (Tris-HCl 50 mmol/L, Trichloroacetic acid 1.5 mol/L in aqua dest.) (5:7, v:v). After 5 minutes exposure to UV light (350 nm), to convert porphyrinogens to porphyrins, the samples were centrifugated for 10 minutes at 14000 x *g* (Eppendorf centrifuge, Merck Nederland BV, The Netherlands), and the fluorescence of the supernatant was measured at 408 nm excitation and 648 nm emission wavelength (Perkin Elmer LS 5B with a red sensitive photomultiplier). Values were calculated according to a standard curve of coproporphyrin III in Tris-HCl buffer (1:1, v:v). Results were expressed as pmol of porphyrins formed per mg protein per hour. Protein was determined according to the method of Lowry *et al.* (1951).

THE PDT POWER INDEX

The ratio of PBG-D to FC, introduced as the PDT power index, was calculated, with the enzyme activities in each tissue sample relative to the activities in normal squamous epithelium per individual. This index was calculated according to the formula:

[PBG-D(tissue)/FC(tissue)] x
[FC(squamous epithelium)/PBG-D (squamous epithelium]

STATISTICAL ANALYSIS

Data are expressed as means ± SD and were analysed for statistical significance using the Wilcoxon matched-pairs signed rank sum test. The enzyme activities of the malignant and pre-malignant tissues were compared to the adjacent normal tissue in the same patient.

Results

All results are shown in ***TABLE 1***. A twofold increase in PBG-D activity was found in Barrett's epithelium (p=0.018) and in adenocarcinoma of the oesophagus (p=0.001) compared to normal squamous epithelium. Regarding the activity of FC, although the mean values were significantly increased compared to normal squamous epithelium, this increase was less marked compared to the PBG-D activity increase. This resulted in a significant increase of the PDT power index in Barrett's oesophagus (p=0.046) and adenocarcinoma (p=0.003) compared to the normal squamous epithelium. Of the five cases of Barrett's oesophagus in which the index was calculated and the grade of dysplasia determined; 4 cases were classified as LGD and 1 case as HGD. The PDT power indexes were 0.8, 1.4, 1.4, 1.6 for LGD and 2.4 for HGD.

TABLE 1 *Enzyme activities and PDT power indexes*

PBG-D activity*			Range
Squamous epithelium	n=27	22.8 ± 7.3	*(10 - 42)*
Gastric mucosa	n=27	24.9 ± 8.6	*(10 - 42)*
Barrett's epithelium	n=7	40.6 ± 13.7†	*(21 - 63)*
Adenocarcinoma	n=17	55.0 ± 19.9‡	*(25 - 93)*
Squamous cell ca.	n=9	37.6 ± 14.1†	*(21 - 67)*
Ferrochelatase activity*			**Range**
Squamous epithelium	n=24	391 ± 152	*(124 - 718)*
Gastric mucosa	n=24	685 ± 265‡	*(336 - 1187)*
Barrett's epithelium	n=6	437 ± 203†	*(176 - 726)*
Adenocarcinoma	n=16	582 ± 220‡	*(230 - 1048)*
Squamous cell ca.	n=7	558 ± 332	*(251 - 1263)*
PDT power index			**Range**
Squamous epithelium	n=24	1.0	
Gastric mucosa	n=24	0.7 ± 0.2‡	*(0.3 - 1.1)*
Barrett's epithelium	n=6	1.8 ± 0.8†	*(0.8 - 3.0)*
Adenocarcinoma	n=16	1.9 ± 1.2‡	*(0.6 - 5.6)*
Squamous cell ca.	n=7	1.1 ± 0.5	*(0.6 - 2.0)*

*pmol/mg protein/hour, mean ± SD; †p<0.05, ‡p<0.01

Discussion

Several groups have shown that porphyrins accumulate in neoplastic tissue after the administration of ALA (Peng *et al.*, 1997, review). Normally, heme synthesis is regulated by substrate availability and by feedback inhibition of the enzyme ALA synthase. The concentration of substrates and intermediates are usually far below the Michaelis constants of the enzymes, in which case intermediates are metabolized to heme (Bottomly & Muller-Eberhard, 1988). When exogenous ALA is administered, normal cells will rapidly produce heme. An excess of exogenous ALA will initially overload the system and porphyrin intermediates will accumulate. The presence of the intermediates contributes to photosensitivity of normal cells, but these intermediates are rapidly metabolised into heme. In malignant and pre-malignant tissue of the oesophagus, we found increased PBG-D and FC activities compared to normal squamous epithelium, and an imbalance between these activities. These results are in line with those found in our previous smaller studies (Hinnen *et al.*, 1997a, 1997b). Due to individual patient and tissue variations in the activities of PBG-D and FC, also described by others (Dailey & Smith, 1984; el-Sharabasy *et al.*, 1992; Navone *et al.*, 1990; Rubino & Rasetti, 1966; Schoenfeld *et al.*, 1987, 1988a, 1988b; Smith, 1987; Van Hillegersberg *et al.*, 1992), the activity of these two heme enzymes can be better described relative to each other. This ratio, which we propose to call the PDT power index, reflects the enzymatic ability of cells to accumulate porphyrins after ALA administration and might predict the susceptibility of tissue to ALA-PDT. The PDT power index was significantly increased in Barrett's epithelium and adenocarcinoma compared to normal squamous epithelium indicating that the FC activity was relatively low compared to the PBG-D activity. Since this index can also be derived from biopsy specimens, e.g. oesophagus or Barrett, it could be applied prior to ALA-PDT to estimate tissue susceptibility. Increased PBG-D activity relative to normal tissue has consistently been found in tumours (el-Sharabasy *et al.*, 1992; Navone *et al.*, 1990, 1991; Schoenfeld *et al.*, 1988a) as well as in rapidly dividing cells, e.g. regenerating liver cells (Schoenfeld *et al.*, 1987, 1988b), suggesting that this phenomenon might be common in situations of increased cell replication. In contrast with the consistent studies concerning the activity of PBG-D, there seems to be a difference in FC activity among different tumour types (Dailey & Smith, 1984; el-Sharabasy *et al.*, 1992; Rubino & Rasetti, 1966; Smith, 1987; Van Hillegersberg *et al.*, 1992). Dailey and Smith (1984) found a decreased FC activity in the Morris hepatoma model, however they also pointed out that some porphyrins can act as inhibitors of FC. Smith (1987) found decreased FC activities in human skin tumours but also in normal skin tissue compared to those in rat liver mitochondria. El-Sharabasy *et al.* (1992) determined the FC activity in whole blood samples of children and adults with acute lymphoblastic leukaemia (ALL), non-Hodgkin's lymphoma (NHL)

or Hodgkin's disease (HD) and found lowered activities in patients with ALL, slightly decreased activities and increased activities of FC in blood of patients with NHL and HD respectively compared to healthy control groups. Compared to liver, which is one of the main heme-synthesizing tissues, most tissues have low enzyme activities (Webber *et al.*, 1997). Our group found the FC activity to be decreased in a colon carcinoma liver metastasis model and we suggested applying ALA-PDT to patients with these liver metastases (Van Hillegersberg *et al.*, 1992). Regarding the effect of the storage temperature on FC activity, interpretation of data from other studies might be biased due to differences in tissue storage. In the gastrointestinal tract, accumulation of porphyrins after ALA administration, is more pronounced in the mucosa than in the underlying submucosa and muscle layers making ALA suitable for treating most mucosal lesions (Bedwell *et al.*, 1992; Loh *et al.*, 1993; Webber *et al.*, 1997). In patients with familial adenomatous polyposis, no differences in PPIX concentrations were found between normal and adenomatous tissue (Mlkvy *et al.*, 1995). In the DMH rat colonic tumour model the same group showed differences in the levels of PPIX between normal mucosa and tumour with a ratio of 1:6 (Bedwell *et al.*, 1992). In another study they showed that higher doses of ALA (60 mg/kg instead of 30 mg/kg) improved the tumour:normal mucosa PPIX sensitisation ratio in patients with colon carcinoma (Regula *et al.*, 1995). Webber *et al.* (1997) showed selective accumulation of PPIX in adenocarcinomas of the gastrointestinal tract in 42 patients. Our biochemical study has characterized the enzymatic capacities of heme biosynthesis in normal, pre-malignant and malignant tissue of the human oesophagus. These results provide evidence for the selectivity of PPIX accumulation between normal and neoplastic tissue of the oesophagus. Whether selective PPIX accumulation creates the possibility of achieving selective necrosis is still in question. Recently, Bown and Millson (1997) have stated that the selectivity of ALA-PDT induced necrosis, in the gastrointestinal tract, is between mucosa and underlying submucosa and muscularis and not between normal mucosa and neoplastic mucosa. In conclusion our study supports the use of ALA for selective PDT in Barrett's oesophagus and early carcinoma. Information about the PDT power index could be useful in predicting the effect of ALA administration on porphyrin accumulation and therefore on the susceptibility of the disorder to ALA-PDT.

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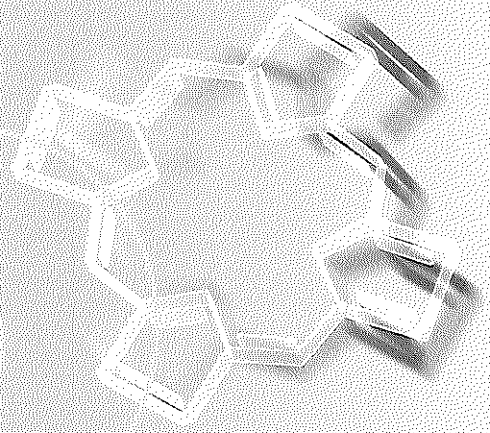
Acrylate yellow filters in operating lights protect against photosensitization tissue damage.



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Abstract

Background: Photosensitized patients are exposed to bright lights when undergoing intraoperative photodynamic therapy or fluorescence measurements. Acrylate yellow filters might reduce unwanted tissue damage. Method: To investigate the protective value of these filters, the spectral power distribution of the operating lights and light energy densities with and without an acrylate yellow filter were measured. Subsequently, the effects of light exposure on the survival of a human hepatocellular carcinoma cell line and the photodamage induced in pig tissues after the administration of 5-aminolevulinic acid were also studied. Results: The light energy density in the ultraviolet and blue light region of the light spectrum emitted by the operating light was reduced up to 50 percent by the acrylate yellow filter. The survival of photosensitized cells was longer and photodamage induced in pig tissues was less when exposed to filtered light. Conclusion: Photodamage induced by operating lights can be reduced by filtering out ultraviolet and blue light by means of acrylate yellow filters.

Introduction

Photodynamic therapy (PDT) has been proposed as an adjuvant to cancer surgery to reduce local recurrence. PDT during operation is complicated by the fact that photosensitized patients are exposed to operating lights in addition to the light source used for PDT (Abulafi *et al.*, 1997; Allardice *et al.*, 1994; van Hillegersberg *et al.*, 1995). The protoporphyrin IX (PPIX) precursor, 5-aminolaevulinic acid (ALA) is currently under study as a potential photosensitizer for gastrointestinal cancer (Hinnen *et al.*, 1998). PPIX is formed by both tumour and normal tissues, and normal organs are also vulnerable if exposed to light of a broad spectrum during the period of PPIX accumulation (Bown and Millson, 1997; Loh *et al.*, 1993).

This investigation aimed to determine whether the use of acrylate yellow filters, which filter out the shorter wavelengths of the spectrum of the operating lights (ultraviolet (UV) and blue region), might reduce tissue damage. Others have also used these filters as a safety measure when operating on photosensitized patients, but their protective value does not appear to have been studied (Fromm *et al.*, 1996; Meerman *et al.*, 1994; Webber *et al.*, 1997).

First, the survival of a human hepatoma cell line, HepG2, was studied after incubation with ALA and subsequent exposure to operating lights with and without an acrylate yellow filter (Iwasa *et al.*, 1989; Visser *et al.*, 1991). Second, the effect of acrylate yellow filters on pigs being exposed to an operating light during a laparotomy following administration of ALA was determined.

Materials and methods

LIGHT SOURCE AND ACRYLATE YELLOW FILTER

An Angenieux operating light type AX 14 (Gambro Medische Apparaten, Breda, The Netherlands) with 2 halogen bulbs (150W) was used for the HepG2 cell line study. The spectral power distribution was determined by a MultiSpec 125 spectrometer attached to a InstaSpec IV CCD camera (Oriel Instruments Corporation, Stratford, CT, USA). A Bechtold C- 571 operating light (Colombus Medical B.V., Zaltbommel, The Netherlands) with essentially the same spectral power distribution was used for the pig experiments. The acrylate yellow 303 filter (4 mm thickness) was obtained from Wientjes (Roden, The Netherlands) and cut to fit the operating lights.

HEPG2 CELL LINE STUDY

HepG2 cells

Hep G2 cells were grown as described previously (Visser *et al.*, 1991), harvested at the moment of exponential growth and seeded in six-well plates at about 0.5 $x10^6$ cells per well.

Porphyrin analysis

Porphyrins were measured by a modification of the method by Chisolm and Brown (Chisolm and Brown, 1975) as described previously (van den Boogert *et al.*, 1998), and expressed as picomoles per 10^6 cells.

Irradiation

Cells were incubated with or without 0.3 mmol/L ALA for 2 h under conditions described by Vonarx et al (Vonarx-Coinsman *et al.*, 1995). Following incubation, cells were cultured for 3 h without ALA. Subsequently, they were exposed for 0, 5, 10, 15 or 20 min to filtered or unfiltered focused light, at 40 cm. Thereafter, cells were cultured until cell viability was determined by the trypan blue exclusion test at 24 h after irradiation.

PIG EXPERIMENTS

ALA administration

Experiments were performed on 2 male pigs weighing 21.5 and 22 kg. After fasting for 24 h and 12 h without water, the pigs were sedated with 5 ml ketamine intramuscularly (100 mg/ml; Apharmo Holland, Arnhem, The Netherlands). ALA 75 mg/kg (Fluka,

Buchs, Switzerland) in 150 ml phosphate-buffered saline was administered by gavage. Blood samples were taken.

Laparotomy and irradiation

Pigs were anaesthetised with 70 percent nitrous oxide and 30 percent oxygen, 2 percent enflurane (Abbott, Amstelveen, The Netherlands), 6 ml fentanyl citrate intravenously (0.05 mg/ml, Janssen-Cilag, Tilburg, The Netherlands) and 2 ml curare intravenously (2 mg/ml; Organon Teknika, Boxtel, The Netherlands). Some 5 h after ALA, liver, small intestine and stomach surfaces were exposed to the focused operating light at 80 cm for 30 min. Other organs were covered. One pig was exposed to filtered light, the other to unfiltered light. Light power densities (mW/cm^2) were measured using sterile photodetectors (coherent model 210 and the UDT model 81; United Detector Technology, Palo Alto, California, USA). Exposed areas were marked. Dark sides of the organs were used to assess dark toxicity.

Post mortem examinations

Some 48 h after laparotomy, pigs were sedated with 5 ml ketamine (100 mg/ml) and the organs were inspected by the same surgeon. The pigs were then killed and tissue samples were taken.

Histological examinations

Tissue samples were fixed with formalin, sectioned, stained with haematoxylin and eosin, and examined.

LABORATORY MEASUREMENTS

ALA and porphobilinogen (PBG) concentrations in plasma were determined using a fluorometric enzyme assay according to a procedure described by de Rooij *et al.* (de Rooij *et al.*, 1987). Levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) in serum were determined (Granutest 25; Merck, Darmstadt, Germany).

Results

SPECTRAL POWER DISTRIBUTION AND ACRYLATE YELLOW FILTER CHARACTERISTICS

The maximum light power density of the operating light was 30 mW/cm^2. The spectral power distribution of the operating lights with and without the acrylate yellow filter and the excitation spectrum of PPIX are shown in ***FIGURE 1***. The filter eliminates nearly all UV and blue light below a wavelength of about 520 nm.

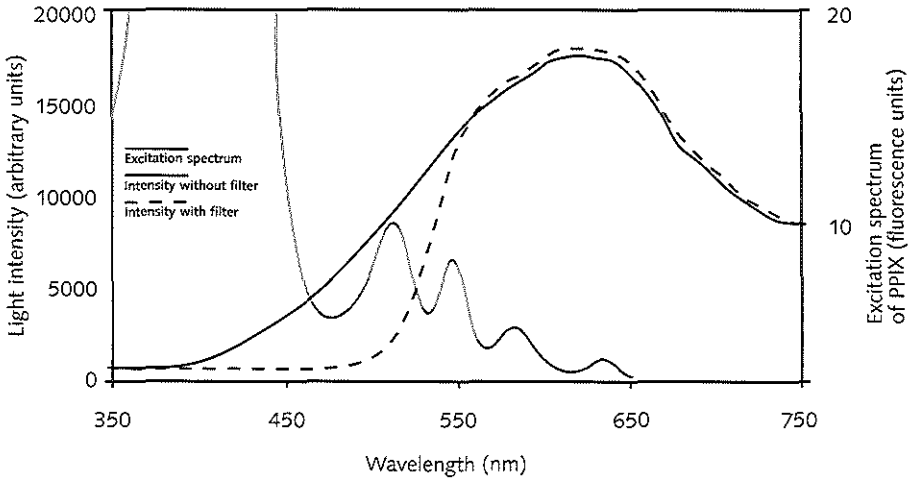

FIGURE 1 *Spectral power distribution of an operating light with and without acrylate yellow filter, and the relative excitation spectrum of protoporphyrin IX (PPIX)*

HEPG2 CELL LINE STUDY

HepG2 cells accumulated PPIX from exogenous ALA (results not shown). Exposure of cells to filtered light (protected cells) for 5 min resulted in a surviving fraction of 89 percent 24 h later (***FIGURE 2***). Only 39 percent of the unprotected cells survived. This initial sixfold reduction in cell death decreased after longer exposure times. After 10 min exposure, 35 percent of protected cells survived, compared to 18 percent of unprotected cells, and after exposure for 20 min the surviving fractions were 13 and 5 percent for protected and unprotected groups respectively. At all time points twice as many protected cells survived compared to unprotected cells. Non-irradiated ALA incubated as well as irradiated non-ALA-incubated cells multiplied throughout the experiment. The temperature of the medium remained constant in all groups.

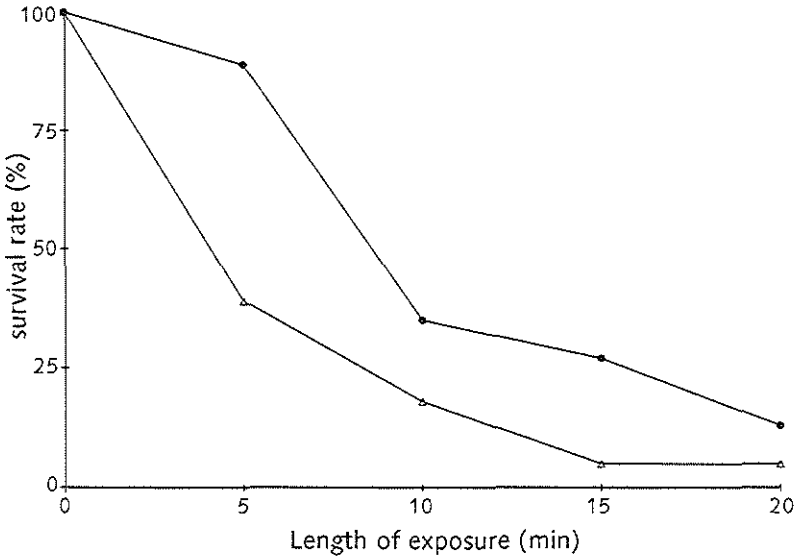

FIGURE 2 Survival of photosensitized HepG2 cells, 24 h after 0, 5, 10, 15 and 20 min of exposure to an operating light with (•) and without (Δ) an acrylate yellow filter

PIG EXPERIMENTS

ALA plasma concentrations, determined at various intervals, peaked at or before 4 h after ALA administration. At 24 h, ALA was not detectable in plasma.

Light power densities in peripheral fields and in fields directly in focus were measured during the laparotomy, and varied from 0.1 to 21 mW/cm^2 without the filter and from 0.1 to 10 mW/cm^2 with the filter. The total light energy densities reaching the liver, small intestine and stomach surfaces consisted of direct (focused) illumination for 30 min and indirect illumination (preparation time and peripheral light during exposure of other organ) for 75 min. Some 66-70 J/cm^2 reached the organs without the filter and 34-36 J/cm^2 reached the organs with a filter. Therefore, a 50 percent reduction of total light energy density, mainly of UV and blue light (wavelength less than 520 nm; ***FIGURE 1***), was caused by the filter.

