SARI GRÖNLUND-PAKKANEN

Photodynamic Therapy with Aminolevulinic Acid Induced Protoporphyrin IX in the Treatment of Superficial Bladder Cancer

An Experimental Study

Doctoral dissertation

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ABSTRACT

A series of experimental studies were carried out in order to study photodynamic therapy and to improve the currently available treatment modalities in superficial bladder cancer. Photodynamic therapy (PDT) is a new anticancer technique directed at the selective destruction of neoplastic tissue. It is an alternative intravesical therapy modality for the treatment of superficial bladder cancer. Aminolevulinic acid (ALA), a precursor in the biosynthesis of heme, induces the production of the endogenous photosensitizer protoporphyrin IX (PpIX). In the present study, the fluorescence distribution of ALA-induced PpIX was first investigated in the normal rat bladder wall by fluorescence microscopy. The fluorescence studies showed that PpIX concentrates effectively in bladder mucosa with the highest fluorescence difference in PpIX accumulation in mucosa to the muscularis layer being achieved at 4 hours after intravenous administration.

We also compared intravenous versus intravesical administration of ALA in the bladder of a rat model with orthotopic urothelial carcinoma. The highest fluorescence difference in PpIX accumulation in tumor and the normal epithelium to the muscularis layer was achieved at 2 hours with intravenous administration (3:1 to 5:1). After intravesical administration, no difference in PpIX accumulation in tumor and normal urothelium was observed. However there was a 7:1 ratio with regard to the muscularis layer at 4 hours. According to the results of this study a difference in PpIX accumulation in urothelial carcinoma or normal urothelium and the muscular layer of the bladder can be achieved by each route of ALA administration.

The second goal of the present study was to assess the optimum light energy dose with the used dosage of ALA to induce only superficial bladder wall damage during PDT. PDT on urinary bladder of rats was performed using an argon-ion laser as an energy source, with ALA-induced PpIX photosensitizer. Four hours after the intravesical administration of ALA, the bladders were intravesically exposed to 20-80 J/cm² light. ALA 20-40 J/cm² light caused mainly superficial damage whereas 80 J/cm² produced full-thickness injuries to the bladder wall. Three weeks after PDT, the histology showed full-thickness injuries in half of those animals treated with 80 J/cm² light.

We also studied the functional changes in the bladder after PDT. Before and after PDT, the bladder capacity and pressure changes in filling cystometry were assessed. The light dose of 20 and 40 J/cm² with ALA caused no reduction in bladder capacity whereas 80 J/cm² light dose produced up to 50% reduction in the capacity at 3 weeks postoperatively. These results indicate that the proper dosing of photosensitizer and light energy, PDT is a safe treatment modality. The functional impairment of urinary bladder function is only transient.

The major objective of the present study was to assess the effect of PDT to treat superficial bladder cancer. The animals were instilled intravesically with AY-27 tumor cells and at day 15 the cytologic examination of the sediment of urine was made in order to detect the presence of tumors of the bladder just before PDT. After the intravenous or intravesical administration of ALA, the bladders were intravesically exposed to 40 J/cm² light. The morphological changes induced in the bladder wall were analyzed using light microscopy after 3 days or 3 weeks. The results of intravesical administration of ALA were controversial. PDT with intravesical administration of ALA was an effective method for treating superficial bladder carcinoma. These findings support the proposal that PDT can be viewed as a safe therapy of superficial bladder cancer.

National Library of Medicine Classification: WB 480, WJ 504, WJ 166
Medical subject headings: photochemotherapy; aminolevulinic acid; 5-aminolevulinate; fluorescence; bladder neoplasms; cytology; rats
To Valtteri and Joonatan
ACKNOWLEDGEMENTS

This study was carried out in the Department of Surgery of Kuopio University Hospital. I am grateful to Professor Esko Alhava, who has given his firm support and has trusted that this study would be ready after all those technical troubles and hardships during these years. It is a privilege for me to be worthy of this confidence.

Docent Martti Talja was the main supervisor of this thesis. His experience and practical solutions have been of enormous benefit to me in these experimental studies. His perfectionism and demanding attitude have upset me at times but perhaps this was the only way to initiate me into the scientific world. I am greatly indebted to him.

I wish to thank Docent Martti Ala-Opas, an another vital supervisor of this study. His ability to be a constant source of encouragement has helped me greatly in completion of this work.

I am deeply grateful to Professor Ronald B. Moore from University of Alberta, who provided the bladder tumor model which made it possible to bring this study to fruition. His great experience and our pleasant co-operation have taught me great deal about PDT.

My warmest thanks belong to Timo for being such a good father to our lovely sons during these years. Timo always was able to solve all of my computer problems. His patience in guiding me through those technical labyrinth has taught me to take my time and I have now learned many useful skills for the future work with computers.

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During these years of this study I have gone through many changes in my every day life. I became mother to little twinsons, Valtteri and Joonatan. The first two years after their birth were full of fear, hopelessness, anxiety and concern about Valtteri’s life and growth. These years taught me more than anything in my entire life not to give up as long as there is any hope left. The joyful and superb sons I now have are constant reminders to me of the real meaning of life. With all my heart, I thank our Maija for the loving and endless care which our boys and the whole family have received. Without her, my career and also this study would never have come to fruition. My warmest thanks belong also to Ulla ja Matti for being the most unselfish and loving grandparents to the boys during all these years.

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Finally, loving thanks to my parents Sinikka and Gideon and to my brother Jari for their support, encouragement and love over the years. You have inspired me with your own example to believe and trust in the future.

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Kuopio, April 2002

Sari Grönlund-Pakkanen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>aminolevulinic acid</td>
</tr>
<tr>
<td>AISPC</td>
<td>aluminium sulfonated phthalocyanine</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BPD MA</td>
<td>benzoporphyrin derivative monoacid</td>
</tr>
<tr>
<td>CASPC</td>
<td>chloro-aluminium sulfonated phthalocyanine</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CCD</td>
<td>charge coupled device</td>
</tr>
<tr>
<td>DHE</td>
<td>dihematoporphyrin ether</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HpD</td>
<td>hematoporphyrin derivative</td>
</tr>
<tr>
<td>INF</td>
<td>interferon-α2b</td>
</tr>
<tr>
<td>KTP</td>
<td>potassium-titanyl-phosphate</td>
</tr>
<tr>
<td>MMC</td>
<td>mitomycin C</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>neodymium:yttrium aluminium garnet</td>
</tr>
<tr>
<td>NPe6</td>
<td>n-aspartylchlorine e6</td>
</tr>
<tr>
<td>PII</td>
<td>Photofrin II</td>
</tr>
<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
</tr>
<tr>
<td>PpIX</td>
<td>protoporphyrin IX</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SnEt2</td>
<td>tin etiopurpurin</td>
</tr>
<tr>
<td>TCC</td>
<td>transitional cell carcinoma</td>
</tr>
<tr>
<td>Tis</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>TmHPC</td>
<td>tetra(m-hydroxyphenyl)chlorin</td>
</tr>
<tr>
<td>TUR</td>
<td>transurethral electroresection</td>
</tr>
<tr>
<td>ZnPc</td>
<td>zinc phthalocyanine</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

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1. INTRODUCTION

Bladder cancer is the fifth most common malignancy in Europe (Jensen et al. 1990), and the fourth most common in the USA. There are over 250,000 new cases reported annually on a worldwide basis (Thrasher et al. 1993). In Finland, bladder cancer is the fourth most common malignancy among men and the fifteenth most common form of cancer in women. A total of 800 new cases were diagnosed in Finland in 1999 (Cancer Registry 2002). The workload created by bladder cancer is however greatly increased by its prevalence, since 60-70% of superficial bladder tumors will recur in spite of appropriate treatment (Greene et al. 1973; Soloway 1980; Hisataki et al. 2001). Indeed, many patients have frequently recurring tumors requiring multiple treatments over many years. In the future, it is to be anticipated that with ageing of the developed world’s population along with factors such as smoking, this upward trend will continue to increase (Messing et al. 1998).

Superficial bladder cancer represents approximately 80% of primary bladder tumors and includes those lesions which are limited to the urothelium (stage Ta) and submucosa (stage T1). In the main these have a good prognosis despite their high recurrence rate (van der Meijden 1998). While most of the superficial tumors have no significant effect on the life expectancy of the patients, approximately 10-15% progress to a muscle-invasive and thus potentially lifethreatening disease (Klemeny et al. 1993; Levi et al. 1993).

Treatments other than surgery, radiotherapy and adjuvant chemo-or immunotherapy are not established in clinical practice for treatment of superficial bladder cancer. Nevertheless, there are many innovative approaches currently being investigated with the hope of reducing recurrence whilst causing less treatment morbidity. Of these, photodynamic therapy (PDT) is one of the most promising (Shackley et al. 1999). PDT is based on an interaction between a photosensitizer and absorbed light in the presence of oxygen to destroy tissue by lipid peroxidation. The monochromatic light is produced by laser devices. The relative selective retention of photosensitizer within or around tumor cells may allow more selective destruction of neoplasm and limited damage to adjacent or systemic normal tissues (Manyak 1991). The endogenous photosensitizer protoporphyrin IX (PpIX) is an immediate precursor of heme in the biosynthetic pathway of heme. The rate of synthesis of PpIX is determined by the rate of synthesis of 5-aminolevulinic acid (ALA). Exogenous ALA bypasses the feedback control, and thus induces the intracellular
accumulation of photosensitizing concentrations of PpIX. The resulting tissue-specific accumulation of photosensitizer forms the basis for using ALA-induced PpIX for PDT (Kennedy et al. 1992).

The purpose of this experimental study was first to determine the best delivery of ALA, i.e. either intravenous or intravesical administration to achieve the optimal distribution of photosensitizer in the normal bladder layers and in orthotopic urothelial carcinoma tissue. The second aim was to assess the optimum light energy needed to induce only superficial bladder wall damage during PDT. These results open up possibilities for further clinical work to provide an effective alternative to current treatment modalities in treating recurrent and aggressive superficial bladder cancer.
2. REVIEW OF THE LITERATURE

2.1 Urinary bladder cancer

2.1.1 Epidemiology

2.1.1.1 Incidence and prevalence
The incidence of carcinoma of the urinary bladder is rising in western countries. Thus in Finland, the age-adjusted incidence rate was 8.0 in men/100 000 inhabitants in the period 1963 to 1967 but has increased to 14.7 by 1998. Lower urinary tract cancers were the fourth most common malignant disease in Finnish men in 1999 and 600 reports of new lower urinary tract cancers in men and 200 in women were filed in Finland in that year. The male:female ratio is approximately 3:1. The peak of incidence of the bladder cancer occurs in patients aged 65 to 75 years. The annual number of deaths from bladder cancer was 210 in 1997 (Pukkala et al. 1997; Cancer Registry 2002).

2.1.1.2 Etiology
Smoking is the most prominent risk factor related to the development of urothelial cancer of the bladder. Smoking increases the risk 4-10 fold in comparison to nonsmokers. The risk correlates with the number of cigarettes smoked, the duration of smoking, and the degree of inhalation of smoke (Salminen et al. 1994; Sorahan et al. 1994; Raitanen et al. 1995a). The results of various studies have suggested approximately 40 occupations in which workers could be at an increased risk for bladder cancer. In particular, aromatic amines and some mineral oils has been reported to induce bladder cancer (Howe et al. 1980; Vainio 1987; Steineck et al. 1990; Pirola et al. 1991; Cordier et al. 1993). Further, alkylating agents such as cyclophosphamide, may increase the risk of transitional cell bladder cancer (Durkee et al. 1980; Fernandes et al. 1996).

Chronic cystitis in the presence on indwelling catheters or calculi is associated with an increased risk for squamous cell carcinoma of the bladder (Locke et al. 1985). Patients with schistosomiasis develop a chronic inflammation and regenerative process of the urinary bladder which may also later lead to the development of squamous cell cancer (Lucas 1982; Stock et al. 1994).
Pelvic irradiation is also a risk factor, since women treated with radiation therapy for carcinoma of the uterine cervix have a nearly fourfold risk of developing bladder cancer (Sella et al. 1989).

2.1.2 Staging of bladder cancer

2.1.2.1 TNM system and staging

The TNM system for clinical staging of bladder tumors, formulated by the International Union Against Cancer (UICC), facilitates comparison of treatment results at different centres. The third edition of the TNM classification (UICC 2001) is presented in Table 1.