During exposure to unfiltered light, macroscopic changes included redness of the gastric serosa, a white contracting serosa of the small intestine, a colour change of the liver from brown to yellow, and a change to a wrinkled aspect of the liver surface. These changes were less pronounced after exposure to filtered light.

At post-mortem examination, macroscopic changes after illumination without the filter were more pronounced. Haemorrhagic spots existed and the tissue was swollen com-

pared with the dark control side of the same organ or tissue illuminated using the filter. Histologically, no changes were found in the dark control tissues of both pigs. There were clear differences in liver histology between areas illuminated with or without the filter (***FIGURES 3*** and ***4***). Liver necrosis was twice as deep when the filter was not used (1.0 versus 0.5 mm). Necrosis was not seen in the stomach and small intestine, but oedema, leucocyte infiltration and disintegration of muscle cells were observed. Transient (for 48 h) increases in ASAT concentrations were more pronounced in the pig operated on without the filter. There was no skin damage or abnormal behaviour.

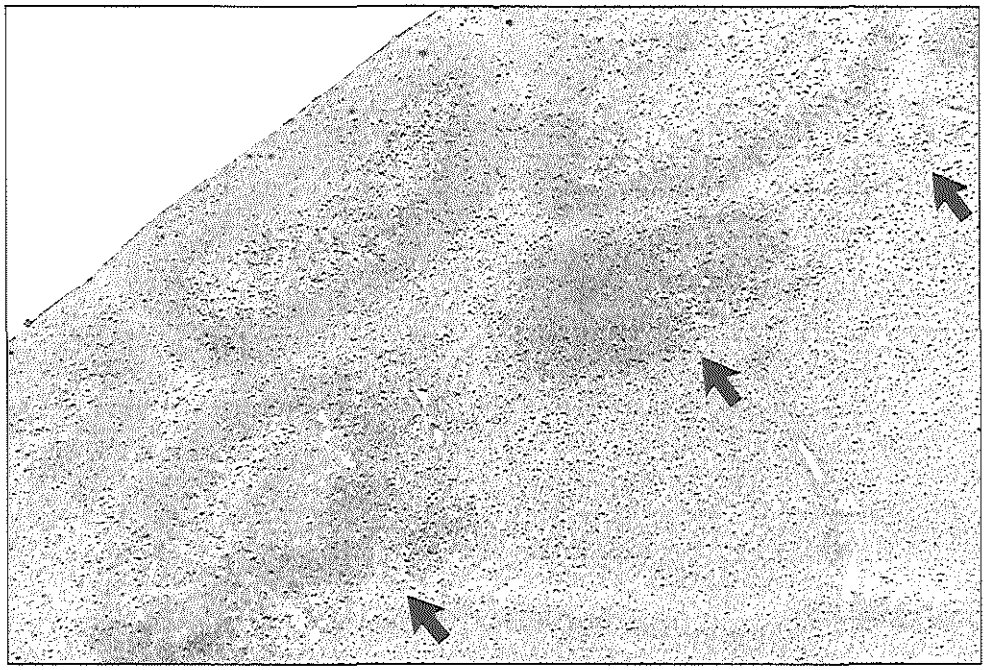

FIGURE 3 *Necrotic zone (1 mm) at the illuminated site of the liver surface without the filter. Arrows indicate towards the liver surface and demarcate the necrotic zone (original magnification x 20)*

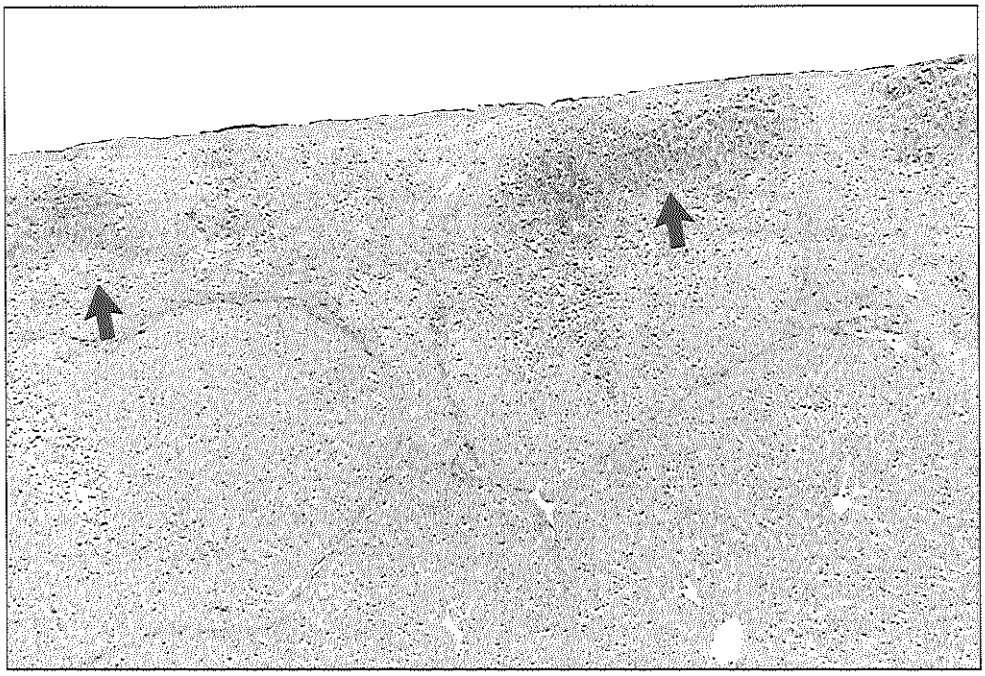

FIGURE 4 Necrotic zone (0.5 mm) at the illuminated site of the liver surface with the filter. Arrows indicate towards the liver surface and demarcate the necrotic zone (original magnification x 20)

Discussion

PPIX is highly photosensitizing, as shown in the disease erythropoietic protoporphyria. Photochemical reactions are induced by UV and blue light (Todd, 1994). It seems reasonable to protect patients undergoing surgery who have been photosensitized after ALA administration, by using filters that block UV and blue light. Acrylate yellow filters, which cut off wavelengths shorter than 520 nm, reduce the light energy density by 50 percent, mainly in the UV and blue light range (***FIGURE 1***). These filters were found to protect photosensitized HepG2 cells when exposed to operating lights for less than 5 min. With an exposure time exceeding 5 min, the filter failed to prevent cell death. However even after longer exposure, the survival rate of cells protected by the filter during irradiation was still twice as high as that of cells irradiated without a filter. Acrylate yellow filters also had a protective effect in pigs after ALA pretreatment. Both macroscopic and microscopic damage was less pronounced when the light was filtered. Energy densities in these

experiments were higher than the known energy densities provided to human tissues during operation as measured by Allardice *et al.* (Allardice *et al.*, 1989). These authors studied the safety of intraoperative PDT with haematoporphyrin derivative; however they defocused the operating lights at 1.30 m from the investigated area, creating minimum illuminance. Allardice and co-workers concluded that operating lights can reach unacceptably high energy levels and that an additive dose given by intraoperative PDT might cause unwanted side-effects. They mentioned the possible use of yellow filters but concluded that yellow light may be unacceptable to surgeons. The present study demonstrates that acrylate yellow filters have a protective effect. However, after longer exposure to filtered light, tissue damage is likely to occur and surgeons should probably still cover organs that are not in the immediate operating field to prevent unnecessary light exposure and damage. Some anatomical structures (e.g. ureters and hypogastric nerves) might have their colour appearance changed and surgeons should be aware that a colour change may hinder their recognition of structures. Meerman *et al.* (Meerman *et al.*, 1994) used acrylate yellow filters during liver transplantations in patients suffering from erythropoietic protoporphyria. The abnormal colour perception did not hamper the surgeons and other personnel.

In the present authors' hospital, these filters were used in ten photosensitized patients during resection and reconstruction of the oesophagus. There was no deterioration in the visibility of structures and the use of these filters was fully acceptable to the surgeons. Surrounding lights in the operating theatre do not add much to the light energy density reaching the exposed organs (results not shown), so these lights can be kept on for the convenience of the nursing and anaesthetic personnel.

In conclusion, the use of acrylate yellow filters is recommended when photosensitized patients are operated on, and also when porphyrin concentrations are to be measured in tissue obtained at operation, to minimize photoactivation.

Acknowledgements

We thank Dr PE Zondervan, pathologist, for help in the interpretation of the findings. We also thank Dr H de Wilt for operating on the pigs, Dr HJ van Staveren for his help concerning the physics of light and Mrs A Edixhoven for laboratory assistance. We acknowledge Gambro Medische Apparaten (Breda, The Netherlands) for kindly providing an Angenieux operating light to perform our in vitro experiments.

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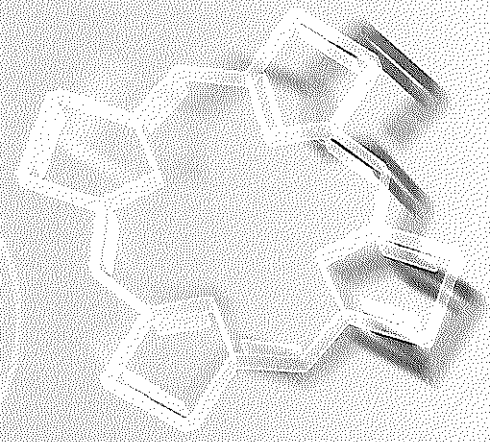
Porphyrin biosynthesis in human Barrett's oesophagus and adenocarcinoma after ingestion of 5-aminolaevulinic acid.



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Summary

5-Aminolaevulinic acid (ALA) induced porphyrin biosynthesis, which is used for ALA based photodynamic therapy (ALA-PDT), was studied in tissues of 10 patients with Barrett's oesophagus (BE) and adenocarcinoma of the oesophagus (AC) undergoing oesophagectomy at a mean time interval of 6.7 hours after the ingestion of ALA (60 mg/kg). In BE, AC, squamous epithelium (SQ) and gastric cardia, the activities of the haem biosynthetic enzymes, porphobilinogen deaminase (PBG-D) and ferrochelatase (FC) and the PDT power index - the ratio between PBG-D and FC in BE and AC in comparison with SQ - were determined before ALA ingestion. Following ALA administration, ALA, porphobilinogen, uroporphyrin I and PPIX were determined in tissues and plasma. The PDT power index did not predict the level of intracellular accumulation of PPIX found at 6.7 hrs. In BE, there was no selectivity of PPIX accumulation compared to SQ, whereas in half of patients with AC selectivity was found. Higher haem biosynthetic enzyme activities (i.e. PBG-D) and lower PPIX precursor concentrations were found in BE and AC compared to SQ. It is therefore possible that PPIX levels will peak at earlier time intervals in BE and AC compared to SQ.

Introduction

Barrett's oesophagus (BE) is a pre-malignant condition in which progression from metaplasia to low-grade dysplasia and high-grade dysplasia could lead to invasive adenocarcinoma of the oesophagus (AC) (Drewitz *et al.*, 1997; Hameeteman *et al.*, 1989; van der Burgh *et al.*, 1996). High-grade dysplasia is often regarded as an indication for oesophagectomy (Cameron and Carpenter, 1997; Clark *et al.*, 1996; Edwards *et al.*, 1996). A possible alternative, which is less mutilating and also applicable in patients with a high surgical risk is 5-aminolaevulinic acid-induced photodynamic therapy (ALA-PDT). Two relevant clinical studies have been performed, in which patients with high-grade dysplasia or early cancer in BE received an oral dose of ALA (60 mg/kg), followed by photoactivation 4-6 hours later (Barr *et al.*, 1996; Gossner *et al.*, 1998). Both high-grade dysplasia and early cancer were eradicated allowing regeneration of squamous epithelium without scarring or stricture formation. However, the presence of islands of columnar cells remaining beneath regenerating squamous epithelium created the concern that superficial healing could mask underlying dysplasia. These results suggest that ALA-PDT needs to be improved.
Haem biosynthesis, an essential process in every cell, is the basis of ALA-PDT (***FIGURE 1***).

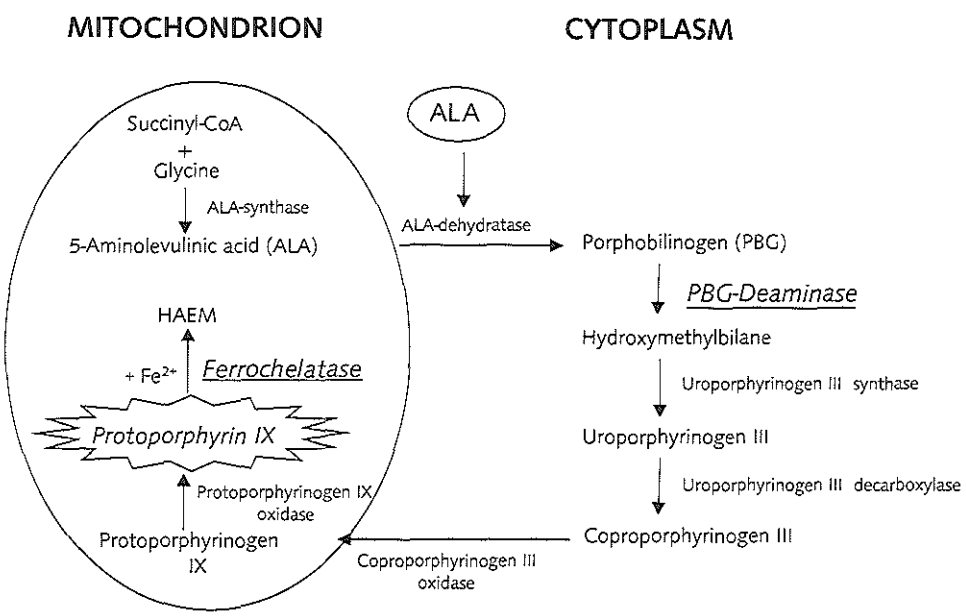

FIGURE 1 Haem biosynthetic pathway

ALA is the first intermediate, and two molecules of ALA are converted to porphobilinogen (PBG) which is metabolised to porphyrinogen intermediates by porphobilinogen deaminase (PBG-D). The last step of haem biosynthesis is the insertion of iron into PPIX by ferrochelatase (FC). Normally, haem synthesis is regulated by feedback inhibition of the enzyme ALA synthase. Exogenous ALA bypasses this feedback inhibition and the activities of PBG-D and FC and the intracellular iron pool become rate-limiting factors. As a result porphyrins, predominantly PPIX, will accumulate (Bishop and Desnick, 1982; Kennedy and Pottier, 1992). Previously, we observed an imbalance between the activities of PBG-D and FC in BE and AC (Hinnen *et al.*, 1998). The ratio between PBG-D and FC activities, normalised for squamous epithelium, was found to be significantly higher in BE and AC. In that study, we suggested that this ratio, which we have called the PDT power index, might be a useful parameter for predicting the accumulation of PPIX in tissues after the administration of ALA.

In this study, we examined the relation between the PDT power index and the intracellular concentration of PPIX in tissues of patients with BE and AC at approximately

6 hours after ALA ingestion (60 mg/kg) as this is the clinically most frequently used time interval. We determined the intracellular concentrations of ALA and other haem intermediates by biochemical extraction methods rather than fluorescence microscopy as used by others (Barr *et al.*, 1996; Regula *et al.*, 1995). In addition, plasma pharmacokinetics of ALA and porphyrins were studied and side effects were monitored.

Materials and methods

PATIENTS

In total 10 patients (2 women and 8 men; age 44 - 81 years; mean age 65 years) gave their written informed consent to participate in this study, which was approved by the Medical Ethical Committee of the University Hospital Rotterdam. Nine patients with histologically proven AC in BE and one patient with high-grade dysplasia in BE underwent an oesophageal resection with a gastric tube interposition. One patient was excluded from analysis because the tissue samples taken from BE were contaminated with AC as samples were taken at the border between BE and AC.

STUDY DESIGN

Biopsy samples (pre-5-aminolaevulinic acid administration)

Apart from one patient, all patients underwent an endoscopy with biopsies taken from BE, AC as well as from normal gastric cardia mucosa (GC) and normal squamous epithelium (SQ). Biopsies were embedded in formalin, sectioned, and stained with hematoxylin and eosin. The grade of tumour differentiation and the grade of dysplasia in Barrett's mucosa were described according to Haggitt (Haggitt, 1994). In addition, adjacent biopsies were kept at -70°C until the activities of PBG-D and FC and porphyrin concentrations were determined (Hinnen *et al.*, 1998).

5-Aminolaevulinic acid administration

Six hours before the oesophageal resection, ALA (Fluka, Buchs, Switzerland, 60 mg/kg) was dissolved in orange juice (10 ml, at room temperature) and given to the patient. Following this, all patients drank an additional 30 ml of water.

Photodegradation of porphyrins and photosensitization tissue damage during exposure to the operating lights was prevented by covering the tissues not in the immediate operating field with gauzes and shielding the operating lights with acrylate yellow filters (Wientjes B. V, Roden, The Netherlands), which eliminate nearly all UV and blue light below a wavelength of about 520 nm (Hinnen *et al.*, 2000a). For 48 hours after the

administration of ALA, patients were kept in subdued light. Side effects were monitored by questionaires and physical examination.

Blood samples
Venous blood samples were collected prior to and at 1, 3, 6, 9, 12, 24 and 48 hours after the administration of ALA. Whole blood was collected in tubes wrapped in aluminum foil to prevent photoconversion and photodamage, and kept on ice. The blood samples were centrifuged at 1300 x g for 10 min, then the plasma was removed, protected from light and stored at -70°C until the determinations of ALA, porphobilinogen (PBG), uroporphyrin (URO) and PPIX. In addition, samples were collected for routine biochemistry (urea, creatinine, sodium, potassium, albumine, alkaline phosphatase, bilirubin, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT)).

Tissue samples (post-5-aminolaevulinic acid administration)
Immediately after the oesophageal resection, tissue samples were taken from BE, AC, SQ and GC for histological examination. Adjacent tissue samples were kept at -70°C until the determinations of ALA, PBG, URO and PPIX. It was not always possible to take tissue samples at exactly 6 hours after the administration of ALA as in some patients the start of the operation was delayed by the prolonged anaesthetic preparations and in others the anaesthetic procedure was complicated by hypotension (see side effects). However 6 of 9 patients were sampled at about 6 ± 0.5 hours.

LABORATORY ASSAYS

Chemicals
PPIX disodium salt, Zinc-PPIX and PBG were obtained from Porphyrin Products (Logan, UT, USA). Coproporphyrin, URO and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tris-HCL was obtained from Boehringer Mannheim (Mannheim, Germany) and all other chemicals were obtained from Merck (Darmstadt, Germany).

Porphobilinogen deaminase and ferrochelatase assays
Tissue samples, kept on ice, were homogenised in water (1/5, wt/wt) using a Potter Elvehjem homogenizer (Kontess Glass Co., Vineland, NJ, USA). PBG-D and FC activities as well as the PDT power index - the ratio between PBG-D and FC in BE and AC in comparison with SQ - were determined as described previously (Hinnen *et al.*, 1998). Data were expressed as pmol/ mg protein/ hour. Protein was determined according to the method of Lowry *et al.* (Lowry *et al.*, 1951).

Determinations of 5-aminolaevulinic acid, porphobilinogen, uroporphyrin and protoporphyrin IX in plasma and tissue

The analysis of ALA and PBG was performed as described previously (van den Boogert *et al.*, 1998). URO was extracted from 25 µl tissue homogenate or plasma (twofold diluted in NaCl (150 mmol/L)) by addition of 200 µl of URO extraction buffer (UEB; Tris-HCl 50 mmol/L, pH 8.0; trichloroacetic acid, 1.5 mol/L in aqua dest., (3/5, v/v)). After 5 min exposure to UV light (350 nm), to convert porphyrinogens into porphyrins, the samples were centrifuged for 7 min at 3000 x *g*.

The fluorescence of the supernatant was measured at an excitation wavelength of 410 nm and an emission wavelength of 656 nm using a LS 50B spectrofluorometer with a red sensitive photomultiplier (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). Values were calculated according to a standard curve of URO I in UEB. Recovery of porphyrins during the extraction was determined by adding standard URO to the samples and in this study recoveries were found in the range of 85-100%.

PPIX was extracted from tissue by adding 50 µl PPIX extraction buffer (PEB; Tris-HCl 50 mmol/L, pH 8.0; 425 µl dimethylsulfoxide/methanol, (DMSO/MeOH, 30/70, v/v)) to 25 µl tissue homogenate. The diluted homogenate was mixed vigorously using a vortex and left for about 30 min at room temperature. Samples were then centrifuged for 10 min at 3000 x g. 100 µl of supernatant was injected on a HPLC as described previously (Van Hillegersberg *et al.*, 1992), however using an excitation wavelength of 415 nm and an emission wavelength of 630 nm. For the extraction of PPIX from plasma, 950 µl of PEB was added to 50 µl plasma. Values were calculated according to standard curves of Zinc-PPIX and PPIX in DMSO/MeOH (30/70, v/v). Recovery of porphyrins during the extraction was determined by adding standard Zinc-PPIX and PPIX to the samples and in this study recoveries were found in the range of 90-100%. Plasma levels were expressed in nmol/L and tissue levels in pmol/ mg protein.

PROTOPORPHYRIN IX AND ITS PRECURSORS

At 6 hours after ALA administration, tissues not only contained PPIX but also other haem synthesis intermediates, which are the precursors of PPIX and therefore considered as potential PPIX. Since the concentration of URO was very low in tissue and plasma samples compared to PPIX, the URO data were omitted from further analysis. PBG is formed from two molecules of ALA and 4 molecules of PBG form a PPIX molecule. To calculate the potential PPIX molecules, present at 6 hours after ALA administration, the concentrations of ALA and PBG were divided by 8 and 4 respectively and we called this "PPIX equivalents".

STATISTICAL ANALYSIS

Data are expressed as means ± sem and were tested for statistical significance using Student's *t*-test for paired values. Enzyme activities and concentrations of haem intermediates in BE, AC and GC were compared to SQ. Pearson correlation coefficients were calculated to study possible correlations. P-values <0.05 were considered significant.

Results

PORPHOBILINOGEN DEAMINASE AND FERROCHELATASE ACTIVITIES AND PDT POWER INDEX

Before oral ALA administration, PBG-D and FC activities were determined in endoscopically derived biopsy samples taken the oesophagus (BE, AC and SQ) and the proximal stomach (GC). A twofold increase in PBG-D activity (pmol/ mg protein/ hr) was found in BE (39.18 ± 5.67, p = 0.013) and in AC (38.76 ± 3.98, p = 0.001) compared with SQ (19.72 ± 2.85), whereas the activity in GC (21.46 ± 1.27) was not different from the activity in SQ (***TABLE 1***). The activities of FC (pmol/ mg protein/ hr) were not significantly different in BE (696 ± 89, p = 0.06) and AC (532 ± 68, p = 0.36) compared to SQ (444 ± 49), whereas the FC activity in GC (688 ± 38, p = 0.02) was significantly increased. In BE, the PDT power index (1.4 ± 0.2, p = 0.18) was not significantly different from SQ (1.0). In AC, this index was significantly increased (1.9 ± 0.3, p = 0.01) whereas in GC the index was significantly decreased (0.7 ± 0.1, p = 0.01) compared to SQ.

PROTOPORPHYRIN IX AND PROTOPORPHYRIN EQUIVALENTS CONCENTRATION IN TISSUE

Tissue samples of 9 patients were collected at a mean time interval of 6.7 ± 0.5 hours (range 5.25 - 10) after the administration of ALA. All tissue types contained the same concentrations of ALA-PPIX equivalents (***TABLE 1***). In BE, AC and GC, the intracellular concentration of PBG-PPIX equivalents were significantly lower than in SQ.

TABLE 1

Haem biosynthetic enzyme activities before ALA ingestion and the concentrations of PPIX and "PPIX equivalents" (pmol/mg protein) at a mean time interval of 6.7 hrs after ALA ingestion (60 mg/kg) in gastro-oesophageal tissues of 9 patients.

tissue type	"PPIX equivalents" ALA / 8	"PPIX equivalents" PGB / 4	PPIX	PBG-D activity	FC activity	PDT power index
SQ	134 ± 38	312 ± 59	92 ± 15	20 ± 3	444 ± 49	1.0
BE	132 ± 39	201* ± 38	77 ± 17	39* ± 6	696 ± 89	1.4 ± 0.2
AC	101 ± 38	126* ± 30	112 ± 45	39* ± 4	532 ± 68	1.9* ± 0.3
GC	61 ± 19	129* ± 29	57* ± 10	21 ± 1	688* ± 38	0.7* ± 0.1

SQ, squamous epithelium; BE, Barrett's oesophagus; AC, adenocarcinoma; GC, gastric cardia; PBG-D, porphobilinogen deaminase; FC, ferrochelatase, enzyme activities in pmol/ mg protein/ hour; PDT power index, ratio between PBG-D and FC normalised for SQ.

** p< 0.05 compared to SQ*

The individual variability in the concentration of PPIX is demonstrated in ***TABLE 2*** together with the patients and tissues characteristics. PPIX was the main metabolite of ALA found in tissue. Undetectable levels of porphyrins were found in tissue samples of any of the 9 patients when taken before the oral administration of ALA (results not shown).
The concentration of PPIX in BE (77 ± 17) was not significantly different from SQ (92 ± 15), whereas the concentration in GC was significantly lower (57 ± 10, p = 0.01) (***TABLE 1***). Only one patient (TABLE 1, patient 5) showed a selective accumulation of PPIX in BE compared with SQ. Levels of PPIX did not depend on the grade of dysplasia found in BE.
The concentration of PPIX in AC (112 ± 45) was not significantly different from SQ (92 ± 15) (***TABLE 1***). Selective accumulation of PPIX was seen in four cases of AC. Of the remaining four cases of AC, in one patient tissue was obtained at 10 hours after the administration of ALA and in the other three cases the AC was histologically found to be poorly differentiated.
The PDT power index did not correlate with the levels of PPIX found.

TABLE 2

Patient and tissue characteristics of 9 patients after the oral administration of 60 mg/kg ALA.

	Age (yrs)	Sex	Tumour diff grade	Grade of dysplasia	Sampling time (hrs)	PPIX SQ	PPIX BE	PPIX AC	PPIX GC
1	51	M	moderately	LGD	10	86	46	31	67
2	45	M	moderately	ND	5.25	55	55	**82**	60
3	79	F		HGD	6.16	172	173		125
4	73	M	well	LGD	6	51	40	**78**	36
5	69	M	poorly	ND	7.83	85	**107**	**142**	54
6	72	M	poorly	ND	6	80	84	60	44
7	77	F	poorly	ND	6.67	68	24	50	36
8	63	M	poorly	LGD	7.83	60	34	37	26
9	45	M	moderately	LGD	6	168	127	**414**	64

ND, no dysplasia; LGD, low grade dysplasia; HGD, high grade dysplasia. Sampling time (hrs), sampling time in hours after ALA. PPIX concentrations (pmol/mg protein) in SQ, squamous epithelium; BE, Barrett's oesophagus; AC, adenocarcinoma; GC, gastric cardia. Data in bold, selective accumulation of PPIX compared to other tissue samples of the same patient.

PHARMACOKINETICS OF 5-AMINOLAEVULINIC ACID, PORPHOBILINOGEN AND PROTOPORPHYRIN IX IN PLASMA

Plasma ALA, PBG, and PPIX kinetics are shown in ***FIGURE 2***. After the initial absorption and distribution phase the decrease in ALA, PBG and PPIX followed first-order kinetics with half lives of 1.8, 5.9 and 6.7 hours respectively. The range in the half lives of ALA, PBG and PPIX between different patients was considerable: 1.1-2.5 hours for ALA, 4.1-11.6 hours for PBG and 2.5-12.8 hours for PPIX. In all patients, peak concentrations of ALA were detected at 1 hour and concentrations declined to baseline levels at 24 hours after administration. There was a considerable variability between patients in the time to achieve the peak plasma concentrations of PBG and PPIX (range: 6-12.3 hours). Plasma concentrations of PBG and PPIX declined to baseline levels at 48 hours after ALA ingestion.

SIDE EFFECTS

Side effects were vomiting, skin photosensitivity, hypotension and transient increases of ASAT and ALAT. Eight patients suffered from at least one of these side effects. Three patients vomited incidentally between 2.5 - 4.5 hours after the administration of ALA. ASAT and ALAT were elevated 2 to 3-fold above normal levels in 6 patients and peaked

at day 2 after ALA administration. Mild skin photosensitivity, characterised by itching and mild erythema was present in 7 patients. One patient had severe edema of his facial skin, lips and tongue. The symptoms improved spontaneously within 24 hours. Hypotension was found in 5 patients, in 4 patients intra-operatively, within 6 hours after the administration of ALA. The mean systolic blood pressure of these patients dropped from 125 mm Hg to 70 mm Hg and the mean diastolic blood pressure from 70 mm Hg to 40 mm Hg.

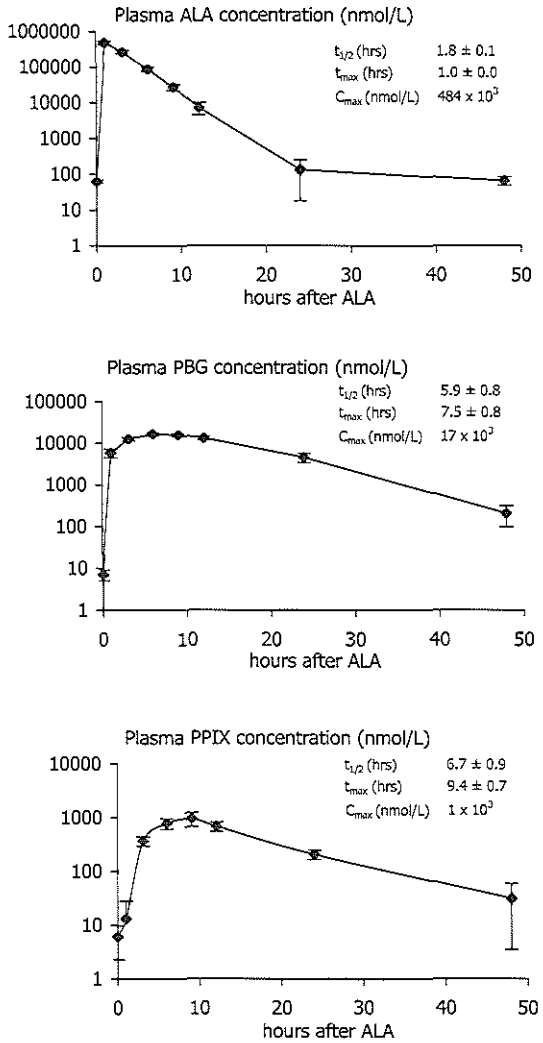

FIGURE 2 *Pharmacokinetics of ALA, PBG and PPIX in plasma of 10 patients after ingestion of ALA (60 mg/kg). The plasma concentrations are expressed as log-values (y-axis, means ± sem).*

Discussion

In patients with AC in BE we previously found increased activities of PBG-D and FC in endoscopic biopsies of BE and AC compared with SQ (Hinnen *et al.*, 1998). Based on this observation we proposed a PDT power index, the ratio between PBG-D and FC activity in BE and AC in comparison with SQ. We suggested that this index could be of value in predicting porphyrin concentrations in these tissues after ALA administration.
Under the conditions chosen in the present study, however, the PDT power index did not predict the level of intracellular PPIX accumulation found at a mean time interval of 6.7 hours after ALA administration in BE, AC, SQ and GC in these patients. It is possible that a relationship still exists between the PDT power index and PPIX accumulation at another time interval as we found higher enzyme activities and lower PPIX precursor concentrations in AC and BE compared to SQ (***TABLE 1***). It is therefore possible that PPIX levels could have peaked at earlier time intervals in BE and AC compared to SQ.
As found by others, who determined PPIX by fluorescence microscopy, an indirect, semi-quantitative method (Barr *et al.*, 1996; Regula *et al.*, 1995), there seemed to be little selectivity of PPIX accumulation in BE. In contrast, we found selective accumulation of PPIX in 4 of 8 cases of AC. The other four AC samples contained only low levels of PPIX, as compared to SQ. In three of these cases the histology showed a poorly differentiated tumour. It has been reported that the grade of differentiation can have a negative or positive effect on the ability of cells to accumulate porphyrins, depending on the type of tissue (Li *et al.*, 1999). In the fourth patient without selectivity between AC and SQ, tissue was collected at a rather late time (10 hrs) after the administration of ALA, at which time PPIX could already have been converted into haem.
Not only is the absolute intracellular PPIX concentration an important factor for the effect of PDT but also the intracellular localisation of PPIX at the time of application of PDT, the duration of illumination and the flux of PPIX in cells (Hinnen *et al.*, 2000b; Iinuma *et al.*, 1994). If oxygen levels are high enough, more PPIX molecules per time unit result in a greater oxygen radical yield, and therefore will have a more pronounced effect (Henderson and Dougherty, 1992).
The rapid kinetics of ALA and PPIX found in plasma (***FIGURE 2***) explain why ALA is an attractive pro-drug for PDT. PPIX in plasma is derived from liver and other cells and the decline in plasma levels reflect a decline in tissue levels (van den Boogert *et al.*, 1998). Because of this rapid decline in PPIX levels, skin photosensitivity is only short-lasting (Barr *et al.*, 1996; Gossner *et al.*, 1998).
A severe side effect observed in this study was hypotension. Herman *et al.* recently studied the hemodynamic effects of ALA. A relevant observation in that study was a significant decrease in the systolic and diastolic blood pressure in all 6 patients (Herman *et al.*,

1998). Goldberg *et al.* found in animal studies evidence for an ALA-triggered histamine release, which could result in vasodilatation and in that way hypotension (Goldberg and McGillion, 1973). Based on these findings we treated four of our patients with anti-histaminic agents and corticosteroids prior to ALA administration, but this failed to prevent hypotension in all of them. Haemodynamic stability was restored by infusion of isotonic fluids and plasma. It is presently not clear whether ALA, PPIX or a metabolite is responsible for this side-effect.

In conclusion, this study describes the photodynamic potential of the haem biosynthetic pathway in tissues of patients with BE and AC. At a mean time interval of 6.7 hours after ALA administration, PPIX accumulation could not be predicted from the PDT power index. Selectivity of PPIX accumulation was found in half of the cases of AC but not in BE. The optimum time interval is still not established but is possibly found at an earlier time interval after ALA administration. Side effects after ingestion of 60 mg/kg ALA can be serious and a hypotensive response can occur. Optimising the results of ALA-PDT in the treatment of BE and AC requires further effort in studies concerning the kinetics of ALA and its products in target tissues.

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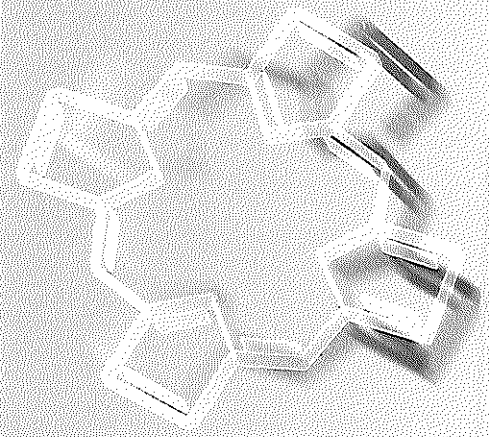
Timing of 5-aminolaevulinic acid-induced photodynamic therapy for the treatment of patients with Barrett's oesophagus.



Submitted

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Abstract

Background & Aims: 5-Aminolaevulinic acid- induced photodynamic therapy (ALA-PDT) is being used as an experimental treatment of Barrett's oesophagus (BE), a pre-malignant lesion in the distal oesophagus. Knowledge of the pharmacokinetics of ALA and the photosensitizer protoporphyin IX (PPIX) in tissues and plasma of patients with BE could help to determine the optimal time interval between the administration of ALA and illumination.

Methods: Twenty six patients with BE were randomized to varying time intervals between ingesting 60 mg/kg ALA and undergoing an endoscopy with biopsies of BE and normal oesophageal and gastric mucosa. At 1, 2, 7, 8 and 24 h, two patients at each time and at 3, 4, 5 and 6 h, 4 patients at each time after ALA ingestion were included. ALA, porphyrin intermediates and PPIX were determined in all biopsy and plasma samples.

Results: The maximum concentration of PPIX was found earlier in BE (4.6 ± 0.5 h) than in SQ (6.6 ± 2.2 h) ($p<0.05$). PPIX concentrations were higher in SQ than in BE especially at longer time intervals. In addition, tissue ALA concentrations were found to be 20-fold higher than the plasma concentrations at 1 hour after ALA ingestion, suggesting uptake from the oesophageal lumen. Skin photosensitivity is short-lasting but often debilitating.

Conclusions: Our results provide a rationale for the use of ALA-PDT for the treatment of BE at 4-5 h after ALA ingestion and for local application of ALA in the oesophagus.

Introduction

Barrett's (columnar-lined) oesophagus (BE) is a pre-malignant condition which is induced by chronic gastro-oesophageal reflux (Winters *et al.*, 1987). Patients with BE have a 30-125 fold increased risk for oesophageal cancer, compared with the general population (Drewitz *et al.*, 1997; Hameeteman *et al.*, 1989; van der Burgh *et al.*, 1996). Adenocarcinoma of the oesophagus results from a multistep process of progression from metaplasia to low-grade dysplasia, high-grade dysplasia and ultimately invasive cancer (Hameeteman *et al.*, 1989; Hamilton and Smith, 1987). A way to interfere in this sequence of increasing risk of developing malignancy is to apply 5-aminolaevulinic acid-induced photodynamic therapy (ALA-PDT), an experimental non-invasive therapy, to remove Barrett's mucosa.

To date, two clinical studies have been performed, in which patients with high-grade dysplasia or early cancer in BE received an oral dose of ALA (60 mg/kg), followed 4-6 h later

by photoactivation. Both high-grade dysplasia and early cancer were eradicated and this was followed by regeneration of squamous epithelium under proton pump inhibiting therapy (Barr *et al.*, 1996; Gossner *et al.*, 1998). As ALA induces higher concentrations of PPIX in mucosa than submucosa or muscularis mucosa, superficial damage is achieved, leaving the muscle layer intact (Loh *et al.*, 1993). However, the presence of islands of columnar cells beneath regenerating squamous epithelium created the concern that superficial healing could mask underlying dysplasia (Barr *et al.*, 1996; Biddlestone *et al.*, 1998). The basis of ALA-PDT is the heme biosynthetic pathway (***FIGURE 1***). Two molecules of ALA are converted to porphobilinogen (PBG), which is metabolized to porphyrinogen intermediates by porphobilinogen deaminase (PBG-D). The last step of the heme synthesis is the insertion of iron into PPIX, catalysed by ferrochelatase (FC). Normally, heme synthesis is regulated by feedback inhibition of the enzyme ALA synthase. Exogenous ALA bypasses this feedback inhibition and the activities of PBG-D and FC and the intracellular iron pool become rate-limiting factors. As a result porphyrins, predominantly PPIX, will accumulate (Bishop and Desnick, 1982; Hinnen *et al.*, 2000a; Kennedy and Pottier, 1992).

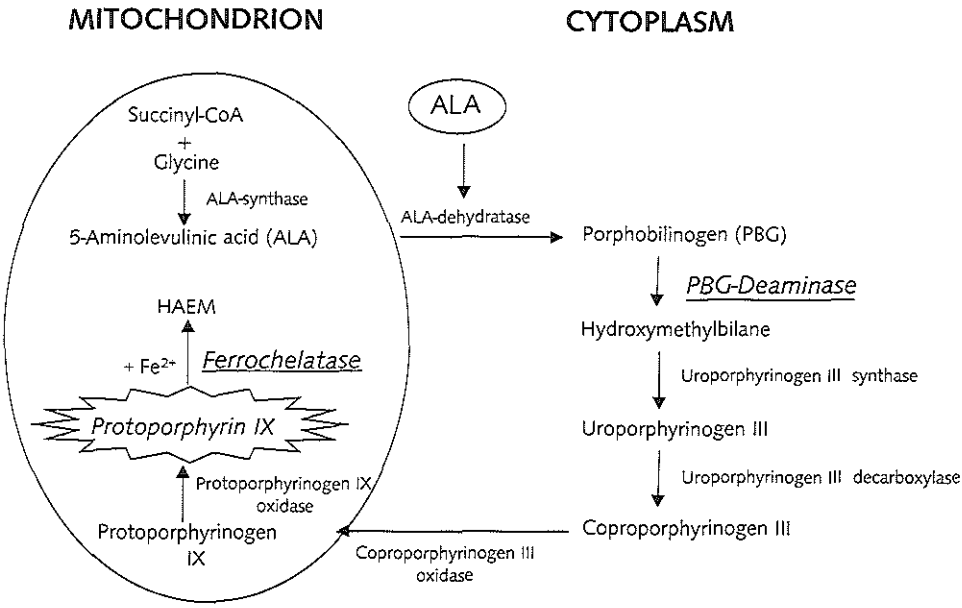

FIGURE 1 *The haem biosynthetic pathway*

In a previous study, we observed an increased ratio between PBG-D and FC activities in BE and adenocarcinoma of the human oesophagus (AC) compared to normal squamous epithelium (SQ) (Hinnen *et al.*, 1998). In a subsequent study we found that PPIX concentrations in BE and AC at a mean time of 6.7 h after ALA ingestion (60 mg/kg) were not higher than those in SQ. The results also suggested that the rate of porphyrin biosynthesis was higher in BE compared to SQ (Hinnen *et al.*, 2000a). This lead us to hypothesize that maximum PPIX concentrations could be reached at an earlier time than 6-7 h after the oral administration of ALA in BE than in SQ. Little detailed knowledge of the pharmacokinetics of ALA and PPIX in BE, SQ and normal gastric mucosa (GC) as well as in plasma of patients with BE is available. Such information could help to optimize treatment parameters such as the interval between the administration of ALA and illumination. For that reason, we performed a time sequence study and determined ALA, PBG, uroporphyrin and PPIX in tissue and plasma of patients with BE at varying time intervals after the oral administration of ALA.