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Pre-invasive carcinoma, carcinoma in situ</td>
</tr>
<tr>
<td>Ta</td>
<td>Papillary non-invasive carcinoma</td>
</tr>
<tr>
<td>T1</td>
<td>Carcinoma with microscopical infiltration of the lamina propria but not the detrusor muscle. May be palpable as a free mobile mass before, but not after transurethral resection</td>
</tr>
<tr>
<td>T2</td>
<td>Carcinoma with microscopical infiltration of the superficial muscle. A mobile induration may be palpable before but not after transurethral resection</td>
</tr>
<tr>
<td>T3a</td>
<td>Carcinoma with microscopical infiltration of the deep muscle</td>
</tr>
<tr>
<td>T3b</td>
<td>Carcinoma with microscopical infiltration through the bladder wall. A mobile mass is palpable after transurethral resection of the exophytic tumor</td>
</tr>
<tr>
<td>T4a</td>
<td>Carcinoma involving the prostate, uterus or vagina</td>
</tr>
<tr>
<td>T4b</td>
<td>Carcinoma fixed to the abdominal or pelvic wall</td>
</tr>
<tr>
<td>N</td>
<td>Lymph node involvement (N0 without, N1-4 with)</td>
</tr>
<tr>
<td>M</td>
<td>Distant metastases (M0 without, M1 with)</td>
</tr>
</tbody>
</table>

2.1.2.2 Histopathology

In the WHO classification, TCCs are placed into three grades based on the degree of anaplasia (Mostofi et al. 1973). In this grading system, grade 1 tumors have the least atypia, grade 2 tumors consist of an intermediate group and grade 3 tumors are anaplastic.
A new grading system has been the basis for the revised grading in the new edition of the WHO classification of bladder tumors (Epstein et al. 1998). The term TCC has been replaced by the term urothelial carcinoma. Also, the assessment of tumor differentiation has been changed. Within papillary tumors, a new group of papillary urothelial neoplasms with low malignant potential has been added between benign papillomas and low grade papillary urothelial carcinomas. In this grading system, it was also suggested that urothelial carcinomas should be divided into two grades, low grade and high grade carcinomas. In the new edition of the WHO classification, however, the division of urothelial carcinomas into three grades has been maintained (Mostofi et al. 1999).

2.1.2.3 Carcinoma in situ (Tis)

Tis is a flat proliferation of malignant urothelial cells confined to the epithelium of the mucosa which is verified cytologically or histologically by the TNM classification (UICC 2001). Tis presents clinically in three different ways (Torti et al. 1984). The lesion may appear de novo in patients with no prior history of bladder cancer, so called primary Tis. More commonly Tis appears immediately adjacent to a current superficial papillary tumor or nodular invasive tumor, concomitant Tis. Secondary Tis occurs on a follow-up of an earlier urothelial carcinoma. Unlike papillary tumors, Tis is an aggressive carcinoma and has a considerable potential for invasiveness (Althausen et al. 1976; Wolf et al. 1983; Koss et al. 1985).

2.1.3 Prognostic factors

2.1.3.1 General

The importance in prognostic factors lies in the need to identify the patients who will have tumor recurrence and, more importantly, those who will develop progression into an invasive or metastatic disease requiring more aggressive therapy. The available prognostic factors in superficial bladder cancer are useful for identifying low and high-risk subgroups but the predictive value of tumor stage, grade and multiplicity has been found to be inaccurate (Kiemenej et al. 1993; Herr 1997).
2.1.3.2 Stage

Tumor stage is the most commonly used method for prognosis. The degree of bladder wall invasion, and the presence of lymph node and distant metastases are the most important determinants of the treatment regimen and outcome (Heney et al. 1983; Malmström et al. 1987; Abel et al. 1988). The 5-year risk of stage progression from Tis to invasiveness or metastases is 60-70% without adjuvant treatment modalities (Utz et al. 1980; Herr et al. 1986), and the 5-year survival rate is approximately 60% (Utz et al. 1980). Lamina propria invasion (T1) has a clear impact on the risk of first recurrence (Kieneck et al. 1993), the recurrence-free rates (Heney et al. 1983; Parmar et al. 1989), and the risk for the development of muscle invasion (Malmström et al. 1987). It has been proposed that pT1 tumors should be substaged to pT1a, pT1b and pT1c tumors according to the level of lamina propria invasion (Holmång et al. 1997; Hermann et al. 1998; Smits et al. 1998).

2.1.3.3 Grade

The tumor's histological appearance (grade) can indicate its potential to infiltrate and spread. The subjective grading methods are based on the evaluation of special cellular features and some estimates of the presence of mitotic figures in the sections (Mostofi et al. 1973). The significance of grade as a prognostic factor is, however, controversial. In superficial bladder cancer, the risk of progression and mortality is 2-5% in grade 1, 11-16% in grade 2 and 24-45% in grade 3 (Heney et al. 1983; Flamm et al. 1990).

2.1.3.4 Prognostic tumor markers

To obtain a better estimate of the behavior of an individual superficial bladder cancer, the use of a prognostic panel including the assessment of cell proliferation activity and determination of p53, bcl-2, as well as epidermal growth factor receptor expressions seems to be beneficial in the selection of appropriate treatment and follow-up strategies (Liukkonen 2000).

2.1.3.5 Mucosal dysplasia

The risk for progression is greatly elevated by the presence of concomitant urothelial abnormalities, severe dysplasia and Tis. From Ta/T1 tumors, 33% with moderate to
severe dysplasia in the adjacent mucosa progressed to muscle-invasive disease compared to 8% with normal adjacent mucosa (Heney et al. 1983).

2.1.3.6 Tumor multiplicity and location

The patients with multiple tumors show higher rates of progression (Lutzeyer et al. 1982; Kiemeney et al. 1993). The recurrence time is shorter and recurrence count higher in patients with multifocal tumor at diagnosis (Heney et al. 1983; Parmar et al. 1989). The recurrence of multiple tumors during follow-up predicts also a poor outcome of the disease (Holmäng et al. 1995).

The bladder neck involvement, tumor grade and stage were the best predictors of progression in an independent manner. The progression-free rate at 10 years was 99% when a tumor was found of pTa, grade 1-2 and without bladder neck involvement. In the case of pT1G3 tumor with bladder neck involvement, the 10 year progression rate was 37% (Fujii et al. 1998). Papillary status in addition to tumor grade is also a predictor of progression. Flat tumors are more aggressive at invading muscular tissue (Lipponen et al. 1991a)

2.1.3.7 Recurrence rate

The recurrence rate, i.e. the number of recurrences per patient-year prior to adjuvant instillation treatment has a significant impact on the subsequent recurrence rate and progression (Lutzeyer et al. 1982; Jauhiainen et al. 1990). The presence or absence of recurrence at the 3 month check cystoscopy provides an important due to the future progress of a patient’s disease. If no recurrence has been found at 3 months, there was virtually an 80% chance that the patient would never have another recurrence. On the other hand, if there was a recurrence at 3 months, there was only a 10% chance that the patient would have no further recurrences but also a 35% chance having recurrences consistently (Fitzpatrick et al. 1986).

2.1.3.8 Smoking

Few reports on the effect of smoking on the prognosis of bladder cancer have been published. Thompson et al. (1987) found a significant association between smoking and
stage and grade. A higher risk for recurrences among smokers has been suggested and cessation of smoking is associated with a clear drop in the risk of bladder cancer development (Thompson et al. 1987; Carpenter 1989). Quitting smoking was followed by a reduction in risk of about 50% within 3 years, suggesting that smoking might act as a tumor promotor in bladder carcinogenesis (Vineis et al. 1988). Raitanen et al. reported that 27% of the non-smokers and 40% of the smokers died during the first 10 years after diagnosis (Raitanen et al. 1995a).

2.1.3.9 ABO(H) blood groups

The ABO(H) blood group antigens are found not only on blood cells but also on the surface of many endo- and epithelial cells. They have been reported to be absent or reduced in a number of malignant tumors (Hakomori 1975). Patients with type O blood with superficial bladder tumors have a higher incidence of high-grade tumors and cancer progression (Orhuela et al. 1987). Also a higher mortality rate (Srinivas et al. 1986) and a higher incidence of tumor invasion (Kvist et al. 1989) have been observed in patients with the blood group O.

2.1.3.10 Cell proliferation rate and mitotic indices

The proliferation rate in superficial bladder cancer cells seems to provide the best prognostic estimates (Lipponen et al. 1992). The cell proliferation has been analysed with flow cytometry, mitotic indices, and expression of various cell cycle regulating proteins (Wijkström et al. 1987; Lipponen et al. 1991b; Lipponen et al. 1992). The number of mitotic figures in neoplasms has also prognostic significance. Lipponen and co-workers stated that in superficial TCC bladder cancer, the mitotic index is the best histological prognostic variable (Lipponen et al. 1992).

2.1.3.11 Flow cytometric analysis (DNA ploidy and S-phase fraction)

Grade 1 tumors have usually a diploid DNA content while almost every poorly differentiated tumor is aneuploid; grade 2 tumors are virtually evenly split between aneuploid and diploid. Ploidy is also strongly correlated with T-category (Tribukait et al. 1982; Blomjous et al. 1988). DNA-ploidy is also strictly correlated with histological grading
(Campanella et al. 1992). Tis is uniformly found to be aneuploid (Tribukait et al. 1982). A total of 75% of diploid and 45% of aneuploid tumor patients survived 10 years (Lipponen et al. 1993). S-phase fraction (SPF) is based on the fraction of tumor cells in the DNA synthesis phase. Flow cytometric quantitation of cell proliferation is very cell cycle-specific, semiautomatic and fast. It also uses a large number of nuclei in the analysis. However, the analysis lacks morphological control and though intratumor variation, tumor necrosis and non-tumoral cells may significantly weaken the reliability of this method (Sallinen et al. 1994). A high SPF has been associated with decreased survival, even in patients with superficial cancer, as well as with recurrence-free survival (Lipponen et al. 1993).

2.2 Diagnostic methods

2.2.1 Symptoms

Intermittent and painless hematuria is the commonest presenting symptom of bladder cancer, occurring in approximately 85% of patients (Varkarakis et al. 1974). In reality, nearly all patients with cystoscopically detectable bladder cancer have at least microhematuria. Hematuria is often intermittent so that a negative result in one or two specimens has little meaning in ruling out the presence of bladder cancer (Messing et al. 1998). Urinary frequency, dysuria, lower abdominal pain, urgency, irritability in the absence of infection and interstitial cystitis are reported by one third of patients with high grade bladder cancer (Utz et al. 1980).

2.2.2 Urine cytology

Urine cytology is a non-invasive test to the diagnosis of bladder cancer. Cystoscopy of the bladder may be normal, but the bladder epithelium, upper urinary tract, prostatic ducts or stroma, or urethra may still produce neoplastic cells which may be found with cytology (Bane et al. 1996). The sensitivity of cytology varies from 37-50% (low-grade tumors) to near 100% (TIS, high grade sessile neoplasms) (Utz et al. 1980; Murphy et al. 1984; Koss et al. 1985). The rate of false positive cytological findigs ranges currently from 3 to 15%. Urinary calculi, infection, instrumentation, and radiotherapy may impact on the accuracy of cytology. Also, instillation treatment can cause changes in the normal urothelium which may mimic malignant cytology (Murphy et al. 1984).
2.2.3  Urine tumor markers

The BTA stat test and its newer modification BTA TRAK are one-step, non-invasive and rapid immunochromatographic assays that detect bladder-tumor-associated antigen in human urine (Raitanen et al. 1999). The antigen detected by the BTA stat test has been identified as human complement factor H related protein (hCFHrp). The BTA stat test can be performed in 5 min and without pretreatment of the voided urine sample (Sarosdy et al. 1997). The sensitivity of this test is superior to that of urine cytology in detecting primary and recurrent tumors of the urinary bladder. To avoid unnecessary, costly invasive further investigations in patients with a false-positive BTA stat test, it is suggested that the test should not be used in patients who have received Bacillus Calmette-Guérin (BCG), and those with any kind of instillation treatment (Raitanen et al. 2001).

2.2.4  Cystoscopy, biopsy and photodynamic diagnosis

Urethrocystoscopy is a basic and obvious method for diagnosing the bladder cancer. It can assess the extent of the bladder cancer and the condition of the bladder and urethral mucosa. Accurate pathological staging requires enough deep biopsy taken with a resectoscope including the muscle of the bladder wall (See et al. 1992; Soloway et al. 1992). If a papillary tumor is present within suspicious areas of dysplasia or TIS and there is a malignant cytology of the urine, random biopsies are indicated. TIS is a tumor which cannot be seen macroscopically in 30-50% of the patients using conventional cystoscopy under white light (Wolf et al. 1983; Wolf et al. 1987; Norming et al. 1991).

ALA-derived PpIX fluorescence cystoscopy may be useful for detecting the precise sites of bladder urothelial lesions, especially in cases of suspicious or positive urine cytologic findings (Peng et al. 1997a; Zaak et al. 2001). In a clinical work with bladder carcinoma, the sensitivity of the ALA-derived PpIX fluorescence cystoscopy in detection of neoplastic urothelium was 96.9%, which was significantly higher than that of conventional white light cystoscopy (72.7%) (Kriegmair et al. 1996). Intravesical instillation of a pH-neutral 3% ALA solution is used for 2-3 hours followed by fluorescence cystoscopy with violet light from a krypton ion laser (406.7 nm) for excitation of ALA-derived PpIX. The sharply marked red fluorescence induced from ALA in the urothelial lesions can be easily observed with the naked eye during the fluorescence cystoscopy. The mean ratio of fluorescence intensity between urothelial carcinoma and normal urothelium is 17:1. The fluorescence microscopy reveals that the PpIX fluorescence is limited mainly to the
urothelial layer and little is detected in the submucosal or muscle layers of the bladder wall (Kriemlar et al. 1994; Steinbach et al. 1994; Kriemlar et al. 1995a).