Materials and methods

PATIENTS

Twenty six patients (9 women and 17 men; age 34 - 74 years; mean age 55 years) with histologically proven BE gave their written informed consent to participate in this study. The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam.

STUDY DESIGN

5-Aminolaevulinic acid administration

Patients were randomized to varying time intervals to undergo an endoscopy with biopsies after ingesting 60 mg/kg ALA (Fluka, Buchs, Switzerland, 60 mg/kg) dissolved in orange juice (10 ml) and about 30 ml of water for rinsing. At 1, 2, 7, 8 and 24 h, two patients at each time and at 3, 4, 5 and 6 h, 4 patients at each time after ALA ingestion were included.

All patients were kept in a darkened room until 9 h after the ingestion of ALA and then left the hospital with an umbrella and sunglasses to protect them from ALA-induced skin and eye photosensitivity. Since we observed that the first patients participating in the study experienced skin photosensitivity, we advised the other patients to apply a sunscreen with high UV protection (Nivea Sun® Sunblock 70, containing titanium dioxide, Beiersdorf AG, Hamburg, Germany) during their stay in the hospital and during their way

home. Photodegradation of porphyrins and photosensitisation tissue damage during exposure to the light of the endoscope was prevented by covering the light emitting part of the endoscope with an acrylate yellow filter (Wientjes B. V, Roden, The Netherlands). This filter eliminates nearly all UV and blue light below a wavelength of about 520 nm (Hinnen *et al.*, 2000b). Side effects were monitored by interviews with the patients.

Biopsy samples

Apart from one patient, who was unable to undergo the endoscopy at the planned time because of vomiting, abdominal pain and diarrhea, all patients underwent an endoscopy at the randomized time with biopsies taken from BE, normal squamous epithelium (SQ) and normal gastric cardia mucosa (GC). Biopsies were embedded in formalin, sectioned, and stained with hematoxylin and eosin. The grade of dysplasia in Barrett's mucosa was described according to Haggitt (Haggitt, 1994). In addition, adjacent biopsies were frozen (-70°C) for the determinations of ALA, porphobilinogen (PBG), uroporphyrin (URO) and PPIX.

Blood and urine samples

Blood samples were collected prior to and at 0.5, 1, 2, 4, 6 and 8 h after the administration of ALA. Whole blood was collected in heparinized tubes wrapped in aluminum foil to prevent photoconversion and photodamage, and kept on ice. The blood samples were centrifuged at 1300 x g for 10 minutes, then the plasma was removed, protected from light and stored at -70°C for the determination of ALA, PBG, URO and PPIX.
Some 24 h after the administration of ALA, an early morning urine sample was collected by the patient and sent to the laboratory for the determination of ALA, PBG and URO.

LABORATORY ASSAYS

Chemicals

PPIX disodium salt, zinc PPIX and PBG were obtained from Porphyrin Products (Logan, UT, USA). Coproporphyrin, URO and Triton X -100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tris-HCL was obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

Determinations of ALA, PBG, URO and PPIX in plasma, tissue and urine

Determinations of ALA and PBG in plasma, tissue and urine and determinations of porphyrins in urine were performed as described previously (van den Boogert *et al.*, 1998). Urine concentrations were expressed in µmol/mmol creatinine.
URO was extracted from 25 µl tissue homogenate or plasma (twofold diluted in NaCl

(150 mmol/L)) by addition of 200 µl of URO extraction buffer (Tris-HCl 50 mmol/L, pH 8.0; trichloroacetic acid, 1.5 mol/L in aqua dest., (3/5, v/v)). After 5 minutes exposure to UV light (350 nm), to convert porphyrinogens into porphyrins, the samples were centrifuged for 7 minutes at 3000 x g. The fluorescence of the supernatant was measured at an excitation wavelength of 410 nm and an emission wavelength of 656 nm using a LS 50B spectrofluorometer with a red sensitive photomultiplier (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). Values were calculated according to a standard curve of URO I in URO extraction buffer. Recovery of porphyrins during the extraction was determined by adding standard URO to the samples and recoveries were found in the range of 85-100%.

PPIX was extracted from tissue by adding 50 µl PPIX extraction buffer (Tris-HCl 50 mmol/L, pH 8.0; 425 µl dimethylsulfoxide/methanol, (DMSO/MeOH, 30/70, v/v)) to 25 µl tissue homogenate. The diluted homogenate was mixed vigorously using a vortex and left for about 30 minutes at room temperature. Samples were then centrifuged for 10 minutes at 3000 x g. One-hundred µl of supernatant was injected on a HPLC as described previously (van Hillegersberg *et al.*, 1992), however using an excitation wavelength of 415 nm and an emission wavelength of 630 nm. For the extraction of PPIX from plasma, 950 µl of PPIX extraction buffer was added to 50 µl plasma. Values were calculated according to standard curves of Zinc-PPIX and PPIX in DMSO/MeOH (30/70, v/v). Recovery of porphyrins during the extraction was determined by adding standard Zinc-PPIX and PPIX to the samples and recoveries were found in the range of 90-100%. Plasma levels were expressed in nmol/L and tissue levels in pmol/mg protein. Tissue concentrations were also calculated in nmol/L for comparisons with plasma concentrations.

STATISTICAL ANALYSIS

Data are expressed as means ± sem. The analysis of statistical significance was performed using the Wilcoxon signed-rank test or the Wilcoxon rank-sum test for within, respectively, between patients comparisons. For the determination of the time interval at which maximum PPIX concentrations were found in the different tissues, and the maximum concentration itself, a piece-wise linear model was used (Neter and Wasserman, 1974). In this analysis, the PPIX concentrations were logarithmically transformed. From the plasma ALA concentrations of the individual patients, the ALA concentration in plasma at the time of biopsy of each patient was calculated. To determine the standard errors and the statistical significant differences between times of maximal concentrations in the different tissues, the bootstrap method was used (Efron and Tibshirani, 1993). To study possible correlations, Spearman correlation coefficients were calculated or linear regression analyses were performed. Two-sided P-values <0.05 were considered significant.

Results

PPIX was the main metabolite of ALA found in tissue and plasma samples. Since the concentration of URO was very low compared to PPIX in both tissue and plasma samples, the URO data were omitted from further analysis. No porphyrins were detected in plasma samples before the administration of ALA (***FIGURE 2***).

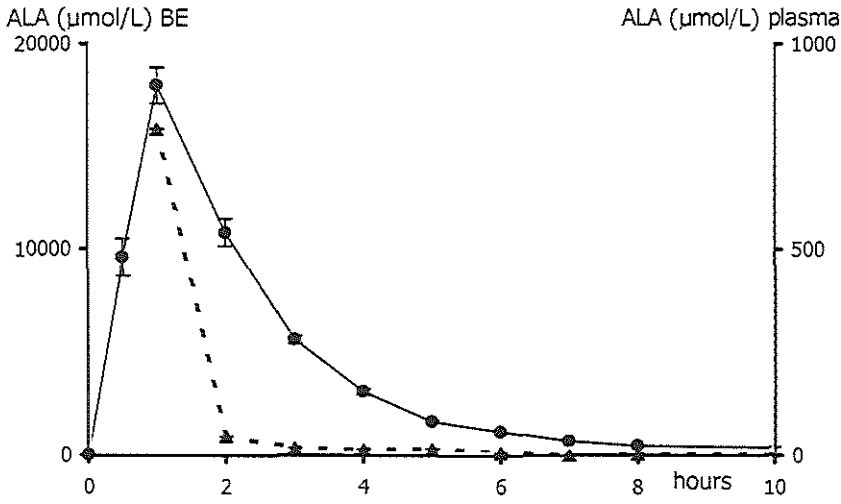

FIGURE 2 Pharmacokinetics of 5-aminolevulinic acid (ALA) in tissue (▲) and plasma (●) of patients with Barrett's oesophagus (BE) after ingestion of 60 mg/kg ALA. Concentrations (µmol/L) in means ± sem; BE, n=2 at 1, 2, 7 and 8 h, n=4 at 3, 4, 5 and 6 h; plasma n=26 at all time intervals. Please note the different axes for BE and plasma and the 20-fold difference at 1 hour between the tissue and plasma ALA concentrations.

PPIX CONCENTRATIONS IN TISSUES

Maximum concentrations of PPIX after ALA ingestion were found at different time intervals (mean ± sem) in the various tissue types (***FIGURE 3***). The maximum concentration of PPIX was found earlier in BE (4.6 ± 0.5 h) than in SQ (6.6 ± 2.2 h) ($p<0.05$, 95% confidence interval for the time difference: 0.1 - 4.4 h, ***FIGURE 3***). In GC, the maximum concentration of PPIX was found at 4.8 ± 1.0 h.

The maximum concentration was 122 pmol/mg protein in BE, 136 pmol/mg protein in SQ and 117 pmol/mg protein in GC. At all time intervals before the maximum concen-

tration of PPIX was found in BE (t = 1, 2, 3 and 4 h, n=11), the concentration of PPIX in BE was not different from the PPIX concentration in SQ (p=0.86). At longer time intervals after ALA ingestion (t=5, 6, 7, 8 and 24 h, n=14), the PPIX concentration was significantly lower in BE compared to SQ (p=0.013). The longer the time interval after ALA ingestion, the greater the difference between the PPIX concentrations in BE and SQ ($p<0.001$) .

PLASMA ALA VERSUS TISSUE ALA CONCENTRATIONS

At 1 hour after ALA ingestion, the intracellular ALA concentration was about 20 times higher in all types of tissue than in plasma. The pharmacokinetics of ALA in BE and plasma is shown as a time course in ***FIGURE 2***. After 1 hour, tissue concentrations of ALA rapidly decreased and at 2-8 h and 24 h, tissue ALA concentrations were not significantly different from plasma ALA concentrations (p=0.26).

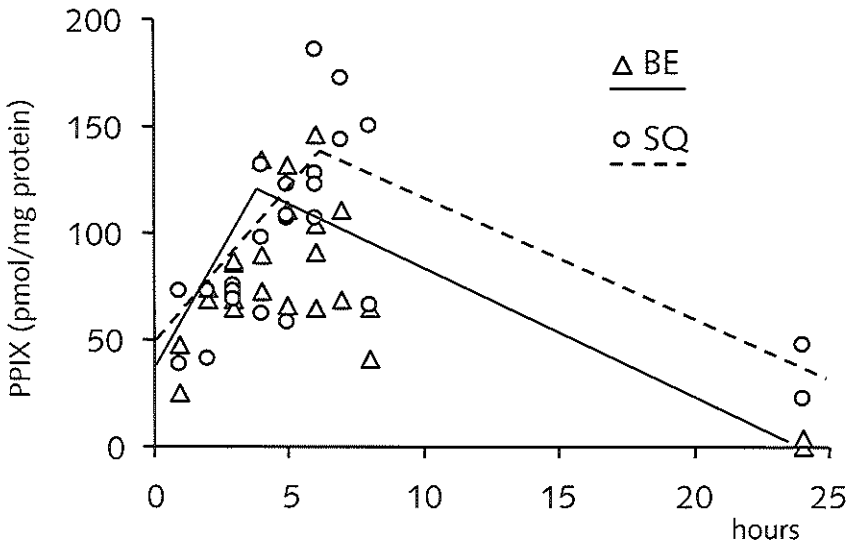

FIGURE 3 *Protoporphyrin IX (PPIX) kinetics in tissues of 26 patients with Barrett's oesophagus after ingestion of 5-aminolevulinic acid (ALA) (60 mg/kg). Peak PPIX levels occur at a significantly earlier time point in BE (–) than in SQ (---) (4.6 ± 0.5 versus 6.6 ± 2.2 hours, p<0.05, piece-wise linear model)*

PHARMACOKINETICS OF 5-AMINOLAEVULINIC ACID, PORPHOBILINOGEN AND PROTOPORPHYRIN IX IN PLASMA

Maximum plasma concentrations of ALA (898 ± 45 µmol/L, mean ± sem) were found at 1 hour after ALA ingestion in all patients (***FIGURE 2***). After the initial absorption and distribution phase the ALA concentration decreased significantly and followed first-order kinetics with a mean half-life of 1.3 ± 0.1 h (range: 0.7 - 1.9 h). At 8 h, the plasma ALA concentration had returned to base-line levels. At 6 h, maximum plasma concentrations of PBG (20 ± 1 µmol/L) and PPIX (942 ± 129 nmol/L) were found.

ALA IN URINE

At 24 h after ALA ingestion, the urine concentration of ALA was found in the normal range in 21 patients (4.1 ± 0.9 µmol ALA/mmol creatinine). However, in 5 patients the concentration of ALA in urine after 24 h was still above the normal range (152.7 ± 20.7). In all 26 patients the plasma ALA concentrations found at 1 hour after ALA ingestion correlated with urine ALA concentrations found at 24 h (Spearman, p=0.02) The five patients with high urine ALA concentrations at 24 h appeared to have a significantly higher plasma ALA concentration at 1 hour after ALA ingestion than the patients with normal urine ALA concentrations at 24 h (1100 µmol/L versus 815 µmol/L, p=0.015). However, the decrease in plasma ALA concentrations was not different between the two groups (high or low ALA concentration in urine, p=0.82).

SIDE EFFECTS

The most common side effect was skin photosensitivity. During the first 9 h after ALA administration, 9 of 26 patients (35%) developed painful erythema of the facial skin. The next day, another 11 patients (42%) complained of redness and painful sensations in their face. In 5 of 20 patients (25%) with skin photosensitivity, the erythema was accompanied by edema, i.e., around the eyes. One of these patients also had severe edema of his hands. The painful sensations subsided within 48 h and erythema within a few days after ALA ingestion.

One patient had severe abdominal cramps accompanied by vomiting and diarrhoea from 4.5 until 10 h after ALA ingestion and was therefore not subjected to an endoscopy. Two other patients also vomited after ALA ingestion, one at 5 h and the other at 9 h after ALA administration.

Discussion

Knowledge of the pharmacokinetics of ALA and ALA-induced PPIX in BE and surrounding normal tissues could help to optimize treatment parameters such as the interval between administration of ALA and illumination. Up to now, accumulation of PPIX in these tissues has only been quantified by fluorescence microscopy (Barr *et al.*, 1996; Regula *et al.*, 1995). We previously determined the intracellular concentrations of ALA and PPIX in tissues of patients with BE (Hinnen *et al.*, 2000a). There appeared to be no selectivity in the accumulation of PPIX in BE compared to SQ at a mean time interval of 6.7 h after ALA ingestion. However, we found evidence that the rate of porphyrin biosynthesis was higher in BE compared with SQ.

In view of this, we hypothesized that maximum PPIX concentrations could be reached at an earlier time in BE than in SQ. In the present study, we confirmed that maximum PPIX concentrations occurred at a significantly earlier time point in BE than in SQ (4.6 versus 6.6 h, ***FIGURE 3***). After the time that the maximum PPIX concentration was found in BE, PPIX concentrations in biopsies from SQ became significantly higher compared with BE. It was shown that the difference in PPIX concentrations between SQ and BE was greater at longer time intervals after ALA ingestion. A selective PDT effect can therefore only be achieved by local illumination of the Barrett's epithelium as this study emphasizes the fact that selectivity in PPIX accumulation lies not in the difference between the accumulation of PPIX in BE and SQ (Hinnen *et al.*, 2000a), but in the difference between the accumulation in mucosa and underlying muscle as has been demonstrated in recent studies (Barr *et al.*, 1996; van den Boogert *et al.*, 1999). Since squamous mucosa builds up significantly higher levels of PPIX at longer time intervals than BE, unwanted photodamage to SQ will be reduced when PDT is performed early within the time window, i.e. at 4-5 hours after ALA administration. So far, PPIX has only been quantified by biochemical extraction methods in one other study (Ackroyd *et al.*, 1999). In that study biopsies were taken at 2, 4 and 6 hours after ALA ingestion. The authors suggested that peak levels of PPIX occured at 4 hours after ALA administration in normal oesophageal tissue and at 6 hours after ALA administration in Barrett's mucosa. In our opinion, maximum concentrations of PPIX and the time interval at which this concentration is reached can not be accurately calculated from only three time intervals as levels at earlier and later time intervals were not determined in that study.

At 1 hour after ALA ingestion, concentrations of ALA in BE, SQ and GC were found to be 20-fold higher than the concentrations in plasma at that time. Only two patients were included at that time interval, but as levels were very high at that time they could be measured with great accuracy. This phenomenon suggests local absorption of ALA at the luminal site of oesophageal and gastric tissues. The acidic environment of orange juice, in which ALA was dissolved, leaves a part of the ALA molecules uncharged. This may have facilitat-

ed direct absorption (passive diffusion) of ALA by the epithelial cells. Local absorption of ALA under acidic conditions by passive diffusion is an advantage in the treatment of skin and bladder abnormalities with ALA-PDT (Kennedy and Pottier, 1992; Kriegmair *et al.*, 1996). In contrast to ALA, the tissue concentrations of PBG and PPIX never reached such high levels. This might be due to the fact that the cells were overloaded with ALA for only a short period of time. This is supported by the fact that tissue ALA concentrations decreased rapidly from 1 hour to equal plasma concentrations at 2-8 h. Our findings provide support for the concept that local use of ALA in the oesophagus might be advantageous. By this approach, the cellular heme biosynthetic pathway could be overloaded with ALA for a longer exposition time. Application of ALA in a gel or a spray has been used by Vonarx *et al.* in mice (Vonarx *et al.*, 1997) and by Ortner *et al.* (Ortner *et al.*, 1997) in patients. This may possibly induce an even higher local PPIX accumulation in the exposed tissue.

The optimism about negligible photosensitivity of the skin after ALA (Barr *et al.*, 1996; Gossner *et al.*, 1998) was not confirmed by our study. Thirty-five percent of our patients developed painfull erythema of their facial skin already during the first 9 h after administration of ALA and being kept in a room with subdued light. In total, 77% of the patients developed skin photosensitivity. Additional preventive measures such as the application of sunscreen protection factor 70 did not prevent the symptoms of photosensitivity. Moreover, one should also be aware that exogenous ALA might provoke porphyria-like symptoms as was demonstrated by one patient in our study.

As we demonstrated rapid kinetics of ALA and PPIX in all patients, the abnormal concentrations of ALA in the urine samples of 5 patients (19%) were unexpected. Despite thorough biochemical and clinical investigations which only revealed a higher ALA concentrations in plasma at 1 hour after ALA administration, these patients were clinically and biochemically not different from the other patients.

In conclusion, this is the first study in which PPIX concentrations were determined at sufficient time intervals in tissues and plasma of patients with BE after ALA ingestion to allow reliable pharmacokinetics of PPIX . Maximum PPIX concentrations occured at a shorter time interval after ALA ingestion in BE than in SQ. Concentrations of PPIX were higher in SQ especially at longer time intervals. In addition, tissue ALA concentrations were found to be 20-fold higher than the plasma concentrations at 1 hour after ALA ingestion. Side effects, i.e., skin photosensitivity is short-lasting but can be serious. These results provide a biological rationale for the use of ALA-PDT for the treatment of BE at 4-5 h after ALA ingestion (60 mg/kg) and for the local application of ALA in the oesophagus.

Acknowledgements

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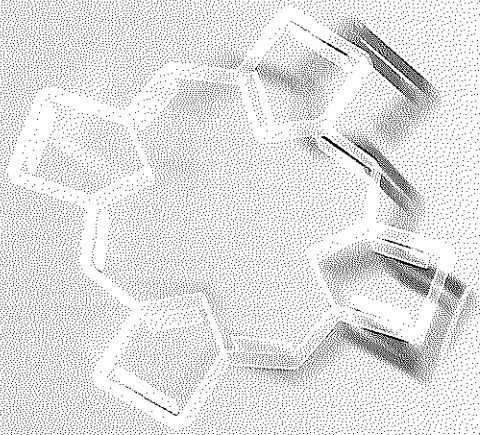
Ferrochelatase activity inhibition by 5-aminolaevulinic acid-induced photodynamic therapy.