Suspicion of a tumor in the bladder neck, prostate or urethra should be followed by an examination with transurethral electroresection (TUR)-biopsies (Herr et al. 1990; Soloway et al. 1992).

2.2.5 Radiographic urinary tract evaluation

To exclude concomitant upper tract disease and ureteric obstruction an intravenous urography is necessary. Ureteral obstruction caused by a bladder tumor is normally a sign of a muscle invasive cancer (Messing et al. 1998).

To confirm upper tract lesions or a site of obstruction retrograde pyelography may be helpful (Messing et al. 1998). Transabdominal ultrasound can detect bladder tumors but has been of minimal value and it cannot replace cystoscopy (Brun et al. 1984). Intravesical ultrasound during bladder endoscopy has value in evaluating the degree of tumor invasion into the bladder wall (Salo 1987). Computed tomography scanning assess the extent of the primary tumor and also provides information about the presence of pelvic and para-aortic lymphadenopathy and the possible presence of liver and/or adrenal metastases (Lantz et al. 1984). However, it can detect only gross extravesical tumor extension and is rather inaccurate in detecting nodal metastases (Nurmi et al. 1988). Magnetic resonance imaging scanning is not much more helpful than computed tomography scanning in the diagnosis of bladder cancer (Messing et al. 1998).

2.3 Treatment of superficial bladder cancer

2.3.1 General

TUR and fulguration are usually performed for diagnosis and treatment of superficial bladder cancer (Soloway 1983a). The initial treatment decision is based on the histopathological grading of the tumor and on tumor multiplicity (Gilbert et al. 1978). Patients with a single low grade tumor have a minimal risk of recurrence and progression and may be treated with TUR alone (Soloway 1983b; Prout et al. 1992). However, the risk of recurrence for patients treated only with TUR is 50-70% (Lutzeyer et al. 1982;
Parmar et al. 1989; Soloway et al. 1992; Kiemenej et al. 1993; Hudson et al. 1995). Intravesical instillation treatment is the mainstay in the management of recurrent superficial bladder cancer with three specific goals. Those include treating existing/residual tumor, preventing recurrence of tumor after complete bladder tumor resection, and preventing progression of disease (Utz et al. 1980; Klän et al. 1991; Lamm et al. 1992; Rajala et al. 1999).

When a tumor is invasive, or superficial but not controllable with conservative measures, radical treatment is indicated. Total cystectomy has been recommended even as the initial treatment of Tis (Utz et al. 1980; Droller et al. 1985). However, it appears that PDT can enable 31% of patients whose only remaining option is radical cystectomy to retain their bladders for up to one year without undue risk of disease progression (Nseyo et al. 1998).

2.3.2 Intravesical chemotherapy

Differences in patient selection, treatment regimens and response criteria all complicate any comparison between chemotherapeutic agents. The benefit of thiopeta is 12%, of doxorubicin 13% and of mitomycin C (MMC) 15% for the prevention of recurrence compared to controls according to a review of controlled studies (Lamm et al. 1995).

Thiotepa was the first modern intravesical chemotherapeutic agent introduced in the early 1960’s (Jones et al. 1961). The efficacy of thiopeta in inhibiting recurrence following TUR has been demonstrated (Oravisto 1972; Prout et al. 1983; Zinke et al. 1983). However, thiopeta has no value in the treatment of Tis (Koontz et al. 1981).

Doxorubicin is an antineoplastic anthracycline antibiotic which is effective in the treatment of Tis (Jakse et al. 1980; Jauhiainen et al. 1987). Epirubicin, 4-epi-doxorubicin, have both been reported to be similarly effective, but they are less myelotoxic and cardiotoxic and thus have better tolerance than doxorubicin (Whelan et al. 1991; Raitanen et al. 1995b). However, also a 32% incidence of side effects necessitating therapy withdrawal in patients treated with epirubicin has been shown (Cumming et al. 1990).

MMC is an alkylating antibiotic that affects cell function by inhibiting DNA synthesis (Soloway 1983b). MMC has been found to have a significant benefit to TUR alone with regard to the recurrence rate and reduced tumor progression (Huland et al. 1985; Jauhiainen et al. 1987). MMC may eradicate residual tumors and is effective in ablative therapy of superficial bladder cancer in 69% of the patients (Maffezzini et al. 1996). MMC
has also been shown to be effective in patients who have failed to benefit from thiotepa treatment and complete responses of 20-27% have been reported. MMC is generally well tolerated (Soloway 1983b; Jauhiainen et al. 1987).

2.3.3 Intravesical immunotherapy

BCG is a tuberculosis vaccine and has been administered intravesically to treat superficial bladder cancer since 1976 (Morales et al. 1976). The precise mechanism of action of BCG is still largely unknown, but it is assumed that the anti-tumor effect is produced by both an inflammatory reaction and an immune response (Brosman 1984; Böhle et al. 1993). The effect of BCG is approximately 20% higher compared with the most effective chemotherapeutic agents (Rajala et al. 1999). BCG instillations seem to be superior to doxorubicin and MMC in terms of complete response in treating carcinoma in situ of the bladder. It has also been considered to be the most effective intravesical agent in high risk patients providing long-term protection against tumor recurrences (Lamm et al. 1991; Malmström et al. 1999; Rajala et al. 1999).

The most common side-effect of BCG therapy is bladder irritability. These mild or moderate irritative bladder symptoms resolve generally without treatment in few days and occur in 50 to 90% of patients on BCG instillations (Lamm et al. 1989). More severe side-effects requiring antituberculous therapy occur in up to 6% (Lamm et al. 1986). In general, BCG treatment must be withdrawn in 4 to 20% of the patients due to adverse events, which are more common with BCG than the other chemotherapy agents (Martinez-Pineiro et al. 1990; Malmström et al. 1999).

2.3.3.1 Interferon-α2b

Interferon-α2b (INF) has antiproliferative, antiangiogenic and immunostimulatory properties. The role of INF in the intravesical treatment of bladder cancer is still controversial. INF has been used as a single therapeutic agent or in combination with chemotherapeutic agents or BCG. Some advantage in terms of tumor recurrence has been found with supplemental administration of epirubicin and INF (Raitanen et al. 1995b). The same is true for the combination of INF and MMC over the drugs used alone (Engelmann et al. 1992). However, a supplemental combination of BCG and INF compared to standard BCG instillation showed no benefit (Cockett et al. 1991). Flu-like
symptoms and dysuria are the most common side-effects of INF, being usually mild and transient (Kalble et al. 1994).

2.3.4 Laser therapy

Although there is clear evidence that lasers can successfully eradicate bladder tumors by tissue vaporization, there is still scepticism about their clinical application. Different types of lasers are readily available for surgical use (Ruston et al. 1991). The argon laser can be directed via a fine quartz fibre and is minimally absorbed by water. It can therefore be applied quite well in an endoscopic system. The result, however is a rather superficial zone of tissue destruction. The argon laser has been used in solid tumors and Tis but there does not seem to be any single convincing benefit over the more conventional treatments. It does have the advantage of being suitable for use with the flexible cystoscope, thus making feasible out-patient treatment of bladder cancers (Biserte et al. 1989). For endoscopic surgery of bladder tumors, the laser beam of the Neodymium: Yttrium Aluminium Garnet (Nd:YAG) is conducted through a quartz fiber of between 0.4 and 0.6 mm diameter. The tip of the fibre is held between 0.25 and 0.75 cm distant from the tumor while the laser is activated. The laser is fired in short bursts of less than 5 s, while moving the point of application over the surface of the tumor as the visible changes of tissue necrosis become apparent (Hofstetter et al. 1981). The Nd:YAG laser competes with surgical diathermy as a means of producing thermal tissue destruction. The fact that tissue destruction occurs without physical contact with the tissue has been put forward as one of the major theoretical advantages of Nd:YAG surgery over conventional techniques (Ruston et al. 1991). It may be that the dispersion of viable cells into the irrigant and their subsequent implantation are important factors in the generation of recurrent bladder tumors ( Howe et al. 1977). It is also assumed that this “no touch” method is less likely to be associated with intramural lymphatic dispersion of tumor cells (Hofstetter 1986). These advantages are negated with most new bladder tumors whose diameter exceeds 1 cm. Since the depth of penetration of the Nd:YAG laser is limited to 0.5 cm, large tumors must be treated in the first instance by transurethral resection. The laser is used at a second sitting to coagulate the base of the tumor when any intraluminal tumor spread will already have occurred (Ruston et al. 1991). The main disadvantage is that tumor staging will be partly lost, because most of the tumor will be necrotic or vaporized.
The results have been quite controversial. Early reports of uncontrolled studies gave cause for optimism (Hofstetter et al. 1981; Okada et al. 1982). More recently, several studies have stated that they are unable to demonstrate a reduced recurrence rate (Beisland et al. 1986; Arkell et al. 1988). An alternative justification of the Nd:YAG laser is that it can be used without the need for general or spinal anaesthesia. The laser therapy is thermal coagulation of collagen but the risk of bladder perforation is minimal because the penetration of therapy is less than 0.5 cm and it produces little bleeding. The therapy is suitable for use in an out-patient basis. The cost benefits from this are enormous and the potential benefits in terms of patient convenience should not be underestimated (Ruston et al. 1991).

2.4 Photodynamic therapy

2.4.1 History

The first clinical use of PDT was in 1903, when von Tappeiner and Jesionek treated skin tumors with topical eosin followed by exposure to white light. The technique has slowly evolved since that date (Pope et al. 1991a). The great interest in porphyrinoid compounds, the most commonly used photosensitizers, stems from Policard’s observation in 1924 of reddish fluorescence in certain human and animal tumors attributed to endogenous porphyrin accumulation from hemolytic bacterial infection (Manyak 1991). In 1960, Lipson and coworkers described porphyrin tumor localization after injection of a synthetic porphyrin mixture called hematoporphyrin derivative (HpD) (Lipson et al. 1961). HpD was the most commonly used photosensitizer until it was further purified to a combination of ethers and esters known as dihematoporphyrin ether (DHE), sold commercially as Photofrin II (Manyak 1991).

The treatment of urologic malignancy was one of the earliest clinical applications for PDT since interest was regenerated for this modality in the 1970s. It was in 1975 that the potential of PDT in bladder carcinoma was demonstrated by Kelly, Snell and Berenbaum. They administered HpD to 11 patients and looked at tissue fluorescence under ultraviolet light, noting a good correlation between areas of tumor and the observed fluorescence, particularly for carcinoma in situ. One patient had an area of bladder illuminated by a mercury vapour lamp transmitted through a rigid quartz rod. Several papillary tumors were necrosed but the authors commented that improved techniques for illumination were
needed (Kelly et al. 1975; Kelly et al. 1976). Since this photosensitization techniques have been directed toward diagnosis and treatment of bladder carcinoma. Fluorescence has been used in vitro with bladder cancer cells to study uptake, subcellular localization, and molecular aggregation of porphyrin photosensitizers. The photodynamic destruction of bladder cancer cells has also been successfully accomplished in vitro and bladder cancer cell death has been correlated with fluorescence (Hisazumi et al. 1984; Bellnier et al. 1985; Williams et al. 1987). However, photosensitization of the skin, lasting up to 6 weeks, diminishes the acceptability of PDT with porphyrins (Dougherty et al. 1992). Moreover, there is a significant risk of damaging the bladder muscle and causing bladder shrinkage, because of reduced mucosal selectivity (Harty et al. 1989).

A novel approach to PDT involves endogenous photosensitization by administration of 5-aminolevulinic acid (Kennedy et al. 1990; Kennedy et al. 1992). ALA is converted by the heme biosynthetic pathway to protoporphyrin IX. PpIX is an efficient photosensitizer, the accumulation of which is particularly evident in certain malignant cells because of their enzymatic constitution that favors PpIX production (Peng et al. 1997a). Thus, ALA may be a more reliable approach to photosensitization (Loh et al. 1993a; Sroka et al. 1996) and result in a more selective tumor accumulation of the photosensitizer than can be achieved with other agents (Kennedy et al. 1990; Kennedy et al. 1992).

### 2.4.2 Mechanisms of action of PDT

The primary mechanism of tumor destruction is mediated by cytotoxic singlet oxygen free radicals. The photodynamic action begins with the absorption of light by a photosensitizer followed by a cascade of photochemical reactions resulting in the generation of singlet molecular oxygen by energy transfer. Singlet oxygen is very reactive and diffuses in the surrounding tissues to destroy many targets, such as the cell wall, mitochondria, and lysosomes (Dougherty et al. 1998). PDT may also destroy the tumor indirectly by damaging the vascular endothelium, which results in hypoxia and tissue death (Waldow et al. 1984). This process appears to be mediated through various cytokines such as prostaglandins, lymphokines and thromboxanes. Support for an immunologic involvement in cytotoxicity by PDT comes from studies of immunosuppression, modulation of cyclooxygenase and cytokine release (Henderson et al. 1989; Lynch et al. 1989; Evans et al. 1990; Fingar et al. 1990; Nseyo et al. 1990a).
2.4.3 Laser light administration

The main requirement for the therapeutic light is that the wavelength used should match the absorption peak of the photosensitizer. Another limiting point for successful therapy is the limited tissue penetration of the therapeutic light. The most penetrating wavelengths for use are in the red and near infrared part of the spectrum, but even using these wavelengths, the maximum depth of penetration and resulting necrosis in most tissues is typically no more than about 5 mm. HpD and DHE both have only a minor absorption peak for red light (at 630 nm), although newer sensitizers generally have their major absorption bands at longer wavelengths for greater tissue penetration (e.g. aluminium sulphonated phthalocyanine at 675 nm, benzoporphyrin at 700 nm and silicon naphthalocyanine at 776 nm) (Pope et al. 1991a).