Submitted

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Abstract

Administration of 5-aminolaevulinic acid (ALA) leads to porphyrin accumulation in tumour cells, mostly as protoporphyrin IX. ALA is used as a pro-drug for photodynamic therapy (ALA-PDT). The effect of ALA-PDT, using UV light and 633 nm laser light, on the activities of two haem biosynthetic enzymes porphobilinogen deaminase and ferrochelatase was studied in human EBV-transformed lymphoblastoid cell lines as a model of human tumour cells. Ferrochelatase activity was significantly inhibited by ALA-PDT, whereas the porphobilinogen deaminase activity remained unchanged. The extent of ferrochelatase inhibition was related both to the intracellular protoporphyrin IX concentration and to the time of UV light exposure. ALA-PDT therefore causes a relative block in haem biosynthesis by inhibiting ferrochelatase. A short period of pre-illumination of ALA-treated porphyrin-loaded tissues might enhance porphyrin accumulation and a second illumination might result in a more pronounced ALA-PDT effect.

Introduction

5-aminolaevulinic acid (ALA) induced porphyrin accumulation, which is predominantly protoporphyrin IX (PPIX), is used for ALA-based photodynamic therapy (ALA-PDT) (Kennedy and Pottier, 1992; Peng *et al.*, 1997). During illumination of porphyrin- containing tissues with light of an appropriate wavelength tissue destruction results through oxidative processes that affect specific cell structures adjacent to the production site of porphyrins (Iinuma *et al.*, 1994; Moan, 1990; Weishaupt *et al.*, 1976).

It is of interest to follow the steps leading to the production of PPIX. Two molecules of ALA are converted to porphobilinogen, which is metabolised to porphyrinogen intermediates by porphobilinogen deaminase (PBG-D). The last step of the haem synthesis is the introduction of iron into PPIX, catalysed by ferrochelatase (FC). Exogenous ALA administration bypasses the first and rate-limiting step of porphyrin synthesis, catalysed by ALA-synthase, and PBG-D then becomes the rate-limiting enzyme in haem formation (Bishop and Desnick, 1982). Both normal and tumour tissues accumulate porphyrins after administration of ALA (Kennedy and Pottier, 1992). However, in human tumours we have observed an increased ratio between the activities of PBG-D and FC compared to normal tissues (Hinnen *et al.*, 1998). The changed balance between these enzyme activities might contribute to a relative increase of porphyrin accumulation in these tissues compared to normal tissues.

ALA-PDT is at present being used in the treatment of several premalignant and malignant disorders (Peng *et al.*, 1997). However results in skin tumours and Barrett's oesophagus

suggest that ALA-PDT needs to be improved (Barr *et al.*, 1996; Gossner *et al.*, 1998). An option currently being explored to optimize ALA-PDT is to interfere in the haem biosynthetic pathway to increase the cellular PPIX concentration (Curnow *et al.*, 1999; Gaullier *et al.*, 1997; Tan *et al.*, 1997). The mitochondrial enzyme FC is of especial interest as during the insertion of iron into PPIX, it envelops PPIX. It has been demonstrated in vitro that the primary site of phototoxic damage by ALA-PDT is the mitochondrion (Iinuma *et al.*, 1994; Liang *et al.*, 1998; Peng *et al.*, 1996). Considering the diffusion length of oxygen radicals formed during ALA-PDT (Moan, 1990) and the close interaction between PPIX and FC, it is likely that FC will be more prone to photodamage than cytoplasmatic enzymes such as PBG-D. Illumination of porphyrin- containing cells shortly after starting incubation with ALA, might therefore damage FC and leave PBG-D intact, and could be a means of further increasing porphyrin levels for ALA-PDT.
In this study we examined the effect of UV light (365 nm) and red laser light (633 nm) after ALA administration on both the FC and PBG-D activity in a human tumour cell model. We used human EBV-transformed lymphoblasts as a tumour cell model. Two different light sources were used. Firstly, UV ligth as porphyrins have their absorption maximum at these wavelengths (the so-called Søret band). Secondly, we used red laser light (633 nm), the weakest absorption band of porphyrins, however with deeper tissue penetration then UV light and therefore better clinical properties.

Materials and methods

CHEMICALS

RPMI-1640 medium and penicillin-streptomycin were supplied by Biowhittaker (Maryland, MD, USA) and fetal calf serum (FCS) by Gibco Laboratories (Grand Island, NY, USA). The following reagents were obtained from Porphyrin Products (Logan, UT, USA): PPIX disodium salt, zinc PPIX, uroporphyrin and porphobilinogen. ALA-HCl and Triton X-100 were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Tris-HCL was purchased from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade and used as received.

CELL LINES AND CULTURE CONDITIONS

EBV-transformed human lymphoblastoid cells of six healthy individuals were cultured in 75 cm^2 flasks (Costar, Corning Incorporated, Corning, NY, USA) at 37°C in a humidified atmosphere (5% CO_2 in air) on RPMI-1640 medium supplemented with 15% heat-inactivated FCS, 10^5 I.U./l penicillin and 100 mg/l streptomycin.

INCUBATION CONDITIONS

Exponentially proliferating cells were harvested and used for the experiments. During the first series of experiments UV light used. In these experiments cells were resuspended in a medium containing NaCl (138 mmol/L), Na_2HPO_4 (8 mmol/L), $CaCl_2$ (1.0 mmol/L), KCl (2.7 mmol/L), KH_2PO_4 (1.5 mmol/L) and MgCl (0.5 mmol/L). In the second series of experiments, using red laser light, standard RPMI culture medium was used for practical reasons. To compensate the pH decrease due to ALA in the medium the pH was adjusted to 7.6 before the incubations.
In pilot experiments, we found that the ability to accumulate porphyrins from ALA was the same in all cell lines (data not shown).

ILLUMINATION EXPERIMENTS

General conditions

Before and after illumination, samples of incubated cells were drawn and washed in saline (NaCl 150 mmol/L) by centrifugation at 55 x g, to remove ALA and to isolate the intact cells in the pellet. In addition the porphyrin concentration, the protein concentration and the activity of FC and PBG-D were determined. The FC and PBG-D activity of illuminated cells was expressed as a percentage relative to the activity of their non-illuminated controls (100 %). The effect of ALA or PPIX on FC and PBG-D activities of non-illuminated cells and the effect of light exposure on FC and PBG-D in cells incubated in the absence of ALA was determined as controls in each experiment.

UV light experiments

Each cell line was incubated with 0.4 mmol/L ALA, and 19 samples were drawn at each time interval of 0, 30, 60 and 120 minutes. Subsequently, these samples were illuminated with UV light (365 nm, 7 mW/cm^2) generated by a UV transilluminator L 2734 (LabCenter, Breda, The Netherlands) for 0, 2.5, 5 or 10 minutes. Cell samples with a protein content below the level of the minimum protein standard were excluded from further analysis. The intracellular porphyrin concentration, the protein concentration and the activity of FC were then determined.

633 nm laser light experiments

Cell lines were incubated in the presence of various ALA concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.2 mmol/L) for 2 hours. Subsequently, cells were exposed for 125 seconds to 633 nm laser light (100 mW/cm^2) generated by a 600 Series Dye module pumped by a KTP/532 surgical laser (Laserscope, San José, CA, USA). The intracellular porphyrin concentration, the protein concentration and the activity of FC and PBG-D were then determined.

FERROCHELATASE AND PORPHOBILINOGEN DEAMINASE ASSAYS AND PORPHYRIN ANALYSIS

Ferrochelatase (FC) activity was measured by a modification of the method of Li *et al.* as described previously (Li *et al.*, 1987; Van Hillegersberg *et al.*, 1992). Cells were mixed with water and kept on ice before being used for the assay.

Porphobilinogen deaminase (PBG-D) was measured as described previously (Hinnen *et al.*, 1998; Wilson *et al.*, 1986). The following adaptions were made;

(a) One percent of bovine serum albumin (BSA) was added to the solution of porphobilinogen in Tris-HCL buffer (pH 8.0) to prevent binding of enzyme molecules to the plastic of the tubes.(b) The fluorescence of the supernatant, uroporphyrin-I, was measured at an excitation wavelength of 410 nm and an emission wavelength of 654 nm (Perkin Elmer LS 50B with a red sensitive photomultiplier, Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands).(c) Values were calculated according to a standard curve of uroporphyrin-I.

Results of the FC and PBG-D activities were expressed as pmols of porphyrins formed per mg protein per hour.

PPIX accumulation was determined spectrofluorometrically (excitation wavelength: 417 nm and emission wavelength: 636 nm) in the LS 50B spectrofluorometer. This result was divided by the protein content and expressed as arbitrary fluorescence units (F.U.). The protein content was determined according to the method of Lowry *et al.* (Lowry *et al.*, 1951). All experiments were performed in subdued light in duplicate.

STATISTICAL ANALYSIS

Data were expressed as means ± sem and analysed for statistical significance using the Student's t-test for paired values. Pearson correlation coefficients were calculated to study possible associations. Multiple regression analysis was used to examine the effect of the duration of exposure (min) to UV light and the intracellular porphyrin concentration on the activity of FC. P-values <0.05 were considered significant.

Results

ALA or PPIX had no effect on enzyme activities in the dark and light exposure did not affect the activities of cells not incubated with ALA (***FIGURE 1*** and ***FIGURE 2***).

UV LIGHT EXPERIMENTS

The lymphoblastoid cells accumulated porphyrins linearly in relation to the incubation time (Pearson $r = + 0.98$, $p < 0.001$). In cells exposed to UV light, FC activity was inhib-

ited and this inhibition was more pronounced at higher intracellular concentrations of porphyrins as well as after longer UV light exposure (***FIGURE 1***). The inhibition of FC depended on both the time of UV light exposure ($p < 0.001$) and the intracellular concentration of porphyrins ($p = 0.001$). Significant photoinactivation of FC by UV light was seen only with an illumination time of at least 5 minutes and with the porphyrin concentration that could be achieved by at least 60 minutes of incubation with ALA.

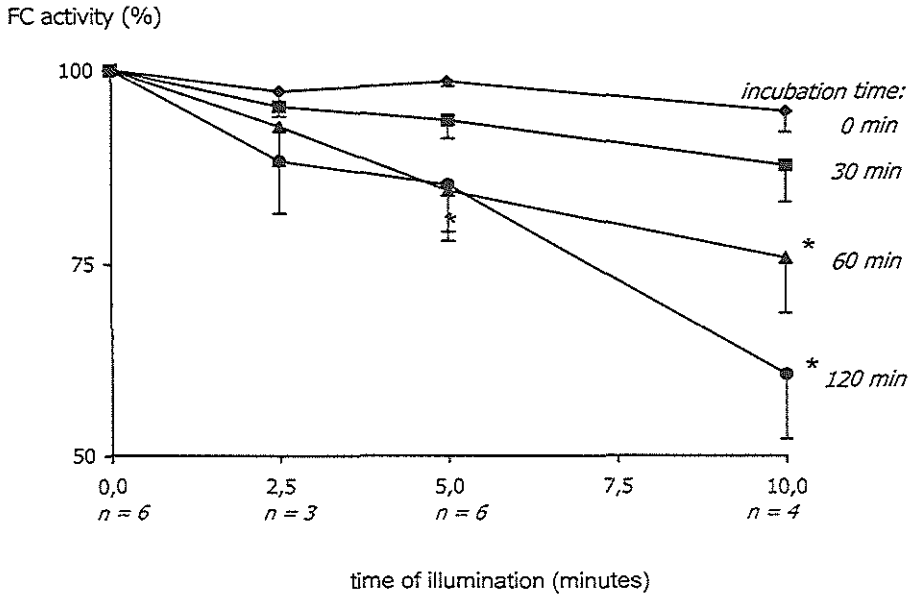

FIGURE 1 *The effect of the time of exposure to UV light on ferrochelatase (FC) activity in six human EBV- transformed lymphoblast cell lines incubated with 0.4 mmol/L ALA. Cell lines incubated for the same time period (min) and therefore with similar intracellular porphryin concentrations were grouped and within these groups the effect of the time of exposure to UV light was studied. Cell samples with a protein content below the level of the minimum protein standard were excluded from further analysis. The mean ± sem enzyme activity of illuminated cells was expressed as a percentage, relative to the activity of their non-illuminated controls (100%).* $p \leq 0.001$.*

633 NM LASER LIGHT EXPERIMENTS: FC ACTIVITY

FC activity of cells incubated with 0.6 mmol/L ALA for 2 hrs and subsequently illuminated by 633 nm laser light was found significantly lower than that of non-illuminated controls (615 versus 750 pmol ZnPr/ mg protein/ hour; 82 ± 2% versus 100%, mean ± sem, n=22, $p<0.001$). When results of all incubations at all ALA concentrations were analysed, the activity of FC was found to be inversely correlated to the intracellular porphyrin concentration (***FIGURE 2***, Pearson, r= - 0.80, $p<0.001$).

633 NM LASER LIGHT EXPERIMENTS: PBG-D ACTIVITY

Illumination of cells following incubation with 0.6 mmol/L ALA for 2 hrs had no effect on the mean PBG-D activity. We found no difference in PBG-D activity compared to the non-illuminated controls (63.0±7.5 versus 63.7±6.5 pmol URO/ mg protein/hour, 100%, n=22).

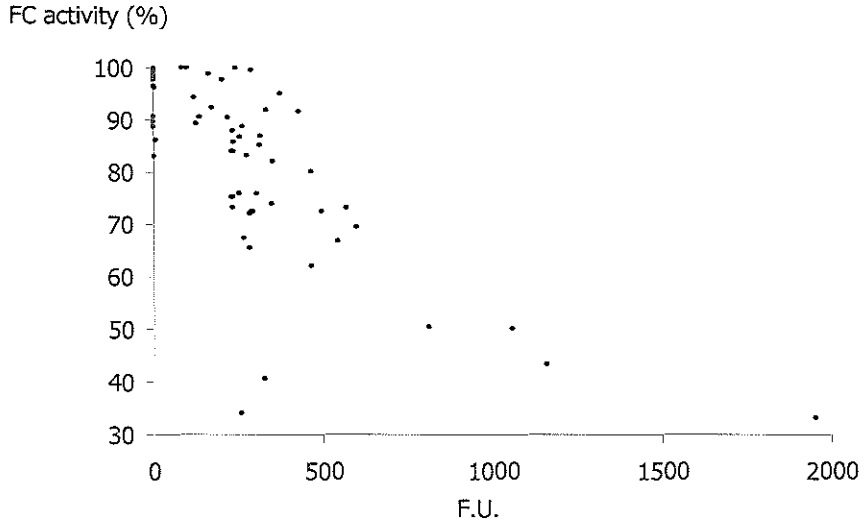

FIGURE 2 The effect of red laser light (633 nm) on the activity of ferrochelatase (FC) in six human EBV-transformed lymphoblast cell lines, used as a tumour cell model, incubated for 2 hours with various ALA concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.2 mM). The enzyme activity of illuminated cells was expressed as a percentage, relative to the activity of their non-illuminated controls (100%). Porphyrin concentrations are expressed as arbitrary fluorescence units (F.U.).

Discussion

In this study, we investigated the effect of ALA-PDT on the activities of PBG-D and FC. In view of the limited diffusion length of oxygen radicals produced during exposure of PPIX to light (Moan, 1990; Moan and Berg, 1991) and the moment of close interaction between FC and PPIX (FC activity enholds the chelation of ferrous iron into PPIX), we expected the mitochondrial enzyme FC to be damaged during ALA-PDT, whereas cytoplasmic enzymes such as PBG-D would remain intact. In porphyrin- containing human EBV-transformed lymphoblasts, used as a tumour cell model, we found that exposure of these cells to UV light and 633 nm laser light selectively damaged FC. Photoinactivation of FC depended on both the concentration of cellular PPIX and on the time of exposure to UV light (***FIGURE 1*** and ***FIGURE 2***).

Apart from direct inactivation of FC, it is also possible that FC activity was decreased secondary to destruction of mitochondria since this organel is considered to be the primary target of ALA-PDT (Iinuma *et al.*, 1994; Peng *et al.*, 1996). However, in this study we found that human lymphoblastoid cells were still able to accumulate PPIX after ALA-PDT when re-cultured in the presence of ALA. This suggests that direct inhibition of FC occurs even in the absence of mitochondrial destruction (data not shown). This is also supported by studies of He *et al.*, who found the same FC inhibiting effect of UV light, after culturing A431 human epidermal carcinoma cells with ALA. The effect of UV light on FC was found to result from direct damage to FC, since mRNA levels of FC remained stable (He *et al.*, 1995; He *et al.*, 1993). Iunima *et al.* found evidence that the subcellular localisation or even the submitochondrial localisation of PPIX might be important for the efficacy of ALA-PDT. In two cell lines that differed in PDT-sensitivity despite containing equal amounts of PPIX, mainly located in the mitochondria, they found differences in submitochondrial staining (Iinuma *et al.*, 1994).

As expected from its cytoplasmic localisation, PBG-D was not damaged by ALA-PDT. In contrast, Gibson *et al.* found an increased PBG-D activity after the administration of ALA in rat mammary adenocarcinoma (R3230 AC) and human mammary (MCF-7) and mesothelioma tumour cell lines (H-MESO-1) (Gibson *et al.*, 1998). They suggested that ALA administration induced enzyme synthesis *de novo*. They administered cycloheximide to support this. However, cycloheximide inhibits the synthesis of all proteins and therefore also of regulatory proteins. As mRNA levels were not determined in that study it is not sure yet, whether PBG-D was induced *de novo* by the administration of ALA or that ALA only influenced the stability of the enzyme. In another study, Gibson *et al.* showed that the activity of PBG-D was inhibited by ALA-PDT in a rat mammary adenocarcinoma cell line (Gibson *et al.*, 1999). Since kinetics of PPIX and factors influencing PPIX generation and PDT sensitivity can be different in different cell types, these factors might pos-

sibly contribute to the conflicting findings (Wyld *et al.*, 1997).
In conclusion, ALA-PDT causes a relative block in haem biosynthesis by inhibiting FC and sparing the activity of PBG-D. The ability of ALA-PDT pre-treated cells to accumulate porphyrins could be enhanced by two-phase ALA-PDT. A short period of pre-illumination of ALA-treated porphyrin-loaded Barrett's mucosa might enhance selective porphyrin accumulation, resulting in a more pronounced ALA-PDT effect after a second illumination.

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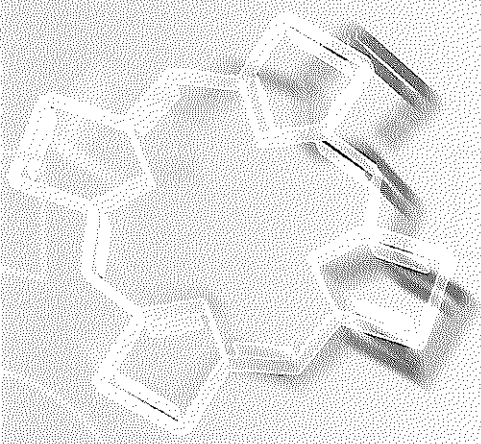
A two-phase illumination scheme in ALA-PDT: improvement of the clinical outcome?



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Abstract

5-Aminolaevulinic acid-induced photodynamic therapy (ALA-PDT) is used for the treatment of premalignant and malignant disorders. Excess exogenous ALA leads to intracellular protoporphyrin IX (PPIX) accumulation. ALA-PDT by itself inhibits the activity of the haem biosynthetic enzyme ferrochelatase (FC), which is responsible for the conversion of PPIX into haem. To examine the effect of ALA-PDT on PPIX accumulation and the effect of one or two laser treatments (two-phase ALA-PDT) on cell survival, we used EBV-transformed lymphoblastoid cells as a model of human tumour cells. ALA-treated cells were illuminated ones or twice with 633 nm laser light (100 mW/cm^2) or kept in the dark for the same time period. At selected times, the porphyrin and protein concentrations were determined. Porphyrin biosynthesis remained intact in EBV-transformed lymphoblastoid cells after a first episode of ALA-PDT. Following the first illumination, cells were able to accumulate significantly more PPIX than their non-illuminated controls. Two illuminations resulted in more cell death than one illumination. A two-phase illumination scheme may improve the clinical outcome of ALA-PDT.

Introduction

5-Aminolaevulinic acid-induced photodynamic therapy (ALA-PDT) is based on the concept that the administration of ALA bypasses the rate-limiting enzyme of porphyrin synthesis, ALA-synthase, and induces the accumulation of intracellular porphyrins, mostly protoporphyrin IX (PPIX) (Hinnen *et al.*, 1999; Hinnen *et al.*, 2000; Hinnen *et al.*, 1998; Kennedy and Pottier, 1992). After absorption of light by PPIX, energy is transferred to oxygen molecules (Weishaupt *et al.*, 1976), leading to tissue destruction through oxidative processes that affect cell structures adjacent to the production site of porphyrins (Iinuma *et al.*, 1994; Moan, 1990).