The current primary source of light for PDT is the laser, because of its efficient fiberoptic coupling (Nseyo 1992). In PDT light energy is not used to create a thermal effect, instead it initiates the above described photochemical reaction (Ruston et al. 1991). The red light is coupled into a 400 μm quartz fiber, which is used to deliver the power efficiently and conveniently through a normal 21 Ch cystoscope into the bladder. The end of the fiber may be fitted with either a microlens, for focal treatment of bladder tumor, or a spherical (bulb) tip, for whole-bladder (isotropic) light treatment (Nseyo 1992).

The four basic lasers used for cutting, coagulation, and evaporating purposes in urological surgery are carbon dioxide (CO\(_2\)), neodymium-yttrium aluminum garnet (Nd:YAG), Nd:YAG passed through a potassium-titanyl-phosphate (KTP) crystal, and holmium:YAG laser. The tissue interactions produced by each laser depend on laser wavelength, output power, and whether the laser is pulsed or continuous. However, the tissue reaction to the laser depends not only upon the power density of the laser and the duration of exposure but also on the water content and the color of the tissue (Bhatta 1995).

Although PDT employs argon and dye lasers as energy sources, it does not rely on a thermal effect to cause tumor destruction as does CO\(_2\) or Nd:YAG laser therapy (Nseyo 1992).
2.4.3.1 Nd:YAG laser

The Nd:YAG is a solid-state laser that utilizes a crystal seeded with neodymium ions to furnish the active medium. This near-infrared laser is absorbed poorly by water, tissue proteins, and blood. Therefore, this laser produces highly tissue-penetrating radiation capable of reaching to a depth of 5 mm penetration depth (Orihuela et al. 1994). It has good hemostatic capabilities, although perhaps not as effective as the argon laser (Bhatta 1995).

2.4.3.2 KTP crystal laser

The KTP crystal laser is generated by passing a rapidly pulsed Nd:YAG beam through a crystal, which doubles the frequency and halves the wavelength to 532 nm. Unlike the Nd:YAG, however, the KTP laser has a bright green, clearly visible beam. This beam has coagulative properties similar or even identical to the argon laser, but compared with Nd:YAG laser, there is a shallow depth of penetration (0.3-1 mm) (Bhatta 1995).

2.4.3.3 Argon laser

The argon laser produces wavelengths in the blue-green portion (458-515 nm) of the spectrum. The visible light emitted by argon lasers will transmit directly through water and is absorbed very strongly by pigmented molecules such as hemoglobin. The result is a rather superficial zone of tissue destruction (Staehler et al. 1976). Although the argon laser output can be transmitted through a flexible quartz fiber, an aiming beam is often necessary for accurate direction. Advantages include the versatility allowed by transmission through a flexible fiber, paucity of plume with vaporization, and excellent coagulative capabilities. The disadvantages are that the argon laser requires a water hookup for cooling and tinted filters to protect the eyes of operating room personnel (Bhatta 1995).
2.4.3.4 Diode lasers

Semiconductor lasers are smaller, potentially cheaper, and much more efficient than traditional lasers. Fibers easily transmit diode laser radiation, which can be pulsed or continuous. There is some possibility to change the wavelength to 805 nm by adding aluminium or to 1000 nm by adding indium (Bhatta 1995). The principle of diode lasers can be considered as analogous to a light emitting diode. A current is passed via an appropriate semiconductor and spontaneous light is emitted, which is called light emitting diode. A laser light is produced by using the same principles but adding reflecting mirrors to form a resonator where the stimulated light can reflect back and forth, allowing only a specific wavelength to be emitted. The wavelength is determined by the active compound used (Anson et al. 1994).

2.4.3.5 Dye lasers

The active medium is an organic liquid dye, which has to be optically excited by another light source (e.g. another laser or flash lamp). Liquid dyes are brightly colored and have complex sets of electronic and vibrational energy levels, and the wavelength emitted depends on the type of dye used. Their main advantage is that their emission covers a whole range of wavelengths in the visible part of spectrum, between 400-700 nm, and they can be tuned, i.e., the emission wavelength can be changed. The dye in these lasers must be changed at regular intervals, and the use of dyes typically makes these lasers difficult and expensive to maintain when compared to solid-state lasers (Bhatta 1995).

The most common source of red light is a tunable-dye laser with an argon laser, which produces high pump power (10 to 20 W) of blue-green (414-520 nm) light that excites krypton red dye to emit red light (630 nm) (Nseyo et al. 1990b).

2.4.4 Photosensitising drugs

2.4.4.1 General

The ideal characteristics of an effective photosensitizer are:

It should be a single pure compound, non-toxic, have a short half life and specific binding to tumor and not to normal tissue, possess a high quantum yield of singlet oxygen and activation spectrum with a peak between 700 and 800 nm. Unfortunately though many of
the photosensitizing drugs which have been used in much of the clinical work have shown themselves to be effective sensitizers, they do not meet all of the above criteria. Consequently there has been an intensive effort to develop new drugs (Ash et al. 1993).

2.4.4.2 Hematoporphyrin derivative

In 1960 Lipson et al described porphyrin tumor localization after injection of a synthetic porphyrin mixture called hematoporphyrin derivative (Lipson et al. 1961). HpD was the most commonly used photosensitizer until it was further purified to a combination of ethers and esters known as DHE, marketed commercially as Photofrin II (Quadra Logic Technologies, Vancouver, BC) (Manyak 1991; Nseyo 1992). The drug has a very long half life in tissue and accumulates in the skin so that patients are rendered photosensitive for 6 to 8 weeks. In the skin, the retention is probably secondary to increased binding by mast cells, macrophages, and Langerhans’ cells (Nseyo et al. 1988; Lin 1991). The preferential localization and prolonged retention by tumor tissue may be attributable to DHE’s chemical properties, including its lipophilicity, hydrophobicity, and molecular charge (Lin 1991). Although the optimal time interval required between administration and treatment has not been determined, most investigators wait 48-72 hours before treatment to allow clearance from nonmalignant tissues (Manyak 1991).

Photofrin II can be activated in tissue by the visible (400-630 nm) spectrum. However, red light at a wavelength of 630 ± 3 nm is used in Photofrin II-mediated PDT. Only, activation by 630 nm light uses a small peak of the activation spectrum which results in a relatively low yield of singlet oxygen. This wavelength has been used primarily to achieve maximal tissue penetration, that is, an effective biologic depth of 0.5 to 1 cm in bulky tumors, and to minimize light (primarily green) absorption by hemoglobin (Nseyo 1992; Ash et al. 1993).

Although nearly all human clinical trials of PDT have used either HpD or DHE they are far from ideal photosensitizers. For curative treatments, the drugs should be effective for thicker tumors while for palliation they need to have less extensive and shorter duration skin photosensitivity (Ash et al. 1993).

2.4.4.3 Benzoporphyrin derivative monoacid (BPD MA)

BPD MA is a chlorin compound with an absorption peak at 690 nm which should almost double the effective depth of penetration compared to that which can be achieved at 630
nm. Due to its insolubility it has been formulated in an unilamellar liposome (Lui 1994). The half life in plasma is approximately 6 h and skin photosensitivity returns to normal within a few days (Sternberg et al. 1993).

2.4.4.4 Tetra(m-hydroxyphenyl)chlorin (TmHPC)

This is one of a series of tetrahydroxyphenyl porphyrin derivatives which have been prepared synthetically. It has the advantage that it can be prepared as a pure compound and the absorption spectrum shows a maximum activation at 650 nm (Ash et al. 1993). Tumor to normal tissue ratios of 10-15 have been demonstrated and the duration of skin photosensitivity seems to be less than that of Photofrin II. TmHPC has been used intraoperatively to treat inoperable malignant mesothelioma and has shown selective necrosis at up to 1 cm depth with low doses of light (10-20 J/cm²) (Windahl et al. 1993).

2.4.4.5 N-aspartylchlorine e6 (NPe6)

Npe6 has been derived directly from naturally occurring chlorophyll. The maximum absorption is at 664 nm (Ash et al. 1993). According to early results it is effective as a photosensitizer and the skin photosensitivity is minimal one week after drug administration. The drug is maximally effective when light is given 2 hours after dosing and it seems to have a predominantly vascular effect in vivo (Nelson et al. 1987; Roberts et al. 1989).

2.4.4.6 Tin etiopurpurin (SnEt2)

SnEt2 is a synthetic drug which is a chlorin with a fused ring. Its absorption maximum is 700 nm with a quantum singlet yield of 0.6-0.7. The drug appears to be maintained at fairly constant levels for 24 h but is cleared from the skin over several days rather than several weeks as for Photofrin II (Ash et al. 1993).

2.4.4.7 Phthalocyanine derivates (AlSPc, ZnPc, CASPc)

Phthalocyanines have been studied as potential PDT drugs for several years and in animal studies aluminium and zinc phthalocyanine compounds have shown advantages over Photofrin II. Zinc phthalocyanine is being considered for clinical trial by delivery in a liposomal system. Its absorption maximum is 680 nm. There is potentially a slight
disadvantage in that the drug may induce a grey or green-blue tinge to the skin of patients for a brief time after intravenous injection (Ash et al. 1993).

According to Pope and Bown, AlSPc is a promising photosensitizer for bladder PDT. They gave AlSPc intravenously and intravesically and used intravesical red light (675 nm) from a copper vapour pumped dye laser as their energy source. With low doses of the drug, it was possible to produce a superficial necrosis without muscle damage across a range of light doses. Direct absorption of this photosensitizer following intravesical administration was unreliable (Pope et al. 1991a).

The microscopic biodistribution of chloro-aluminium sulfonated phthalocyanine (CASpC) after intravenous injection or intravesical instillation in a rat bladder carcinoma model has also been compared. After intravenous injection, the photosensitizer was distributed within the whole tumor with increased fluorescence around the microvasculature. In the normal bladder wall, weak fluorescence was seen in the area of the vasculature in the submucosa and the muscularis. After intravesical instillation, strong fluorescence was detected only at the tumor surface and in normal urothelium; no fluorescence was found in other areas of the tumor or in submucosa or muscularis. According to one study, intravesical administration of CASpC may be a feasible alternative to intravenous injection with the potential for reduced systemic and non-malignant tissue toxicity (Bachor et al. 1992).

2.4.5 Aminolevulinic acid-based PDT

2.4.5.1 General

The tissue photosensitizer PpIX is an immediate precursor of heme in the biosynthesis pathway for heme. In certain types of cells and tissues, the rate of synthesis of PpIX is determined by the rate of synthesis of 5-aminolevulinic acid, which in turn is regulated via a feedback control mechanism governed by the concentration of free heme. The presence of exogenous ALA bypasses the feedback control, and thus may induce the intracellular accumulation of photosensitizing concentrations of PpIX. However, this occurs only in certain types of cells and tissues. The resulting tissue-specific photosensitization provides the foundation for using ALA-induced PpIX for PDT (Kennedy et al. 1992).

While the photosensitization process involved in ALA-PDT is believed to be essentially the same as that of most preformed photosensitizing agents (such as porphyrin derivatives,
chlorins, phthalocyanines, merocyanines, etc.), there are notable differences in ALA-PDT that may be exploited. First, PpIX is in every cell in the body, and thus has a natural clearance mode by conversion into heme. Second, ALA is a relatively small molecule that can be introduced topically, intravesically and orally, as well as intravenously. Third, PpIX is one of the most photolabile porphyrins known, and is rapidly destroyed during the PDT process. Consequently, the dosimetry of light is less critical than is the case for other pre-formed photosensitizers (Pottier et al. 1997).

2.4.5.2 Enzymatic control

The porphyrin that usually accumulates in cells when tissues are exposed to an excess of exogenous ALA is PpIX. Uroporphyrins I and III and coproporphyrins I and III are formed by oxidation of the corresponding porphyrinogens. These non-specific oxidative processes take place in the cytoplasm. PpIX normally is formed from protoporphyrinogen IX by a highly specific process of oxidation which involves protoporphyrinogen oxidase, an enzyme which is present only in the mitochondria (Pottier et al. 1997) (Fig.1.).

![Diagram of heme biosynthesis pathway](image)

*Figure 1. Schematic figure of the heme biosynthesis pathway. Gly = Glycine, Suc-CoA = Succinyl-CoA, ALA = 5-aminolevulinic acid, PBG = porphobilinogen, U'GEN I/II = uroporphyrinogen I/II, C'GEN I/III = coproporphyrinogen I/III, P'GEN IX = protoporphyrinogen IX, PpIX, UP I/III = uroporphyrin I/III, CP I/III = coproporphyrin I/III, a = 5-aminolevulinic acid synthase, b = 5-aminolevulinic acid dehydratase, c = uroporphyrinogen III cosynthase, d = uroporphyrinogen decarboxylase, e = coproporphyrinogen oxidase, f = protoporphyrinogen oxidase, g = ferrochelatase Fe++, h = porphobilinogen deaminase.*
In all 95% of the ALA dose is converted to PpIX after intravenous administration in tissues (Loh et al. 1993b). Although there are multiple enzyme-catalysed steps between ALA and heme, in the liver and certain other tissues the whole process appears to be regulated by a feedback mechanism in which the presence of free heme inhibits the synthesis of ALA, a distant precursor of heme (Sinclair et al. 1975; Iwasa et al. 1989; Mamet et al. 1990).

Under normal conditions, the maximum rate at which ALA is synthesized and enters the biosynthetic pathway for heme is always less than the maximum rate of the slowest of the subsequent steps in that pathway. Consequently, each step always has ample reserve capacity and intermediates do not accumulate. If the ALA/heme feedback control is bypassed by the presence of a large excess of exogenous ALA, the rate of synthesis of the first intermediate (porphobilinogen) is determined primarily by the maximum capacity of the enzyme system that is responsible for that specific step. What happens at each subsequent step may vary from tissue to tissue, since this will be determined by the relative capacities of the various processes that are involved (Kennedy et al. 1992). PpIX may accumulate as a result of a partial block at the level of either protoporphyrinogen oxidase or ferrochelatase. A partial deficiency of either of these enzymes in malignant or premalignant cells might be responsible for their abnormally large accumulation of ALA-induced PpIX. Alternatively, a relative deficit of transferrin receptors on the malignant cells or a relative increase in the capacity of the malignant cells to synthesize PpIX could lead to the same phenotype (Pottier et al. 1997). It is possible that the biosynthetic pathways for heme in normal and malignant cells of the hemopoietic system may differ in a manner that might permit the induction of a useful degree of differential photosensitization (Arsov et al. 1981).

2.4.5.3 Mechanism of action

In tissue, the photosensitiser is activated by absorbing light. One of three competing processes can then occur.
The activated photosensitiser molecules can react with tissue oxygen to produce singlet oxygen, which is thought to be the cytotoxic agent (Dougherty et al. 1992). This is a threshold effect and a certain quantity of singlet oxygen must be produced before a cell is killed. The photosensitiser molecules then return to the ground state and can be activated again. The activated molecules can fluoresce (emit light) and then return directly to the ground state, whereupon they can be activated again (Pope et al. 1991a) (Fig.2.).

The activated molecules can go through a different process in which they are destroyed by the same activating light (photodegradation). This means that the total quantity of photosensitiser in tissue is steadily reduced as the tissue is exposed to light. This has important implications for protecting tissues with low concentrations of photosensitiser (Dougherty et al. 1992).

PDT is often portrayed as a “catalytic” type of reaction in which the photosensitizer is regenerated upon transferring its energy to oxygen. However, porphyrins themselves can be photomodified, either by direct photolysis or as a result of oxidation by singlet oxygen. Such photomodifications can lead to chlorin type photoproducts that may themselves act as potent photosensitizers. Alternatively, the photosensitizer may be totally photodegraded (photobleached) (Rotomskis et al. 1996; Georgakoudi et al. 1998). The rapid photobleaching of ALA-induced PpIX is one of its most important properties because it
greatly reduces the problem of light dosimetry. In the presence of rapid photobleaching, the phototoxic effect on a tissue is determined almost entirely by the concentration of PpIX in the cells of that tissue, and after a certain dose of light has been given, the phototoxicity does not increase with any further increase in light dose. Since tissues that contain only a small amount of ALA-PpIX will not be harmed by exposure to even an extremely large overdose of light, it is not necessary to reduce the light given to the malignant tissue in order to protect adjacent or overlying non-malignant tissues. This permits great simplification of the light dosimetry in many clinical situations (Fingar et al. 1987; Kennedy et al. 1992).

2.4.5.4 Optimal wavelength for ALA-PDT

A wavelength of photosensitizer absorption maximum and, correspondingly, of exciting laser source is one of the most important parameters determining the depth of photodynamic action in tissue. In most cases of ALA-PDT, light at 630 nm is applied whereas 635 nm light would be optimal for depths exceeding 2 mm (Peng et al. 1997a). Basically, the choice of the optimal wavelength for PDT should be made on the basis of the appropriate action spectrum. One convenient method would be to measure the action spectrum of photobleaching of the dye, since that process is caused by generation of singlet oxygen, which is also the cytotoxic photoproduct (Peng et al. 1997a). As photosensitizing photoproducts with an absorption peak around 670 nm are formed during ALA-PDT, it may be advantageous to use a broad-band light source with an emission spectrum that also covers part of the action spectrum of the photoproducts (Peng et al. 1997b).

2.4.5.5 Biodistribution of ALA-induced PpIX in the bladder

Following intravenous administration, ALA leads to a rapid and even build up of PpIX in the mucosa of hollow viscera while the underlying muscularis layer is sensitised to a much lesser extent. The photosensitisation produced is transient lasting less than 24 hours and consequently prolonged skin photosensitivity is not a problem (Loh et al. 1993a; Leveckis et al. 1994; Linuma et al. 1995). The ALA-induced PpIX accumulation in the rat bladder is highest at 4 hours after ALA administration. Peak levels of fluorescence are achieved earlier with lower doses of ALA. Both maximum fluorescence and time to maximum fluorescence is dose-dependent in the mucosa (Loh et al. 1993a; Leveckis et al. 1994).
The biodistribution of PpIX in rat bladder tumor models has also been examined. The tumor to normal bladder wall ratio was 2:1 at 4 hours following intravenous or intravesical administration of ALA when a quantitative comparison of PpIX biodistribution by extraction and fluorescence spectroscopy was made (Linuma et al. 1995). According to Xiao and coworkers (Xiao et al. 1998) the PpIX ratios of tumor-to-bladder mucosa, submucosa and muscle layers were 3:1, 5:1 and 8:1, respectively, at 4 hours following ALA injection. Thus, ALA might be superior in selective accumulation to conventional sensitizers which are known to be localized also in endothelial cells of the tumor stroma. The data appear to hold great promise for PDT in bladder cancer, as phototoxicity should be limited to mucosal lesions. Bladder shrinkage due to photodamage of subepithelial structures even in case of high light doses is not expected (Steinbach et al. 1994).

2.4.5.6 Clinical experience for the PDT of superficial bladder cancer

At present, PDT is primarily suggested as being one of the therapy methods of papillary urothelial carcinoma and refractory Tis, and prophylaxis of recurrent superficial (Ta, T1) urothelial carcinoma in those patients who have failed intravesical chemo- or BCG immunotherapy (Nseyo 1996).

BCG immunotherapy has decreased recurrence, progression, and mortality rates. An initial course of intravesical BCG immunotherapy induces a durable response in 31-88% of Tis patients (Lamm et al. 1992; Lamm et al. 1995). However, only 39-58% of BCG failures will respond to a second course of BCG immunotherapy (Bretton et al. 1990; Coplen et al. 1990). Therefore, it is important to investigate alternative intravesical therapies, including PDT, for the management of superficial bladder cancer (Nseyo et al. 1996; Nseyo et al. 1998).

In small-scale clinical trials over the last 15 years Photofrin II induced PDT has been used as a treatment for papillary cancer with 70-95% response rate (Hisazumi et al. 1983; Prout, Jr. et al. 1987; Shumaker et al. 1987; Benson, Jr. 1988; Nseyo 1992; Walther et al. 1997) and for carcinoma in situ with 33-100% response rate (Jocham et al. 1989; Manyak 1991; Nseyo 1992; Kriegmair et al. 1995b; Nseyo et al. 1996; Walther et al. 1997). Considering that these PDT responses have been obtained in urothelial carcinomas resistant to chemo- and immunotherapy, PDT should be considered as an alternative after the failure of either of these primary treatments. It also appears that PDT can enable 31% of patients whose only remaining option is radical cystectomy to retain
their bladders for up to one year without undue risk of disease progression (Nsreyo et al. 1998).

Although PII has proved to be an effective photosensitizer, there are limitations associated with PII-based PDT. These include prolonged cutaneous phototoxicity and lack of tumor selectivity following systemic administration of PII. One major complication in whole bladder PDT with PII has been the development of fibrotic, contracted bladders with upper tract obstruction due to damage of the muscularis (Nsreyo et al. 1985; Harty et al. 1989). These major complications may be avoided by using ALA as a photosensitizer because of its high epithelium and tumor selectivity. However, there are only three published studies investigating the clinical use of intravesical ALA in the treatment of bladder cancer. All of these studies have been published within the last five years. The PDT protocols are variable between studies, though all patients treated had disease that was resistant to conventional therapy.

Kriegmair and co-workers studied PDT with intravesically applied ALA in 10 patients (Kriegmair et al. 1996). Light irradiation was used at doses of 15 to 60 J/cm² and light of wavelength 633 nm or 633 nm and 514 nm was used. Four of the ten patients with papillary tumors had a complete response, 2 had a partial response (more than 50% reduction in number or area of tumors) and 4 had no response at 3 month follow-up cystoscopy. All 4 patients with co-existing Tis had no evidence of Tis at follow-up. All patients experienced bladder irritability which regressed by 2 weeks in 80% and in the remainder by 4 weeks. There were no reported photodermatoses or bladder contractures.

Stenzl and colleagues published a series of 6 patients (5 with Tis, 1 with papillary superficial cancer) (Stenzl et al. 1996). ALA solution was administered intravesically for 30 minutes in sedated patients. By using a specialised catheter containing a stainless steel electrode and a pulsed current (DC) of 15 mA, the polar ALA molecules were driven into the bladder wall by iontophoresis, a technique known as electromotive drug administration. The light dose used was 30-50 J/cm². On the follow-up, there was a 3-month curative rate of 100%, with 5 of the 6 patients still tumor free at 10-16 months.

D’Hallewin and co-workers presented data on 6 patients with resistant Tis (D’Hallewin et al. 1997). They used ALA solution intravesically to sensitize the bladder and irradiated with red light at an incident dose of approximately 12.5 J/cm². Eradication of Tis was seen
in 67% of the patients treated at the 3-month follow-up. There was no bladder shrinkage or cutaneous photosensitivity reaction reported.

Recent work by Datta and colleagues (Datta et al., 1997) shows a supra-additive effect of ALA-PDT in combination with mitomycin when used on various bladder cell lines. In future, such combination treatment may allow a significant breakthrough in superficial urothelial bladder cancer treatment.

Since there is a very limited experience with human bladder ALA-PDT, many questions about the treatment procedure remain open to debate such as the ALA dose, the light dose, in addition to whether it should be continuous or fractionated light dosage. The results described above show that PDT offers potential in the treatment of superficial bladder cancer. In particular, the treatment of PDT may improve on current therapy modalities by offering an effective alternative to radical treatment of aggressive superficial disease such as that occurring in carcinoma-in-situ of the bladder.
3. **AIMS OF THE STUDY**

The general aims of this study were to study PDT experimentally and to find out its suitability for the treatment of superficial bladder cancer. More specifically the following questions were studied:

1. How does ALA-induced PpIX concentrate in the normal rat bladder wall as measured with fluorescence distribution? What is the optimum ALA dosage?
2. What is the optimum light energy dose with the used ALA dosage to induce only superficial bladder wall damage during PDT?
3. How does bladder function recover after PDT depending on light energy doses used?
4. What is the difference in PpIX accumulation between urothelial carcinoma and normal urothelium and the muscular layer of urinary bladder after intravenous or intravesical administration of ALA?
5. Is PDT with ALA-induced PpIX an effective and safe treatment modality for superficial bladder carcinoma?
4. MATERIAL AND METHODS

4.1 Animals and anaesthesia

Female Wistar (studies I-III) rats weighing approximately 200 g and Fischer F344 (studies IV-V) rats were obtained from the National Laboratory Animal Centre (University of Kuopio). All animal protocols were approved by the Animal Care and Use committee at University of Kuopio. The animals were anaesthetized with a mixture of phentanylfluanisone (Hypnorm, Janssen Pharmaceutica, Beersse, Belgium) and midazolam (Dormicum, Roche, Basel, Switzerland) by subcutaneous injection and weighed. Body temperature was maintained with an euthermic blanket.