ALA-PDT is used clinically for certain skin lesions (Peng *et al.*, 1997). For the treatment of Barrett's esophagus (Barr *et al.*, 1996; Gossner *et al.*, 1998), a pre-malignant lesion, and for colorectal neoplasms (Fromm *et al.*, 1996; Mlkvy *et al.*, 1995; Regula *et al.*, 1995) it is still in an experimental stage. The effects of ALA-PDT are heterogeneous; in Barrett's esophagus, the presence of islands of columnar cells remaining beneath regenerating squamous epithelium has created the concern that superficial healing could mask underlying dysplasia (Barr *et al.*, 1996; Biddlestone *et al.*, 1998).Therefore, ALA-PDT needs to be improved.

In a previous study, using EBV-transformed lymphoblastoid cell lines as a model of human tumour cells, we showed that ALA-PDT by itself caused a relative block in haem biosyn-

thesis by inhibiting ferrochelatase, which catalyses the synthesis of haem by insertion of iron into PPIX (Hinnen *et al.*, 1999). In addition, the activity of porphobilinogen deaminase remained unchanged after ALA-PDT. This enzyme was shown to be the rate-limiting enzyme of haem biosynthesis after ALA synthase (Bishop and Desnick, 1982).
An important factor influencing the efficacy of PDT is the concentration of photosensitizer in the target tissue (Iinuma *et al.*, 1994; Tan *et al.*, 1997). A method to increase the concentration of PPIX in tissues could be the use of a two-phase illumination schedule in which the first illumination is used to inhibit FC causing an increase in PPIX accumulation and this could result in the second illumination to be more effective than only a single illumination. In this study we examined the effect of ALA-PDT on PPIX accumulation and on cell survival after one or two illuminations in the EBV-transformed lymphoblastoid cell line model.

Materials and methods

CHEMICALS

RPMI-1640 medium and penicillin-streptomycin were supplied by Biowhittaker (Maryland, MD, USA) and fetal calf serum (FCS) by Gibco Laboratories (Grand Island, NY, USA). ALA-HCl, was purchased from Sigma Chemical Corporation (St. Louis, MO, USA) and Tris-HCL from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade and used as received.

CELL LINES AND CULTURE CONDITIONS

Human EBV-transformed lymphoblastoid cell lines of five healthy individuals were cultured in 75 cm^2 flasks (Costar, Corning Incorporated, Corning, NY, USA) at 37°C in a humidified atmosphere (5% CO_2 in air) on RPMI-1640 medium supplemented with 15% heat-inactivated FCS, 10^5 I.U./l penicillin and 100 mg/l streptomycin. Exponentially proliferating cells were harvested, resuspended in fresh medium and incubated with or without 0.6 mM ALA. To compensate the pH decrease due to ALA in the medium the pH was adjusted to 7.6.

INCUBATION AND ILLUMINATION EXPERIMENTS

General conditions

Four different illumination experiments (I, II, III, IV) were performed using 633 nm laser light (Laser-series, L cells) together with control experiments using an equal time period in the dark (Dark-series, D cells) (***FIGURE 1***). Apart from the laser treatment, all cells were incubated in the dark at 37°C. Cells incubated with ALA were exposed to 0.6 mM ALA. At selected times, cells were washed with NaCl solution (150 mM) and centrifugated at 55 x g to remove culture medium and ALA. For porphyrin determinations the cell pellets were resuspended in 1 ml NaCl (150 mM) and samples of 150 µl were drawn. Subsequently, the cell suspensions were centrifugated at 55 x g and the cell pellets were resuspended in 110 µl aqua dest. for protein determinations.

The laser treatment consisted of an exposure time of 125 s to 633 nm laser light (100 mW/cm^2) generated by a Dye module pumped by a KTP/532 surgical laser (Laserscope UK Ltd, Gwent, UK).

Experiments

All experiments started with an incubation period of 2 h in the presence of ALA (L1 → L2 or D1 → D2 in ***FIGURE 1***) followed by a laser or dark treatment (L3, D3).

Experiment I and II: The effect of two courses of ALA-PDT followed by incubation in medium containing ALA (Exp. I) or in medium without ALA (Exp. II) on porphyrin concentration and cell death. After the initial PDT treatment, cells were re-incubated with ALA (Exp. I) or without ALA (Exp. II) for 3 h, then washed and subjected to a second laser treatment and again re-incubated with ALA (Exp. I) or without ALA (Exp. II) for another 17 h (Exp. I: D/L3 → D/L4a → D/L6a, Exp. II: D/L3 → D/L4b → D/L6b).

Experiment III and IV: The effect of a single course of ALA-PDT followed by incubation in medium containing ALA (Exp. III) or without ALA (Exp. IV) on porphyrin concentration and cell death. After the initial PDT treatment, cells were re-incubated in medium with ALA (Exp. III) or without ALA (Exp. IV) for 20 h (Exp. III: D/L3 → D/L5a, Exp. IV: D/L3 → D/L5b).

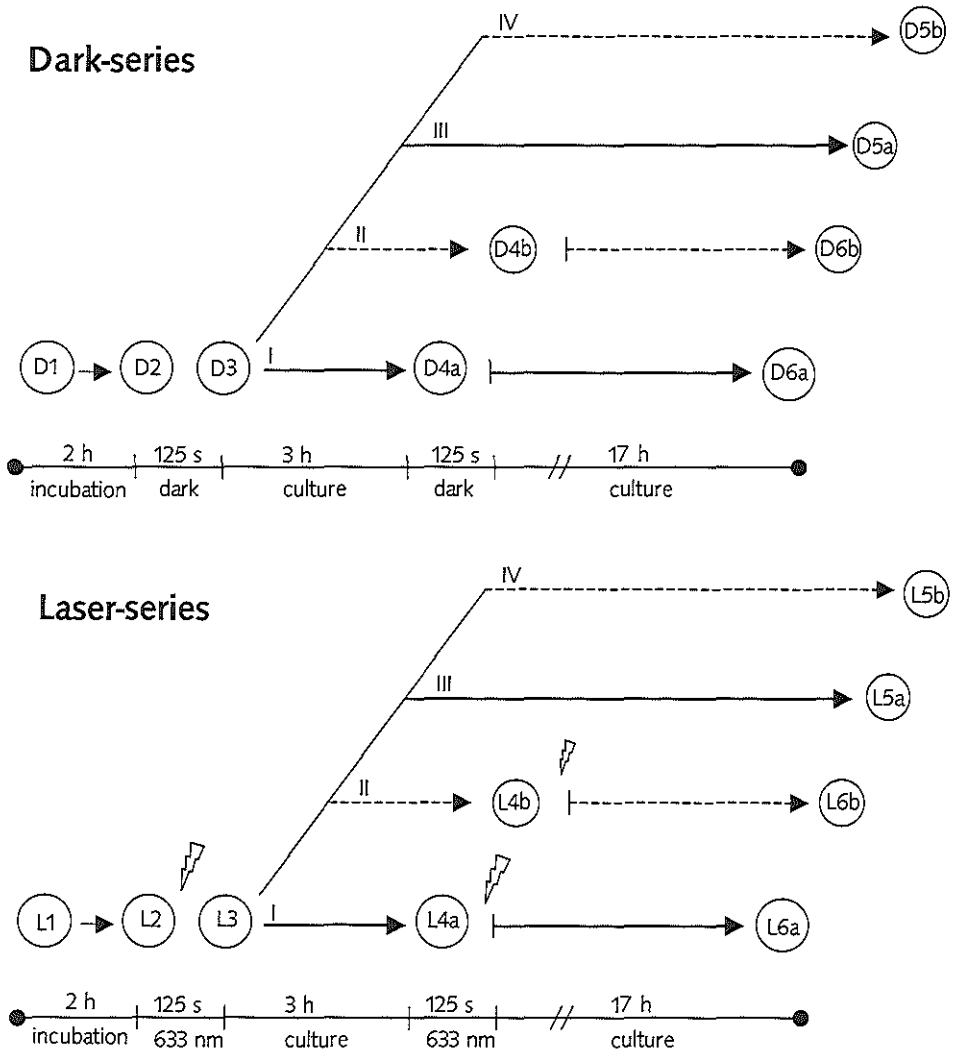

FIGURE 1 *Flow chart of experiments I, II, III and IV to study the effect of (two-phase) ALA-PDT on PPIX accumulation and cell death in cultured EBV-transformed lymphoblastoid cells. The different experiments are explained in detail in the Materials and Methods section. Laser series; 125 s, 633 nm, 100 mW/cm² ( ); arrow, with 0.6 mM ALA; dashed arrow, without ALA (control)*

PORPHYRIN ANALYSIS AND PROTEIN DETERMINATION

PPIX accumulation was determined spectrofluorometrically at an excitation wavelength of 417 nm and an emission wavelength of 636 nm in a LS50B spectrofluorometer with a red sensitive photo multiplier (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). Porphyrin emission was expressed as mg of protein. After the first 2 h incubation period (D2, L2), the resulting porphyrin concentration was set at 100%. The porphyrin content of D and L cells was expressed as a percentage of the concentration in D2 and L2 cells, respectively.
The protein content was determined according to the method of Lowry *et al.* (Lowry *et al.*, 1951). In this study we used the protein content in the cell pellet as a measure of cell death. In all experiments it was observed that the protein content had not changed after the first illumination with 633 nm laser light or an equal time period in the dark. We therefore chose to set the protein contents of the D3 and L3 cells at 0%, suggesting that cell death and/or cell loss was not apparent at this time point. As a consequence, the protein content of D and L cells was expressed as a percentage of the protein content of the D3 and L3 cells, respectively.

STATISTICAL ANALYSIS

Data are expressed as means ± sem and were analysed for statistical significance using the Student's *t*-test for paired values. P-values <0.05 were considered significant.

Results

The effect of ALA-PDT (Laser-series) on the ability of human lymphoblastoid cells to accumulate porphyrins and the efficacy of one or two laser treatments is shown in ***TABLE 1.*** Results of statistical comparisons within and between the Laser- and Dark-series are shown in ***TABLE 2.***
The presence of ALA or PPIX had no effect on the survival of non-illuminated cells and light exposure did not affect the survival of cells incubated without ALA (***TABLE 2***). Cell loss in the Dark-series starting from D4a/D4b, is most likely the result of loss of cells due to the washing procedures. As a substantial amount of cells was lost due to handling during the experiments, together with the individual spread in cell loss per experiment, we decided to compare means of groups of repeated light and dark experiments.
During exposure of ALA-treated cells to 633 nm laser light, the intracellular concentration of porphyrins per mg protein decreased from 100% to 57% (L2 → L3, $p<0.001$), whereas porphyrin concentration did not change when ALA-treated cells were kept in the dark (D2 → D3). Immediate cell death after ALA-PDT was not observed (L2 → L3). The

percentage of cell death (and cell loss due to the washing procedure) after a single laser treatment (Exp. I and III) was found to be 66% after 3 h of re-incubation with or without ALA (L3 → L4a/b) and this increased up to 80% after 20 h of re-incubation with or without ALA (L3 → L5a/b). More cells had died after 20 h than after 3 h of incubation (L4a/L4b vs L5a/L5b, $p < 0.001$). In addition, more cell death was found after two laser treatments than after one (L2 → L6a vs L2 → L5a, $p < 0.001$). When cultured without further ALA, all cells showed a significant decrease in porphyrins from 100% to undetectable levels (Exp. II and IV of the Dark- and Laser-series). An additional period of 3 h re-incubation with ALA after a first course of ALA-PDT resulted in higher porphyrin levels in comparison to cells which had not been exposed to laser light (L4a vs D4a, $p < 0.001$). When cells were cultured with ALA for 20 h after the initial course of ALA-PDT, the porphyrin concentrations increased from 100% to 220% (L2 → L5a, $p = 0.001$), whereas porphyrin concentrations decreased from 100% to 55% in their non-illuminated controls (D2 → D5a, $p < 0.001$). Porphyrin contents could not be determined in the cells that were treated twice by the laser as only a few cells survived and the protein content was far below the lowest standard (L6a).

TABLE 1 *The effect of ALA-PDT or darkness on the percentage of cell loss, cell death and intracellular porphyrins in human EBV-transformed lymphoblastoid cell lines.*

Dark-series[a]	*n*	*cell loss (%)*	*porphyrin concentration (%)*	*Laser series*[a]	*n*	*cell loss & death (%)*	*porphyrin concentration (%)*
D1	23	0	ND[b]	L1	23	0	ND
D2	23	0	100	L2	23	0	100
D3	23	0	100	L3	23	0	57 ± 2
D4a	23	33 ± 3[d]	110 ± 7	L4a	23	66 ± 3	119 ± 12
D4b	18	32 ± 3	ND	L4b	18	68 ± 3	ND
D5a	23	36 ± 2	55 ± 5	L5a	23	80 ± 2	220 ± 30
D5b	21	35 ± 2	ND	L5b	21	77 ± 2	ND
D6a	18	54 ± 2	46 ± 4	L6a	18	97 ± 1	NR[c]
D6b	17	55 ± 2	ND	L6b	17	87 ± 1	ND

[a] *See* ***FIGURE 1*** *for the different experiments*
[b] *ND, not detectable*
[c] *NR, not reliable due to a too low protein content*
[d] *means ± SE*

TABLE 2 *Comparisons between ALA-PDT and darkness effects on cell loss, cell death and porphyrin concentration in human EBV-transformed lymphoblastoid cell lines.*

Dark-series[a]	*cell loss*	*porphyrin concentration*	*Laser series*[a]	*cell loss & death*	*porphyrin concentration*
D2 vs D3	NS[b]	NS	L2 vs L3	NS	$p < 0.001$
D2 vs D4a	$p < 0.001$	NS	L2 vs L4a	$p < 0.001$	NS
D2 vs D5a	$p < 0.001$	$p < 0.001$	L2 vs L5a	$p < 0.001$	$p = 0.001$
D2 vs D6a	$p < 0.001$	$p < 0.001$	L2 vs L6a	$p < 0.001$	
D4a vs D4b	NS	$p < 0.001$	L4a vs L4b	NS	$p < 0.001$
D4a vs D5a	NS	$p < 0.001$	L4a vs L5a	$p < 0.001$	$p < 0.01$
D4a vs D6a	$p < 0.001$	$p < 0.001$	L4a vs L6a	$p < 0.001$	
D5a vs D5b	NS	$p < 0.001$	L5a vs L5b	NS	$p < 0.001$
D5a vs D6a	$p < 0.001$	NS	L5a vs L6a	$p < 0.001$	NS
D6a vs D6b	NS	$p < 0.001$	L6a vs L6b	$p < 0.001$	
L vs D	*cell loss*	*porphyrin concentration*	*L vs D*	*cell loss*	*porphyrin concentration*
L3 vs D3	NS	$p < 0.001$	L5a vs D5a	$p < 0.001$	$p < 0.001$
L4a vs D4a	$p < 0.001$	$p < 0.001$	L6a vs D6a	$p < 0.001$	

*a See **FIGURE 1** for the different experiments*

b NS, not statistically significant

Discussion

Cultured human EBV-transformed lymphoblastoid cells were used as a model of human tumour cells to study the effect of ALA-PDT on porphyrin accumulation and the effect of one or two laser treatments (two-phase ALA-PDT) on cell survival. In a previous study we used the same cell model and demonstrated that these cells accumulated porphyrins after the administration of ALA and that ALA-PDT (UV and 633 nm laser light) caused a relative block in haem biosynthesis by inhibiting FC (Hinnen *et al.*, 1999). He *et al.* demonstrated that the inhibition of FC activity in a human epidermoid carcinoma cell line resulted from direct damage to FC since mRNA levels of FC remained stable. They

also found that re-incubation with ALA, following UV light exposure, resulted in an increase in PPIX accumulation (He *et al.*, 1995; He *et al.*, 1993).
In this study, it was shown that illumination with red laser light (633 nm) had an immediate effect on the intracellular porphyrin concentration, which significantly dropped from 100 % to 57 % (L2 → L3, ***FIGURE 1*** and ***TABLE 2***). After ALA-PDT, part of the haem biosynthetic pathway remained intact as cells were still able to accumulate porphyrins (L2 → L4a and L5a). The FC inhibiting effect of ALA-PDT increased the accumulation of PPIX from newly administered ALA as was shown by significantly different intracellular porphyrin concentrations in the L4a and L5a cells compared to their non-illuminated controls (D4a and D5a). The finding that ALA-PDT increased PPIX accumulation, at least in part through FC inhibition, was further supported by the finding that the intracellular porphyrin concentration in non-illuminated cells dropped from 100% to 55% during 20 h of subsequent incubation with ALA (D2 → D5a). We previously demonstrated that FC activity remained unchanged in these non-illuminated cells (Hinnen *et al.*, 1999). As PPIX was not detectable in the medium (results not shown), the decrease in porphyrins suggests that these cells were able to convert porphyrins into haem during the 20 h of re-incubation. This was also supported by the finding that porphyrin concentrations in porphyrin-loaded cells in the second and fourth experiment of the Dark- and Laser-series decreased to undetectable levels after 3 h of re-incubation without ALA.
In contrast to the instant decrease in intracellular porphyrins following ALA-PDT, cell death was first detected at 3 h (arbitrary second sampling time) after the laser treatment. Cell death increased in time and was significantly higher after two laser treatments than after a single laser treatment. Direct cytotoxicity has been shown to be insufficient to explain the effects of PDT. Cells from murine tumours remaining in situ after PDT undergo necrosis, whereas those explanted immediately after PDT remain viable in vitro (Henderson *et al.*, 1985). This suggests that local tissue factors, for instance an effect on microcirculation (oxygen supply) (Leveckis *et al.*, 1995) and/or the presence of neutrophils may play an important role (de Vree *et al.*, 1996). In this study we only used the protein content as a measure of cell survival and local tissue factors were not present, possibly explaining the delayed process of cell death in the cultured cells studied.
Previously, van der Veen *et al.* reported immediate photobleaching of PPIX after PDT and re-appearance of fluorescence in a transplantable rat mammary tumour model (van der Veen *et al.*, 1994). As an explanation for this observation they suggested the occurrence of inhibition of FC after illumination (He *et al.*, 1993) or the release of PPIX from damaged cells. In another study, these authors observed an improvement of the effect of ALA-PDT, in terms of an increased tumour volume doubling time of transplanted rat rabdomyosarcoma, using a light fractionation time interval of 75 min (de Bruijn *et al.*, 1999). They proposed that new PPIX was formed during the interval of 75 min enhanc-

ing the effect of the second illumination. The mechanism of the re-appearance of PPIX fluorescence may indeed be related to the decreased FC activity in cells after a single treatment. The decrease in FC activity caused by the initial course of ALA-PDT reduces the conversion of PPIX into haem, increases the accumulation of PPIX and provides an explanation for the increased PDT effect after two illuminations. In conclusion, our results strongly suggest that the haem biosynthetic pathway remains intact after ALA-PDT and that ALA-PDT enhances the ability of human EBV-transformed lymphoblastoid cells to accumulate PPIX. This can at least in part be explained by the inhibiting effect of ALA-PDT on the activity of FC. This effect of ALA-PDT on FC activity (He *et al.*, 1995; He *et al.*, 1993; Hinnen *et al.*, 1999) supports the observations that the primary target of ALA-PDT is the mitochondrion (Iinuma *et al.*, 1994; Liang *et al.*, 1998; Peng *et al.*, 1996). In addition the latter makes it unlikely that ALA-PDT interferes in haem biosynthesis by inhibiting the cytoplasmatic enzyme PBG-D as suggested by Gibson *et al.* (Gibson *et al.*, 1999). Cell death becomes apparent after some hours and the effect of PDT in terms of cell death is significantly better after two laser treatments than after a single treatment. Two-phase ALA-PDT may therefore be of clinical value in the efficacy of the treatment of malignant and pre-malignant disorders like Barrett's oesophagus. A local first phase of illumination of Barrett's mucosa, following oral ingestion of ALA, might induce damage to FC. As continuous influx of ALA from plasma exists, these illuminated cells will accumulate greater amounts of porphyrins compared with normal surrounding cells. Subsequently, a second phase of illumination may result in a more complete and selective ablation and therefore improve the results of ALA-PDT for the treatment of disorders such as Barrett's oesophagus.