Table 2 Summary of animals and groups used in articles I-V

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Total number</th>
<th>Grouping</th>
<th>Article</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar rat</td>
<td>60</td>
<td>3 x 4 x 5</td>
<td>I</td>
</tr>
<tr>
<td>Wistar rat</td>
<td>64</td>
<td>4 x 4 x 4</td>
<td>II</td>
</tr>
<tr>
<td>Wistar rat</td>
<td>64</td>
<td>4 x 4 x 4</td>
<td>III</td>
</tr>
<tr>
<td>Fischer F 344 rat</td>
<td>40*</td>
<td>36: (3x4x3) + 4 contr.</td>
<td>IV</td>
</tr>
<tr>
<td>Fischer F 344 rat</td>
<td>36 + 40*</td>
<td>36: (3x12) +40* contr.</td>
<td>V</td>
</tr>
</tbody>
</table>

4.2 Aminolevulinic acid administration

Aminolevulinic acid as the hydrochloride (Sigma Chemical Company, St. Louis, MO, USA) with a purity of 98% was prepared immediately prior to intravenous or intravesical administration. ALA was dissolved in physiological saline and injected at a concentration 1393 mg/ml intravenously into the tail vein via a 25 G needle at doses 100 mg/kg (0.075 ml), 200 mg/kg (0.150 ml) and 300 mg/kg (0.225 ml) or intravesically (studies IV and V) at a concentration of 100 mg/ml 0.25 ml. In the latter case, the clamped urethral catheter was left indwelling for two hours after instillation. The catheter was then removed and animals were allowed to void.
4.3 Preparation of specimens for fluorescence scanning and SEM (Studies I, II, IV)

At determined intervals after ALA dosage, the animals were sacrificed. The urinary bladder was catheterized with a fine gauge cannula so that any urine which might contain photosensitizer could be gently washed out. The bladders were then distended to 0.3 ml with freezing medium (OCT compound, Miles Scientific Inc., Elkhart, IN) and dissected out. The specimens were immediately frozen in a bath of isopentane cooled in liquid nitrogen and stored in a freezer at -70 °C temperature. Several 10 μm transverse sections of the distended bladder were cut using microtome for fluorescence studies (studies I and IV). Processing of specimens was done in subdued light to minimize photobleaching of PpIX and slides for fluorescence microscopy were allowed to thaw just prior to microscopic examination. 5 μm thick sections adjacent to frozen section (studies II and IV) were cut and processed for light microscopy (studies I, II, IV and V). From these hematoxylin and eosin (H&E) samples, the borders of the tissue structures (studies II and IV) and presence or absence of neoplastic tissue (studies IV and V) was noted, along with the degree of tumor invasion.

The specimens for SEM analysis (study II) were washed with 0.1 M phosphate buffer (pH 7.4) before fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h. These specimens were dehydrated in ethanol and dried in a Balzers Union CPD 020 Critical Point Dryer. The specimens were coated with 200 Å thick gold in a Jeol Fine Coat Ion Sputter JFC-1100 (1.2 kV, 5-10 mA, 6 min) and viewed in a Jeol JSM-820 SEM (7-12 kV acceleration). The findings were photographed and analyzed. The changes in epithelium were assigned to three categories based on the grade of injury as seen in the light microscopic samples.

4.4 Fluorescence detection (Studies I and IV)

Microscopic fluorescence photometry of the frozen sections was carried out using an inverted fluorescence microscope (Nikon Planfluor, Tokyo, Japan, magnification 20x / 0.5 numerical aperture) with the charge coupled device (CCD) camera. The camera exposure time was 30 s. Xenon flash lamp (FX-249, EG&G Inc., Wellesley, USA) was used as an excitation light source. The excitation light was filtered with an interference filter (Omega Optics, USA) which only lets the light from 600 nm to 620 nm to pass (study I). The emitted fluorescence light was first separated from the reflected and scattered light with a
dichroic mirror (Omega Optics, USA) which allows the light that has a wavelength longer than 645 nm to pass, and after this it is filtered with an interference filter (Omega Optics, USA) which is permeable to the light from 650 to 690 nm.

In study IV, the excitation light was filtered with an interference filter (Nikon Corp., Tokyo, Japan), which is only permitted transmission of 510 nm to 560 nm wavelengths. The emitted fluorescence light was first separated from the reflected and scattered light with a dichroic mirror (Nikon Corp., Tokyo, Japan) which allows the light that has a wavelength longer than 575 nm to pass, and after this it is filtered with an interference filter (Nikon Corp., Tokyo, Japan) which only transmits wavelengths longer than 590 nm.

In study I the imaging was made with the cooled scientific CCD camera (Wright Instruments, London, UK). The fluorescence intensities in the different parts of the samples were measured for comparison with the camera control software (AT1, Wright Instruments, London, UK). Three areas over the mucosa, submucosa and muscularis were chosen for analysis on each section. The signal was processed by a personal computer into a falsely color-coded microscopic image of the section, depicting the mean signal in counts per pixel. The software also allowed quantitative analysis of the image by calculating the mean fluorescence count and standard deviation within any chosen area of the fluorescence image. The fluorescence levels were corrected for autofluorescence for each respective layer of tissue, as determined using control specimens.

In study IV, the images were captured with a next generation CCD camera (Perkin Elmer Life Sciences, Cambridge, UK) and fluorescence intensities compared in the different regions of the samples with image analysis software (Image Pro Plus v.4.0, Media Cybernetics, Silver Spring, MD, USA). This software also allowed semi-quantitative analysis of the image by calculating the mean fluorescence count and standard error of mean within any chosen area on the fluorescence image. Three separate areas of the urothelium, submucosa and muscularis were chosen for analysis on each section. The fluorescence levels were corrected for autofluorescence from each respective layer of tissue, as determined using control specimens.

### 4.5 Filling cystometry (Study III)

In study III, filling cystometry was performed for each animal at three weeks before PDT and after treatment at days 1, 3, 7 or 21 according to grouping. Each animal served as its
own control. For cystometry, the animals were anesthetized and the rat bladder was cannulated with a 20 gauge Venflon R (BOC Ohmeda, USA) cannula. This was connected via a three-way tap to an invasive pressure measurement transducer (Braun, Germany) and to a syringe pump. The lines were rinsed with normal saline and carefully checked to exclude all air bubbles at the line. To minimize resistance of flow in the lines, the system was calibrated to zero in the beginning of bladder filling. The pressure measurement transducer was connected to an AS/3R (Datex, Helsinki, Finland) intensive care monitor, which was connected to a computer (Toshiba T 1000 PC) that collected the measured data at a sample rate of every three seconds to a file. A constant infusion rate of 0.2 ml/min was maintained with a syringe pump. Physiological saline at room temperature was used as the filling fluid. The following criteria were used as end points of the cystometric measurement: 2 ml infused volume or 16.3 cmH₂O filling pressure or uninhibited bladder contractions seen as visible urine leakage from the urethra.

4.6 Tumor cells and induction of orthotopic urothelial cancer (Studies IV and V)

_Tumor cells:_ The rat bladder urothelial cancer cell line AY-27 (Xiao et al. 1999) was obtained from Dr. R.B. Moore, Department of Surgery, University of Alberta, Canada. The cell line was cultured in RPMI-1640, supplemented with 10% fetal bovine serum. For implantation, the subconfluent cell monolayers were trypsinized, suspended in the growth medium and counted. After 2 washes with 40 ml of OptiMEM (Life Technologies, Gaithersburg, MD, USA) the cells were suspended in OptiMEM to yield a density of 3 million/ml and kept on ice. In this study, 0.15 ml of this cell suspension was used for each implantation (equivalent to 4.5 x 10⁶ cells per bladder).

_Tumor induction:_ The rat bladder was catheterized via the urethra with an 18 or 20 gauge plastic intravenous cannula. This size of cannula was optimal for administration of the various agents, providing a snug fit, without leakage around the catheter.

To facilitate tumor seeding, the bladder urothelium was first conditioned with an acid rinse. A volume of 0.15 ml of 0.1 N hydrochloric acid (HCl) was kept in the bladder for 15 s and neutralized with 0.15 ml of 0.1 N potassium hydroxide (KOH) for 15 s. Subsequently the bladder was drained and flushed several times with sterile phosphate-buffered saline. Immediately after bladder conditioning, the AY-27 cells (4.5 x 10⁶) were instilled and left indwelling for at least one hour. The rats were turned 90° every 15 minutes to facilitate
whole bladder exposure to the tumor cell suspension. After one hour, the catheter was removed and the rats were allowed to void the suspension spontaneously. After recovery from the anaesthetic, the rats were placed into standard cages and monitored daily for general health status including weight.

4.7 Urinary Cytology (Study V)

Cytologic examination of the sediment of urine was made in animals at 15 days post cancer cell implantation in order to detect the tumors of the bladder before PDT. About 1 ml of voided urine was catheterized with a cannula and fixed with 70% ethanol, Millipore filtered, and stained with Papanicolaou stain.

4.8 Laser equipment

A Spectra-Physics Model Stabilite 2016 continuous wave Argon ion laser served as a pumping source for a Spectra-Physics water-cooled dye laser. The generally accepted absorption maximum of PpIX in the red is at 635 nm but PpIX absorbs light up to 700 nm. DCM ((2-(2-(4-(dimethylamino)phenyl)ethenyl))6-methyl-4H-pyran-4ylidene)-propanedinitrile) was used as a dye solution. The dye was lasered at 638-650 nm wavelengths. These wavelengths were measured with Kratos Model GM 100-1 monochromator driven by Kratos GMA Motor drive. The spectrum was depicted on a Servogor type SE 120 plotter. The pumping argon laser had a maximum output power of 4 W, but the actual maximum power was 1.6 W (measured with laser power measurement system, Scientech Mentor MAIO indicator and Scientech Mentor MC 2500 calorimeter). Losses, due to absorption and reflections of dye, mirrors and resonator, fiber junction and fiber itself decreased maximum power to 250 mW measured at the fiber tip. The fiber used was a 200 µm optical fiber with a ball-shaped tip (diameter 300 µm). The irradiation times varied from 4 min 49 s up to 19 min 16 s depending on the totally applied light dose. The laser equipment was installed on Zero-G vibration isolation Systems -table, thus the measurements were carried out at high stability.

4.9 Photodynamic therapy (Studies II, III and V)

Animals were sensitized with ALA (300 mg/kg) intravenous administration 4 hours prior to light exposure (studies II and III) and for infection prophylaxis all animals were given 15
mg benzylpenicillin procaine (Ilcocillin 300 mg/ml, Orion-Farmos) subcutaneously. For PDT, the rat bladder was catheterized with a 20 gauge cannula and filled to a volume of 0.3 ml with saline and 0.02% soybean emulsion (Intralipid) diffusion medium (studies II and III). The bladder was assumed to be spherical in calculations for light energy dosing 20, 40 and 80 J/cm². The bladder was exposed through a lower abdominal incision (studies II and III). The urethral cannula was removed and the optical fiber with a ball-shaped tip was positioned centrally within the bladder via urethra in visual control. Laser power of 140-150 mW was used with lasering times of 4 min 49 s, 9 min 38 s and 19 min 16 s, to achieve total energy doses of 20, 40 and 80 J/cm². The temperature of the bladder walls during PDT was monitored by placing the thermometer in the central part of the bladder anterior wall in all animal groups to exclude the thermal effects.

In study V, PDT was given 15 days post cancer cell implantation. After sedation the animals were sensitized with intravenous (300 mg/kg) or intravesical (100 mg/ml, 0.25 ml) administration of ALA 4 h prior to light exposure. For PDT, the rat bladder was catheterized with a 20 G cannula and filled to a volume 0.2 ml with saline. The bladder was assumed to be spherical in calculations for light energy-dosing. The urethral cannula was removed, and the 200 μm optical fiber with a ball-shaped tip positioned centrally within the bladder blindly via the urethra to a depth of 1.6 cm from the outer orifice of the urethra. This depth was measured before by exposing the bladder of ten similar sized rats as in this study through a lower abdominal incision and the central part of bladder examined with a cannula. A laser power of 100 mW was applied for 11 min, to achieve a total energy dose of 40 J/cm² light.

4.10 Statistical analysis

The results are expressed as mean ±SD or SEM. Group comparisons were analyzed using the Mann-Whitney U-tests and the Pearson’s χ² test with exact probabilities. The differences between histological samples before and after the treatments were calculated using the Wilcoxon signed rank and matched-pairs test. The results were considered statistically significant at p < 0.05.
5. RESULTS

5.1 Microscopic biodistribution of ALA-induced PpIX

PpIX induced fluorescence was significantly higher in the superficial layers of the bladder in samples taken 2 and 4 hours after ALA administration at all dose levels than in the control group (p<0.05, Mann-Whitney U-test). Analysis also showed significant differences in fluorescence in samples taken 1 hour after 200 mg/kg and 300 mg/kg ALA administration (p<0.05, Mann-Whitney U-test). At the same dosage level, fluorescence reached a peak in mucosa at 4 hours then and declined in 24 hours to the control level at all doses. A clear trend in difference between the fluorescence intensity between superficial layers and muscularis was found in all samples taken after 1, 2, 4 and 6 hours of ALA administration (Fig. 3, 4 and 5), however, the difference was statistically significant only in samples taken 4 hours after 100 mg/kg ALA administration (p<0.05, Wilcoxon matched-pairs test) (Study I).

![Fluorescence intensity in mucosa and muscularis](image)

*Figure 3. Mean fluorescence levels (±SD) in the mucosa and muscularis of the bladder at various times after ALA (100 mg/kg) administration. (Reproduced from J Photochem. Photobiol. B: biol., 38: 269-273, 1997 by copyright permission of Elsevier Publishers)*
Figure 4. Mean fluorescence levels (±SD) in the mucosa and muscularis of the bladder at various times after ALA (200 mg/kg) administration.


Figure 5. Mean fluorescence levels (±SD) in the mucosa and muscularis of the bladder at various times after ALA (300 mg/kg) administration.

The fluorescence distribution of PpIX was also measured after intravesical (0.25 ml, 100 mg/ml) or intravenous (100 or 300 mg/kg) administration of ALA. Generally, the highest fluorescence levels were seen in tumor tissue (Figure 6). All samples also demonstrated higher fluorescence intensity in the urothelium and lamina propria (superficial layers) than in the muscular layer of the bladder. In the latter tissue, the fluorescence levels increased above the autofluorescence level 2 hours after the highest and 4 hours after the lowest intravenous dose (Study IV).

Figure 6. Fluorescence photomicrography 8 hours after 300 mg/kg ALA intravenously reveals the highest fluorescence intensity in tumor tissue and mucosa with a virtual absence of fluorescence in the bladder muscle layer. Reduced from x 20.

(Reproduced from J Urol 167: 2002 by copyright permission of Lippincott Williams & Wilkins)

PpIX fluorescence was higher in tumor tissue samples obtained 4 hours after intravesical ALA administration than in those obtained at 2 hours (Figure 7). At 2 and 4, hours the difference in fluorescence in tumor tissue and the muscular layer as well as in normal urothelium and the muscular layer of the bladder was statistically significant (p<0.05,
Wilcoxon’s signed rank test). There was no difference between tumor and normal epithelium. The absolute fluorescence level 4 hours after ALA administration was 7-fold higher in tumor than in the muscular layer (Study IV).

![Fluorescence levels diagram](image)

*Figure 7. Fluorescence levels (mean ± SEM) after 100 mg/ml (0.25 ml) ALA intravesically in 3 or 4 animals per group. Asterisk indicates p < 0.05 (Reproduced from J Urol 167: 2002 by copyright permission of Lippincott Williams & Wilkins)*

After intravenous administration, the highest difference in fluorescence intensity in tumor tissue and normal epithelium was measured at 2 hours at each administered concentration of ALA (Figures 8 and 9). The difference varied between 2.3 and 3.1-fold. With time this difference decreased and was even negative at 8 hours after the highest dose. The difference in tissue fluorescence in tumor tissue and the bladder muscular layer as well as in normal urothelium and bladder muscle tissue was statistically significant 2 and 4 hours after ALA administration (p<0.05, Wilcoxon’s signed rank test). Absolute PpIX fluorescence intensity levels rose with the increasing ALA dose when the intravenous administration route was used. The highest levels were measured 4 hours after 300 mg/kg of ALA administration. Four hours after 300 mg/kg of ALA, the difference in absolute fluorescence intensity was 6-fold higher in tumor tissue than in muscle tissue and 4-fold higher in normal urothelium than in the bladder muscular layer (Figure 9). The greatest difference in PpIX fluorescence concentration in tumor tissue and the muscle layer was seen at 2 hours with the lower intravenous dose (Study IV).
5.2 Morphological changes after PDT (Study II)

There were no macroscopic differences in the bladder walls between the control and treatment groups irradiated with 20 and 40 J/cm² light. After 80 J/cm², two of 16 animals
were lost during the follow-up. In these animals the adjacent perivesical fat and peritoneum appeared purulent around the necrotic bladders, but there was no damage to adjacent organs. In two more animals in the same group, the bladders were flaccid and oedematous.

In the SEM analysis, 20 J/cm² with no photosensitizer caused no changes in the bladder wall (Figure 10). Histology showed only three cases of superficial tissue destruction in this group (n=16). The maximum PDT effect occurred after 1-3 days of irradiation. After ALA with 20 J/cm², the tissue damage effects were transient; such that after 1 week there was no further muscular layer destruction. After 40 J/cm², in all rats the acute phase of destruction reached the muscular layer; this damage also subsided within 7 days in nearly all rats and after 3 weeks there was no muscular necrosis or fibrosis in any of the animals. In rats receiving 80 J/cm², the destruction of the muscular layer increased during the first 3 days (Figure 11) and in half of the animals there was still damage to the muscular layer 3 weeks after therapy.

Both SEM and light microscopy showed a significant difference (p<0.05, Pearson χ² test) in bladder wall lesions induced by fluences of 40 and 80 J/cm² with ALA, compared with the control group. The difference between ALA plus 20 J/cm² and the control group was significant in the SEM analysis. The correlation between the findings from SEM and light microscopy was significant (Pearson correlation 0.78).

Figure 10. Bladder epithelium of a control animal treated with 20 J/cm² light with no photosensitizer; the cells are smooth and the junctions tight. SEM x 500.

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5.3 Filling cystometry (Study III)

In pretreatment analysis the intravesical pressure increased gradually with infused volume and reached 8-11 cmH₂O end-filling pressure. The infused volumes varied from 1.2 ml to 2.0 ml saline at room temperature. In some cases, urine leakage was observed also at lower pressure values and fluid infusion was stopped immediately (Figure 12).

The animals which received only 20 J/cm² light without ALA served as controls. At day 1, the cystometry curve was similar to pre-treatment measurements, but in later measurements (3, 7 and 21 days) the bladder pressure was significantly increased. Even at 21 days after treatment, the bladder pressure was higher than in all PDT treatment groups (p<0.05, Mann-Whitney U-test) and the change to pre-treatment measurement was significant (p<0.05, Mann-Whitney U-test).

Animals receiving light doses 20 J/cm² and 40 J/cm² together with ALA had a similar outcome. In both groups at day 1, the pressure showed a sharp rise (p<0.05, Mann-Whitney U-test), but returned to the pre-treatment level before day 7 and stayed there at day 21.

Those animals which received the highest light doses 80 J/cm², did not recover during the 21 day follow-up period from PDT therapy. Bladder pressure rose up to the 7th postoperative day though at day 21 was at a slightly lower level than that measured at day 3. The changes were significant (p<0.05, Mann-Whitney U-test) at all measurement points.
Figure 12. Filling cystometry curves in rat bladder after PDT. Mean ± SEM in the same animals prior to PDT (●) and 1, 3, 7, and 21 days after PDT using intravenous ALA photosensitizer (300 mg/kg) and 20 J/cm² (▲), 40 J/cm² (○), and 80 J/cm² (△) energy doses. Control group received 20 J/cm² (●) energy without ALA pretreatment. Asterisks indicate statistical significance (p < 0.05).
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5.4 Bladder capacity (Study III)

The cystometric capacities of the test animals after laser treatment were expressed as percentage of the ratio to the measurements of the same animals prior to PDT (Figure 13). The bladder volume was assessed as the volume infused when in post-treatment measurement, the intravesical pressure exceeded the preoperative end filling pressure in each animal group. At day 1, the bladder capacity was reduced in every group, but after that time point, recovery started in animals treated with ALA and 20 or 40 J/cm² light dose. In the groups of ALA and 80 or 20 J/cm² light only, the bladder capacity was still declining at 3 days and recovery started at day 7.

In the 20 J/cm² light group, the animals did regain only 34% of their bladder original capacity of control values at 21 days, whereas 50% of the capacity was regained in the
group of 80 J/cm² light with ALA. A more complete recovery was seen with lower doses of light and ALA. Values as high as 100% and 97% of control capacity values were registered in some animals treated with ALA and 20 or 40 J/cm² light.

Figure 13. Changes in rat bladder capacity after PDT using intravenous ALA photosensitizer (300 mg/kg) and 20 J/cm² (▲), 40 J/cm² (○), and 80 J/cm² (×) energy doses. Control group received 20 J/cm² (□) energy without ALA pretreatment. Bladder capacity is expressed as a percentage of that in the same animals prior to PDT. (Reproduced from Urol Res 29: 205-209, 2001 by copyright permission of Springer-Verlag)

5.5 Characteristics of the bladder tumor model (Study IV and V)

A total of 36 rats were instilled intravesically with AY-27 cells (Study IV). All 36 rat bladders were histologically examined by a series of tissue sections. The presence of the tumor was noted. In all, a tumor was detectable in 30 of the 36 animals (83%) sacrificed 15 days after implantation. Histological examination of the tumor specimens confirmed them to be grade II to III superficial TCC (Figure 14).

In the last study (study V), a total of 40 rats were inoculated with AY-27 cells intravesically. As controls served 36 animals from study IV for TCC induction (83%). After PDT, all 40 rat bladders were histologically examined by a series of tissue sections and the presence of the tumor was noted.
5.6 Cytology samples (Study V)

Cytologic examination of the sediment of urine was made in animals (n=36) at day 15 post cancer cell implant in order to detect the tumors of the bladder before PDT. Overall, the cytohistologic correlation for the animals with bladder cancer was poor. The histological analysis showed 9 tumors and only in 5 of these animals cytology was malignant (56%).

5.7 Treatment effects (Study V)

After PDT with intravesical (100 mg/ml) administration of ALA and laser therapy (40 J/cm²) carcinoma was seen only in 8% (1/12) of treated animals according to histologic examination. The treatment result was statistically significant (p=0.000, Pearson’s χ² test). As many as 92% (11/12) of the animals in this treatment group were tumor free 3 or 21 days after PDT (Figure 15).
On the other hand in the intravenous ALA administration group irradiated with 40 J/cm² light the viable carcinoma was found in 45.5% (5/11) (p=0.063, Pearson’s χ² test). In that group one animal was lost after 7 days with no apparent cause of death.

In the group of rats treated only with 40 J/cm² light carcinoma was found in 25% (3/12) of the animals according to histologic examination after 3 or 21 days (p=0.001, Pearson’s χ² test).

The maximum histological effect of PDT occurred after 3 days of irradiation. Necrosis, inflammation, oedema and haemorrhage visible in the mucosa and submucosa were seen in all (6/6) of the samples in the intravesical group seen 3 days after PDT. The destruction reached the muscular layer with abscess in one animal of six (17%) in the intravenous group.

At 3 weeks after PDT there was muscular necrosis with perforation and abscess in 33% (2/6) of the animals in the intravesical group. This was felt to be related to perforation during catheterization (technical complication) due to the location (dome) and focal nature of finding. Histologically the bladder wall was normal in 50% (3/6) of the animals and mild superficial damage was still seen in one animal at 3 weeks post PDT with intravesical
ALA. In the intravenous group bladder walls were normal (2/5) or only mild superficial damage (3/5) was seen at 3 weeks of PDT.
6. DISCUSSION

At present, PDT is primarily suggested for the therapeutic management of refractory transitional-cell carcinoma in situ and prophylaxis of recurrent superficial (Ta,T1) transitional-cell carcinoma of the urinary bladder in those patients who have not benefitted from intravesical chemotherapy or immunotherapy (Waidelich et al. 2001). The advantage of PDT is that the entire mucosa of the bladder can be treated simultaneously. PDT requires a photosensitizer and light. There is a tissue- and tumor-specific photodynamic dose threshold for damage, which means that the tissue concentration of the photosensitizer and the activating light dose must reach a threshold level to evoke tissue damage (Chattani et al. 1992). In addition to the correct dose level of the photosensitizer, the most suitable laser light dose and optimal timing of light are essential in PDT to minimize muscle fibrosis and permanent reduction of bladder capacity.

6.1 The distribution and kinetics of ALA-induced PpIX in the bladder

The major objective of study I was to determine the optimum amount of ALA administration to achieve the highest PpIX fluorescence intensity in mucosa. Another aim was to determine the time period when the difference in fluorescence between mucosa and muscularis would be at its highest. Aminolevulinic acid-induced PpIX appears to localize preferentially in the mucosa rather than the muscle of hollow organs (Bedwell et al. 1992). This has been reported experimentally in the rat bladder (Loh et al. 1993b; Linuma et al. 1995) and suggests that ALA might have distinct advantages over hematoporphyrin derivative photosensitizer in patient use, since detrusor muscle damage and subsequent fibrosis may be expected to be much less of a clinical problem. Following intravenous administration, ALA leads to a rapid and even build up of PpIX in the mucosa of hollow viscera while the underlying muscularis layer is sensitised to a much lesser extent. The photosensitisation produced is short lived, lasting less than 24 h and consequently prolonged skin photosensitivity is not a problem (Divaris et al. 1990).

In the present study, the ALA-induced PpIX accumulation in the rat bladder was highest by 4 hours after ALA intravenous administration and returned to background level by 24 hours (Study I). The results correlate with those of Loh and coworkers (Divaris et al. 1990; Loh et al. 1993b). In this study at a dose of 100 mg/kg ALA, maximum fluorescence level was achieved by 2 hours, but at doses of 200 mg/kg and 300 mg/kg the time to maximum
fluorescence was 4 hours. Peak levels of fluorescence were achieved earlier with lower doses of ALA also in the study of Loh and coworkers (Loh et al. 1993b). Both maximum fluorescence and time to maximum fluorescence were dose dependent in the mucosa. The muscularis layer did not exhibit PpIX fluorescence at all.