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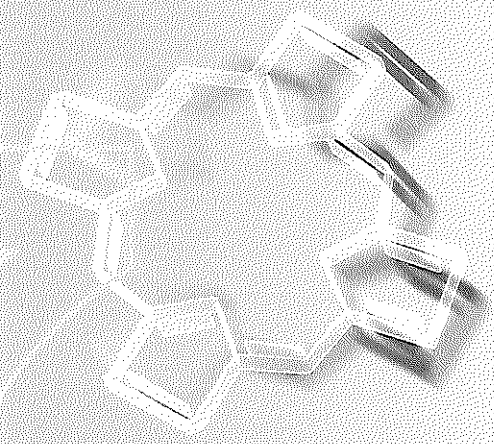
General Discussion



As stated in the introduction (*chapter 1*), the aim of this thesis was to study porphyrin and haem biosynthesis in (pre)malignant tissues of the human oesophagus before and after the administration of 5-aminolaevulinic acid (ALA). Two haem biosynthetic enzymes, porphobilinogen deaminase and ferrochelatase, are studied in more detail for their crucial role respectively in the secondary regulation of haem synthesis and the formation of PPIX.

I. PORPHOBILINOGEN DEAMINASE AND FERROCHELATASE ACTIVITIES IN HUMAN (PRE)MALIGNANT CELLS: DO THEY PREDICT PORPHYRIN ACCUMULATION?

There are a limited number of publications about the activities of the two haem biosynthetic enzymes porphobilinogen deaminase (PBG-D) and ferrochelatase (FC) in normal as well as in (pre)malignant tissues. Increased PBG-D activity relative to normal tissue has consistently been found in tumours (el-Sharabasy *et al.*, 1992; Navone *et al.*, 1990; Schoenfeld *et al.*, 1988) as well as in rapidly dividing cells, such as regenerating liver cells (Schoenfeld *et al.*, 1988; Schoenfeld *et al.*, 1987) suggesting that this phenomenon might be common in situations of increased cell replication. In contrast with the consistent studies concerning the activity of PBG-D, there seems to be a difference in FC activity among different tumour types (Dailey and Smith, 1984; el-Sharabasy *et al.*, 1992; Rubino and Rasetti, 1966; Smith, 1987; van Hillegersberg *et al.*, 1992). In this thesis the main theme is the activities of PBG-D and FC in different tissues, as these activities probably play an important role in determining the rate of PPIX synthesis and accumulation after ALA administration. We were the first to measure these activities in human normal oesophageal (SQ) and gastric mucosa, Barrett's epithelium (BE) and adenocarcinoma of the oesophagus (AC) (*Chapters 2 and 4*) (Hinnen *et al.*, 2000a; Hinnen *et al.*, 1998). The concept that the ratio between these enzyme activities would predict PPIX accumulation in tissue after ALA administration was appealing. This would mean that patients could undergo an endoscopy with biopsies at one time and drink ALA only when the ratio would predict high PPIX levels, suggesting that this patient could be a candidate for ALA-PDT. Although the ratio, which we called the PDT power index was higher in BE and AC compared to SQ, this index was not found to predict PPIX accumulation in these tissues under the conditions chosen in *Chapter 4* (Hinnen *et al.*, 2000a). However the higher PBG-D activities and lower PPIX precursor concentrations in AC and BE compared to SQ do suggest that the PDT power index might possibly have predicted PPIX levels at earlier time intervals (this was not studied) or with another ALA dose (Brand *et al.*, 2000; Saidi *et al.*, 2000) and that PPIX levels would have reached their maximum in BE at an earlier time interval after ALA ingestion than in SQ (studied in *Chapter 5*).

From the above it becomes clear that a simple relationship between enzyme activities and PPIX accumulation seems not very likely. This was supported by more recent studies, which show that PPIX accumulation also depends on many other variables like the temperature and pH in cells/tissues, cell cycle and differentiation, tissue oxygenation and the availability of iron (Gannon and Brown, 1999; Li *et al.*, 1999; Moan *et al.*, 1999; Tan *et al.*, 1997; Wyld *et al.*, 1997; Wyld *et al.*, 1998).

II. SELECTIVE PPIX ACCUMULATION

From fluorescence studies it appears that controversy exists as to whether the selectivity of ALA-induced PPIX accumulation lies in the difference between premalignant and normal epithelium or in the difference between epithelium and underlying muscle in the digestive tract (Barr *et al.*, 1996; Bedwell *et al.*, 1992; Regula *et al.*, 1995; van den Boogert *et al.*, 1999). In *Chapter 4*, we determined the intracellular concentrations of PPIX by biochemical extraction methods rather than fluorescence microscopy (Hinnen *et al.*, 2000a). It was repeatedly (*Chapter 4 and 5*) found that there was no selectivity in PPIX accumulation, when using therapeutic doses of ALA, between Barrett's epithelium and surrounding normal squamous epithelium. At subtherapeutic doses of ALA (2 and 10 mg/kg) preliminary data have shown a difference in PPIX fluorescence between Barrett's mucosa and normal squamous mucosa (Saidi *et al.*, 2000).
Transmural fluorescence studies of the oesophagus have shown a selectivity in PPIX accumulation between mucosa and underlying muscle. Moreover, the effects of ALA-PDT have proven to be superficial, sparing the muscle layer, which leads to the conclusion that PPIX selectivity lies in the difference between mucosa and muscle but not between Barrett's and squamous mucosa.
Porphyrin extraction from Barrett's and normal squamous mucosa at different time intervals after ALA ingestion (1-8 h after ALA ingestion, *Chapter 5*) have shown that PPIX levels are significantly lower in Barrett's mucosa than in normal squamous mucosa. The only finding that might be of clinical use lies in the fact that maximum PPIX concentration occured significantly earlier after ALA ingestion in Barrett's oesophagus (4.6 h) than in the normal squamous mucosa (6.6 h). This leads to the conclusion that the most selective damage to Barrett's mucosa can be achieved early within the time window (4-5 h after ALA ingestion) of maximum PPIX levels in Barrett's mucosa.

III. PHOTOSENSITIVITY: THE SIDE EFFECT

Photosensitivity of all tissues, including skin and mucosa, is a side effect of the administration of ALA to patients as porphyrins not only accumulate in cells of the target area but in all haem producing cells.
The optimism about negligible photosensitivity of the skin after ALA administration (Barr *et al.*, 1996; Gossner *et al.*, 1998) was not confirmed by our studies (*Chapter 4 and 5*). In *Chapter 4*, 7 of 10 patients complained of some skin photosensitivity. In the study mentioned in *Chapter 5*, 35 percent of the patients developed painful erythema of their facial skin already during the first 9 h after administration of ALA despite being kept in a room with subdued light. In total, 77% of the patients developed skin photosensitivity. Additional preventive measures such as the application of sunscreen factor 70 did not prevent the symptoms of photosensitivity.
Unwanted photosensitization tissue damage can be prevented in part by the use of acrylate yellow filters as shown in *Chapter 3*. For the use of intra-operative PDT as an adjuvant to curative tumour resection to destroy residual tumour the use of these filters is recommended. Perhaps the use of these filters in front of the windows could have reduced this side effect in *Chapter 5*.
Compared to the morbidity of oesophagectomy or the side-effects of other cancer treatments such as chemotherapy, the skin photosensitivity after ALA-PDT is acceptable.

IV. OPTIMIZATION OF ALA-PDT : TWO-PHASE ALA-PDT

As mentioned before, PDT requires the presence of three components for its action, namely: a photosensitizer, light and oxygen. These three have to meet the following criteria for any effect; (1) the concentration of the photosensitizer (PPIX) has to exceed a threshold (Hinnen *et al.*, 2000b), (2) a certain quantum of light energy -fluence rate and illumination time- has to be absorbed by photosensitizer molecules (PPIX) to create a minimum triplet status yield (see chapter 1, section IIb, (Hinnen *et al.*, 2000b; Iinuma *et al.*, 1999)) and (3) tissue oxygenation should be optimal as hypoxia diminishes the PDT effect (Georgakoudi *et al.*, 1999). Despite this knowledge the effects of ALA-PDT in tissues of patients are still limited to partial necrosis (Barr *et al.*, 1996; Fan *et al.*, 1996; Gossner *et al.*, 1998; Mlkvy *et al.*, 1995; Regula *et al.*, 1995). Optimization studies focus on the three components mentioned above (see *Chapter 1*).
The idea of using light fractionation schedules to improve the outcome of PDT was prompted by the realization that rapid oxygen consumption during the photochemical process can lead to an oxygen deficit within a few seconds, especially in tumour areas

distant from the vessels, thereby protecting cells from further damage from PDT (Foster *et al.*, 1991 ; McIlroy *et al.*, 1998). Moreover tumour vascular shutdown may also occur during ALA-PDT and this will further prevent an optimal PDT effect (Leveckis *et al.*, 1995; Roberts *et al.*, 1994; van der Veen *et al.*, 1994). Recovery of vascular constriction during the dark period and as a result re-oxygenation of the tissues (Curnow *et al.*, 1999) will provide more oxygen to quench the triplet-state of PPIX and results in higher production of oxygen radicals and ultimately in an increased PDT efficacy (Curnow *et al.*, 1999; Hua *et al.*, 1995; Messmann *et al.*, 1995). From a theoretical study, it was shown that the optimal fractionation period depends only on the oxygen diffusion time (10 s) and it was speculated that long dark periods would probably be inefficient by allowing time for sublethal repair processes (Pogue and Hasan, 1997). At present, most light fractionation schedules have been with equal times of light on and off, with an interval varying between 1 and 60 s, and the maximum tumoricidal effect has been observed with 30-s and 60-s fractions (Foster *et al.*, 1991; Iinuma *et al.*, 1999). Previously, de Bruijn *et al.* observed an improvement of the effect of ALA-PDT, in that the doubling time of the tumour volume of transplanted rat rabdomyosarcoma was prolonged using a light fractionation time interval of 75 minutes (de Bruijn *et al.*, 1999). They proposed that new PPIX was formed during the interval of 75 min enhancing the effect of the second illumination. Another way that proved succesful in our *in vitro* study described in Chapter 7, was the use of a light fractionation schedule with a dark period of 3 hours (a two-phase illumination scheme) as opposed to a continuous illumination schedule in EBV-transformed lymphoblastoid cells (Hinnen *et al.*, 2000c). Following the first illumination, cells were able to accumulate significantly more PPIX than their non-illuminated controls when continuously incubated with ALA. Two illuminations resulted in more cell death than one illumination. This is, at least in part, caused by the inhibitory effect of ALA-PDT on the haem biosynthetic enzyme ferrochelatase as we described in *Chapter 6* (He *et al.*, 1995; He *et al.*, 1993; Hinnen *et al.*, 2000b). So the mechanism of the re-appearance of PPIX fluorescence observed by van der Veen *et al.* (van der Veen *et al.*, 1994) and the observed improvement of PDT after a second illumination by de Bruijn *et al.* (de Bruijn *et al.*, 1999) may indeed be related to the decreased FC activity in cells after a single treatment. The decrease in FC activity caused by the initial course of ALA-PDT reduces the conversion of PPIX into haem, increases the accumulation of PPIX and provides an additional explanation for the increased PDT effect after two illuminations. In conclusion, increased PDT-sensitivity, which is the starting point for the concept of light fractionation schedules to enhance the ultimate outcome of PDT is not only determined by tissue reoxygenation but probably also by the renewed PPIX synthesis after ALA-PDT induced FC inhibition (Hinnen *et al.*, 2000b; Hinnen *et al.*, 2000c). Other factors that might play a role are the subcellular localization of PPIX and the cell cycle phase at the time of illu-

mination. PPIX is synthesized in the mitochondrion, but diffuses to other cellular sites (Iinuma *et al.*, 1994; Steinbach *et al.*, 1995). The subcellular localization of PPIX at the time of PDT might therefore play a role in the effect of one or two illuminations. The metabolic activity of a cell varies with the phase of the cell cycle. Cells in certain phases of the cell cycle have been shown to produce different amounts of PPIX and their PDT sensitivity correlated with the PPIX concentration (Wyld *et al.*, 1998). One should realise that besides the intracellular factors and vascular supply, other local tissue factors like the presence and action of neutrophils probably play an important role in the PDT effect as well (de Vree *et al.*, 1996).
Whether the clinical outcome of ALA-PDT will improve using long-term light fractionation remains uncertain. Only the study by Fan *et al.* demonstrated a benificial effect of long term light fractionation in ALA-PDT in patients with premalignant and malignant lesions of the oral cavity (Fan *et al.*, 1996). Despite a light fractionation schedule, they observed partial necrosis in all cases, however their timing was not ideal. Considering the fractionated ALA doses that they used and the pharmacokinetics of ALA and PPIX that we studied in oesophageal mucosa (*Chapter 5*), PPIX levels where probably suboptimal at the time of treatment.

V. FUTURE RESEARCH

The basic concept of the approach described here is that the best way to prevent progression of Barrett's epithelium to oesophageal cancer is to selectively remove the Barrett's epithelium and, by suppresing reflux of (acid)stomach contents, allow the normal squamous epithelim to regrow. Unfortunately, support for this approach is at present largely theoretical or limited to shortterm clinical studies. Before this approach can be recommended for clinical practice it is obvious that many studies still need to be done. A few clinical studies of ALA-PDT for the treatment of Barrett's oesophagus, which are not well documented in terms of exact timing and fluence rates, have been reported (Barr *et al.*, 1996; Gossner *et al.*, 1998; Tan *et al.*, 1999). Before performing large scale studies on ALA-PDT to remove Barrett's epithelium, further studies on the histological effects are needed. One possibility to gain insight in the extent of the histological effect of PDT is to subject patients who have to undergo an oesophagus resection because of the existence of an adenocarcinoma or high-grade dysplasia in Barrett's oesophagus to ALA-PDT a few days prior to the resection.
In addition, the improved damaging effect of a second illumination found in cell lines need to be confirmed by studies in patients with Barrett's oesophagus, preferably in patients shortly before they undergo oesophagectomy. Pathogenetic studies on Barrett's

oesophagus will provide us with knowledge on how to optimize the treatment of Barrett's oesophagus. In addition, such studies can even provide us with insight in the possible prevention of Barrett's oesophagus or the development of adenocarcinoma within it.

As stated in the introduction, Barrett's oesophagus is almost certainly a consequence of chronic reflux of stomach contents into the oesophagus. Not only acid but also bile reflux is damaging to the squamous epithelium as is reviewed by Byrne *et al.* (Byrne and Attwood, 1999). The latter is often forgotten as is shown by the concept of medical anti-reflux therapy with proton pump inhibitors. In general neither the length of the Barrett's segment nor the grade of dysplasia within this segment is altered by medical treatment. Proton pump inhibitors (PPI) are often prescribed for patients with Barrett's oesophagus as they control symptoms, and create an environment in which ulcers heal and stricture formation is prevented (Shepherd, 2000). Despite the fact that treatment with PPI's does not result in total regression of Barrett's epithelium, partial regression has occasionally been demonstrated with prolonged PPI treatment (Peters *et al.*, 1999). However, the most important question is not whether complete regression will result but whether neoplastic progression will be stopped.

Anti-reflux surgery such as Nissen fundoplication prevents both bile and acid reflux and may theoretically prevent the development of adenocarcinoma in Barrett's oesophagus. However in practice this therapy does also not seem to influence the incidence of carcinoma in Barrett's oesophagus. This may be because that by the time of operation, molecular and cellular events leading to the eventual development of malignancy have already occurred. Further studies are needed to investigate whether early Nissen fundoplication can prevent the development of adenocarcinoma within Barrett's oesophagus. However the very limited effects, if any, of anti-reflux treatment alone on replacement of Barrett's epithelium by squamous epithelium, suggest that removal of Barrett's epithelium will be an essential step in preventing adenocarcinoma of the lower oesophagus. It therefore seems highly worthwhile to continue to develop therapies such as PDT to safely remove Barrett's epithelium. Once a more consistently effective means of ablation has been defined, long-term studies in which PPI therapy alone or anti-reflux surgery alone is compared with either ALA-PDT alone or a combination of ALA-PDT with PPI's or surgery, would be necessary to answer the question whether Barrett's oesophagus should be treated by photodynamic therapy at all.

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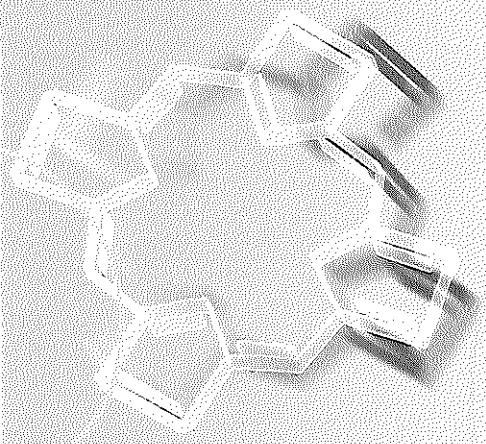
Summary



The incidence of oesophageal adenocarcinoma is increasing more rapidly than that of any other cancer in the western world. Adenocarcinoma is assumed to arise in Barrett's oesophagus, a metaplastic epithelial transformation as a reaction to chronic gastro(duodenal)-oesophageal reflux. Despite excellent anti-reflux medication and modern anti-reflux surgery, neither the length nor the grade of dysplasia of Barrett's oesophagus is influenced, so malignant transformation can still proceed.
A promising non-invasive treatment of Barrett's oesophagus studied in this thesis is photodynamic therapy (PDT). For its destructive effect, PDT requires a photoactive agent - in our studies, protoporphyrin IX (PPIX) formed from administered 5-aminolaevulinic acid (ALA)-, light of an appropriate wavelength - 633 nm- and oxygen. To date results of clinical trials are promising but more research needs to be performed before ALA-PDT can be used as a standard therapy.
The studies in this thesis were set up to explore underlying mechanisms of ALA-induced PPIX accumulation in tissues of patients with Barrett's oesophagus. In addition *in vitro* studies were performed to find tools to optimize ALA-PDT and provide a basis for further clinical studies.

In **chapter 1**, the pathophysiology, diagnosis and management of Barrett's oesophagus are described. Barrett's oesophagus is a metaplastic lesion in the distal oesophagus. The diagnosis is based on histology of endoscopically derived biopsies. Barrett's oesophagus seems to result from chronic gastro(duodeno)oesophageal reflux and has the potential to undergo further changes. Barrett's oesophagus can lead to the development of adenocarcinoma of the oesophagus through a sequence of progression from metaplasia to low-grade dysplasia, high-grade dysplasia and ultimately to invasive cancer. Although reflux is considered the major metaplasia-inducing factor, reducing acid exposure of the Barrett's segment does not influence the length nor the grade of dysplasia of Barrett's oesophagus and malignant degeneration can still proceed. Patients with low-grade dysplasia may undergo regular endoscopies. The majority of patients with high-grade dysplasia are often subjected to a prophylactic oesophagectomy. A non-invasive endoscopic ablative therapy could be an alternative to surgical resection.
The history of PDT dating from the end of the 19th century and its fundamentals are described. PDT requires the presence of a light sensitive compound (photosensitizer), light and oxygen. Absorption of light of an appropriate wavelength by the previously administered and accumulated photosensitizer leads to the formation of oxygen radicals, which oxidize cellular structures and ultimately lead to tissue destruction.
5-Aminolaevulinic acid (ALA), a naturally occurring intermediary of the haem biosynthetic pathway is a promising pro-drug for the photodynamic treatment of Barrett's oesophagus. Exogenous administration of the pro-drug ALA leads to intracellular

accumulation of the photosensitizer protoporphyrin IX (PPIX). Clinical studies into ALA-PDT in patients with Barrett's oesophagus showed promising results but also suggest that ALA-PDT needs to be improved.

Chapter 2 describes the results of a study performed in 27 patients undergoing an oesophageal resection. Nine patients had a squamous cell carcinoma, 18 had an adenocarcinoma of the distal oesophagus and in nine of these patients Barrett's epithelium was present. Samples from histological proven Barrett's mucosa, squamous cell carcinoma and adenocarcinoma as well as normal gastric mucosa and normal squamous epithelium were taken immediately after the resection. The grade of tumour differentiation and the grade of dysplasia in Barrett's epithelium were determined. The activities of two haem biosynthetic enzymes, porphobilinogen deaminase (PBG-D) and ferrrochelatase (FC) were measured. A PDT power index for ALA-induced porphyrin accumulation, the ratio between PBG-D to FC normalised for normal squamous epithelium of the oesophagus, was calculated to evaluate the intertissue variation in the ability to accumulate porphyrins.
A twofold increase in PBG-D activity was found in Barrett's epithelium and adenocarcinoma of the oesophagus compared with normal squamous epithelium. The increase in FC activity in these tissues was less marked than the increase in PBG-D activity, resulting in a significantly increased PDT power index in Barrett's oesophagus and adenocarcinoma compared with normal squamous epithelium.
This biochemical study has characterized the enzymatic capacities of haem biosynthesis in normal, premalignant and malignant tissue of the human oesophagus and provides evidence for selective accumulation of porphyrins after ALA administration. We suggested that the PDT power index might be a useful parameter for predicting the accumulation of porphyrins in tissues after ALA administration. Whether this will indeed be the case was studied in further studies described in **chapter 4** and **5**.