It is not clearly understood why ALA is absorbed by carcinomatous tumor tissue; it is assumed that ALA is metabolized to PpIX more efficiently in malignant cells (Kennedy et al. 1990; Kennedy et al. 1992; Kriegsmair et al. 1996). The properties of carcinomatous tissue may contribute to this selective distribution. These include elevated numbers of low-density protein receptors, the presence of macrophages, and a decreased pH value. The abnormal structure of tumor stroma characterized by its large interstitial space, a leaky vasculature, compromised lymphatic drainage, a high amount of newly synthesized collagen (which can bind porphyrins), and the high amount of lipid present in tumors may all contribute to the preferential distribution of sensitizers (Dougherty et al. 1998).

The orthotopic superficial bladder tumor model provides a good opportunity to investigate systematically the biodistribution of ALA induced PpIX in the bladder. Its close resemblance to human superficial bladder cancer is an advantageous feature of this model, making it relevant for studying whole bladder PDT.

In the rat model with orthotopic urothelial carcinoma, 4 hours after intravesical ALA installation the PpIX fluorescence tumor-to-bladder muscularis ratio was 7:1 (Study IV). However, intravesical instillation provided tumor labeling comparable to that of the intravenous route but with a loss of selectivity for PpIX accumulation in tumor and normal urothelium. The results agree with those of Xiao et al. (Xiao et al. 1998). Linuma et al. made a quantitative comparison of PpIX biodistribution by extraction and fluorescence spectroscopy following intravesical ALA administration. The tumor to normal bladder wall ratio was 2:1 at 4 hours as measured by PpIX content (μg/g) in tissue, not by fluorescence intensity (counts per pixel) as in our study. Fluorescence microscopy revealed very intense levels of fluorescence in the urothelium and diffuse moderate fluorescence in the tumor cells. The relative intensity of the fluorescence in the urothelium was higher than in the tumor cells. This suggests a diffusion limitation barrier of ALA at the basement membrane of urothelium (Linuma et al. 1995). Our data support this finding as no difference in fluorescence intensity was observed for normal urothelium and tumor tissue after intravesical ALA administration.
After intravenous ALA administration, the peak levels of tumor fluorescence intensity were achieved at 2 hours with the lower dose of 100 mg/kg and at 4 hours with the higher dose of 300 mg/kg ALA. According to this study, the intravenous dosing route was the only method that induced a difference in PpIX fluorescence in tumor tissue and normal bladder urothelium. The fluorescence difference started to diminish 2 hours after ALA administration. Our data are consistent with previous studies (Linuma et al. 1995; Xiao et al. 1998). Xiao and co-workers reported that 4 hours after 1000 mg/kg ALA injection intravenously, PpIX ratios of tumor-to-bladder urothelium and muscle layers were 3:1 and 8:1 (Xiao et al. 1998). Linuma and colleagues found that the distribution ratio of tumor and normal bladder urothelium of PpIX was 2:1 at 4 h after intravenous administration of ALA (Linuma et al. 1995). In the current study, the differences in fluorescence at each dose level for the tumor-to-urothelium and tumor-to-muscular layers were highest 2 hours after administration for the lower and higher ALA doses (3:1 and 50:1, and 2:1 and 8:1, respectively). Previous studies have shown that a PpIX ratio of 5:1 for urothelium and bladder muscle is sufficient to achieve selective destruction of the urothelium and spare the bladder muscle layers in PDT (Chang et al. 1996; Xiao et al. 1998).

6.2 The optimum laser light dose

The most serious potential complication of whole bladder PDT is permanent bladder contracture, which has been reported in 13-40% of the patients treated with PI1 induced PDT (Nseyo 1996; Nseyo et al. 1998). When treating the whole bladder the light intensity given must be sufficient to produce damage to the superficial layers of the bladder wall, which then heals by regeneration of the remaining islands of the healthy basal layers of mucosa. This would make it possible to eradicate carcinoma in situ. It would be beneficial if tumor tissue were more prone to therapy than normal epithelial tissue. The higher concentration of photosensitizer in tumor than in other tissues would improve the therapy results. The other goal for PDT is to leave the muscular layer intact, to avoid irreversible damage to bladder wall or to cause any functional impairment. The purposes of the studies II and III were to determine the optimum amount of laser light energy needed to induce reversible damage to the bladder wall and allow a return of functional capacity.

In study II, a light energy dose of 20 J/cm² with ALA (300 mg/kg) induced an uneven, superficial mucosal destruction, which subsided within 7 days. When the light energy dose was doubled to 40 J/cm², the mucosal destruction was equal in all animals 1 day after
therapy. After 1 week, the bladder epithelium started to recover and after 3 weeks there was no muscular layer damage and the epithelium had almost totally recovered. When the light energy dose was increased to 80 J/cm² there was complete epithelial damage in all animals 3 days after PDT. Also two animals died or had a major necrosis of the bladder in this group. The muscular damage was long-lasting, as after 3 weeks there was deep muscular necrosis in one of the remaining four animals. Thus the dose of ALA was too high or at the upper limit of the optimum because of the deep muscular destruction induced.

According to the results in study III, a light dose of 20 J/cm² without ALA induced a 66% reduction in bladder capacity at three weeks. Barr and co-workers found that the bursting strength of the colon following PDT is not reduced despite full thickness damage to the colonic wall. However after thermal damage, immediate colon perforations were seen in 20% of cases. The reduced bursting strength lasts for 2 weeks. Selective collagen strain studies indicated that the submucosal colonic collagen may not be damaged by PDT with ALSPc, but the architecture is severely disrupted by thermal injury (Barr et al. 1987). Our results also suggest that the light energy is linked to the chemical process where the photosensitizer works as a catalyst in oxygen radical production, inducing tissue destruction. If the photosensitizer is not present, the light energy is transformed into thermal energy.

A wide range of light doses are used in clinical series of PDT for superficial bladder cancer (Pope et al. 1991a; Dougherty et al. 1992; Nseyo 1992; Nseyo et al. 1996; Nseyo et al. 1998). Our results revealed that the light dose of 20 J/cm² and 40 J/cm² with ALA induced only irritative changes that subsided after day 1, but there were no functional changes. Pope and Bown also concluded that even quite low doses of PDT still produced a marked initial reduction in bladder capacity due to the acute inflammation, but these improved to within normal limits after about 1 month (Pope et al. 1991b).

When the light dose was increased to 80 J/cm² with administration of ALA (300 mg/kg) there was a marked reduction seen in bladder capacity. The animals treated did not regain more than half of their bladder capacity at 3 weeks. Both the light energy and photosensitizing chemical dosages are limiting factors for the final effect of PDT. The dosage of light is more difficult to control than that of chemicals but this study shows that it can be adjusted.
6.3 PDT efficiency

In study V the level of 83% tumor establishment was adopted from study IV because of the difficulty to histologically examine the tumor presence before PDT. After intravesical instillation with AY-27 cells, histological examination of the tumor specimens confirmed the presence of grades II to III superficial TCC. All tumors were superficial and no invasion to the muscle layer of bladder wall was noted (study IV). As PDT is a promising therapeutic modality for patients with superficial bladder cancer (Nseyo 1996) the bladder tumor model should mimic the clinical disease entity as closely as possible if one is to draw relevant conclusions.

Cytologic examination of the sediment of voided urine was used in this study as a noninvasive method to detect and diagnose the presence of tumors before PDT. The results indicated that cytology of voided urine is highly reliable in the diagnosis of high-grade tumors, with a sensitivity of 94.2%. The method fails almost invariable in the recognition of grade I papillary tumors and in about one-third of grade II tumors (Utz et al. 1980; Murphy et al. 1984; Koss et al. 1985). Cytology of voided urine before PDT failed to identify bladder cancer in about half of tumors subsequently detected in this animal model. Maybe the effects of catheterization or even simple manipulation of the bladder on the make-up of the urinary sediment influenzed the results. The histological examination of the bladder is possible after PDT in animal models and the lack of reliable method to detect tumors before therapy makes it difficult to analyse the treatment effects in a totally reliable manner. Newer tumor markers like BTA Stat should has been considered to be used instead of urine cytology for detection of orthotopic tumor existence (Raitanen et al. 2001).

The major objectives of this study were to compare intravenous and intravesical administration of ALA in order to evaluate the effect of PDT in treating bladder cancer. PDT with intravesically applied ALA was effective in 92% (11/12) of the animals in destroying superficial urothelial carcinoma of the bladder. After PDT, the carcinoma was found in 8.3% of the treated animals as opposed to 83% of controls. Previous studies have shown that a fluorescence distribution ratio of 5:1 between the urothelium and bladder muscle is sufficient to obtain selective destruction of the urothelium and to spare the bladder muscle layers in PDT (Chang et al. 1996; Xiao et al. 1998). In the present study, a fluence of 40 J/cm² with ALA induced mainly a transient and superficial mucosal destruction, which subsided within three weeks. After three weeks there was muscular
layer damage with abscess due to bladder perforation in 12% (2/17) of the treated animals. Both of these animals were treated with intravesical administration of ALA and PDT. The reason for this could be attributed to technical complications of bladder catheterization and resulting bladder perforation. Histologically in this same group normal bladder wall was evident in 50% (3/6) and in one animal (1/6) mild superficial damage was seen after three weeks. No muscular layer damage was evident excluding the regions suspicious for catheter perforation in the intravesical group.

According to our results (Study IV) four hours after intravesical installation of ALA, the PpIX fluorescence between tumor and bladder muscularis was 7:1. However, no difference in fluorescence intensity was observed between normal urothelium and tumor tissue following intravesical ALA administration. Intravesical instillation provided tumor labeling comparable to the intravenous route, but with a loss of selectivity of PpIX accumulation between tumor and normal urothelium. However, the treatment effect of PDT after intravesical administration of ALA was good. It seems that it may even be beneficial to sensitize also the normal urothelium together with the tumor tissue to produce damage to the superficial layers of the bladder wall.

After intravenous administration of ALA followed by PDT, the carcinoma was found in 45% of the animals. The results of this study agree with earlier findings that intravenous injection of ALA induces a significant difference in PpIX fluorescence in tumor tissue and normal bladder epithelium (Tinuma et al. 1995; Xiao et al. 1998). The accumulation of photosensitizer in tumor tissue may leave the concentration in the normal mucosa below that needed level to induce the PDT effect. If the concentration of ALA is below the threshold level needed to initiate PDT, ALA will be broken down in the photobleaching process without any appreciable tissue effects. Invasion into the lamina propria has been considered an important threshold barrier in assessing a tumor’s potential to progress and metastasize (Heney et al. 1983; Abel et al. 1988). When the superficial layers of the bladder wall with the lamina propria are totally destroyed with intravesically applied ALA and PDT, the risk of recurrence and progression can be minimized and the treatment effect remains good.

Our results suggest that PDT with intravesical ALA administration is an effective and safe treatment for superficial bladder cancer. The results of intravenous administration of ALA were controversial. However the promising treatment results with intravesical administration favor further clinical testing of ALA-PDT for superficial bladder cancer.
7. SUMMARY AND CONCLUSIONS

In this experimental study, we studied PDT in the treatment of superficial bladder cancer. Subsequent to our results with normal rat bladder PDT, we continued these studies with a rat model of orthotopic urothelial carcinoma.

On the basis of the present study the following conclusions can be drawn:

1. The fluorescence studies showed that PpIX concentrates in bladder mucosa and that the highest fluorescence levels from analysed doses are achieved four hours after intravenous (300 mg/kg) administration of ALA. At the same time, the highest difference in PpIX distribution in mucosa and muscularis is attained.

2. The optimum laser energy with ALA (300 mg/kg) was 40 J/cm² to achieve the maximum PDT effect in bladder mucosa. At one week after PDT, the bladder epithelium started to recover and there was no muscular layer damage. After three weeks, the epithelium had totally recovered. When the dose was increased to 80 J/cm², the muscular layer damage was long-lasting, since at three weeks there was still muscular necrosis in 25% of the treated bladders.

3. The light dose 20 J/cm² and 40 J/cm² together with ALA (300 mg/kg) caused no permanent reduction in bladder capacity, whereas 80 J/cm² light dose produced up to 50% reduction in capacity at 3 weeks. Our results indicate that with proper dosing of photosensitizer and light energy, the functional impairment of urinary bladder may be minor and transient.

4. After intravenous administration, the highest difference in fluorescence intensity between tumor tissue and normal epithelium was measured at 2 hours with both administered concentrations of ALA (100 mg/kg and 300 mg/kg). The difference varied between 2.3 and 3.1 fold. Absolute PpIX fluorescence intensity levels rose with increasing ALA dose, when the intravenous administration route was used. Four hours after ALA (300 mg/kg) administration, the difference in absolute fluorescence intensity was 6 times higher in tumor tissue than in muscle tissue and 4 times higher in normal urothelium than in muscular layer of bladder. After intravesical administration of ALA, PpIX fluorescence was higher in tumor tissue samples taken at 4 than at 2 hours and there was no difference in
normal epithelium and tumor tissue. The absolute fluorescence level at 4 hours after administration of ALA was 7 times higher in tumor tissue than in the muscular layer.

5. PDT with intravesical administration of ALA is an effective and safe modality for the treatment of superficial bladder cancer. The promising results favor further clinical testing of ALA based PDT in the therapy of superficial bladder cancer.
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