Photosensitivity of all tissues, including skin and mucosa, is a side effect of the administration of ALA to patients as porphyrins not only accumulate in cells of the target area but in all haem-producing cells. Before subjecting photosensitized patients to the bright lights in the operating room, we performed the study described in **chapter 3**. Acrylate yellow filters could theoretically reduce unwanted tissue damage when photosensitised patients are subjected to operating lights. In this study, the spectral power distribution of the operating lights and light energy densities with and without acrylate yellow filters were measured. Subsequently, the effects of light exposure on the survival of a human hepatocellular carcinoma cell line and the photodamage induced in pig tissues after the administration of 5-aminlaevulinic acid were studied. The light energy density in the ultraviolet and blue region of the light spectrum emitted by the operating light was

reduced by up to 50 per cent by the acrylate yellow filter. The survival of photosensitised cells was longer and photodamage induced in pig tissues was less when exposed to filtered light. It was therefore concluded that photodamage induced by operating lights could be reduced by filtering out ultraviolet and blue light by means of acrylate yellow filters.

In **chapter 4**, 5 -Aminolaevulinic acid-induced porphyrin biosynthesis was studied in tissues of 10 patients with Barrett's oesophagus (BE) and adenocarcinoma of the oesophagus (AC) undergoing oesophagectomy at a mean time interval of 6.7 hours after the ingestion of ALA (60 mg/kg). In BE, AC, squamous epithelium (SQ) and gastric cardia, - the activities of the haem biosynthetic enzymes, porphobilinogen deaminase (PBG-D) and ferrochelatase (FC) and the PDT power index - the ratio between PBG-D and FC in BE and AC in comparison with SQ - were determined before ALA ingestion. Following ALA administration, ALA, porphobilinogen, uroporphyrin I and PPIX were determined in tissues and plasma. The PDT power index did not predict the level of intracellular accumulation of PPIX found at 6.7 hrs. In BE, there was no selectivity of PPIX accumulation compared to SQ, whereas in half of patients with AC selectivity was found. Higher haem biosynthetic enzyme activities (i.e. PBG-D) and lower PPIX precursor concentrations were found in BE and AC compared to SQ. We therefore speculated that it is possible that PPIX levels will peak at earlier time intervals in BE and AC compared to SQ.

In **chapter 5** we investigated whether peak levels of PPIX actually occur at earlier time intervals after ALA ingestion in Barrett's oesophagus compared to normal oesophagus. Twenty six patients with BE were randomized to varying time intervals between ingesting 60 mg/kg ALA and undergoing an endoscopy with biopsies of BE and normal oesophageal and gastric mucosa. At 1, 2, 7, 8 and 24 hrs, two patients at each time and at 3, 4, 5 and 6 hrs after ALA ingestion, 4 patients at each time were included. ALA, porphyrin intermediates and PPIX were determined in the biopsy samples and also in plasma samples derived at various time intervals after ALA administration.
The maximum concentration of PPIX was found significantly earlier in BE (4.6 ± 0.5 hrs) than in SQ (6.6 ± 2.2 hrs) ($p < 0.05$). Concentrations of PPIX were higher in SQ than in BE especially at longer time intervals after ALA ingestion. In addition, tissue ALA concentrations were found to be 20-fold higher than the plasma concentrations at 1 hour after ALA ingestion, suggesting uptake from the oesophageal lumen. Skin photosensitivity is short-lasting but was often debilitating. These results provide a biological rationale for the use of ALA-PDT for the treatment of BE at 4-5 hrs after ALA ingestion (60 mg/kg) and for the local application of ALA in the oesophagus.

Chapter 6 describes an in vitro study in which the effect of ALA-PDT, using UV light and 633 nm laser light, on the activities of two haem biosynthetic enzymes porphobilinogen deaminase and ferrochelatase was studied in human EBV-transformed lymphoblastoid cell lines as a model of human tumour cells. Ferrochelatase activity was significantly inhibited by ALA-PDT, whereas the porphobilinogen deaminase activity remained unchanged. The extent of ferrochelatase inhibition was related both to the intracellular protoporphyrin IX concentration and to the time of UV light exposure. ALA-PDT therefore causes a relative block in haem biosynthesis by inhibiting ferrochelatase. We hypothesised that a short period of pre-illumination of ALA-treated porphyrin-loaded tissues might enhance porphyrin accumulation and a second illumination might result in a more pronounced ALA-PDT effect.

The hypothesis at the end of chapter 6 was explored in vitro for its credibility in **Chapter 7**. To examine the effect of ALA-PDT on PPIX accumulation and the effect of one or two laser treatments (two-phase ALA-PDT) on cell survival, we used EBV-transformed lymphoblastoid cells as a model of human tumour cells. ALA-treated cells were illuminated ones or twice with 633 nm laser light (100 mW/cm^2) or kept in the dark for the same time period. At selected times, the porphyrin concentrations and protein contents as a measure of cell death were determined. Porphyrin biosynthesis remained intact in EBV-transformed lymphoblastoid cells after a first episode of ALA-PDT. Following the first illumination, cells were able to accumulate significantly more PPIX than their non-illuminated controls. Two illuminations resulted in more cell death than one illumination. A two-phase illumination scheme may improve the clinical outcome of ALA-PDT.

In **Chapter 8** the findings described in this thesis are discussed.

Samenvatting

De incidentie van slokdarmkanker (m.n het adenocarcinoom van de slokdarm) neemt sneller toe dan van enig andere vorm van kanker in de Westerse wereld. Adenocarcinoom ontstaat in een Barrett slokdarm, een metaplastische verandering in het onderste deel van de slokdarm als reactie op chronische reflux van maag- en darmsappen. Anti-reflux medicamenten en moderne anti-reflux chirurgie beïnvloeden noch de lengte van het Barrett segment noch de graad van dysplasie en kwaadaardige ontaarding wordt niet voorkómen.
Een veelbelovende niet-invasieve behandelingsmethode voor Barrett slokdarm, die in dit proefschrift wordt bestudeerd, is fotodynamische therapie (PDT). Voor een weefselbeschadigend effect van PDT zijn de volgende factoren noodzakelijk: een lichtgevoelige stof -in onze studies protoporfyrine IX (PPIX) gevormd uit toegediend 5-aminolevuline zuur (ALA)- , licht van een specifieke golflengte -633 nm- en zuurstof.
De resultaten van de klinische studies, die tot nu toe zijn verricht, zijn veelbelovend. Het is echter noodzakelijk verder onderzoek te verrichten alvorens ALA-PDT standaard in de klinische praktijk kan worden toegepast.
De studies in dit proefschrift zijn verricht om de onderliggende mechanismen van ALA-geïnduceeerde stapeling van PPIX in weefsels van patiënten met Barrett slokdarm te bestuderen. Daarnaast zijn *in vitro* studies verricht om ALA-PDT verder te optimaliseren.

In **hoofdstuk 1** werd de pathofysiologie, de diagnostiek en de behandeling van Barrett slokdarm beschreven. Barrett slokdarm is een metaplastische verandering in het distale deel van de slokdarm. De diagnose is gebaseerd op de histologische diagnose in biopten die verkregen zijn d.m.v. endoscopisch onderzoek. Barrett slokdarm lijkt het resultaat te zijn van chronische reflux van maag- en darmsappen en heeft de potentie verdere veranderingen te ondergaan, die volgens de sequentie metaplasie, laaggradige dysplasie en hooggradige dysplasie kunnen leiden tot de vorming van een invasief adenocarcinoom. Hoewel reflux de belangrijkste metaplasie-inducerende faktor is, lijkt de reductie van zuurexpositie in de Barrett slokdarm, noch de lengte van de Barrett slokdarm, noch de gradering van dysplasie te beïnvloeden en kan een kwaadaardige verandering optreden. Patienten met laaggradige dysplasie ondergaan periodieke endoscopische controles. Het merendeel van de patienten met hooggradige dysplasie ondergaat momenteel een preventieve slokdarmresectie. Een niet-invasieve endoscopische therapie ter verwijdering van het Barrett slijmvlies zou een klinisch significant alternatief voor chirurgie kunnen zijn.
Voor een effect vereist PDT de aanwezigheid van een lichtgevoelige stof ("fotosensitiser"), licht en zuurstof. Absorptie van licht van een specifieke golflengte leidt tot de vorming van zuurstofradicalen, welke de oxidatie van allerlei celstucturen veroorzaken en leiden tot weefseldestructie.
5-Aminolevuline zuur (ALA), een natuurlijk voorkomende intermediair van de heemsyn-

these is een veelbelovende stof voor de fotodynamische behandeling van de Barrett slokdarm. Toediening van ALA leidt tot intracellulaire stapeling van de "fotosensitiser" protoporfyrine IX (PPIX). De resultaten van klinische studies naar de toepassing van ALA-PDT voor de behandeling van patiënten met Barrett slokdarm zijn veelbelovend, maar suggereren tevens dat ALA-PDT verder verbeterd dient te worden.

In **hoofdstuk 2** werden de resultaten beschreven van een studie, die verricht is bij 27 patiënten die een slokdarmresectie ondergingen. Negen patiënten hadden een plaveiselcelcarcinoom van de slokdarm, 18 hadden een adenocarcinoom van de slokdarm en bij 9 van deze laatste groep patiënten was tevens Barrett slijmvlies aanwezig. Biopten werden direct na de resectie genomen uit histologisch bewezen Barrett slijmvlies, plaveiselcelcarcinoom en adenocarcinoom alsmede van normaal maag- en slokdarmslijmvlies. De differentiatiegraad van de tumor en de graad van dysplasie van het Barrett slijmvlies werden microscopisch vastgesteld. Tevens werden de activiteiten van twee enzymen van de heemsynthese, nl. porfobilinogeen deaminase (PBG-D) en ferrochelatase (FC) gemeten. De "PDT power index" voor ALA-geïnduceerde porfyrine stapeling, dat is de ratio tussen PBG-D en FC genormaliseerd voor normaal slokdarmslijmvlies, werd berekend in bovengenoemde weefsels ter evaluatie van de variatie in de mate waarin deze weefsels porfyrines stapelen na ALA toediening.
Een tweevoudige stijging van de activiteit van PBG-D werd gevonden in Barrett slijmvlies en in adenocarcinoom vergeleken met normaal slokdarm slijmvlies. De toename van de activiteit van FC was minder uitgesproken dan die van PBG-D wat resulteerde in een significant verhoogde "PDT power index" in Barrett slokdarm en adenocarcinoom van de slokdarm vergeleken met normaal slokdarmslijmvlies.
Deze biochemische studie karakteriseerde de enzymatische capaciteit van de heemsynthese in normaal, premaligne en maligne weefsel van de humane slokdarm en leverde aanwijzigen voor een selectieve stapeling van porfyrines na ALA toediening. Op grond van deze studie was onze hypothese dat de "PDT power index" een waardevolle parameter zou kunnen zijn voor het voorspellen van de stapeling van porfyrines in weefsels na ALA toediening. Of dit inderdaad zo is, werd bestudeerd en beschreven in de **hoofdstukken 4** en **5.**

Lichtgevoeligheid van alle weefsels, inclusief huid en slijmvliezen, is een complicatie van het toedienen van ALA aan patiënten omdat porfyrines niet alleen in het te behandelen weefsel maar in alle heemproducerende cellen stapelen.
Voordat de lichtgevoelige patiënten werden blootgesteld aan het felle licht van de operatielampen tijdens een slokdarmresectie, zoals noodzakelijk was in de studie genoemd in hoofstuk 4, werd een studie verricht die beschreven is in **hoofdstuk 3**. Theoretisch zouden acrylaat geelfilters ongewenste weefselschade kunnen verminderen, die optreedt als lichtgevoelige patiënten worden blootgesteld aan het licht van operatielampen. In deze

studie werden het lichtspectrum en de lichtenergie die operatielampen uitstralen met en zonder geelfilter gemeten. Vervolgens werd het effect van de belichting op de overleving van een humane leverkankercellijn en de door licht geïnduceerde schade aan varkensweefsels na het toedienen van ALA bestudeerd. De hoeveelheid lichtenergie in het ultraviolette en blauwe deel van het lichtspectrum van de operatie lamp werd tot 50 procent gereduceerd door het acrylaat geelfilter. De overleving van lichtgevoelige cellen was langer en de schade aan varkensweefsels was minder uitgesproken wanneer deze waren blootgesteld aan gefilterd licht. Op grond van deze studie werd geconcludeerd dat de door operatielampen geïnduceerde weefselschade kan worden verminderd door ultraviolet en blauw licht uit het lichtspectrum te filteren middels een acrylaat geelfilter.

In **hoofdstuk 4** werd de door 5-aminolevuline zuur geïnduceerde porfyrine synthese bestudeerd in weefsels van 10 patiënten met een Barrett slokdarm (BE) en adenocarcinoom van de slokdarm (AC), die een slokdarm resectie ondergingen op een gemiddeld tijdtip van 6.7 uur na het drinken van ALA (60 mg/kg). In BE, AC, plaveiselepitheel (SQ) en maagcardia werden de activiteiten van de heemsynthese enzymen porfobilinogeen deaminase (PBG-D) en ferrochelatase (FC) en de "PDT power index" -de ratio tussen PBG-D en FC in BE en AC in vergelijking met SQ- vóór het toedienen van ALA bepaald. Na de toediening van ALA werden ALA, porfobilinogeen, uroporfyrine I en PPIX bepaald in bovengenoemde weefsels en in plasma. De "PDT power index" bleek geen voorspellende waarde te hebben voor de concentratie van gestapeld PPIX op het tijdstip van 6.7 uur na toediening van ALA. Er was tevens geen selectieve stapeling van PPIX in BE vergeleken met SQ, in tegenstelling tot AC, waar in de helft van de gevallen wel selectiviteit werd vastgesteld. In BE en AC werden verhoogde enzymactiviteiten (vooral PBG-D) en verlaagde PPIX voorloper moleculen gevonden in vergelijking met SQ. Op grond van deze studie speculeerden wij dat het mogelijk is dat maximum PPIX concentraties op een eerder tijdstip na het toedienen van ALA kunnen worden bereikt in BE en AC in vergelijking met SQ.

In **hoofstuk 5** werd onderzocht of de maximum PPIX concentraties inderdaad op een eerder tijdstip na het toedienen van ALA worden bereikt in BE in vergelijking met SQ. Zesentwintig patiënten met een Barrett slokdarm werden gerandomiseerd naar verschillende tijdsintervallen tussen het drinken van ALA (60 mg/kg) en het ondergaan van een endoscopisch onderzoek met afname van biopten uit BE en normaal slokdarm- en maagslijmvlies. Op 1, 2, 7, 8 en 24 uur werden 2 patiënten op ieder tijdstip en op 3, 4, 5, 6 uur werden 4 patiënten op ieder tijdstip na het toedienen van ALA geïncludeerd. ALA, porfyrine intermediairen en PPIX werden bepaald in biopten alsmede in plasma monsters afgenomen op verschillende tijden na ALA toediening.

De maximum concentratie van PPIX werd significant eerder gevonden in BE (4.6 ± 0.5 uur) dan in SQ (6.6 ± 2.2 uur) ($p< 0.05$). De concentraties van PPIX waren hoger in SQ dan in BE, vooral op latere tijdstippen na ALA toediening. Daarnaast bleken de weefsel ALA concentraties op 1 uur na ALA toediening 20 maal hoger dan de concentraties in plasma, wat ALA opname vanuit het slokdarmlumen in slokdarmcellen suggereert. Lichtgevoeligheid van de huid bleek kortdurend aanwezig maar werd vaak als zeer belastend ervaren. Deze resultaten verschaffen een biologische onderbouwing voor het gebruik van ALA-PDT voor de behandeling van BE op 4-5 uur na het toedienen van ALA (60 mg/kg) alsmede voor de lokale toepassing van ALA in de slokdarm.

In **hoofdstuk 6** werd een in vitro studie beschreven waarin het effect van ALA-PDT met ultraviolet (UV) licht en 633 nm laser licht op de activiteiten van de twee heemsynthese enzymen, porfobilinogeen deaminase en ferrochelatase werd onderzocht in humane Epstein Barr Virus (EBV)-getransformeerde lymfoblastoïde cellijnen, welke gebruikt werden als een model voor humane tumorcellen. De activiteit van ferrochelatase werd significant geremd door ALA-PDT, terwijl de activiteit van porfobilinogeen deaminase niet veranderde. Er was een relatie tussen de mate van ferrochelatase activiteit remming en de concentratie van PPIX alsook de duur van de belichting met UV licht. ALA-PDT veroorzaakte dus een relatieve blokkade in de heem synthese door de remming van ferrochelatase. Op grond van deze resultaten was onze hypothese dat een korte vóórbelichting van weefsel, dat na ALA-toediening porfyrines heeft gestapeld, de verdere stapeling van porfyrines zal bevorderen en een daaropvolgende tweede belichting zal resulteren in een optimaler ALA-PDT resultaat.

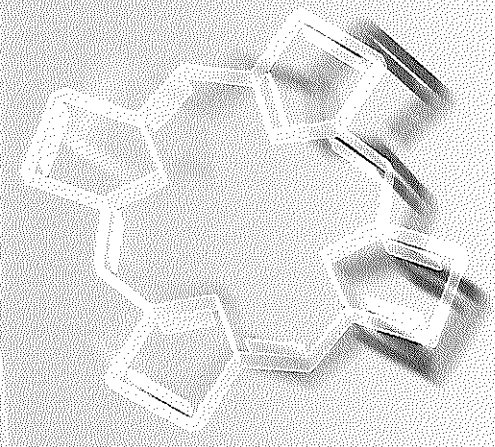
De hypothese opgeworpen aan het einde van hoofdstuk 6 werd getest in **hoofstuk 7**. Om het effect van ALA-PDT op de stapeling van PPIX en het effect van een of twee laserbehandelingen (twee-fase ALA-PDT) op de overleving van cellen te bestuderen, werden EBV-getransformeerde lymphoblastoïde cellen gebruikt als een model voor humane tumorcellen. Cellen die met ALA behandeld waren, werden een of twee maal belicht met 633 nm laser licht of bleven gedurende een zelfde tijdsperiode in het donker. Op geselecteerde tijdstippen werden de porfyrine concentraties en de eiwit gehalten, als maat voor celdood, bepaald. De porfyrine synthese bleef intact na de eerste belichting. Tevens stapelden de belichte cellen meer PPIX dan hun niet-belichte controles. Twee belichtingen resulteerden in meer celdood dan een enkele belichting. Aan het einde van dit hoofdstuk speculeerden wij dat een belichtings schema met twee belichtingen de klinische resultaten van ALA-PDT wellicht zouden kunnen verbeteren in vergelijking met slechts een enkele belichting.

In **hoofdstuk 8** worden de bevindingen, beschreven in dit proefschrift, bediscussiërd.

1 2 3 4 5 6 7 8 9 10

11

Addendum



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Curriculum Vitae

Petra Hinnen was born on March 10, 1970 in Coevorden and lived in Schoonebeek until she started her medical training at the Erasmus University Rotterdam in 1988, where she graduated in 1993. During her medical studies she worked on a research project in the Eye Hospital Rotterdam during nine months, concerning congenital cataract in children. Before obtaining her medical degree in 1996, she traveled for five months in South-East Asia.

In may 1996 she started the work described in this thesis at the Department of Gastroenterology & Hepatology and Internal Medicine II of the Erasmus Medical Centre in Rotterdam in collaboration with the Department of Surgery and Pathology.

In January 2000 she started her residency in Internal Medicine at the Reinier de Graaf Hospital, Delft as part of her specialty training in Internal Medicine.

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List of Abbreviations

AC	adenocarcinoma
ALA	5-aminolaevulinic acid
ALA-S	5-aminolaevulinic acid synthase
ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
BE	Barrett's esophagus
DMSO	dimethylsulfoxide
EBV	Epstein Barr virus
FC	ferrochelatase
FCS	fetal calf serum
F.U.	fluorescence units
GC	gastric cardia
HGD	high-grade dysplasia
HPLC	high performance liquid chromatography
Hp	haematoporphyrin
HpD	haematoporphyrin derivative
KTP	potassium titanium phosphate
LGD	low-grade dysplasia
ND	no dysplasia
Nd-Yag	neodymium:yttrium-aluminium-garnet
PBG	porphobilinogen
PBG-D	porphobilinogen deaminase
PDT	photodynamic therapy
PEB	protoporphyrin IX extraction buffer
PPIX	protoporphyrin IX
SD	standard deviation
SEM	standard error of the mean
SQ	squamous epithelium
UEB	uroporphyrinogen I extraction buffer
URO	uroporphyrinogen
URO-S	uroporphyrinogen III synthase
UV	ultraviolet